E2f4 and *E2f5* are essential for the development of the male reproductive system.

Paul S. Danielian[1], Rex A. Hess[2] and Jacqueline A. Lees[1, 3]

[1] David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA, 02139, USA.

[2] Reproductive Biology & Toxicology, Department of Comparative Biosciences, College of Veterinary Medicine, University of Illinois, Urbana, IL, 61802, USA.

[3]Corresponding author, David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA, 02139, USA

Email address: jalees@mit.edu

Telephone: +1-617-252-1972

Fax: +1-617-253-8749

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Abstract

The E2F transcription factors are primarily implicated in the regulation of entry and exit from the cell cycle. However, *in vivo* studies have established additional roles for E2Fs during organ development and homeostasis. With the goal of addressing the intestinal requirements of *E2f4* and *E2f5*, we crossed mice carrying *Vil-cre*, *E2f4* conditional and *E2f5* germline alleles. *E2f4* deletion had no detectable effect on intestinal development. However, *E2f4f/f;E2f5+/-;Vil-cre* males, but not *E2f4f/f;Vil-cre* littermates, were unexpectedly sterile. This defect was not due to defective spermatogenesis. Instead, the seminiferous tubules and rete testes showed significant dilation, and spermatozoa accumulated aberrantly in the rete testis and efferent ducts. Our data show that these problems result from defective efferent ducts, a tissue whose primary function is to concentrate sperm through fluid absorption. First, *Vil-cre* expression, and consequent E2F4 loss, was specific to the efferent ducts and not other reproductive tract tissues. Second, the *E2f4f/f;E2f5+/-;Vil-cre* efferent ducts had completely lost multiciliated cells and greatly reduced levels of critical absorptive cell proteins: aquaporin1, a water channel protein, and clusterin, an endocytic marker. Collectively, the observed testis phenotypes suggest a fluid flux defect. Remarkably, we observed rete testis dilation prior to the normal time of seminiferous fluid production, arguing that the efferent duct defects promote excessive secretory activity within the reproductive tract. Finally, we also detect key aspects of these testis defects in *E2f5-/-* mice. Thus, we conclude that *E2f4* and *E2f5* display overlapping roles in controlling the normal development of the male reproductive system.

Key words: E2F4, E2F5, efferent ducts, testis, rete, ciliogenesis, multiciliated, fertility, villin, intestine.

Introduction

In mammals the E2F family of transcription factors comprises eight genes that together regulate genes required for cell proliferation throughout the cell cycle. [1-3] E2Fs 1 through 6 function as heterodimers with members of the DP family of transcription factors whilst E2F7 and E2F8 function independently. E2Fs 1 through 3 are thought to function primarily as transcriptional activators whilst E2Fs 4 through 8 repressors of transcription, however, studies of mutant mice indicate that these assignments are not definitive. [4, 5] The activity of E2Fs 1 through 5 is controlled by association with members of the pocket protein family, which includes the tumor suppressor protein pRB and its relatives p107 and p130. These can inhibit the E2Fs transcriptional activity and also form repressive complexes via recruitment of histone deacetylases (HDACs) or the DREAM complex. [6, 7] Mitogenic signaling leads to phosphorylation of the pocket proteins by cyclin dependent kinases and complex dissociation enabling de-repression of E2F-target genes.

Analyses of mutant mice and also cell lines has demonstrated that many E2Fs function redundantly (reviewed in ref. [8]). For example, failure to enter the cell cycle in mouse embryo fibroblasts (MEFs) only occurs following mutation of all three activator *E2fs*, *E2f1, E2f2* and *E2f3,* whilst many of the *in vivo* phenotypes of the single *E2f1* or *E2f3* mutant mice are exacerbated by double mutation of these genes. [9-13] Functional redundancy was thought to explain why mutation of the repressor *E2f4* did not lead to E2F-target gene derepression in MEFs, despite the fact that E2F4 is the most abundant E2F in MEFs. [14-16] Supporting this idea, the analyses of *E2f4* and *E2f5* mutant MEFs showed that only double mutant MEFs fail to cell cycle arrest following expression of the cyclin dependent kinase inhibitor p16INK4a, which functions via preventing pocket proteins dissociation from E2Fs. [17]

Despite the modest effects of *E2f4* knockout on target genes, E2F4 was found to be essential for normal viability. [14, 15] We showed that the postnatal death of the *E2f4* mutant mice was due to a failure of multiciliated cell development in the airway epithelium, which results in chronic rhinitis and an increased susceptibility to

opportunistic infections. [15, 18] The loss of ciliated cells from the airway arose in the absence of detectable changes in cell proliferation suggesting that E2F4 had non-cell cycle related functions.[18] Interestingly this phenotype was also observed in *E2f4+/-;E2f5-/-* double mutants but not *E2f4+/-* or *E2f5-/-* single mutants suggesting functional redundancy between these two structurally related E2Fs in multiciliogenesis (P.S.D, J. Sero and J.A.L, unpublished observations). *E2f4-/-;E2f5-/-* double mutant animals are not viable and die *in utero*, reinforcing the notion that functional redundancy can occur between these two proteins.[17]

E2f4 mutant mice were also reported to exhibit atrophy of the intestine and several cell line studies have suggested that E2F4 plays a role in the proliferation of intestinal epithelial cells. [14, 19, 20] However, we only observed degeneration of the intestinal tract in the moribund *E2f4-/-* neonates, suggesting that it was an indirect consequence of dehydration and starvation of these animals. [15] Accordingly, *E2f4-/-* mice were shown to be fully viable when placed on antibiotics, to prevent respiratory infections, indicating that E2F4 is not essential for intestinal development. [15] However, this did not rule out the possibility that E2F4 and E2F5 exhibit functional redundancy in the intestine. With the goal of addressing this question, we intercrossed mice carrying *E2f4* conditional and *E2f5* germline mutant alleles with an allele that expresses Cre recombinase in the intestine (*Vil-cre)*. We found that E2F4 is not required for normal intestinal development and homeostasis. However, because *Vil-cre* is also expressed in the efferent ducts of the male reproductive tract, we discovered that E2F4 and E2F5 are essential for the normal tract development and fertility.

In the reproductive system of male mammals spermatozoa arise in the seminiferous tubules of the testis, which ultimately coalesce in the rete testis. The rete testis is directly connected to the efferent ducts, which subsequently connect to the epididymis allowing sperm to move from the testis to the Vas deferens and finally the urethra. The primary function of the efferent ducts is considered to be the reabsorption of

50% to 96% of the luminal seminiferous fluid, resulting in the concentration of sperm during their passage to the epididymis. [21] The efferent ducts also contain multiciliated cells with motile cilia that are believed to stir the luminal fluid, rather than regulating its flow from the testis to the epididymis. [21] In this paper we show that mutation of *E2f4* within the efferent ducts in combination with heterozygous mutation of *E2f5* leads to a loss of multiciliated cells from the efferent ducts, dilation of the seminiferous tubules, dilation of the rete testis and infertility. In addition we show that homozygous mutation of *E2f5* results in a partial phenotype indicating again that E2F4 and E2F5 function redundantly. These data therefore further illustrate that the E2F family of transcription factors plays critical roles in organ development in mammals.

Results

Mutation of *E2f4* and *E2f5* in the efferent ducts leads to male sterility.

To analyze the function of E2F4 alone, and in combination with other E2Fs, in a tissue specific manner we generated a conditional (floxed) allele of *E2f4* (manuscript in preparation). This allele was validated by confirming that expressing Cre recombinase throughout the early embryo recapitulated the classic defects of the *E2f4* germline mutant mice (data not shown). We then initiated experiments to determine if E2F4 and E2F5 play overlapping role in the development or homeostasis of the intestine using the *Vil-cre* transgene which expresses Cre recombinase throughout the intestinal epithelium from embryonic day 12.5. [22] We found that *E2f4f/f;Vil-cre* adult mice (n>10) were viable and fertile and that the intestine appeared morphologically normal (Supplementary Figure 1). Immunohistochemical (IHC) analyses confirmed that E2F4 was completely absent from the intestinal epithelium of *E2f4f/f;Vil-cre* mice (Supplementary Figure 1), indicating efficient mutation of *E2f4*. Thus, we conclude that E2F4 is not essential for development or homeostasis of the intestine in this model. We then crossed the *E2f4f/f;Vil-cre* mice with *E2f5* germline mutant animals. [23] In generating these crosses, we discovered that

male *E2f4f/f;E2f5+/-;Vil-cre* mice were sterile despite being able to plug females (n > 5). Males of all other genotypes, including *E2f4f/f;E2f5+/+;Vil-cre*, *E2f4+/f;E2f5+/-;Vil-cre* and *E2f4+/f;E2f5+/+;Vil-cre*, were fertile (n>5 per genotype) indicating that the levels of E2F5 expression are critical for fertility in the absence of E2F4.

E2f5-/- mutant mice develop hydrocephalus and show early lethality. [23] In our hands, these animals require euthanasia by 3 weeks of age (data not shown). Thus, to further investigate the infertility phenotype we analyzed males derived from crossing *E2f4+/f;E2f5+/-;Vil-cre* males with *E2f4f/f* females. Previous studies had reported that *Villin*, in addition to being expressed in the intestine, is expressed in the efferent ducts and fallopian tube epithelium. [24] Since the efferent ducts connect the rete testis to the epididymis [21], it seemed likely that defects within the efferent duct epithelium were causing the observed infertility. To validate the expression of *Vil-cre* in the male reproductive tract, we generated *E2f4+/f;E2f5+/-;Vil-cre* males carrying a reporter allele which expresses the fluorescent protein ZsGreen1 following Cre mediated recombination. [25] We detected strong ZsGreen1 expression in the efferent ducts of the male reproductive system, but no significant expression in the testis or the epididymis, demonstrating that the Cre recombinase was active in cells giving rise to or constituting the efferent ducts (Supplementary Figure 2). *Villin* is also expressed in the ciliated epithelium of the fallopian tube [24] but the *Vil-cre* transgene did not express efficiently in the fallopian tube epithelium of female mice (data not shown), precluding analysis of the role of *E2f4* and *E2f5* in this setting.

Having established that *Vil-cre* was expressed in the efferent ducts we next analyzed the efficacy of recombination at the conditional *E2f4* locus in this epithelium by examining the expression of E2F4 by IHC staining. In control mice E2F4 is expressed sporadically in most cells of the adult efferent duct epithelium at varying levels, with particularly high expression occurring in nuclei furthest from the basement membrane (Supplementary Figure 3). E2F4 expression was absent from the efferent duct epithelium in *E2f4f/f;Vil-cre* adult mice indicating efficient *Vil-cre*-mediated recombination of the locus (Supplementary Figure 3). The efferent duct epithelium of *E2f4f/f;Vil-cre* mice was

morphologically indistinguishable from wild-type epithelium (n>10) demonstrating that loss of E2F4 alone does not significantly compromise efferent duct development.

To ascertain the reason for the infertility, we conducted histological analyses of the reproductive organs of littermate controls (*E2f4+/f;E2f5+/+* or *E2f4+/f;E2f5+/-* mice with or without *Vil-cre*, as well any genotype that lacked *Vil-cre*) and *E2f4f/f;E2f5+/-;Vil-cre* adult male littermates between 2 and 6 months of age. In some crosses mice also carried the *ZsGreen1* Cre-reporter allele. This analysis showed several major pathological features in the *E2f4f/f; E2f5+/-;Vil-cre* adult males, and not the controls, that were outside the efferent ducts. First, the seminiferous tubules of the testis were dilated in all *E2f4f/f;E2f5+/-;Vil-cre* animals (n>12; Figure 1 B, D), relative to littermate controls (Figure 1A, C). In many tubules, the lumen was dilated at the expense of the epithelium, which exhibited atrophy. Measurements showed a statistically significantly decrease in the total epithelium height of the tubule, relative to the total tubule diameter, at both 2 months (controls, 67+/-10.6% v mutants, 39.3+/-12.6%, $p<0.0001$) and 4 months (controls, 66.3+/-9.8% v mutants, 40+/-15.1%, $p<0.0001$) of age (Supplementary Figure 4). Despite this disruption of seminiferous tubule morphology, histological examination indicated that all the stages of spermatogenesis were present. Thus, spermatogenesis is not compromised in these mutant animals (Supplementary Figure 5). Second, the rete testis was also substantially dilated in *E2f4f/f;E2f5+/-;Vil-cre* males (n = 6/6) and often contained many spermatozoa (Figure 1B, F). Spermatozoa were detected in the efferent ducts of 11/13 *E2f4f/f;E2f5+/-;Vil-cre* males indicating that there was not a physical block in the tubules preventing the transition of spermatozoa from the rete testis into the efferent ducts (Figure 1G, H). However, the presence of spermatozoa in the ducts is aberrant, as spermatozoa should not accumulate in the efferent ducts due to the normally rapid transit time. Thus, the passage of sperm from the efferent ducts into the epididymis is compromised in the *E2f4f/f;E2f5+/-;Vil-cre* males. Third, no spermatozoa were observed in the epididymis of 9/12 *E2f4f/f;E2f5+/-;Vil-cre* males (Figure 1J), and very few were seen in the remaining 3/12 males. In addition, the lumen of the epididymis was often filled with material that stains pink following hematoxylin and eosin staining, which is typically observed when spermatozoa fail to reach the epididymis. [26, 27] In

contrast, spermatozoa were detected in the epididymis of littermate males of all control genotypes (n>12; Figure 1I). The primary function of the efferent ducts is considered to be the reabsorption of luminal fluid, with estimates of the amount of seminiferous fluid being reabsorbed ranging from 50% to 96%. [21] Given the above defects, our findings suggest that defective efferent duct function impedes the flow of seminiferous tubule luminal fluid, causing it to back up in the testis and induce seminiferous tubule and rete testis dilation, and impair passage of spermatozoa into the epididymis, yielding infertility.

The efferent duct epithelium of infertile males lacks multiciliated cells.

The efferent duct epithelium is comprised of absorptive cells and multiciliated cells with motile cilia.[21] Since we showed previously that E2F4 is required for multiciliogenesis in the airway [18], we began by assessing the development of the multiciliated cells in the mutants. As indicated above, spermatozoa were detected in the efferent ducts of most mutant animals indicating that the ducts were patent, but the entire efferent duct epithelium in all adult *E2f4f/f;E2f5+/-;Vil-cre* males lacked ciliated cells (n>10; Figure 2A, B). This was verified by IHC staining for acetylated α-tubulin, a component of motile cilia (n = 4/4; Figure 2C, D). IHC analysis verified that E2F4 expression was lost from the efferent duct epithelium in the *E2f4f/f;E2f5+/-;Vil-cre* animals (n = 4/4; Figure 2E, F). We then analyzed the expression of FOXJ1, a transcription factor required for multiciliogenesis and a marker of multiciliated cells. [28, 29] In the efferent duct epithelium of *E2f4f/f;E2f5+/-;Vil-cre* adult males there was a significant reduction in the number of cells that expressed normal levels of FOXJ1, relative to the controls, although many cells expressed very low levels of FOXJ1 (Figure 2G, H). Quantification of the percentage of nuclei in the epithelium expressing normal levels of FOXJ1 in mutant versus control littermates (n=4 for each genotype) showed that this difference was statistically significant (8.4+/-4% versus 27.5+/-6%, $p < 0.0001$). To investigate whether FOXJ1 expression was initiated normally, we examined expression in the efferent ducts at 1 week of age. At this stage of development, where *Vil-cre* deleted E2F4 in the efferent ducts, staining of adjacent sections for FOXJ1 showed substantially

reduced expression levels in *E2f4f/f;E2f5+/-;Vil-cre* males relative to the controls (n=4/5; Figure 3). These results show that efferent duct epithelial development is disrupted at early stages and, even though some cells in the epithelium of *E2f4f/f;E2f5+/-;Vil-cre* adults express normal levels of FOXJ1, multiciliogenesis fails to occur.

Mutation of *E2f4* and *E2f5* disrupts non-ciliated cell development in the efferent ducts.

Since the motile cilia are believed to stir the fluid rather than directing its flow from the testis to the epididymis [21], the loss of motile cilia was unlikely to fully explain the dilation of the seminiferous tubules and rete testis in the *E2f4f/f;E2f5+/-;Vil-cre* males. As E2F4 is expressed in both ciliated and non-ciliated cells of the efferent duct epithelium, we assessed the function of the non-ciliated cells in *E2f4f/f;E2f5+/-;Vil-cre* mice by analyzing the expression of markers associated with efferent duct absorptive cell function. Initially, we examined the expression of Estrogen receptor 1 (ESR1) which is expressed in both ciliated and non-ciliated cells of the efferent duct epithelium and required for reabsorption of luminal fluid (reviewed in refs. [30, 31]). Our analyses showed the ESR1 was still expressed in mutant males, albeit at a slightly lower level (Figure 4A, B). We then examined the expression of the water channel protein aquaporin1 (AQP1), involved in water transport across the efferent ducts, and clusterin (also known as SGP-2 or apolipoprotein J), a marker of endocytosis. [21, 32-34] The majority of clusterin inside efferent duct cells is secreted by the Sertoli cells of the testis, and taken up by the efferent duct epithelium via endocytosis. [21] We found that expression of AQP1 (Figure 4C, D) and clusterin (Figure 4E, F) in the efferent ducts was greatly reduced in 5/5 *E2f4f/f;E2f5+/-;Vil-cre* mice, relative to their littermate controls. This reduction was not uniform but >40% (for 1/5 mice) or >50% (for 4/5 mice) of the efferent duct epithelial cells showed complete loss of both AQP1 and clusterin (Figure 4, Supplementary Figure 6). We also observed reduced apical PAS staining in the mutants relative to littermate controls which is additional evidence of a decreased abundance of endocytic vesicle apparatus (n=5/5; Figure 4G, H). Thus, we conclude that mutation of *E2f4* and *E2f5* also compromised the ability of the efferent ducts to reabsorb seminiferous tubule fluid by disrupting the development of non-ciliated cells.

Mutation of *E2f4* and *E2f5* causes abnormal rete testis development.

Inefficient reabsorption of seminiferous fluid by the efferent ducts would lead to a back up in flow from the testis causing seminiferous tubule and rete testis dilation. To investigate this further, we analyzed the development of the male reproductive tract at 1 week of age, which is before the production of spermatozoa or seminiferous fluid by the seminiferous tubules. If the testis phenotype was due solely to the failure of seminiferous fluid reabsorption rete testis development should be normal at this age. In contrast, we found that the rete testis was dilated in 5/7 *E2f4f/f;E2f5+/-;Vil-cre* males, but in 0/12 controls (Figure 5A, B). In 2/6 animals, we also observed rete testis dilation in double heterozygotes (*E2f4+/f;E2f5+/-;Vil-cre*) at 1 week of age, but this defect was absent in older animals (n>12), suggesting that it resolved at later stage of development (data not shown). Notably, examination of the *Vil-cre* transgene expression using the ZsGreen1 Cre recombinase reporter, a β-galactosidase based Cre recombinase reporter, and also IHC for E2F4 indicated that Cre recombinase was not active in the rete testis (Supplementary Figure 7 and data not shown). Thus, this rete testis dilation is non-cell autonomous. Based on these observations, we hypothesize that disruption of efferent duct development through *E2f4* and *E2f5* mutation causes the efferent duct epithelium, testis and/or rete testis to secrete excess fluid early during development and thereby yielding the rete testis dilation at 1 week of age.

Since mutation of *E2f5* has been shown to cause excess secretion of cerebrospinal fluid from the choroid plexus [23], we further investigated this rete testes phenotype by additionally analyzing pups that were mutant for *E2f5*. For this, we examined testis from one week old pups that were *E2f4+/f;E2f5-/-* or *E2f4f/f;E2f5-/-* and lacked the *Vil-cre* transgene. Notably, the rete testis was dilated in 4/5 *E2f5* homozygous mutant mice (Figure 5C, D), whereas only 1/8 of the control littermates (an *E2f5+/-* animal) showed a minor case of dilation (data not shown). Thus, we conclude that loss of *E2f5* alone is able to cause rete testis dilation. We also examined *E2f5-/-* pups at 3 weeks of age (the oldest they are viable), and observed rete testis dilation in 4/4 *E2f4+/f;E2f5-/-* and

E2f4f/f;E2f5-/- pups, compared with 0/4 control littermates (Figure 5E, F). The dilated rete testis and dilated seminiferous tubule phenotype was also observed in rare surviving adult *E2f5-/-* (2/2) and *E2f4+/-;E2f5-/-* (4/4) mice (data not shown).

Given these results, we next analyzed the development of the efferent duct epithelium to ascertain whether it was also affected by mutation of *E2f5*. At one week of age the levels of FOXJ1 within the efferent duct epithelium were consistently lower in *E2f4f/f;E2f5-/-* and *E2f4+/f;E2f5-/-* pups (without *Vil-cre*) in comparison with control littermates (n=5/5; Figure 5G, H). These data suggested that mutation of *E2f5* impacts the multiciliogenesis developmental program within the efferent duct epithelium at this stage. By three weeks of age the levels of FOXJ1 were moderately reduced in the epithelium of *E2f4f/f;E2f5-/-* or *E2f4+/f;E2f5-/-* pups relative to control littermates (n= 4/4; Figure 5I, J). However, staining for cilia showed a reduction in the abundance, but not a complete loss of multiciliated cells in *E2f4f/f;E2f5-/-* and *E2f4+/f;E2f5-/-* pups at three weeks of age (n=4/4; Figure 5K, L). Notably, expression of E2F4 was not altered by mutation of *E2f5*, as judged by immunohistochemistry (data not shown). These data therefore indicate that loss of E2F5 primarily causes the rete testis dilation but it only partially impairs multiciliogenesis. Conversely, loss of E2F4 only impairs development of the multiciliated cells and promotes rete testis dilation when one copy of *E2f5* is mutant, implying that these transcription factors function redundantly.

Discussion

This study shows that the development of the murine male reproductive system requires the transcription factors E2F4 and E2F5 and that they have overlapping functions. Loss of E2F4 and E2F5 leads to several phenotypes in the reproductive tract that, at least in *E2f4* null, *E2f5* heterozygous context, are directly linked to defective efferent duct development. Within the efferent duct epithelium several cell types are affected. First, multiciliated cell development fails when E2F4 is absent in combination with heterozygosity of *E2f5*. This is illustrated by the absence of cilia and the disrupted

expression of FOXJ1, a marker of multiciliated cells, both early during efferent duct development and in adults. This phenotype most likely arises because E2F4 and E2F5 are involved in the activation of the multiciliated cell program [35-37]). Analyses of *E2f5-/-* efferent ducts indicate that at one week of age, the levels of FOXJ1 are lower in comparison with littermate controls indicating that the multiciliogenesis program is disrupted but by three weeks of age multiciliated cells can develop albeit with reduced efficiency. At the same time, *E2f4* deficiency alone does not disrupt multiciliogenesis in the efferent ducts but the additional deletion of a single *E2f5* allele is sufficient to completely abrogate multiciliogenesis. Taken together, these data provide direct support for overlapping roles of E2F4 and E2F5 in the *in vivo* setting. By extension of this logic, we propose that the failure of multiciliated cell development in the airway epithelium and their normal development in the efferent ducts of *E2f4-/-* mice [18] reflects the fact that E2F5 compensates for the loss of E2F4 in the efferent duct epithelium but not in the airway epithelium. We further predict that loss of E2F4 and E2F5 during the development of other ciliated epithelium, such as that in the fallopian tubes or the ependymal epithelium of the ventricles of the brain, is likely to result in the failure of multiciliated cell development.

Within the efferent duct epithelium the development of the non-ciliated absorptive cells is also disrupted in the *E2f4f/f;E2f5+/-;Vil-cre* males. In this case we see a significantly reduction in the presence of two proteins important for absorptive cell function, Aquaporin 1 and Clusterin, within the efferent ducts suggesting that their absorptive capacity is compromised. This was further supported by the disruption of PAS staining that is indicative of endocytic vesicle apparatus. We hypothesize that the inability of the efferent ducts in the *E2f4f/f;E2f5+/-;Vil-cre* males to appropriately reabsorb seminiferous fluid contributes to the dilation of the rete testes and seminiferous tubules at later stages. However, this cannot fully account for the dilation of the rete testis observed since this is observed in one week old pups, prior to the normal time when the seminiferous tubules produce seminiferous fluid. Since this dilation defect is observed in the *E2f4f/f;E2f5+/-;Vil-cre* males, in which *E2f4* deletion occurs specifically in the efferent ducts, we presume this must involve an efferent duct defect. We speculate that

the efferent ducts, instead of absorbing fluid, are secreting excess fluid, which backs-up into the rete testis, causing it to dilate. Alternatively, disruption of efferent duct epithelium could result in abnormal signaling from the efferent ducts to the rete testis that causes the rete testis itself to secrete excess fluid. Given that the mutant rete testis is substantially larger than normal, there is also overgrowth of the rete testis. This could be a response to the excess fluid and/or altered signaling between the efferent duct and rete testes.

In estrogen receptor 1 (*Esr1*) mutants, which display a similar phenotype in the rete testis and testis (reviewed in [30, 31]), rete testis dilation was observed as early as postnatal day 10 [38] suggesting that it may also occur prior to the stage when the seminiferous tubules are secreting fluid. Interestingly, in the *Esr1* mutant mouse it was clearly established that the efferent ducts have an inability to reabsorb seminiferous tubule fluid and even secreted fluid into the lumen [39], which could account for the observed rete testis dilation. We did not detect a substantial reduction in *Esr1* expression in *E2f4f/f;E2f5+/-;Vil-cre* efferent ducts. However, we cannot rule out that the observed modest reduction of ESR1 levels, and/or an unappreciated defect in ESR1 function, contributes the testis dilation defects in our *E2f4*/*E2f5* mutant models. Interestingly, other transgenic mouse models have been shown to develop rete testis dilation through modulation of *Esr1* expression (*Lgr4)* versus through direct effects upon fluid reabsorption that are likely Esr1-independent Na^+/H^+ *exchanger 3 (Slc9a3)* and *Carbonic Anhydrase II (Car2).* [40-43]

In contrast to our model, *Esr1* mutation does not prevent the development of multiciliated cells, but does decrease the number of formed cilia by more than 50% .[44] It is unclear whether the multiciliated cell defect in *E2f4f/f;E2f5+/-;Vil-cre* males contributes to the observed infertility and/or whether the multiciliated cell and rete testes dilation defects influence one another. Analysis of the male reproductive tract in the *FoxJ1* mutant, which specifically disrupts multiciliated cell development, could help answer this question, but this has not been reported. It is also possible that the stirring or mixing function of efferent ductule cilia is essential for homogenous reabsorption of fluid

by the nonciliated cells, as previously predicted. [45-47] Additionally we speculate that the near absence of sperm from the epididymis could be caused by impaired fluid flow, an occlusion or a defect in smooth muscle contraction, which may rely upon signaling from the efferent ducts.

Irrespective of the contribution of the multiciliogenesis defect, we believe that the primary problem is an accumulation of luminal fluid that reflects both impaired absorption and excess secretion. Interestingly, mutation of *E2f5* lead to excessive secretory activity of the choroid plexus epithelium causing hydrocephalus [23] and transmission electron microscopy and histochemical analysis showed that the nasal respiratory epithelium of *E2f4* mutant embryos resembles that of a secretory epithelium, instead of the normal, predominantly multiciliated epithelium.[18] Taken together with the results presented here, these data suggest that E2F4 and E2F5 play key roles in repressing a secretory cell program for which the cofactors and genes involved are yet to be established. Furthermore, this secretory phenotype has not been observed following mutation of other E2Fs indicating that it is a specific, overlapping role of E2F4 and E2F5.

Materials and Methods

Generation of a conditional, floxed, allele of *E2f4*.

Construction of the *E2f4* conditional allele will be published elsewhere, details are available upon request.

Animal maintenance and genotyping.

For this study mice were maintained on a mixed C57BL/6 x 129Sv background in a conventional facility. All animal procedures followed protocols approved by the MIT Institutional Animal Care and use Committee. Genotyping was performed by PCR using standard procedures and the following primers for the *E2f4* conditional allele: F4cC, gccattaagcctcagctctgtctgg and F4cU, gtgcaccctgagatgtttagtctgg resulting in a 200bp product for the wild-type allele and a 293bp product for the conditional, floxed, allele. F4cC and a separate primer, ctggaacttgcaatgtagacaagg were used to detect the locus following recombination by Cre recombinase (244bp product). Genotyping for the *E2f5*

alleles was performed using the following primers: agacacggagtgggtcagatttggg, tctgtcccctacaagacagacaggc and attcgcagcgcatcgccttctatcg resulting in a 350bp product for the wild-type allele and 520bp product for the mutant allele.
Genotyping for the *Vil-Cre* transgene was performed as described. [48] Genotyping for the *lacZ* and *ZsGreen1* Cre reporter alleles was conducted as described on the Jackson Laboratories' web site (http://www.jax.org/), stock numbers 009427 and 007906 respectively.

In all experiments controls included the following genotypes *E2f4+/f; E2f5+/+* or *E2f4+/f; E2f5+/-* with and without *Vil-cre* as well as animals of any genotype that lacked *Vil-cre*. Mutants in this study are defined as *E2f4f/f; E2f5+/-;Vil-cre* with or without a Cre recombinase reporter allele. Apart from the loss of E2F4 expression we did not detect any phenotype in *E2f4f/f; E2f5+/+;Vil-cre* animals using the assays described in this paper unless specified otherwise, however, these animals were not used as controls. Male *E2f4f/f; E2f5+/-; Vil-cre* mice arose from crosses at approximately Mendelian frequency indicating that this genotype did not cause premature lethality.

Histological analysis and immunohistochemistry.

Collected tissues were fixed in either modified Davidson's fixative or formalin (3.7% formaldehyde in phosphate buffered saline, PBS) for 24 hours at room temperature or overnight at 4^{o}C, respectively and then dehydrated via an ethanol series prior to embedding in paraffin for sectioning at 5µm. For all procedures slides were re-hydrated through an ethanol series following de-waxing in xylenes and rinsed in water or PBS, as required. For each tissue at least two different levels were examined and one section from each level stained with haematoxylin and eosin (H&E) using standard procedures. Periodic acid-Shiff (PAS) staining was performed as described previously. [18] Images were captured on a Nikon Eclipse E600 using a SPOT RT digital camera and SPOT Advanced software.

The quantification of seminiferous tubule dilation was performed using ImageJ software. For each animal a minimum of 10 randomly chosen tubules per testis were measured. The total diameter of each tubule was measured perpendicular and midway along the longest axis of the tubule visible in the section and the diameter of the lumen measured at the same position and angle. The percentage of the tubule diameter comprised of by epithelium was calculated from these measurements (tubule diameter – lumen diameter/tubule diameter) and the data subject to an unpaired Student's t-test. At each time point a minimum of 4 pairs of controls and mutant littermates were analyzed.

For analysis of testis at 1 and 3 weeks of age for each pair of testes four midline sections were cut 300μm apart. If a dilated rete was observed in these sections then this sample was scored as positive.

Immunohistochemistry was performed essentially as described previously [18] using the following antibodies: E2F4 (1:1 LLF4.2 [16]), acetylated α-tubulin (1:8000, http://www.sigmaaldrich.com/catalog/product/sigma/t6793?lang=en&region=US), FOXJ1 (1:400, http://www.ebioscience.com/human-mouse-foxj1-antibody-purified-2a5.htm), ERα (1:1000, http://www.scbt.com/datasheet-542-eralpha-mc-20-antibody.html), AQUAPORIN 1 (1:300, http://www.scbt.com/datasheet-20810-aqp1-h-55-antibody.html), CLUSTERIN (1:200, http://www.scbt.com/datasheet-8354-clusterin-h-330-antibody.html). For each marker analyzed a minimum of four pairs of control and mutant littermate sections were stained and, unless stated otherwise, all scored with the described phenotype. Following immunohistochemistry slides were counterstained with hematoxylin in a Thermo Gemini stainer and coverslips added using the Thermo Consul cover slipper. The percentage of cells exhibiting normal levels of FOXJ1 expression was calculated by counting a minimum of 350 total efferent duct epithelial nuclei per sample from 4 pairs of independent mutant and control littermate samples. The data is presented as mean +/- one standard deviation and was subject to an unpaired Student's t-test.

Acknowledgements

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Figure Legends.

Figure 1. Mutation of *E2f4* and *E2f5* leads to seminiferous tubule and rete testis dilation and a failure of spermatozoa to reach the epididymis. (A) Sagittal sections of adult testis from control (Ctrl) and (B) *E2f4f/f;E2f5+/-;Vil-cre* littermates showing dilation of the seminiferous tubules and rete testis as well as spermatozoa within the rete testis (arrow), and inset image in the mutant. (C) Seminiferous tubules from control and (D) mutant testis showing tubule dilation. (E) Section through the rete testis of control and (F) mutant testis showing rete testis dilation. (G) The efferent ducts of control and (H) mutant testis showing spermatozoa within the efferent ducts of the mutant (arrow). (I) The epididymis of control adults contains spermatozoa in contrast with the mutants (J) where none are detected. All tissue sections were stained with hematoxylin and eosin. Scale bars in A and B, 250µm; E and F, 200µm; C, D, I and J, 100µm; G and H, 20µm.

Figure 2. Efferent ducts of adult *E2f4f/f;E2f5+/-;Vil-cre* mutants lack multiciliated cells. (A) Cilia project into the lumen from the apical surface of multiciliaited cells in controls (Ctrl) but are absent from mutants (B), sections stained with hematoxylin and eosin. (C) Immunohistochemical staining for acetylated tubulin (brown stain) of cilia shows

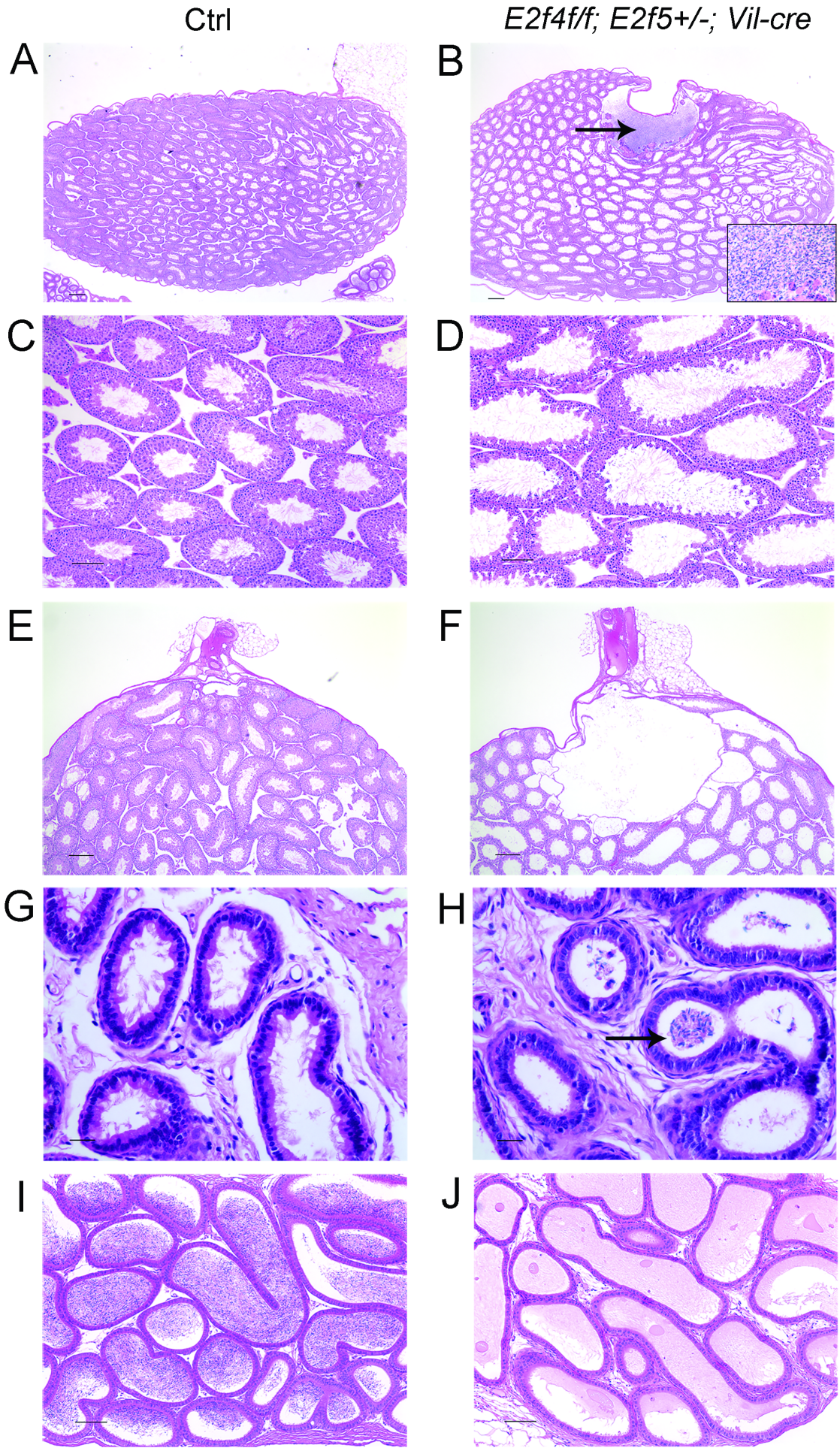
multiciliated cells in controls but not mutants (D). The staining within the lumen of the mutants corresponds to the flagella of spermatozoa. (E) Immunohistochemical staining for E2F4 shows nuclear staining (brown stain) in nuclei of the efferent duct epithelium in the controls but not mutants (F). (G) Immunohistochemical staining for a multiciliated cell expressed transcription factor, FOXJ1 (brown stain) shows nuclear staining in the controls and reduced expression in the mutants (H). Scale bars in C, D, G and H 20μm; A, B, E and F 10μm.

Figure 3. Expression of a multiciliated cell marker, FOXJ1 is reduced early during efferent duct development. Immunohistochemical staining for E2F4 or FOXJ1 (brown stains) in efferent ducts from one week old littermates. (A) Control efferent ducts (Ctrl) showing nuclear expression of E2F4 and (B) the loss of E2F4 from *E2f4f/f;E2f5+/-;Vil-cre* efferent duct epithelium at this stage. Note that mesenchymal expression of E2F4 is not lost in the mutants. (C) Robust expression of FOXJ1 in the controls in comparison with weak expression in the mutants (D). Scale bars: for each set, left hand panel 20μm and the right hand panel 10μm.

Figure 4. Efferent duct marker analyses indicates abnormal development of the adult *E2f4f/f;E2f5+/-;Vil-cre* efferent duct epithelium. (A) Immunohistochemical analysis of estrogen receptor 1 (ESR1) expression (brown stain) in control and (B) mutant efferent ducts showing reduced expression in the mutants. (C) Aquaproin 1 (AQP1) expression (brown stain) is predominantly at the apical surface of control efferent ducts (arrow) but poorly expressed in the mutants (D). (E) Clusterin (CLU) a marker for the endocytic apparatus is located predominantly beneath the apical surface in controls (arrow) but poorly expressed in mutant efferent ducts (F). (G) Sections submitted to the PAS reaction show apical staining in the controls but not in the mutants (H) indicating a loss of endocytic apparatus. Scale bars in all panels 10μm.

Figure 5. Dilation of the rete testis is observed at one week of age in *E2f4f/f;E2f5+/-;Vil-cre* and *E2f5-/-* testes and loss of E2F5 reduces the multiciliated cell population. (A) Sagittal sections of testis from one week old control (Ctrl) and (B) *E2f4f/f;E2f5+/-;Vil-*

cre mutant testis showing extensive dilation of the rete testis in the mutant (arrow). (C) Sagittal sections of testis from one week old control (Ctrl) and (D) *E2f5-/-* mutant testis showing extensive dilation of the rete testis in the mutant (arrow). (E) Sagittal sections of testis from three week old control (Ctrl) and (F) *E2f5-/-* mutant testis showing dilation of the rete testis (arrow) and partial dilation of seminiferous tubules in the mutant. (G) Immunohistochemical staining for FOXJ1, a multiciliated cell marker in one week old control and (H) *E2f5-/-* mutant efferent ducts showing reduced expression in the mutants. (I) Immunohistochemical staining for FOXJ1 in three week old control and (J) *E2f5-/-* mutant efferent ducts showing reduced expression in the mutants. (K) Immunohistochemical staining for acetylated tubulin staining (brown stain) of cilia shows many multiciliated cells in efferent ducts from three week old controls but many fewer multiciliated cells in littermate *E2f5-/-* mutant efferent ducts. Panels A though F hemotoxylin and eosin stained sections. Scale bars: E and F 400μm; A, B, C, D 200μm; I, J, K and L 20μm; G and H 5μm.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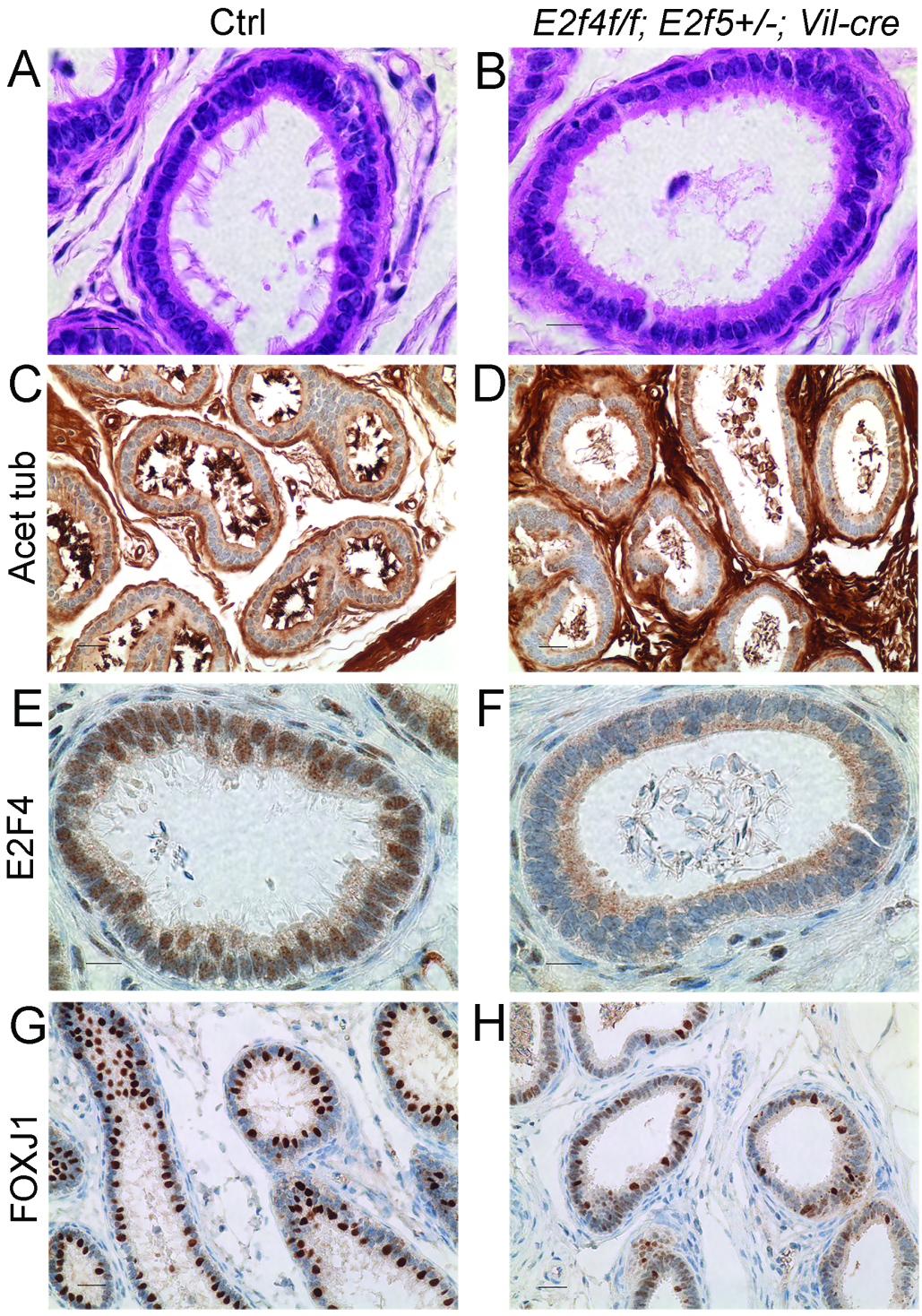
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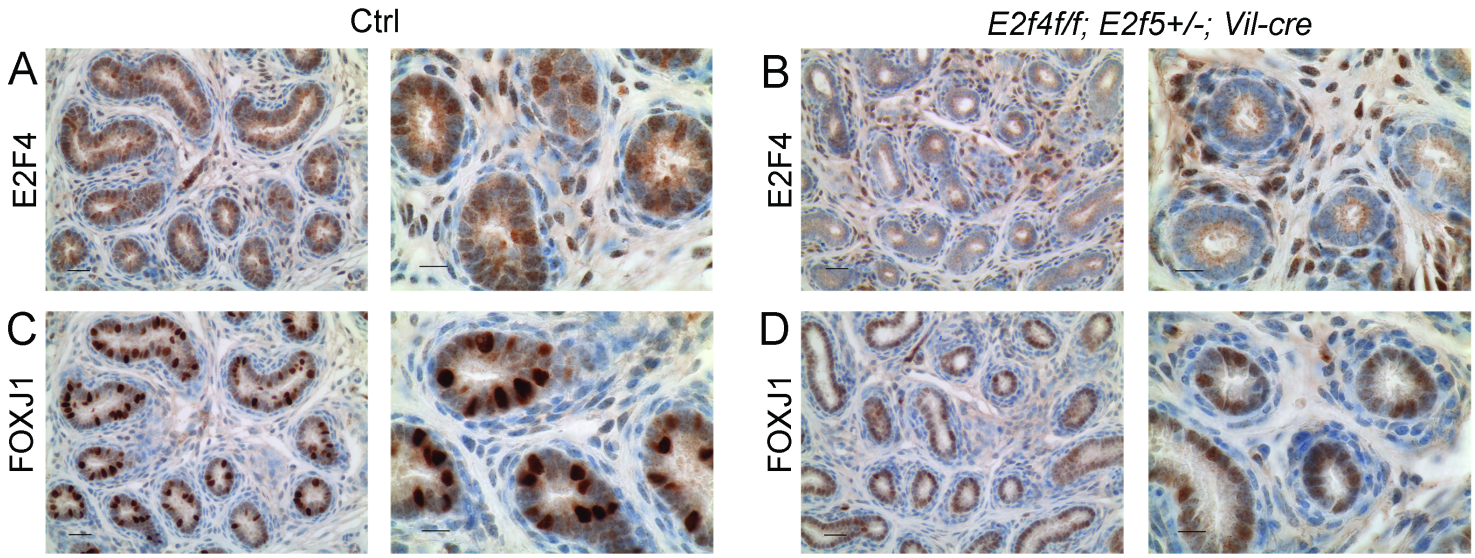
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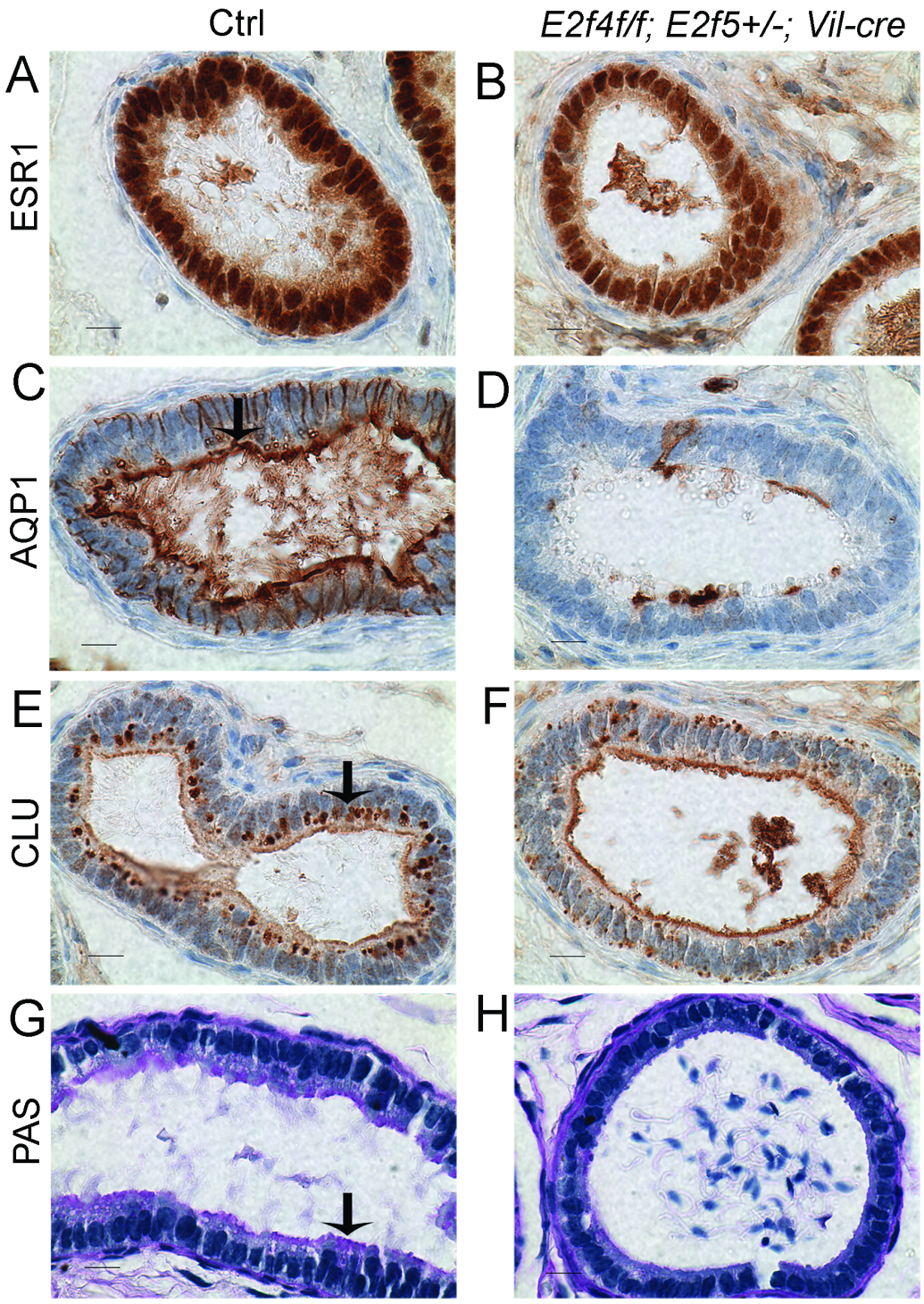
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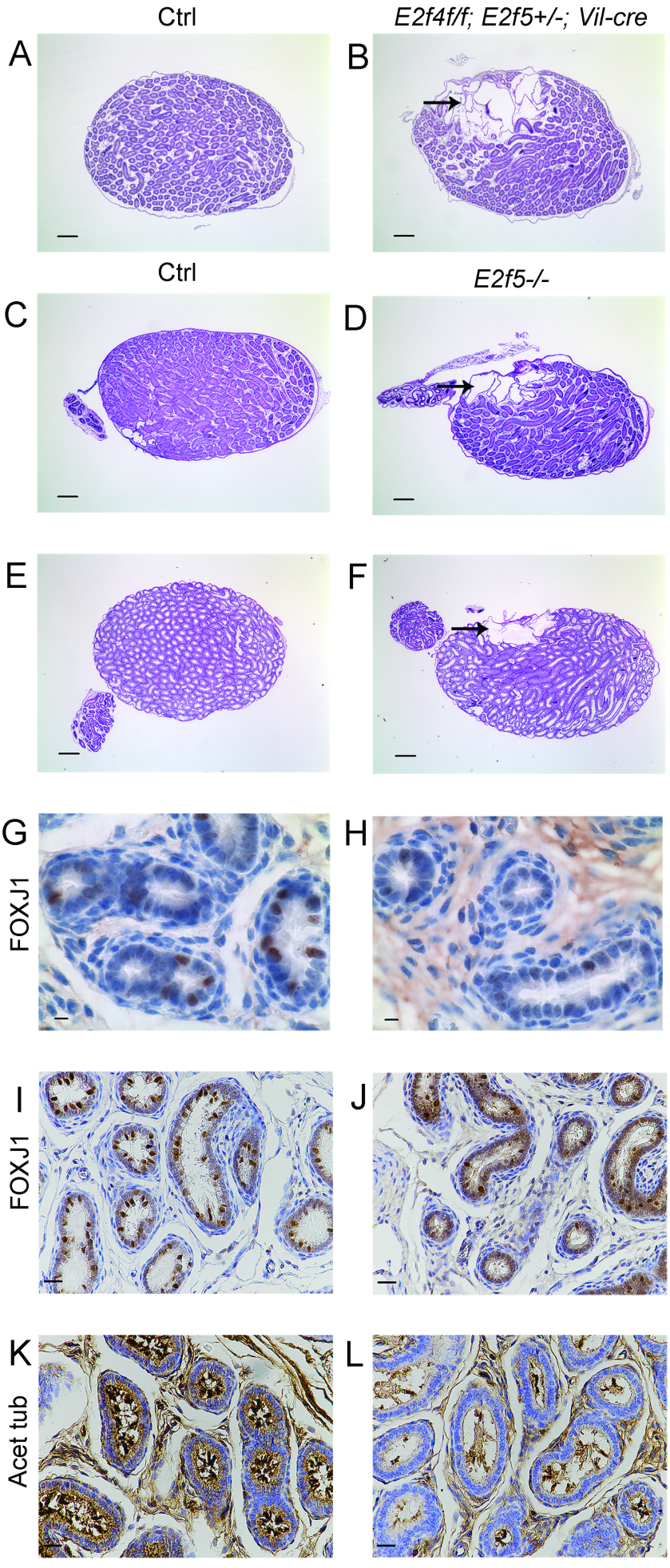
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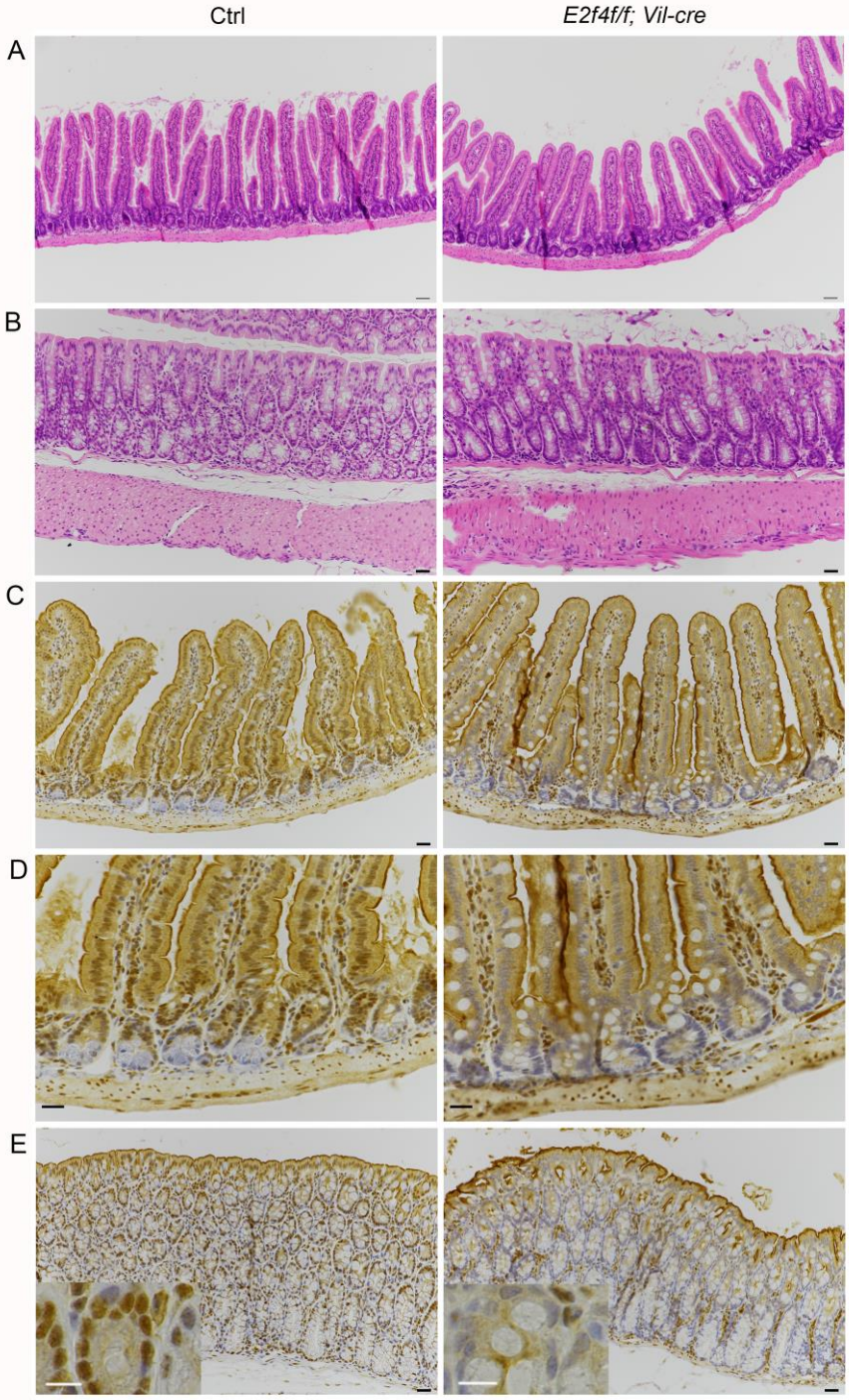

Figure S1. E2F4 is not essential for development of the intestine. (A) Hematoxylin and Eosin (H&E) staining of intestine from control adult (Ctrl) and *E2f4f/f;Vil-cre* mice showing that development of the intestine can occur following loss of E2F4 from the intestinal epithelium. (B) H&E staining of colon from control adult (Ctrl) and *E2f4f/f;Vil-cre* mice showing normal morphology in the mutants. (C) Immunohistochemical staining for E2F4 (brown stain) in the intestinal epithelium from control and *E2f4f/f;Vil-cre* mutant mice showing that E2F4 is expressed in epithelial nuclei of the crypts and villi of controls but is absent from the mutants. Note that *Vil-cre* is only expressed in the intestinal epithelium. (D) Higher power images of the crypts from panel C. (E) Immunohistochemical staining for E2F4 (brown stain) in the colon from control and *E2f4f/f;Vil-cre* mutant mice showing that E2F4 is expressed in epithelial nuclei of the colon but is absent from the mutants. Nuclei are counterstained with hematoxylin (blue). Scale bars: A 50μm, B, C & E 25μm, D 20μm, E inset image 10μm.

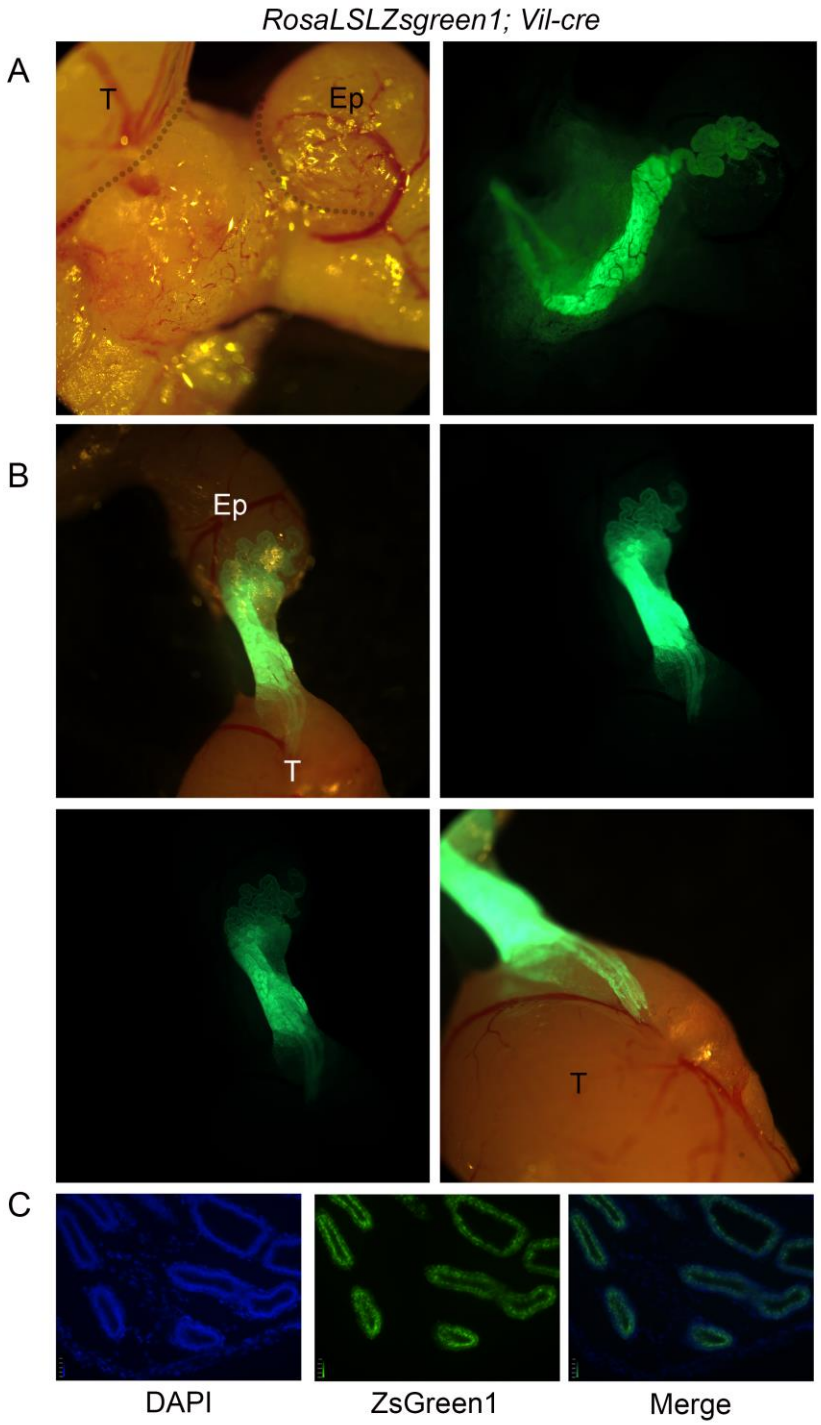

Figure S2. *Vil-cre* is functional in the efferent duct epithelium. (A) The *Vil-cre* transgene mediated recombination of a lox-STOP-lox reporter allele expressing the fluorescent protein Zsgreen1 (*RosaLSLZsGreen1*) within the efferent ducts. The left hand panel shows a bright field image of the testis (T) and epididymis (Ep) and the right hand panel shows a fluorescence image of the same tissue indicating expression of Zsgreen1 in the efferent ducts connecting the testis to the epididymis. (B) Additional images of the efferent ducts at different exposures showing the efferent duct connection with the epididymis and the testis. Note that fluorescence is not detected in the testis. (C) Fluorescence imaging of frozen sections of the efferent ducts showing that *Vil-cre* is functional in the efferent duct epithelium and not the mesenchyme. DAPI staining indicates the nuclei. In no cases was significant fluorescence detected in *RosaLSLZsGreen1* animals without *Vil-cre*.

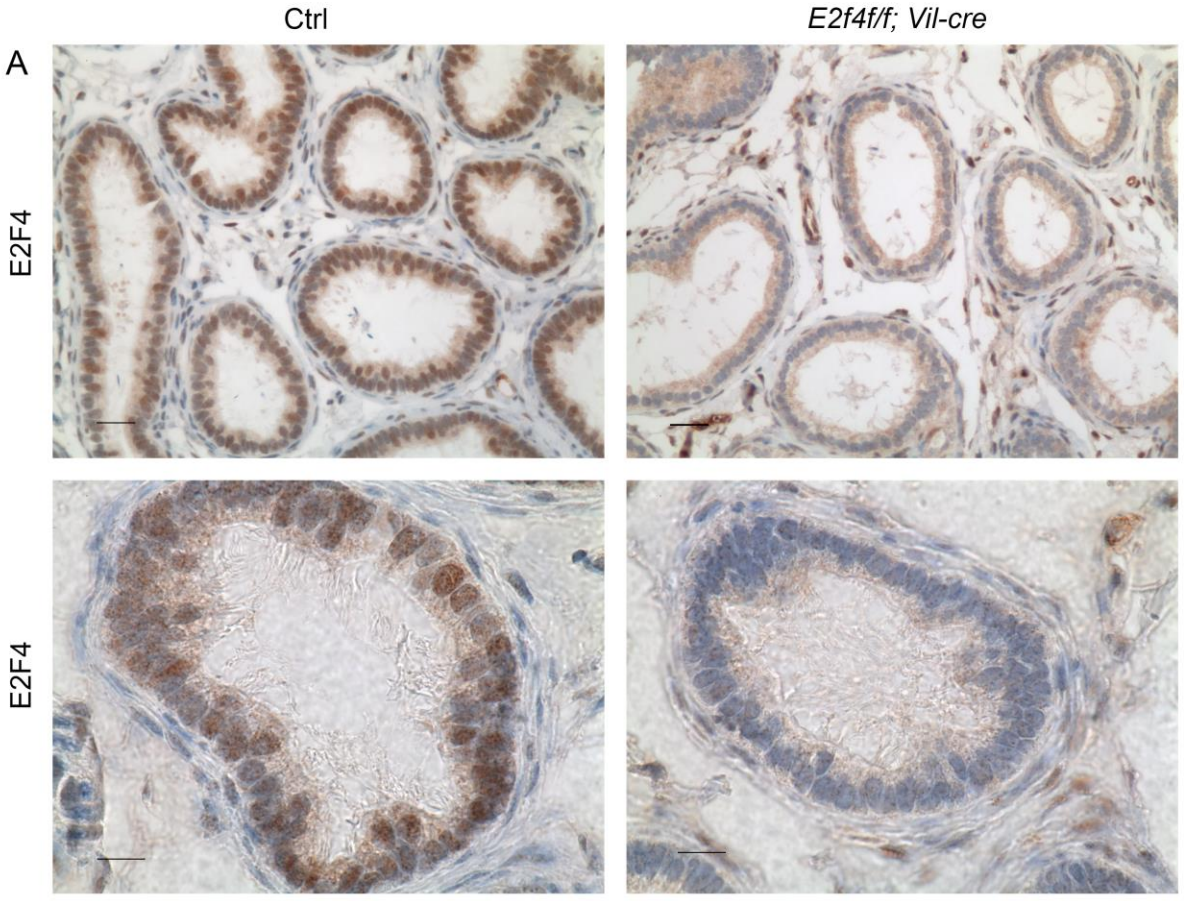

Figure S3. The *Vil-cre* transgene mediates efficient mutation of the conditional *E2f4* allele in the efferent duct. (A) Immunohistochemical staining for E2F4 (brown stain) in the efferent ducts from controls and *E2f4f/f;Vil-cre* mutant mice showing loss of E2F4 expression from the epithelium in the mutant. Bottom panels show high power images of individual efferent ducts. Scale bars: A, top panels 20μm, bottom panels 10μm.

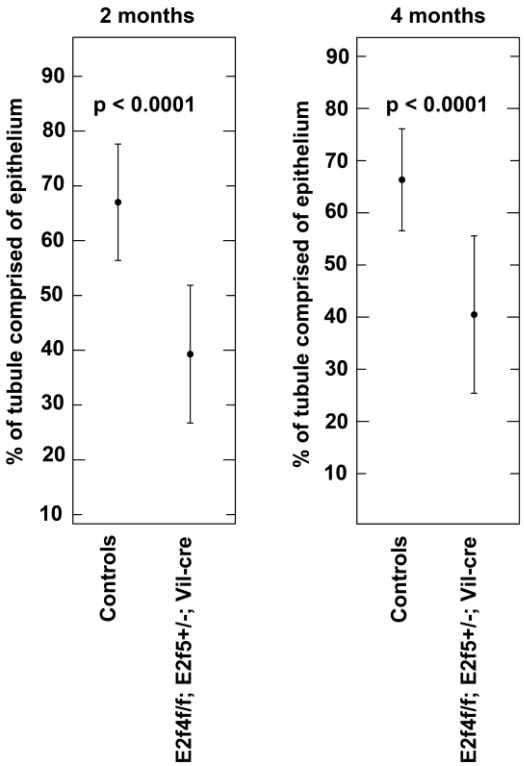

Figure S4. Seminiferous tubules are significantly dilated in *E2f4f/f;E2f5+/-;Vil-cre* testis relative to controls. The percentage of the tubule diameter comprised of by epithelium was calculated from these measurements: (tubule diameter – lumen diameter/tubule diameter) and the data subject to an unpaired Student's t-test. At each time point a minimum of 4 pairs of control and mutant littermates were analyzed and for each animal a minimum of 10 randomly chosen tubules were measured (the minimum number of tubules measured for each group at each time point was 60). The graph shows the mean +/- one standard deviation. At both 2 and 4 months of age the difference was determined to be statistically significant by Student's t-test, p values are shown.

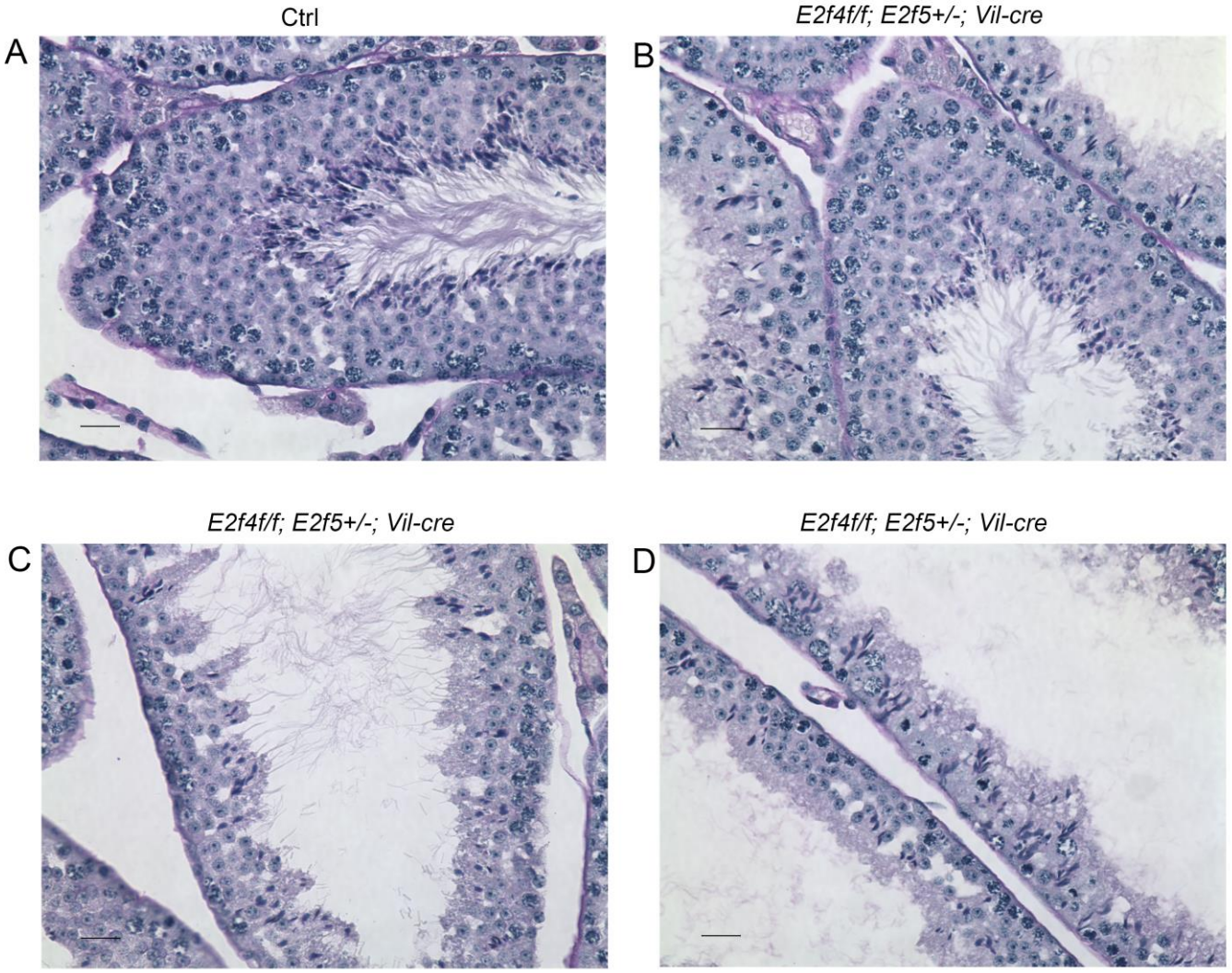

Figure S5. Spermatogenesis is not disrupted in *E2f4f/f;E2f5+/-;Vil-cre* testis. Sections from control (Ctrl) and mutant seminiferous tubules were subject to the PAS reaction. (A) Section through a control seminiferous tubule showing that mature spermatids are present indicating normal spermatogenesis and representative sections (B, C and D) of mutant seminifererous tubules showing that spermatogenesis is complete, even in dilated tubules. Scale bars: all panels 20μm.

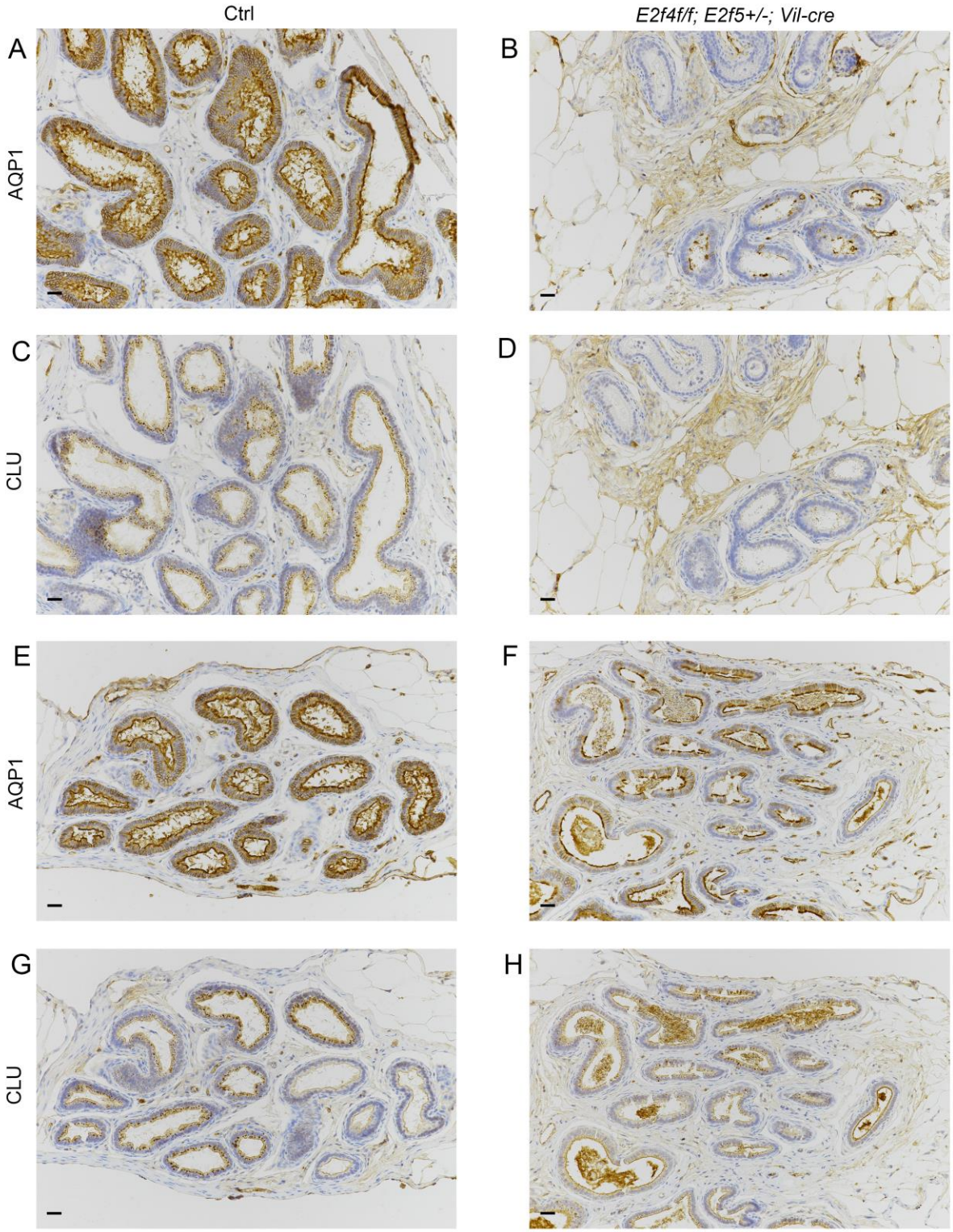

Figure S6. Additional lower magnification images of immunohistochemical staining for Aquaporin1 (AQP1) and Clusterin (CLU) showing disrupted expression in adult *E2f4f/f;E2f5+/-;Vil-cre* efferent ducts, relative to controls. (A and E) Expression of AQP1 in control and (B and F) mutant efferent ducts (brown stain). AQP1 is predominantly expressed on the apical surface of the efferent ducts. (C and G) Expression of CLU in control and (D and H) mutant efferent ducts (brown stain). CLU is expressed in endocytic vesicles beneath the apical surface. Staining in the lumens is background. Scale bars: all panels 25μm.

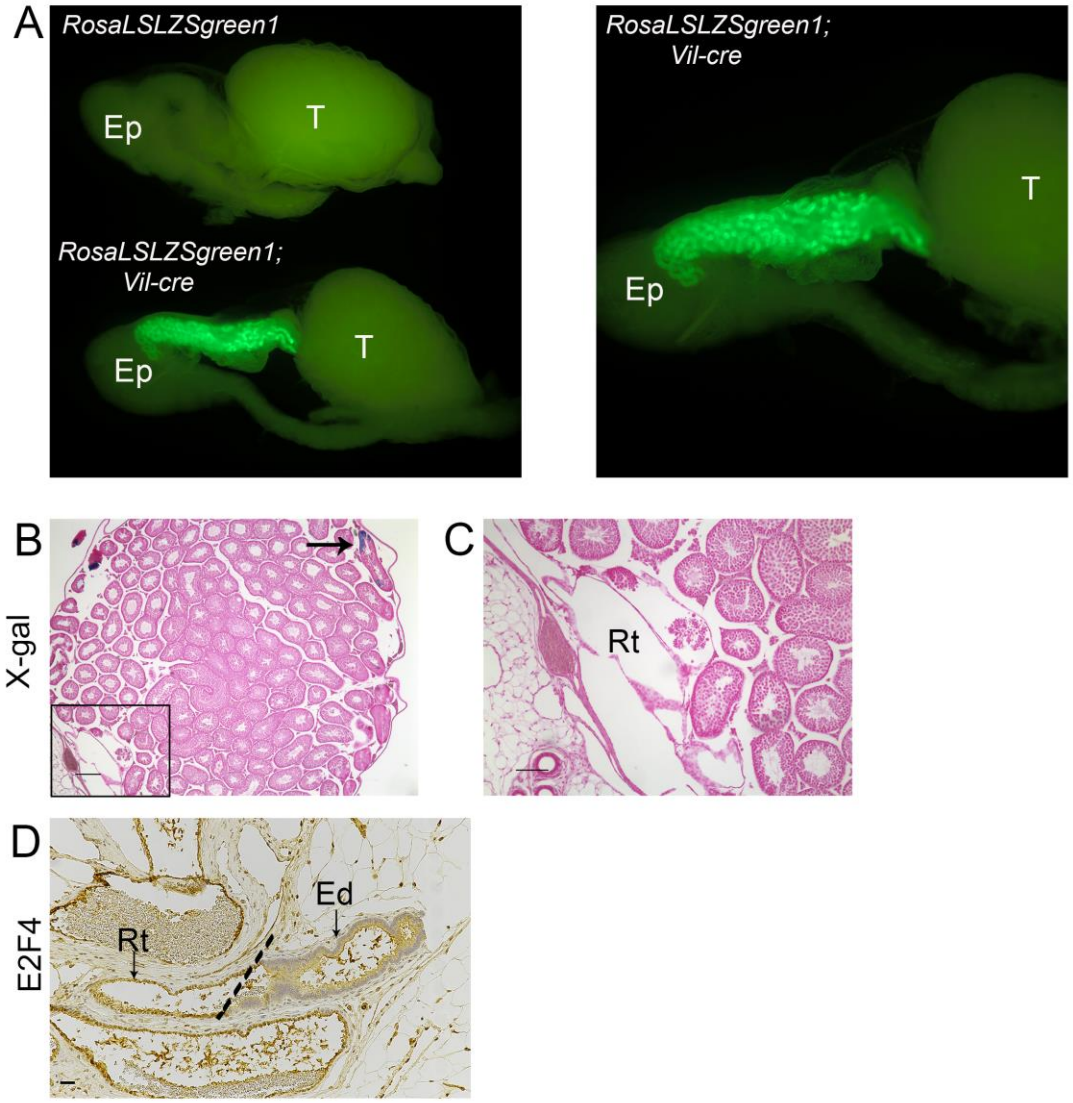

Figure S7. *Vil-cre* is not functional in the rete testis. (A) The *Vil-cre* transgene mediated recombination of a lox-STOP-lox reporter allele expressing the fluorescent protein Zsgreen1 (*RosaLSLZsGreen1*) within the efferent ducts at one week of age. The left hand panel shows a fluorescence image of control (*RosaLSLZsGreen1* alone) at the top and *RosaLSLZsGreen1;Vil-cre* samples at the bottom showing ZsGreen1 expression in the efferent ducts but not within the testis. The testis (T) and epididymis (Ep) are indicated. The right hand panel shows a higher magnification fluorescence image of the same tissue indicating expression of Zsgreen1 in the efferent ducts but not the testis. (B) X-gal stained frozen section of *E2f4f/f;E2f5+/-;Vil-cre; RosaLSLlacZ* adult testis indicating that the rete testis (Rt) is not expressing β-galactosidase. Note the sporadic recombination (arrow), caused by leaky expression of *Vil-cre*, in one seminiferous tubule, which is stained blue (counter stain is nuclear fast red). (C) Immunohistochemical staining for E2F4 showing the junction (indicated by the dashed line) between an efferent duct (Ed) and the rete testis (Rt) in an *E2f4f/f;E2f5+/-;Vil-cre* adult mouse. E2F4 (brown stain) is not detected in the nuclei of the efferent duct epithelium, as expected, but appears present in the rete testis epithelium indicating that *Vil-cre* is not functional in the rete testis. Scale bars: B 200μm, C 100μm, D 25μm.

Supplementary Methods.

X-gal staining of tissue sections.

Tissues were dissected and placed directly into molds containing O.C.T. embedding medium (Tissue Tek, #4583) for 10 minutes then placed onto dry ice until frozen and stored at -80C. Frozen sections were cut at 20μm. The fixative and staining solutions are described in.[1] Sections were thawed at room temperature and fixed for 10 minutes, washed 4 x 5 minutes in phosphate buffered saline (PBS) 0.02% NP40 and then stained overnight. Sections were then washed as before post fixed in formalin and then washed again prior to counterstaining with nuclear fast red.

1) Whiting J, Marshall, H, Cook M, Krumlauf R, Rigby PWJ, Stott D and Alleman RK. Multiple spatially specific enhancers are required to reconstruct the pattern of *Hox-2.6* gene expression. Genes and Development 1991; 5: 2048-2059