

DNA Methylation in Prostate Morphogenesis, Growth and Urinary Function

By

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Dedicated to:

My parents, without your support I would not be where I am today.

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Abstract

Benign prostatic hyperplasia (BPH) is characterized by benign proliferative growth and often associates with lower urinary tract symptoms (LUTS) in aging men. BPH/LUTS are a significant healthcare burden but causative mechanisms are largely unknown. One potential contributor is inappropriate reactivation of prostate developmental signaling pathways. My studies have revealed new roles of DNA methylation in mediating prostate ductal development by regulating prostate androgen responsiveness and epithelial cell adhesion. I extended my studies to test whether perturbations of DNA methylation are responsible for prostatic hypertrophy and voiding dysfunction in a mouse model of BPH. Mice were chronically exposed to exogenous testosterone and estradiol (T+E2) to mimic the hormonal environment of aging men. I observed that T+E2 treatment perturbs global DNA methylation in dorsal root ganglia and prostate while at the same time causing urinary dysfunction. Some T+E2 treated mice were fed a folic acid enriched diet as a potential intervention. The diet normalized global DNA methylation in prostate and dorsal root ganglia and prevented a specific subset of T+E2 induced changes in voiding function, the most noteworthy being a preservation of bladder responsiveness to afferent stimulation. My results prompt the need for future studies to determine whether the folic acid enriched diet mediates its protective actions on voiding function through a mechanism requiring DNA methylation. Together, these studies establish new roles of DNA methylation in the normal process of prostate development and reveal it as a potential therapeutic target for treating BPH/LUTS.

Chapter 1: Introduction

Adapted from Keil et al., Epigenomics (2014). Under review.

Perceived impacts of my efforts to advance the mechanistic understanding of prostate development

The vast majority of my dissertation research has been directed toward identifying prostate development mechanisms - a research area with wide reaching impacts on multiple fields. The developing prostate employs a morphogenetic program involving budding and branching. Similar programs guide development of mammary gland, lung, and kidney. Therefore revealing new prostate ductal developmental mechanisms may inform future studies in these and other organs.

Androgens and estrogens influence development of prostate and other reproductive organs as well as brain and heart (Sebag et al. 2011; Ghahramani et al. 2014). Mechanisms by which these hormones stimulate development are not fully understood. My efforts to examine how androgens and estrogens influence prostate development will potentially shed light on how these hormones mediate morphogenesis of other organs.

One theory for how prostate disease like benign prostatic hyperplasia (BPH) arises is that genes and signaling pathways involved in prostate development are reawakened in the prostate during aging to reactivate proliferative growth (McNeal 1978). My dissertation research revealed new roles of DNA methylation in fetal mouse prostate development. I also tested and confirmed that DNA methylation regulates adult mouse prostate proliferative growth and is critical for acquisition and maintenance of male mouse urinary function in adulthood, endpoints with clear relevance for human BPH.

Contemporary concepts in prostate development

The mouse prostate arises from a sub-compartment of the lower urinary tract (LUT) known as the urogenital sinus (UGS). Androgens instruct UGS epithelium to form prostate ductal precursors called prostatic buds. Prostatic buds begin to emerge at 16 dpc (Lin et al. 2003) as solid cords of a specific type of UGS epithelium (UGE), keratin 14 positive basal UGE. During late prenatal and early postnatal development, prostatic buds continue to elongate and invade into UGM, differentiate and undergo branching morphogenesis. In the mouse these events give rise to an adult prostate ductal network consisting of four bilaterally symmetrical lobes: anterior, dorsal, lateral and ventral prostate (Lasnitzki and Mizuno 1980; Allgeier et al. 2010). The mature mouse prostate glandular architecture consists of prostatic luminal epithelial tubes containing peripheral interspersed basal epithelial cells (Sugimura et al. 1986) and surrounded by smooth muscle interspersed with blood vessels, nerves and other stromal constituents.

There are many reasons why my research on rodent prostate development is likely to provide insight on human prostate development. Like rodents, the human prostate arises from epithelial buds which emerge in an androgen-dependent fashion from ventral, dorsal and anterior surfaces of the UGS (Timms 2008). Prostatic buds in rodent and human prostate arise in a similar pattern (Timms 2008) in response to similar signaling pathways. The mature rodent and human prostates also share histological and molecular similarities (Thielen et al. 2007; Timms 2008).

There are also some noteworthy differences between rodent and human prostate. The adult human prostate consists of histologically discrete zones (peripheral,

transitional and central) and in this regard differs from the mature rodent prostate which consists of discrete lobes. The mature rodent and human prostate also exhibit differences in how they relate to the urethra. The human prostate is encapsulated in a thick fibromuscular layer in close proximity to the urethra, whereas rodent prostate lobes extend outward (Timms 2008). This difference is noteworthy and perhaps clinically significant, since human prostatic hyperplasia is believed to cause LUTS by impinging on the urethra and reducing urinary flow. It is difficult to conceive if and how benign growth of the rodent prostate would impinge on the urethra, and this issue is the basis of a longstanding criticism from many urologic researchers that rodents are not suitable models for identifying the mechanistic basis of BPH-induced urinary dysfunction in humans. Recent evidence, however, links benign prostatic growth in mice to urinary dysfunction (Wood et al. 2006; Nicholson et al. 2012). A careful analysis of the mature male mouse urethra provides a potential mechanism. The mouse prostatic urethra is surrounded by a thick striated muscle urinary sphincter (also known as the rhabdosphincter). The rhabdosphincter ensheaths several hundred microns of the proximal portions of prostatic ducts, which run nearly parallel to the urethra before draining into it. Benign growth in the proximal portion of rodent prostatic ducts, which are bounded by the poorly compliant rhabdosphincter, could potentially impinge upon the urethra and reduce urinary flow. A recent study in which prostate hyperplasia was induced by exogenous hormones in mice showed a relationship between prostate hyperplasia, urethral narrowing, and urinary obstruction (Nicholson et al. 2012). My dissertation research used this model to test whether a dietary DNA methyl donor could protect against urinary dysfunction.

The mechanism by which prostate buds elongate is not understood and could involve many factors including epithelial cell proliferation, UGS stromal composition, and epithelial and stromal cell adhesion (Sugimura et al. 1986; Abler et al. 2011). The underlying mechanisms controlling these events are not well characterized in part because of limitations in the available methods to visualize and quantify prostate bud formation. Several methods have been used to visualize prostatic buds. These include scanning electron microscopy (Lin et al. 2003), serial section reconstruction (Timms 2008) and light microscopy (Cunha 1985; Vezina et al. 2008). Some of these methods are expensive and time consuming, and all rely on a single readout, the presence of protruding buds. None of these methods is capable of distinguishing buds that will form prostate from buds that will recede or form other urethral structures like urethral glands. To address this problem, I developed a combined method of *in situ* hybridization and immunohistochemistry to visualize the UGE and prostatic buds which express a prostate selective marker (NK transcription factor locus 1, *Nkx3-1*) (Keil et al. 2012). The method is inexpensive, high throughput, and provides a multidimensional readout that distinguishes between *Nkx3-1* positive and negative epithelial buds. This method was used to identify the cell adhesion molecule, E-cadherin (*Cdh1*) as an important regulator of prostatic bud elongation and maturation and to demonstrate that *Cdh1* expression in developing mouse prostate is regulated by DNA methylation (chapter 5).

Androgen receptors (ARs) are expressed in UGM and UGE during prostatic bud formation (Takeda et al. 1985). The UGS cell population in which ARs are required for prostatic bud formation was determined using tissue recombination experiments (Cunha et al. 1987). UGS tissues were enzymatically and mechanically separated into UGM

and UGE constituents and recombined and grown *in vitro* or *in vivo* to study prostatic bud formation. The heterotypic tissue recombinants used in these studies derived from testicular feminized mutant mice that carry a spontaneous *Ar* mutation that results in its premature truncation and from wild type mice harboring a functional *Ar*. By combining UGM and UGE of different genotypes, these studies revealed that *Ars* in UGS mesenchyme, not UGE, are required for prostatic bud formation (Cunha et al. 1987). The necessity of AR signaling in prostate mesenchyme suggests androgen-induced paracrine signaling factors, derived from UGM, act on UGE to guide prostate development. These factors have been termed andromedins. Andromedins play an instructive role in prostate development, meaning they can reprogram endoderm-derived epithelium from non-prostate tissues into prostate. For example, fetal UGM is capable of inducing adult bladder epithelium to form prostate (Cunha et al. 1980) and can instruct stem/progenitor spermatogonia to form prostate epithelium (Simon et al. 2009).

Several methods have been employed to identify andromedins. Early studies compared gene expression in whole adult prostate tissue from intact and castrated mice to identify androgen responsive genes (Parker and Mainwaring 1977). A limitation of this approach is that it used whole tissue, making it unable to discern rare transcripts in isolated cell populations. No andromedins were found using this method. Serial analysis of gene expression (SAGE) on developing prostate and microarrays from prostate cancer cell lines have also been used to identify candidate andromedins (Joesting et al. 2005; Zhang et al. 2006). A major drawback of these approaches is that they do not retain information about the location of transcripts within UGM subpopulations, and this

may be the reason that they have not been successful in identifying andromedins. A relatively recent study shows that retinoic acid is capable of inducing a low level of bud formation in female UGSs in the absence of exogenous androgens, raising the possibility retinoids function as andromedins (Bryant et al. 2014). One potential complication of this study is that it was conducted with female UGSs, which are known to form small androgen independent buds that may not necessarily develop into prostatic buds (Allgeier et al. 2010).

Given the longstanding interest in identifying andromedins, and the complications of previous approaches towards meeting this goal, part of my thesis work involved the approach of using *in situ* hybridization to screen for androgen-responsive genes that may serve as andromedins in intact UGS tissues. My search was guided by existing evidence that androgens act in a defined UGM component known as lamina propria mesenchyme which lies adjacent to UGE (Abler et al. 2011). Andromedins are predicted to be enriched in lamina propria UGM because ARs are especially abundant in this region (Takeda et al. 1985) and because radiolabeled testosterone binding localizes to it beginning around 15 dpc, the stage when prostatic buds are specified (Takeda et al. 1985). Further, expression of the androgen responsive gene steroid 5 alpha reductase type 2 (*Srd5a2*) is abundant in lamina propria mesenchyme, indicating that ARs are likely to be active in this UGS cell subset (Abler et al. 2011). I discovered that Wnt inhibitor factor 1 (*Wif1*), a secreted antagonist of Wnt/beta-catenin signaling, localizes to lamina propria UGM. I found that AR activation is both necessary and sufficient for *Wif1* mRNA expression in this population of cells, exogenous WIF1 protein added to UGS organ culture media stimulates prostatic bud formation *in vitro*, and the requirement for

androgens in inducing *Wif1* mRNA expression in UGM can be bypassed *in vitro* by blocking DNA methylation machinery (Chapter 3). My results are among the first to identify a *bone fide* AR target gene in the male UGS during prostate development and raise the possibility that epigenetic mechanisms may govern its expression. The impact of these findings is that they provide insight into the mechanism of prostate development, identify a new marker that can be used to track androgen activity during prostate development, and reveal a potential role of DNA methylation in controlling androgen action in the prostate.

One of the challenging aspects of defining the mechanism of androgen induced prostatic bud formation is that initiation of testicular androgen synthesis in the male mouse fetus does not coincide with initiation of prostate development. Mouse testicular androgen synthesis begins at 13 dpc but prostate bud outgrowth does not begin until three days later, at 16 dpc (Lin et al. 2003). A temporal separation between testosterone synthesis and initiation of prostate development is shared by other mammalian species (Wilson et al. 2003). I postulated that understanding how the initiation of prostate development is timed would provide critical new information about factors that control it. In the sections outlined below I describe the rationale for my hypothesis that DNA methylation is a central mechanism controlling the timing of androgen action in prostate development. In chapter 6 I show that DNA methylation of the AR functions as a developmental checkpoint to regulate the timing of prostate development in mice and in chapter 8 I study the impact of a dietary methyl donor, capable of enhancing DNA methylation, on urinary function in control male mice and in male mice with hormone-induced urinary dysfunction.

Evidence that DNA methylation regulates prostate development

The developing prostate is extremely sensitive to endocrine disrupting chemicals, which perturb DNA methylation patterns in prostate and other tissues. Two estrogenic chemicals receiving considerable research focus are diethylstilbesterol (DES) and bisphenol A (BPA). DES or BPA, given to pregnant CD-1 mice exposed *in utero* to estradiol, change the number of prostate buds formed and change fetal UGS morphology (Timms et al. 2005). Mice exposed during fetal and neonatal development to high doses of DES (200ug/kg) generally develop fewer prostatic buds, while mice exposed to low doses of BPA (10ug/kg) and DES (0.1ug/kg) form more prostatic buds and exhibit increased UGS AR expression (vom Saal et al. 1997; Timms et al. 2005; Richter et al. 2007). How DES and BPA elicit their actions on prostate morphogenesis is not entirely known but evidence supports the hypothesis that BPA-induced changes in DNA methylation alter gene expression to disrupt homeostatic regulation of prostatic growth. This raises the question of whether DNA methylation plays a role in normal prostate development.

Probing the epigenome

Overview of DNA methylation

Epigenetics is the study of chemical modifications to DNA and histone proteins that regulate gene expression without altering DNA sequence. A growing number of epigenetic marks have been identified including DNA methylation, histone tail modifications, non-coding RNAs and others. My research has focused on the epigenetic

mark of DNA methylation which occurs by addition of a methyl group to the 5' position of cytosine. DNA methylation most often occurs in the context of CpG dinucleotides, though non-CpG DNA methylation can occur (Barres et al. 2009; Arand et al. 2012). DNA methylation is classically perceived as a stable and sometimes heritable mark. However, DNA methylation events can also be surprisingly dynamic and added or removed in a spatially and temporally defined context.

DNA methylation is catalyzed by DNA methyltransferases (*Dnmts*) which include maintenance (*Dnmt1*) and *de novo* methyltransferases (*Dnmt3a*, *Dnmt3b*). An additional member, *Dnmt3L*, lacks catalytic activity but participates in *de novo* methylation by interacting with *Dnmt3a* and *Dnmt3b* and other transcription factors (Suetake et al. 2004; Ooi et al. 2007; Pacaud et al. 2014) and uses the histone landscape to recruit *de novo* DNA methyltransferases (Ooi et al. 2007). DNA methylation can also be lost, either through passive mechanisms whereby the methylation pattern is not maintained upon subsequent cell divisions, or through active mechanisms involving base modification, substitution, excision or repair (Feng et al. 2010).

DNA methylation typically regulates gene expression by repressing transcription but in some cases it can be transcriptionally activating. For example, it was recently demonstrated in a study in which I participated, that non-neuronal-derived serotonin increases sonic hedgehog (*Shh*) mRNA abundance in mouse mammary gland by increasing DNA methylation at one site of the *Shh* locus and decreasing DNA methylation at another (Laporta et al. 2014). DNA methylation also acts in concert with methyl-CpG binding proteins (MBDs) and chromatin modifiers to change the chromatin landscape. These events are all influenced by hormones, environment, drugs and other

chemicals, leading to derangement of DNA methylation marks which can perturb development or trigger inappropriate growth later in life, potentially contributing to a host of diseases, including cancer.

It is becoming increasingly clear that environmental and dietary constituents can influence global DNA methylation. These actions are of toxicological relevance (see discussion of BPA and DES above) but can also be leveraged for therapeutic purposes. Folic acid is a dietary factor capable of altering DNA methylation. Folic acid enhances DNA methylation in fetal neural tube and elsewhere (Wolff et al. 1998; Cooney et al. 2002; Waterland et al. 2006). Dietary folic acid supplementation is recommended for women of childbearing age because it protects against fetal neural tube closure defects such as Spina Bifida. It achieves this action by serving as a methyl donor for S-adenosylmethionine, a DNA methyltransferase cofactor used to generate 5-methylcytosine, the methylated nucleotide in methylated DNA. If and how dietary folic acid impacts DNA methylation in the prostate and other organs and whether it can rescue loss of DNA methylation often seen in various diseases or during aging is unknown.

DNA methylation, prostate growth and urinary function in adulthood

Identifying novel prostate developmental growth mechanisms may lead to the identification of new mechanisms responsible for inappropriate prostatic growth during aging, which will provide new drug targets and translate into more effective therapies to curb or alleviate symptoms of BPH.

BPH is characterized by benign growth of the prostate. BPH often associates with lower urinary tract symptoms (LUTS) which include storage or irritative symptoms (increased frequency, urgency, pain, nocturia) and voiding or obstructive symptoms (weak stream, hesitancy, dribbling, incomplete emptying and overflow incontinence) (McVary et al. 2011). Approximately 70% of men over the age of 70 experience BPH and/or LUTS (Berry et al. 1984), which decreases quality of life and posing a significant healthcare burden to the aging population (Saigal and Joyce 2005). Currently available treatment options include drug therapy with alpha adrenergic receptor blockers, 5-alpha reductase inhibitors, phosphodiesterase 5 inhibitors or a combination to alleviate symptoms and reduce prostate volume (Radomski 2014). Some men respond to these treatments while others require invasive surgery to remove prostatic tissue and alleviate severe LUTS. Why some men respond to therapy better than others is unknown, largely because the molecular underpinnings of BPH are not known.

Evidence exists to support the hypothesis that epigenetic changes accompany histological prostate hyperplasia in aging men. Global 5-methylcytosine levels are reduced (Bedford and van Helden 1987) and tumor suppressor genes are often hypermethylated in BPH compared to histologically normal prostate tissue (Henrique et al. 2005). DNA methylation has also been shown to decrease steroid 5 alpha reductase type 2 (*Srd5a2*) expression in prostate (Niu et al. 2011). The possibility that men with highly methylated *SRD5A2* may be more resistant to 5 alpha reductase inhibitor therapy has been raised (Bechis et al. 2014). Together these results indicate that DNA methylation may also play a role in regulating androgen responsive prostate proliferative growth and/or urinary function associated with BPH/LUTS. The role of DNA methylation

in a mouse model of urinary dysfunction is examined in chapter 8 where I identified changes in DNA methylation of the prostate are accompanied by physiological changes in voiding function. Additionally, enhancing DNA methylation using dietary folic acid was sufficient to restore prostate DNA methylation levels and improve aspects of voiding function.

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**Chapter 2: Visualization and quantification of mouse prosate development by in
situ hybridization**

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Introduction

Mouse prostate development begins *in utero* from a transitory developmental sub-compartment of the lower urogenital tract known as the definitive urogenital sinus (UGS). In this manuscript the UGS is defined as the prostatic bud forming region of the male pelvic urethra and the equivalent region of the female pelvic urethra. In most placental mammals, prostate development begins shortly after initiation of fetal testicular androgen synthesis. Androgen receptor (AR) activation in UGS stroma induces prostatic bud formation in UGS epithelium. In C57BL/6J male mice, prostatic bud initiation begins around embryonic day (E) 16.5 and is completed by about E18.5 (Lin et al. 2003). During postnatal development prostatic buds continue to elongate, undergo branching morphogenesis, canalize and undergo differentiation to form the mature prostate ductal network (Cunha *et al.* 1987; Lin *et al.* 2003; Sugimura *et al.* 1986).

Prostatic budding and branching morphogenesis can be activated *in vitro* by incubating the isolated male UGS, female UGS, or neonatal mouse prostate in organ culture media containing testosterone or its more potent metabolite, 5 α dihydrotestosterone (DHT) (Cunha, 1972). Androgens induce formation of bud-like structures in both male and female UGS explants and given sufficient time, many of these budding structures will form primary and secondary branches in a pattern that mimics their development *in vivo* (Cunha *et al.* 1987; Price and Williams-Ashman, 1961). The organ culture model of prostate development can be used to: 1) rescue prostate development in mouse mutants predisposed to mortality during prostatic bud formation, 2) evaluate fetal prostate teratogenicity of chemicals that would otherwise

disrupt pregnancy or harm the pregnant dam or fetus and 3) maintain consistent androgen levels during prostate development.

We and others have quantified prostate development by visualizing and counting the number of prostatic buds and branches formed *in vivo* and *in vitro* (Allgeier *et al.* 2010; Buresh *et al.* 2010; Lin *et al.* 2003; Timms, 2008). Several methods have been developed for this purpose, including light microscopy (Price, 1936), scanning electron microscopy (Lin *et al.* 2003), confocal microscopy (Buresh *et al.* 2010) and three dimensional serial reconstruction of UGS histological sections (Timms *et al.* 1994, 2008). All of these methods are capable of visualizing buds arising from the UGS and urethra and all have advantages and disadvantages. A common disadvantage is many of these methods require specialized equipment (scanning electron or confocal microscopy) or specialized computer programs (to conduct serial section reconstruction), which places them out of reach of some research laboratories.

On the other hand, many molecular biology laboratories are outfitted with a hybridization oven and a dissecting microscope and this equipment in conjunction with other basic research equipment is sufficient for conducting *in situ* hybridization (ISH). Prostatic buds can be visualized in the UGS by ISH staining with a riboprobe against a prostate selective mRNA, NK-3 transcription factor, locus 1 (*Nkx3-1*). In fact, this method has already been used to visualize prostatic buds and assess prostate development qualitatively (Bieberich *et al.* 1996; Chen *et al.* 2005; Gao *et al.* 2005; Ghosh *et al.* 2011; Prins *et al.* 2001, 2006; Tanaka *et al.* 2000; Thomsen *et al.* 2008; Wang *et al.* 2008). Our goal herein was to determine whether a comparable method can

be used to assess prostate development quantitatively by counting ISH-stained prostatic buds.

Nkx3-1 is considered the earliest mRNA marker of mouse prostate identity and was reported to be present in mouse UGS epithelium beginning at E15.5, prior to prostatic bud initiation (Bhatia-Gaur *et al.* 1999). *Nkx3-1* appears to mark all prostatic buds. Importantly, however, *Nkx3-1* is not a prostate bud specific marker because it is also detected in closely apposed urethral and bulbourethral gland buds in males (Bhatia-Gaur *et al.* 1999; Sciavolino *et al.* 1997) and in epithelial outgrowths of the female mouse UGS which do not typically develop into functional secretory structures (Allgeier *et al.* 2010). We recently identified wingless related MMTV integration site 10b (*Wnt10b*) (Abler *et al.* 2011a; Mehta *et al.* 2011) as a selective prostatic bud marker and in this study, characterize ectodysplasin-A receptor (*Edar*) as another newly identified prostatic bud marker. Neither *Wnt10b* nor *Edar* mark urethral gland buds. Whether they mark the same prostatic bud domains as *Nkx3-1* or the same proportion of buds as *Nkx3-1* had not been determined.

There were two goals of this study. The first was to use ISH to stain UGSs with *Nkx3-1*, *Edar*, or *Wnt10b* riboprobes and determine which riboprobe was best suited for visualizing and quantifying prostatic bud formation from the UGS during *in vivo* and *in vitro* development. Our second goal was to determine whether the number and mRNA expression of these markers was comparable between prostatic buds formed in female and male UGS explants cultured in the presence of androgen. To meet these goals, we compared the expression patterns and proportion of prostatic buds marked by *Nkx3-1*, *Edar*, and *Wnt10b* riboprobes after *in vivo* and *in vitro* prostate development. We

determined that *Nkx3-1*, *Edar* and *Wnt10b* mRNAs mark distinct domains of all epithelial buds of the UGS *in vivo* but mark only a subset of buds formed *in vitro*. Furthermore, the proportion of prostatic buds marked by these mRNAs *in vitro* was developmental stage- and gender-dependent. Our experimental results inform a set of guidelines for using ISH to mark and quantify prostatic buds during mouse prostate development *in vitro* and *in vivo*. They also reveal previously unrecognized molecular differences in epithelial buds formed after *in vitro* versus *in vivo* UGS development

Materials and Methods

Animals. C57B/6J mice were purchased from Jackson Labs (Bar Harbor, ME) and housed in polysulfone cages containing corn cob bedding. Mice were maintained on a 12 hr light and dark cycle at 25±5°C and 20-50% relative humidity. Feed (Diet 2019 for males and Diet 7002 for pregnant females, Harlan Teklad, Madison, WI) and water were available *ad libitum*. All procedures were approved by the University of Wisconsin Animal Care and Use Committee and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Female mice were paired overnight with males to obtain timed-pregnant dams. The morning of copulatory plug identification was considered 0.5 dpc. Dams were euthanized by CO₂ asphyxiation.

In situ hybridization (ISH). Detailed protocols are available at www.gudmap.org and were described previously for vibratome-cut UGS sections (Abler *et al.* 2011b). The following modifications were used in this study to stain whole-mount UGS tissues: 0.5% Triton X-100 was added to all solutions and 1mg/mL collagenase (from *Clostridium*

histolyticum # C9891, Sigma-Aldrich, St. Louis MO) was combined with 5ug/mL proteinase K to enhance tissue permeability. Extensive washing with tris-buffered saline (TBS, 24 h, 25°C) facilitated removal of un-bound antibody from whole-mount tissue. BM Purple was used as an alkaline phosphatase chromagen for detection of digoxigenin-labeled riboprobes. *Nkx3-1* (NCBI GeneID: 18095) and *Wnt10b* (NCBI GeneID: 22410) riboprobes were generated as described previously (Abler *et al.* 2011a, Mehta *et al.* 2011). The 649 base pair *Edar* (NCBI GeneID: 13608) probe corresponds to positions 2852 to 3499 of the NCBI reference sequence (RefSeq) NM_010100.3. The staining pattern for each hybridized riboprobe was assessed in at least three litter-independent fetuses. Male and female tissues were processed as a single experimental unit to allow for qualitative comparisons between sexes.

UGS organ culture. UGSs were dissected from male and female fetuses, placed on 0.4 µm Millicell-CM filters (Millipore, Bedford, MA) and cultured as described previously (Vezina *et al.* 2008) in media containing DHT (diluted from an ethanol stock solution to a final media concentration of 10 nM) and 0.1% dimethylsulfoxide (DMSO). UGSs were cultured for 7 days with replacement of media every 2 days.

Immunohistochemistry (IHC). Immunofluorescent staining of ISH-stained vibratome-cut sections was conducted as described previously (Abler *et al.* 2011a). For whole-mount prostate tissues, ISH was conducted first and tissues were fixed at 4°C for 24 hours in 4% PFA. Tissues were washed at 25°C for 15 minutes in TBS containing 0.5% Triton X and 0.1% Tween20 (TBSTx) and blocked at 25°C for 1 hr in TBSTx containing 1% Blocking Reagent (Roche Diagnostics, Indianapolis, IN), 5% normal goat sera, 1%

bovine serum albumin fraction 5 (RGBTx) and 1% DMSO. Tissues were then incubated at 4°C for 24 hours in rabbit anti-CDH1 (#3195 Cell Signaling Technologies, Beverly, MA), diluted 1:750 in RGBTx. Tissues were washed at 4°C for 24 hr in TBS containing 0.5% Triton X, tissues were then incubated at 4°C overnight in biotinylated goat anti-rabbit secondary antibody (BA1000 Vector Laboratories, Burlingame, CA) diluted 1:500 in RGBTx. Tissues were washed at 25°C for 2 hr in TBSTx and incubated in 1x avidin biotin complex (ABC) reagent (PK6100, Vectastain Elite ABC Kit, Vector Laboratories) according to manufacturer's instructions. Following incubation in ABC reagent tissues were rinsed at 25°C for 1hr with TBSTx and incubated in 3,3'-diaminobenzidine substrate (SK-4100, Vector Laboratories) according to manufacturer's instructions. Some tissues were imaged in whole mount. Other tissues were dehydrated into ethanol, cleared in XS-3 xylene substitute (Statlab, McKinney, TX) to preserve BM Purple and DAB color intensities, infiltrated with paraffin, cut into 5 µm sections, dewaxed in XS-3 and mounted with Permount (Fisher Scientific, Fair Lawn, NJ) prior to imaging.

Prostatic bud counting. The number of prostatic buds per UGS was computed as the average count from three persons, each of whom was blinded to the experimental conditions. Only buds within the UGS, as defined in the introduction, were counted (buds on the caudal portion of the urethra, which typically form urethral glands and not prostate gland buds, were excluded from analysis). Additionally, only bud stalks were counted, a bud with one or more branches was only counted as one bud. Statistical analysis was conducted using R version 2.13.1. Student's t-test and analysis of variance (ANOVA) were conducted on untransformed data that passed Bartlett's test for

homogeneity of variance and appeared to be normally distributed. Tukey's Honest Significant Difference (HSD) test was used for post-hoc analysis, and p values of less than 0.05 were considered significant. All results are reported as mean $\pm$ SE, $n \geq 3$ litter independent samples per group.

Results

Edar mRNA selectively marks prostatic buds in developing mouse UGS

We and others previously described *Nkx3-1* and *Wnt10b* mRNA expression patterns in embryonic day (E) 17.5 and postnatal day (P) 0 UGS tissue sections (Abler *et al.* 2011a; Bhatia-Gaur *et al.* 1999). In conjunction with a recent ISH screen, we identified *Edar* as a prostatic bud marker. The *Edar* expression pattern has not been reported previously in prostate and our first goal was to visualize and describe its expression pattern in the UGS during prostatic bud initiation at E17.5. ISH was used to visualize *Edar* mRNA in E17.5 male and female mouse UGS sections. Tissue sections were then immunohistochemically stained with antibodies against anti-cadherin1 (CDH1) and smooth muscle actin alpha 2 (ACTA2) so that the *Edar* expression could be assessed and described in the context of other UGS tissue compartments (Fig. 1). *Edar* was detected specifically in prostatic bud tips (Fig. 1 A: arrowheads) and therefore was not detected in buds where the distal tip was not present in the tissue section. *Edar* was not detected elsewhere in male UGS epithelium or stoma, or in any female UGS tissue compartment at E17.5 (Fig. 1B). Therefore, *Edar*, *Nkx3-1* and *Wnt10b* all appear to be selective mouse prostatic bud markers in E17.5 mouse UGS.

Nkx3-1, Edar, and Wnt10b are specific markers for mouse prostatic buds formed in vivo

We next assessed which selective prostatic bud marker would be best suited for marking prostate during fetal and neonatal development *in vivo*. The most useful marker would be visible in UGS epithelium during prostatic bud specification (E14.5-15.5, prior to the emergence of visible prostatic buds) and would exhibit selective expression in prostatic bud epithelium during bud initiation, elongation and branching morphogenesis. To determine which mRNA marker best fit these criteria, we compared *Nkx3-1*, *Edar* and *Wnt10b* mRNA expression patterns in whole mount E15.5, 16.5, 17.5 and 18.5 male UGSs and in postnatal day (P) 5 prostates. At E15.5, a stage when no prostatic buds are visible by scanning electron microscopic analysis of male UGS epithelium (Lin *et al.* 2003), *Nkx3-1*, *Edar* and *Wnt10b* were detected in UGS epithelium (Fig. 2A-C). *Nkx3-1* mRNA was restricted to fields where ventral, anterior and dorsolateral prostate will arise (Fig. 2A; inset) while *Edar* and *Wnt10b* were expressed in a diffuse pattern throughout UGS epithelium at this stage (Fig. 2B-C; inset). At E16.5, *Nkx3-1* and *Wnt10b* were detected in anterior prostatic buds and both were noticeably concentrated in UGS fields where dorsolateral and ventral prostatic buds are known to form about one day later (Fig. 2D,F; inset), *Edar* remained diffuse in UGS epithelium (Fig. 2 E; inset). At E17.5 and E18.5, *Nkx3-1*, *Edar* and *Wnt10b* were detected in all anterior, ventral and dorsolateral prostatic buds (Fig. 2G-L).

In order to describe *Nkx3-1*, *Edar* and *Wnt10b* mRNA expression patterns in the context of UGS epithelial microanatomy at E18.5 and P5, ISH stained tissue specimens were co-stained by IHC with an anti-cadherin 1 (CDH1) antibody that marks all UGS epithelium. This co-staining procedure allowed us to determine whether each riboprobe

marked all epithelial buds or only a subset and whether each marked the entire length of prostatic buds or only a fractional part. The ISH/IHC co-staining procedure also facilitated improved visualization of urethral gland buds. *Nkx3-1* mRNA was detected in urethral gland buds at E18.5 (Fig. 2J; open arrowheads). Diffuse *Edar* staining was observed in urethral mesenchyme at E18.5, but neither *Edar* nor *Wnt10b* were detected in urethral gland bud epithelium at this stage (Fig. 2K-L). The three mRNAs also exhibited regional expression pattern differences in prostatic buds at E18.5 and in prostatic buds undergoing branching morphogenesis at P5 (Fig. 2J-O). *Nkx3-1* was detected in at least half of the length of the main prostatic buds and in all branched tips (Fig. 2J,M; inset). *Edar* and *Wnt10b* were not detected in main prostatic buds but were instead localized to bud tips distal to primary and secondary branch points (Fig. 2K,L,N,O; inset). *Nkx3-1* was also detected in P5 urethral gland buds (Fig. 2M; open arrowheads) but neither *Edar* nor *Wnt10b* were detected in urethral gland buds at this stage (Fig. 2N-O). Together our results indicate that *Nkx3-1* is the most appropriate mRNA for marking the prostate field at E15.5, prior to bud initiation, and that *Nkx3-1*, *Edar* and *Wnt10b* are equally useful for marking prostatic buds after bud initiation and during branching morphogenesis (E17.5-P5). Our results therefore validate ISH for *Nkx3-1*, *Edar* and *Wnt10b* mRNAs as a tool for 1) distinguishing prostate from urethral gland buds and 2) marking unique domains of the proximodistal prostatic bud axis beginning after E18.5 and extending to at least P5.

Nkx3-1, *Edar* and *Wnt10b* mark the same quantity of prostatic buds formed *in vivo*

One of our overarching goals was to validate ISH as a method for visualizing and quantifying prostate development. To this end, we compared the number of anterior, ventral, dorsolateral and total prostatic buds marked by *Nkx3-1*, *Edar* and *Wnt10b* ISH in the E18.5 male UGS. There were no statistically significant differences among numbers of anterior, ventral, dorsolateral or total prostatic buds detected by each riboprobe (Fig.3, $n=3$). Furthermore, we did not identify any CDH1-positive buds in the UGS that were unmarked by *Nkx3-1*, *Edar* or *Wnt10b*. Care must be taken in quantifying *Nkx3-1* marked buds on or after E17.5 because it marks prostatic and urethral gland buds. However, the distinct anatomical location of urethral gland buds, which are generally caudal to prostatic buds, allows the majority of urethral gland buds to be visually excluded from prostatic bud quantification. Together, these results suggest that all three mRNAs are equally useful for marking and quantifying prostatic buds formed during *in vivo* mouse prostate development.

Nkx3-1, Edar and Wnt10b mark different quantities of prostatic buds formed in vitro

Morphological similarities exist between prostatic buds and branches formed *in vivo* versus those formed *in vitro* (Doles *et al.* 2005). However, it is not known whether mRNA expression in prostatic buds and branches formed *in vivo* is recapitulated during *in vitro* prostate development. Our next goal was to assess the utility of ISH/IHC staining as a method for quantifying prostate development in cultured UGS explants. We wanted this method to be broadly applicable for *in vitro* studies of prostate development. Since some investigators focus such studies on early stages of prostate ductal development (bud formation) and others on later stages (branching morphogenesis), we assessed

Nkx3-1, *Edar* and *Wnt10b* mRNA expression in UGS explants initiated at two different stages: E14.5 (before prostatic bud initiation) and E17.5 (after prostatic bud initiation). UGSs were incubated for 7 days in organ culture media containing 5 α -dihydrotestosterone (DHT, 10nM). UGS explants were stained by ISH to visualize expression patterns of *Nkx3-1*, *Edar* and *Wnt10b* and then by IHC to visualize CDH1 in all bud and non-bud UGS epithelium (Figs. 4-5). This staining method allowed us to quantify mRNA marked epithelial buds, total epithelial buds, and the proportion of buds marked by each mRNA as a fraction of the total. Since *Nkx3-1*, *Edar* and *Wnt10b* mark different bud regions, to maintain consistency in quantification, mRNA marked buds with no branches were counted the same as buds with several mRNA marked branches. In other words, only the number of main ducts that exhibited mRNA staining in one or more regions of the proximodistal axis was counted. The anatomically distinct urethral gland buds were excluded from analysis. We determined that this method did not bias our ability to identify prostatic buds based on the fact that the computed total bud number (mRNA marked + mRNA unmarked) did not appreciably differ among age- and gender-matched *Nkx3-1*, *Edar* and *Wnt10b* stained UGS groups (results not shown, $n \geq 3$ per group).

Our first observation was surprising. Though *Nkx3-1*, *Edar* and *Wnt10b* appear to mark nearly all epithelial buds formed in the UGS during development *in vivo* (Fig. 2), these mRNAs marked only a subset (68% or fewer at E14.5 and 89% or fewer at E17.5) of buds formed *in vitro* (Figs. 4-5). Of particular note, *Nkx3-1* was detected in urethral gland buds after *in vivo* development but was either less abundant or not detected in urethral gland buds of males and females after *in vitro* development (Figs. 4-5; arrows).

This result reveals a potential difference in the molecular composition of prostatic and urethral gland buds formed *in vitro* versus those formed *in vivo*.

The proportion of prostatic buds marked by *Nkx3-1*, *Edar*, and *Wnt10b* mRNAs was developmental stage- and gender-dependent. In E14.5 male UGS explants, *Nkx3-1* was found to mark a greater proportion of buds than *Edar* and *Wnt10b* (Fig. 4A-D). However, in E14.5 female UGS explants, *Nkx3-1* and *Edar* both marked a greater proportion of buds than *Wnt10b* (Fig. 4E-H). In E17.5 male UGS explants, there was no detectable difference in the proportion of buds marked by *Nkx3-1*, *Edar* and *Wnt10b* (Fig. 5A-D). However, in E17.5 female UGS explants, the proportion of *Nkx3-1* and *Edar* marked buds was greater than the proportion of *Wnt10b* marked buds (Fig. 5 E-H). Together, our results indicate that *Nkx3-1* marks the greatest proportion of prostatic buds formed in male UGS explants, while *Nkx3-1* and *Edar* are equally efficient in marking prostatic buds formed in female UGS explants. Furthermore, our results reveal that age and gender of UGS explants at the time of organ culture initiation influences the proportion of buds marked by *Nkx3-1*, *Edar* and *Wnt10b*.

The number of buds formed in vitro and the proportion marked by Nkx3-1 depends on gender and developmental stage at which UGS organ culture was initiated

Mechanistic studies of prostate development are challenged by the fact that the mouse UGS is small and many UGS tissues must often be pooled to obtain sufficient tissue for downstream molecular and biochemical analyses. This problem is amplified in transgenic mouse studies, where dozens of litters are necessary to yield a sufficient number of genotype appropriate UGS tissues. Female UGSs are known to form

prostatic buds in response to androgens (Cunha,1972). As a method to reduce or refine animal usage for prostatic bud formation studies, female UGSs could conceivably be used 1) in combination with male UGS explants to increase experimental sample size and 2) in place of male UGSs to serve as control tissues naïve to high levels of *in vivo* androgen signaling. To determine whether such a use of female UGSs is appropriate, we compared prostatic bud formation in male and female UGS explants.

We first compared total UGS bud number between male versus female UGS explants incubated *in vitro* in the presence of androgens. Only main ducts were counted to eliminate differences in branching morphogenesis between age and gender. The anatomically distinct urethral gland buds were excluded from analysis. In order to achieve the highest statistical power for this analysis we pooled total UGS bud counts among age and gender matched *Nkx3-1*, *Edar* and *Wnt10b* stained groups. This sample pooling was feasible since total UGS bud counts did not detectably differ among age and gender matched ISH stained groups (results not shown). In E14.5 UGS explants, there was no detectable difference between the total number of buds formed in male and female tissues (Fig. 6A). However, E17.5 male UGS explants formed significantly more total buds than female UGS explants (Fig. 6B), an expected result since, at the time of organ culture initiation, prostatic buds were present in E17.5 males and absent in E17.5 females. Since *Nkx3-1* marked the greatest proportion of buds across gender and developmental stage, we next compared the proportion of *Nkx3-1* marked buds to the total buds formed in male versus female UGS explants incubated *in vitro* in the presence of androgens. UGS explants from E14.5 males formed a significantly greater proportion of *Nkx3-1* marked buds compared to those from E14.5

females (Fig. 6C). Yet, E17.5 male and female UGS explants formed the same proportion of *Nkx3-1* marked buds (Fig. 6D). Together, our results reveal that not all buds formed in UGS explants are the same and that composition of the prostatic bud population in UGS explants is influenced by UGS gender and developmental stage.

Discussion

A multitude of drugs, xenobiotics, transgenes, and gene deletions have been shown to impair, delay, reposition or accelerate prostate development by altering the number of prostatic buds formed *in vivo* and *in vitro*. Prostatic bud number therefore represents an important phenotypic endpoint for “quantifying” early stages of prostate development. We described a method that combines ISH and IHC staining to quantify two early prostate development endpoints in the same UGS: total prostatic bud number and proportional bud number marked by prostate-selective mRNAs. This method does not require expensive laboratory equipment and complements existing methods employed to quantify prostate development.

Using this newly validated method for counting prostatic buds, we revealed a noteworthy *in vivo* versus *in vitro* difference in molecular composition of prostatic buds. All prostatic buds formed *in vivo* were marked by *Nkx3-1*, *Edar*, and *Wnt10b*. Yet only a fraction of buds formed *in vitro* were marked by the same prostate-selective markers. Possible explanations are that *in vitro* 1) culture conditions do not completely reproduce the needed battery of prostate morphogenetic signals that are present *in vivo* 2) UGS mesenchyme is impaired in its ability to instruct the appropriate prostate identity onto bud epithelium 3) prostatic bud epithelium is compromised in its ability to respond

appropriately to UGS mesenchyme signals. Our results suggest that sole reliance on prostatic bud number as a metric for *in vitro* prostate development neglects a potentially relevant endpoint – whether or not buds express appropriate prostate marker mRNAs. The fractional number of prostatic buds marked by *Nkx3-1*, *Edar*, and *Wnt10b* mRNAs may also be relevant for assessing prostate development after genetic, pharmacological or toxicological manipulations *in vivo*. While we found that these prostate-selective mRNAs are present in all prostatic buds formed during *in vivo* prostate development in untreated wild type control male fetuses, whether the same is true for transgenic or chemically exposed male fetuses is not known.

Another important aspect of this study is that it establishes, with experimental evidence, that male and female UGS explants cannot be used interchangeably to assess androgen-dependent prostatic bud formation *in vitro*. Androgen-exposed male and female UGS explants varied in both the number of buds formed and the proportion of buds marked by prostate-selective mRNAs. These endpoints were further affected by the developmental stage of the male or female UGS used for organ culture. Though the total number of buds formed did not significantly differ between E14.5 male and female UGS explant cultures, prostatic buds formed in E14.5 male UGSs were more likely to express *Nkx3-1* than those formed in E14.5 female UGSs. On the other hand, while E17.5 male UGS explants formed significantly more buds than E17.5 female UGSs, there was not a significant male versus female difference in the *Nkx3-1*-marked bud proportion at this stage. Our results support the notion that some androgen-induced buds in E14.5 female UGS explants are developmentally delayed or deviate from a normal prostate gene expression program. The latter possibility is consistent with the

fact that, during its peri-natal maturation, the female UGS loses its ability to initiate androgen-dependent prostatic bud formation (Cunha, 1975). Based on our results, the female UGS may begin losing its androgen responsiveness much earlier in development than previously appreciated, as early as E14.5, as evidenced by a failure to express appropriate prostate gene expression in all androgen-induced buds.

We identified *Wnt10b* and *Edar* as additional prostatic bud markers that can be used in conjunction with, or in some cases in place of, the more widely known *Nkx3-1* prostatic bud marker (Bhatia-Gaur et al.1999). All three mRNAs have the benefit of marking prostatic buds over a relatively long prostate development window. Therefore, ISH staining of the UGS with *Nkx3-1*, *Edar*, or *Wnt10b* can be used to visualize and compare prostatic bud number among different experimental groups, even if experimental treatments reduce or delay prostate development.

ISH staining for *Nkx3-1* is particularly useful for visualizing the mouse prostate field prior to prostatic bud initiation. This method could be used to more clearly delineate the mechanism by which a particular experimental condition impairs prostatic bud formation. Absence of detectable *Nkx3-1* ISH staining in the UGS on or after the normal stage of bud initiation (>E16.5) may indicate that specification of the prostate field was delayed or impaired. Alternatively, the presence of *Nkx3-1* and absence of prostatic buds may indicate that the prostate field was specified appropriately but that buds failed to elongate. Distinguishing between these two possibilities refines the genes and signaling pathways likely to be involved in the prostatic bud impairment mechanism.

ISH staining for *Edar* and *Wnt10b* mRNAs can be used to distinguish prostatic from urethral gland buds. *Nkx3-1* was shown here and in previous studies (Abler et

al.2011a, Bhatia-Gaur et al.1999) to mark both prostatic and urethral gland buds. On the other hand, *Edar* and *Wnt10b* were detected in prostate buds and not in urethral gland buds. Visualization of *Nkx3-1*, *Edar*, and *Wnt10b* could be used separately or in conjunction to focus on one particular urogenital bud population or to visualize segmental boundaries in the developing mouse urethra.

An interesting future application for our results is that they can be used to conduct prostate cell lineage analyses and to enrich for highly-specific developing prostate cell populations for gene expression profiling and biochemical studies. It is currently possible to target genes for deletion using an *Nkx3-1-Cre* recombinase gene (Thomsen et al.2008) and conduct lineage tracing of prostate bud epithelial cells using a tamoxifen inducible *Nkx3-1^{CreERT2}* with R26R-YFP or R26R-lacZ reporters (Wang et al.2009). It had not been possible to compare the fate of cells at the tips of prostatic buds versus those in bud proximal regions, or to separate epithelial cells in prostatic bud tips from those in the proximal bud region. Yet, in this report we identified differential expression of *Nkx3-1*, *Edar*, and *Wnt10b* along the axis of prostatic buds. Early in development, all three mRNAs mark the entire length of prostatic buds. During prostate bud elongation and branching morphogenesis *in vivo* (E18.5 until at least P5) and *in vitro* (E17.5 plus at least 7 days in organ culture), *Edar* and *Wnt10b* uniquely mark prostatic bud tips. At the same developmental stages, *Nkx3-1* is present in prostatic bud tips and extends proximally towards the urethra. The specific prostatic bud domain marked by *Edar* and *Wnt10b* represents a unique prostatic bud cell subpopulation because these cells are at the leading edge of prostatic buds as they invade surrounding mesenchyme. It may be possible in future studies to use *Nkx3-1*,

Edar, and *Wnt10b* expression as cell sorting tools to identify mRNAs and proteins that are distinctively expressed or activated in the invasive prostatic growth environment in prostatic bud tips.

Finally, the presence of *Edar* and *Wnt10b* in developing prostate ducts provides insight into the complex signaling pathways involved in prostatic bud specification, initiation and branching morphogenesis. These mRNAs are known members of the tumor necrosis factor (TNF) and WNT signaling pathways and indicate important roles for these signaling pathways in prostate development. Intriguingly, *Edar* and WNT signaling have been shown to interact to establish hair follicle patterning (Laurikkala *et al.* 2002, Zhang *et al.* 2009) whether the same interaction occurs during prostate development is currently unknown.

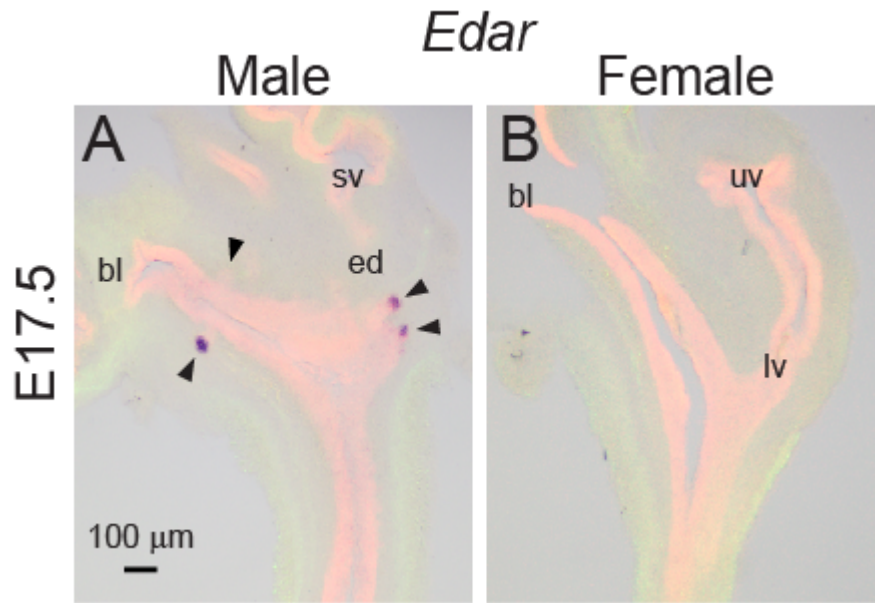


Fig. 1: *Edar* mRNA distribution in E17.5 male and female mouse UGS. (A-B) Near mid-sagittal sections (50 μm) of embryonic day (E)17.5 male and female UGSs were stained by ISH to visualize mRNA expression of ectodysplasin-A receptor (*Edar*). Sections were then immunofluorescently stained with antibodies against smooth muscle actin alpha 2 (ACTA2, green) and cadherin 1 (CDH1, red). Results are representative of three litter-independent samples per group. Abbreviations: bl, bladder; ed, ejaculatory duct; lv, lower vagina; sv, seminal vesicle; uv, upper vagina. Solid arrowheads indicate prostatic buds.

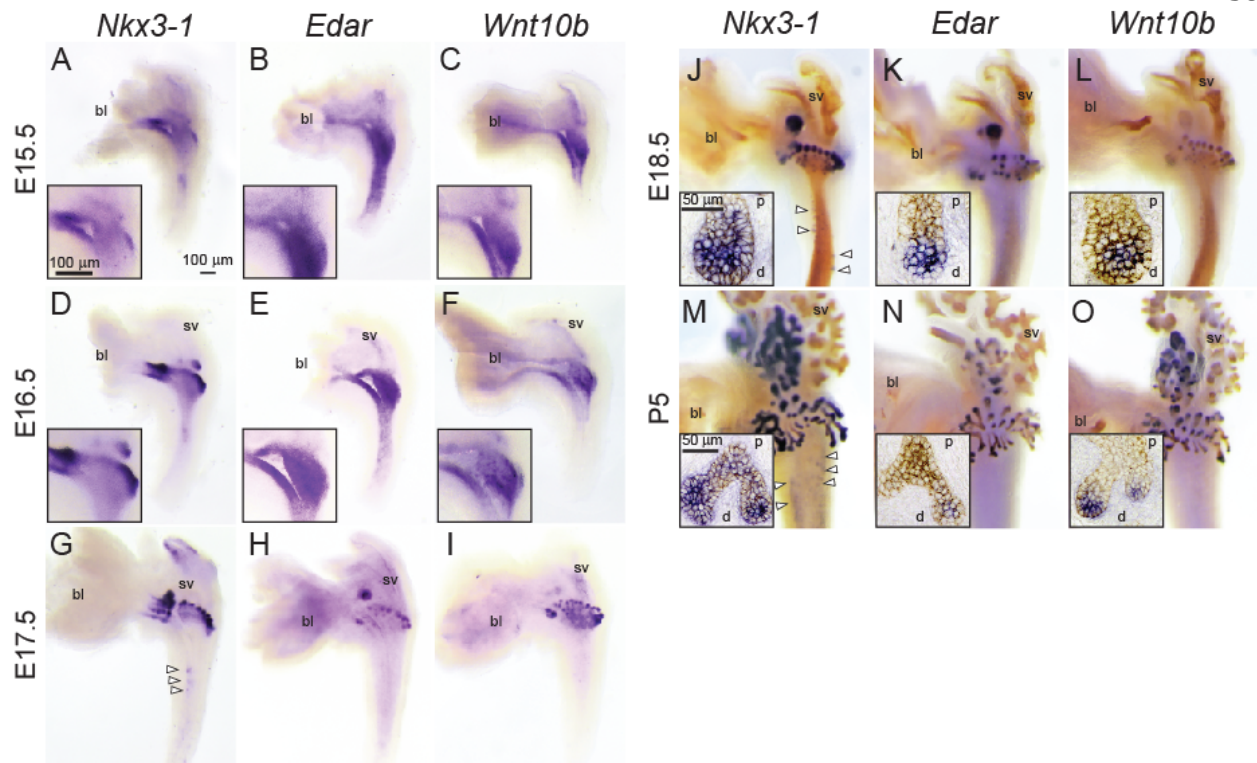


Fig. 2: Developmental time course of *Nkx3-1*, *Edar*, and *Wnt10b* mRNA expression in whole-mount male fetal UGS and neonatal prostate. (A-O) ISH to visualize mRNA expression (purple) of *Nkx3-1*, *Edar*, and *Wnt10b* in embryonic day (E)15.5, 16.5, 17.5, and 18.5 male mouse UGS and postnatal day (P)5 mouse prostate. (J-O) E18.5 UGS and P5 prostate were immunohistochemically stained with anti-cadherin 1 (CDH1, red) to visualize all epithelium. (A-O) Insets are magnified images. (J-O) Insets are 5 μ m stained tissue sections to reveal staining details within the proximal distal axis of prostatic buds. Results are representative of n=3 litter-independent samples/group. Abbreviations: bl, bladder; d, distal; p, proximal; sv, seminal vesicle. Open arrowheads indicate representative urethral gland buds.

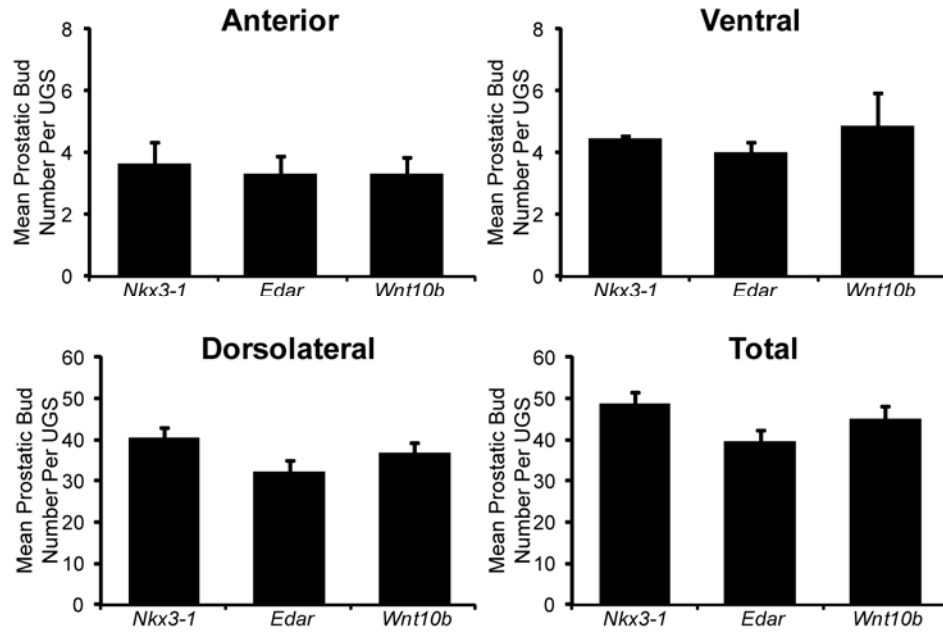


Fig. 3: *Nkx3-1*, *Edar*, and *Wnt10b* mark all macroscopically visible prostatic buds after UGS development *in vivo*. E18.5 male UGS tissues were stained by ISH and IHC as described in Figure 2 and the number of anterior, ventral, dorsolateral and total prostatic buds marked by each mRNA was determined. Results are reported as mean $\pm$ SE, $n \geq 3$ litter independent UGS tissues per group. One-way analysis of variance (ANOVA) was used to determine that there was no statistically significant difference ($p < 0.05$) in the mean prostatic bud number marked by *Nkx3-1*, *Edar* or *Wnt10b*.

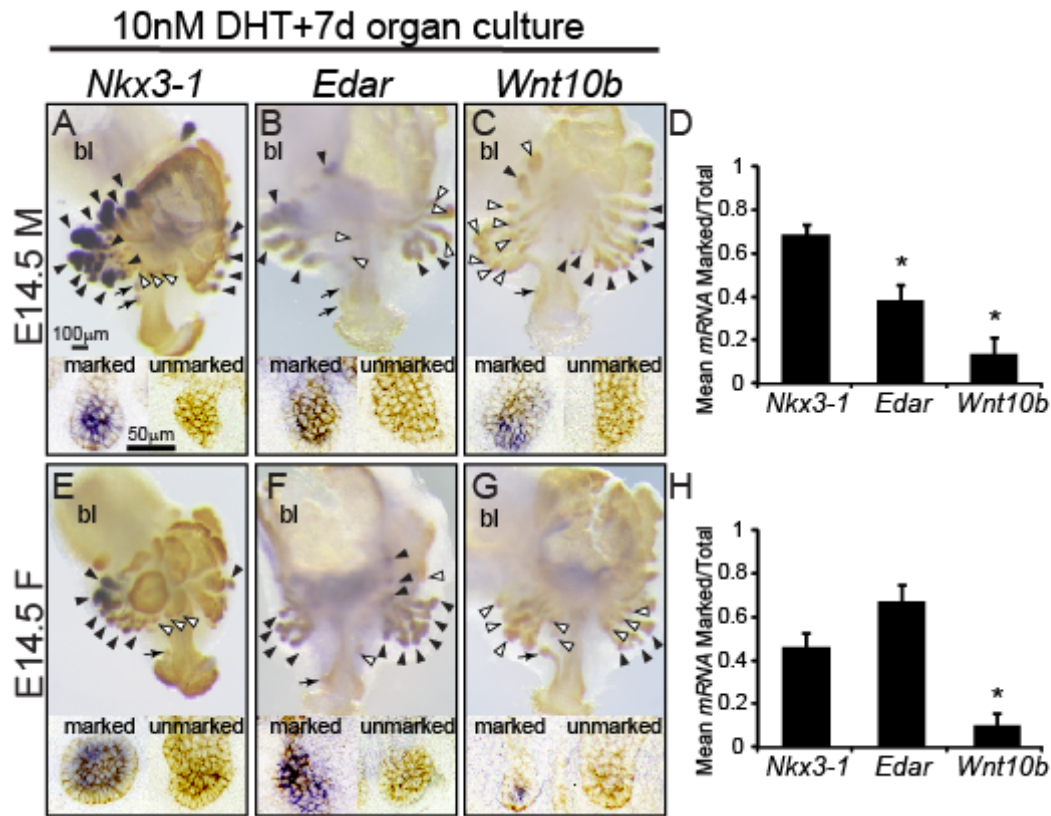


Fig. 4: *Nkx3-1*, *Edar*, and *Wnt10b* mark only a subset of prostatic buds after E14.5 UGS development *in vitro*. E14.5 (A-D) male and (E-H) female UGSs were grown for seven days in organ culture media containing vehicle (0.1% ethanol) or 5 α dihydrotestosterone (DHT, 10 nM). Following culture, UGSs were stained by ISH to visualize mRNA expression (purple) of *Nkx3-1*, *Edar*, and *Wnt10b* and IHC with an antibody against cadherin 1 (CDH1, red) to visualize epithelium and mRNA-unmarked buds. Insets are magnified images of 5 μ m stained tissue sections. Solid arrowheads indicate mRNA stained buds. Open arrowheads indicate buds without detectable mRNA staining. Arrows indicate urethral gland buds. Abbreviations: bl, bladder. Results are reported as mean $\pm$ SE, $n \geq 3$ litter independent UGS tissues per group. Asterisks indicate a significant difference compared to *Nkx3-1* ($p < 0.05$).

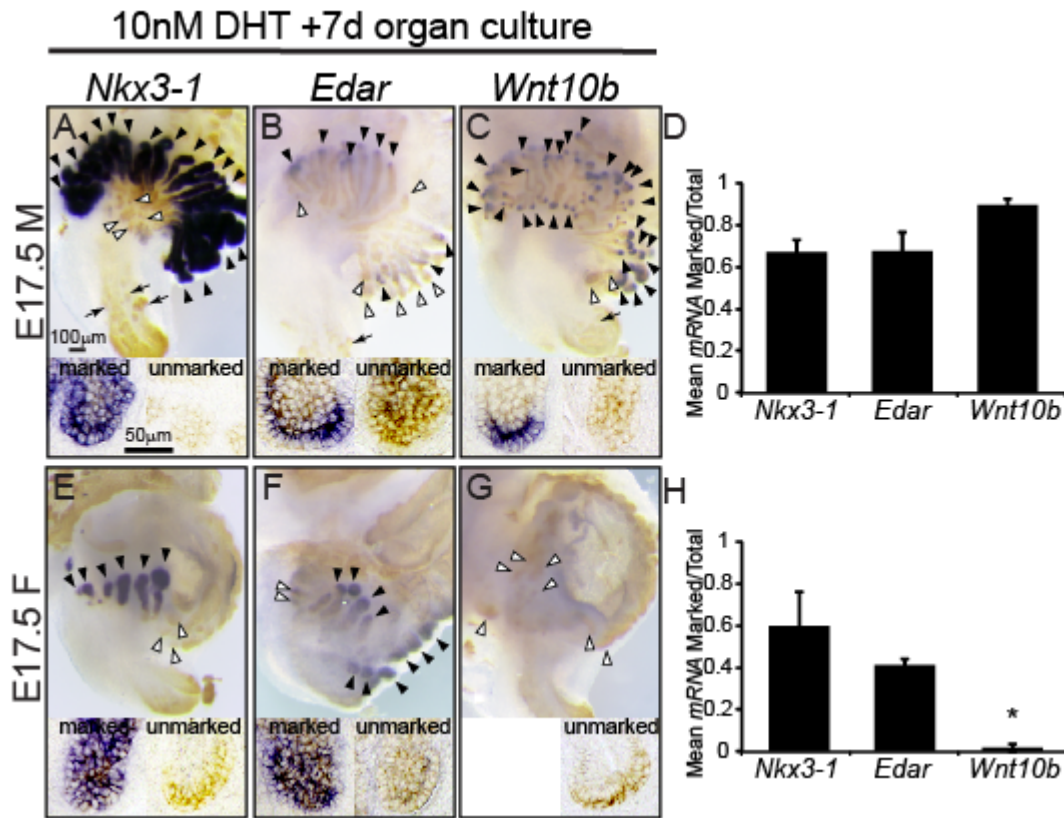


Fig. 5: *Nkx3-1*, *Edar*, and *Wnt10b* mark only a subset of prostatic buds after E17.5 UGS development *in vitro*. E17.5 (A-D) male and (E-H) female UGSs were grown for seven days in organ culture media containing vehicle (0.1% ethanol) or 5 α dihydrotestosterone (DHT, 10 nM). Following culture, UGSs were stained by ISH to visualize mRNA expression (purple) of *Nkx3-1*, *Edar*, and *Wnt10b* and IHC with an antibody against cadherin 1 (CDH1, red) to visualize epithelium and mRNA-unmarked buds. Solid arrowheads indicate mRNA stained buds. Open arrowheads indicate buds without detectable mRNA staining. Arrows indicate urethral gland buds. Insets are magnified images of 5 μ m stained tissue sections. Results are mean $\pm$ SE, $n \geq 3$ litter independent UGS tissues per group. Asterisks indicate a significant difference compared to *Nkx3-1* ($p < 0.05$).

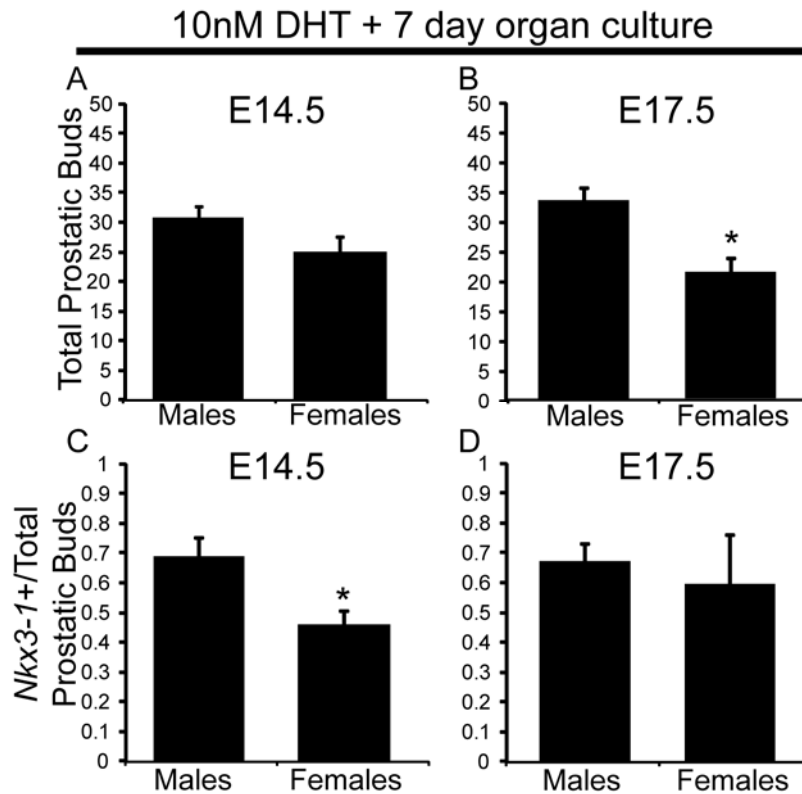


Fig. 6: UGS gender and age at organ culture initiation influence the mean number of prostatic buds formed and the proportion marked by *Nkx3-1*. E14.5 and E17.5 male and female UGSs were stained by ISH and IHC as described in Figure 5 and the number of *Nkx3-1* mRNA-marked and unmarked buds was determined for each UGS. Student's t-test was used to assess male versus female UGS differences in (A-B) the mean number of prostatic buds formed and (C-D) the proportion of buds marked by *Nkx3-1* after *in vitro* UGS development in the presence of 5 α dihydrotestosterone (DHT, 10 nM). Results are mean $\pm$ SE, $n \geq 3$ litter independent UGS tissues per group. Asterisks indicate a significant difference between male and female UGS ($p < 0.05$).

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Chapter 3: Wnt inhibitory factor 1 (*Wif1*) is regulated by androgens and enhances androgen-dependent prostate development

Keil et al., Endocrinol. (2012) 153:6091-6103 with the exception of Figure 9
(unpublished)

Introduction

Androgens initiate prostatic bud formation from the urogenital sinus (UGS) and stimulate prostatic bud elongation, ductal branching morphogenesis and differentiation of mature prostatic ductal epithelium (Sugimura et al.,1986, Hayward et al.,2000). During fetal prostate development, androgens activate androgen receptors (ARs) in UGS mesenchyme (UGM) to induce prostatic bud formation in UGS epithelium (UGE) (Sugimura et al.,1986, Cunha et al.,1980Cunha,1973,Lasnitzki et al.,1980). The developing prostate has therefore been used as a model to assess the role of androgens in mesenchymal/epithelial interactions. A longstanding question in the prostate development field is how ARs in UGM communicate with UGE to establish the pattern and quantity of prostatic buds that will form. Continued investigation is expected to shed light on how the developing prostate microenvironment influences prostate epithelial cell fate. It may also elucidate how adult prostate stromal ARs reactivate developmental signaling pathways to cause inappropriate proliferative growth during prostate disease (Memarzadeh et al.,2007Cantile et al.,2005Sheng et al.,2004Feeley et al.,2005Pienta et al.,1993Lai et al.,2012Niu et al.,2008-Yamamoto et al.,2010).

Numerous gene expression profiling studies have been conducted to identify androgen-responsive mRNAs in the fetal prostate (Pritchard et al.,2008Zhang et al.,2006Parker et al.,1977Parker et al.,1980-Thomsen et al.,2008). The WNT pathway has been identified as a major androgen-responsive pathway in the developing UGS and many *Wnts* have been found to be more abundant in male compared to female mouse UGS (Pritchard et al.,2008, Zhang et al.,2006, Mehta et al.,2011). Several WNT antagonists are also present during prostate development, including but not limited to

WNT inhibitory factor 1 (*Wif1*) and secreted frizzled related proteins (*Sfrps*) 1 and 2 (Pritchard et al.,2008, Zhang et al.,2006, Joesting et al.,2005). Tight regulation of WNT signaling is required for the normal program of prostatic branching morphogenesis (Joesting et al.,2005, Wang et al.,2008).

Despite growing awareness of sexually dimorphic gene expression in the UGS, we are still limited in our knowledge of androgen responsive genes in UGM that initiate prostate ductal development. We conducted a high-throughput *in situ* hybridization (ISH) screen in conjunction with the GenitoUrinary Development Molecular Anatomy Project (GUDMAP) to resolve developing prostate molecular anatomy and identify candidate androgen-responsive mRNAs in fetal mouse UGM (www.gudmap.org). Because mesenchymal AR signaling is necessary for prostate development, we screened for mRNAs that were present in fetal male UGM during prostatic bud formation at E17.5 and were either absent, less abundant or differently patterned in E17.5 female UGM. Our screen revealed a sexually dimorphic expression pattern for the secreted WNT-binding protein *Wif1* which was more abundant in male compared to female UGM. In this manuscript, we tested hypotheses that androgens are necessary and sufficient for *Wif1* mRNA expression in developing and sexually mature mouse prostate and that exogenous WIF1 protein increases androgen-dependent prostatic bud formation in UGS explant cultures. Our results reveal *Wif1* as one of the first androgen induced enhancers of prostatic bud formation and thereby provide new insight into the mechanism of androgen action during mouse prostate development.

Materials and Methods

Animals

Wild type C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). *Wif1*^{tm1Dmth} targeted mutant mice (*Wif1*^{lacZ}) (Kansara et al., 2009) were from Dr. Igor Dawid (NICHD, Bethesda, MD) and were maintained on a C57BL/6J x 129/SV background. Mice were housed in polysulfone cages containing corn cob bedding and maintained on a 12h light and dark cycle at 25±5°C and 20-50% relative humidity. Feed (Diet 2019 for males and Diet 7002 for pregnant females, Harlan Teklad, Madison, WI) and water were available *ad libitum*. All procedures were approved by our institutional Animal Care and Use Committee and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. To obtain timed-pregnant dams, females were paired overnight with males. The next morning was considered embryonic day (E)0.5. Dams were euthanized by CO₂ asphyxiation. *Wif1* mutant embryos were genotyped with PCR primers for the wild type allele (5'-CGAGAACTTCACAAGCAGCACAGG-3', and 5'-CCTGTTACAAATCTGCAGTCAGG-3', 500bp product) and mutant allele (5'-CTGTGGCCGGCTGGGTGTGGCGG-3' and 5'-AGCACTCTAGCCTGATGGGCTC-3', 500bp product). For *in vivo* experiments, wild type timed pregnant dams were injected on E13.5 and E14.5 or on E17.5 and E18.5 with sterile corn oil (5mL/kg s.c. maternal dose) containing 10% ethanol alone or containing flutamide (200mg/kg/day maternal dose, Sigma #F9397-1G, St. Louis MO). UGSs were collected at E15.5 and postnatal day (P)0.

Assay for β -galactosidase activity

LacZ-dependent β -galactosidase activity was assessed as described previously (19).

Castration and testosterone treatment

Forty-eight to fifty day old adult male wild type mice were given buprenorphine (0.1mg/kg) for analgesia and isoflurane (1-2% inhalant) for anesthesia. A scrotal incision was made, the testes and associated fat pads were externalized, ligated and excised and the incision was closed with a suture. Sham castrations were performed as described above except testes and fat pads were not ligated or removed. Some mice received 1cm Silastic® capsule implants (empty or testosterone-filled), which were engineered in our laboratory from Silastic® tubing (1.57mm inside diameter, 3.18mm outside diameter, Dow Corning, Midland MI) and inserted into the subcutaneous space above the scapular fat pad.

***In situ* hybridization (ISH)**

UGSs were fixed overnight in 4% paraformaldehyde (PFA) and either stained in whole mount, cut with a vibrating microtome into 50 μ m sections or frozen in Optimal Cutting Temperature (O.C.T.) medium and cut with a cryotome into 10 μ m sections. Fresh adult prostate was frozen in O.C.T medium and cut with a cryotome into 10 μ m sections. PCR-based riboprobe synthesis (www.gudmap.org) and ISH on vibrating microtome-cut sections and UGS whole mounts are described elsewhere (Keil et al.,2012Abler et al.,2011, 24, www.gudmap.org). Primer sequences for generating PCR-based riboprobes are listed in Supplemental Table 1. For ISH of frozen tissue sections,

tissues were embedded in O.C.T. medium, frozen, sectioned, air dried for 10min and fixed for 1h in PBS containing 4% PFA. ISH was conducted as described previously (Abler et al.,2011) with the following modifications. Washes were conducted in slide mailer tubes with constant agitation. Tissues were permeabilized for 8min at room temperature with 5µg/ml Proteinase K. Slides were coverslipped and placed in a humidified hybridization chamber for prehybridization, probe and antibody solution incubations. The hybridization solution was supplemented with 5% dextran sulfate. The duration of all previously described washes (Abler et al.,2011) were reduced by half. BM Purple alkaline phosphatase precipitating chromagen (Roche Diagnostics, Indianapolis IN) was used for detection of digoxigenin-labeled riboprobes. For some UGS explants, light micrographs of BM Purple-stained tissue sections were captured, inverted, pseudocolored green and merged with immunofluorescent images. The staining pattern for all riboprobes was assessed in at least two tissue sections from at least two litter-independent mice. Tissue sections were processed as a single experimental unit to allow for qualitative comparisons among biological replicates, between males and females and among treatment or genotypic groups. Gene expression patterns were annotated using the GUDMAP anatomical ontology.

Organ Culture

E14.5 wild type male and female UGSs were placed on 0.4-µm Millicell-CM filters (Millipore, Billerica, MA) and cultured for 1d or 4d as described previously (Vezina et al.,2008). Media was supplemented with 5α-dihydrotestosterone (DHT, 10nM), hydroxy-flutamide (OHF, 10nM) (Sigma-Aldrich, St. Louis, MO) and/or recombinant human WIF1

protein (0-2000ng/mL; R&D Systems, Minneapolis, MN). Media and supplements were changed every 2d.

Immunohistochemistry (IHC)

Immunofluorescent staining of ISH-stained, frozen, paraffin and vibrating microtome sections was performed as described previously (Mehta et al.,2011, Abler et al.,2011). Primary antibodies were diluted as follows: 1:200 rabbit anti-cadherin 1 (CDH1, 3195, Cell Signaling Technology, Beverly, MA), 1:250 mouse anti-smooth muscle actin alpha 2 (ACTA2, Leica Microsystems, Buffalo Grove, IL), 1:250 rabbit anti-phospho-histone H3 (PH3, 9701s Cell Signaling Technology) and 1:50 mouse anti-keratin 14 (KRT14, ms-115-p0, Thermo Scientific). Secondary antibodies were diluted as follows: 1:250 Dylight 549-conjugated anti-rabbit IgG (111-507-003, Jackson ImmunoResearch Laboratories, West Grove, PA) and Dylight 488-conjugated goat anti-mouse IgG (115-487-003, Jackson ImmunoResearch Laboratories). Immunofluorescently labeled tissues were counterstained with 4',6-diamidino-2-phenylindole, dilactate (DAPI) and mounted in anti-fade media (phosphate buffered saline containing 80% glycerol and 0.2% *n*-propyl gallate).

RNA isolation and Real-time Reverse Transcriptase PCR

E17.5 UGE was enzymatically and mechanically separated from UGM and homogenized as described previously (25). RNA was purified with the Illustra RNAspin Mini kit (GE Healthcare, Pittsburg, PA) and reverse-transcribed with the SuperScript® III

First-Strand Synthesis System (Invitrogen, Carlsbad CA). Real-time PCR was performed in 10.5µl reactions containing 1X SsoFast EvaGreen Supermix, 0.48µM PCR primers and 3.75µl cDNA and amplified using the CFX96 PCR machine (Bio-Rad, Hercules, CA). PCR primers were as follows: *Wif1*: 5'- GAG AAA GCC CTG TGC ATA CC-3' and 5'-ACT GCT CTC TCC CTC GAG TCC-3', *Krt14* 5'- AAT TCT CCT CTG GCT CTC AGT CAT CC-3' and 5'-AGC TTT AGT TCT TGG TGC GCA GGA C-3', *Acta2* 5'-CTG CCG AGC GTG AGA TTG-3' and 5'-AAT GAA AGA TGG CTG GAA GAG AG-3', and peptidyl prolyl isomerase a (*Ppia*) 5'-TCT CTC CGT AGA TGG ACC TG-3' and 5'-ATC ACG GCC GAT GAC GAG CC-3'. Relative mRNA abundance was determined by the ΔC_t method as described previously (27) and normalized to *Ppia* abundance.

Methylated DNA Immunoprecipitation (MeDIP)

MeDIP was conducted on pooled (5-6 tissues/pool) UGS explants and each experimental group consisted of five pools. DNA was sonicated to 200-1000bp in length, incubated with anti-5-methyl cytosine antibody (Abcam), washed with agarose beads to recover bound antibody/DNA. Precipitated DNA was collected and purified using Qiagen PCR Purification Kit according to manufacturers instructions. Real time quantitative PCR (QPCR) was performed as described previously (Keil et al.,2012) using gene specific primers for mouse *Wif1*: 5'-CTTTGCCAGCATCGTGTCTC -3' and 5'-GCAAGTCTCCAGTCTCCGTTG -3' (Entrez gene ID 24117) spanning the region -266bp to -128bp of the transcription start site (TSS).

Statistical analyses

For RT-PCR, three tissue pools consisting of 2-3 UGSs per pool were used to measure mRNA abundance. For prostatic bud counting, UGSs were stained by ISH for NK3 homeobox 1 (*Nkx3-1*) mRNA and three individuals, blinded to treatment conditions, counted the total number of *Nkx3-1* positive prostatic buds in each of at least four UGSs per experimental group. *Nkx3-1* stained urethral gland buds were excluded from analysis (26). For immunolabeled cell counting, PH3-positive, KRT14-positive and double-positive cells were counted in at least two sections per UGS in at least three UGSs per treatment group. For assessment of castration efficiency, lower urinary tract (LUT, including prostate, urethra, bladder and seminal vesicle) wet weight was measured relative to body weight at time of euthanasia. Statistical analysis was performed using R version 2.13.1. Homogeneity of variance was determined using Bartlett's test. Student's *t*-test or ANOVA, followed by Fisher's least significant difference (LSD) test were used to identify significant differences ($p \leq 0.05$) between or among treatment groups.

Results

Demonstration of *Wif1* riboprobe specificity

We created a *Wif1* riboprobe and demonstrated its ability to recapitulate the *Wif1* expression pattern reported previously for a different *Wif1* riboprobe (28) in E13.5 mouse handplate mesenchyme (Supplemental Fig. 1A). To further establish selectivity of our riboprobe, we also showed that the *Wif1* expression pattern in mouse UGS as

revealed by ISH (described in the section below) was similar to the *lacZ* expression pattern in *Wif1*^{lacZ/+} UGS (Supplemental Fig. 1B).

***Wif1* mRNA is more abundant in male compared to female UGM and overlaps androgen-responsive *Srd5a2* mRNA from the earliest stages of mouse prostate development**

Our next objective was to analyze the *Wif1* expression pattern over a broad developmental period, to include stages E14.5 to P5. This period spans the four earliest stages of prostate ductal development: prostatic bud specification, when molecular signals specify where prostatic buds will form from UGE (E14.5-16.5); prostatic bud initiation, when buds first project from UGE (E16.5-18.5); prostatic bud elongation, when prostatic buds extend into UGS stroma and prostatic ductal branching morphogenesis (E16.5-P5) (Sugimura et al.,1986, Vezina et al.,2008, Lin et al.,2003).

Fetal and neonatal mouse UGS sections were stained by ISH to visualize *Wif1* mRNA. To determine whether *Wif1* expression in mouse UGS is associated with AR activity, we compared its temporal and spatial expression to that of a known androgen-responsive gene, *Srd5a2* (Abler et al.,2011, Matsui et al.,2002). To determine whether *Wif1* is present in UGS prior to prostatic specification, we compared its temporal expression pattern to that of the earliest known mRNA marker of the mouse prostate field, *Nkx3-1* (24, Bhatia-Gaur et al.,1999). To determine the onset and duration of sexually dimorphic *Wif1* mRNA expression, we compared its expression in male and female UGS at all examined developmental stages (Fig.1). Tissues were also stained by IHC to visualize epithelium with cadherin 1 (CDH1, red) and smooth muscle actin

alpha 2 (ACTA2, green) so that *Wif1* expression could be assessed and described in the context of other tissue compartments (Abler et al.,2011).

Wif1 mRNA was detected in male and female UGS at E14.5, prior to prostate field specification as noted by the onset of *Nkx3-1* expression (Fig.1 A-E). *Wif1* was more abundant in male compared to female UGS stroma beginning at E15.5 (Fig.1 F,G) and continuing until at least P5 (Fig.1 U,V). Intriguingly, the onset and duration of sexually dimorphic *Wif1* expression in UGS stroma coincided temporally with onset and duration of sexually dimorphic *Srd5a2* mRNA expression in UGS stroma (Fig.1 H-I). Beginning at E15.5 and continuing until at least P5, *Wif1* and *Srd5a2* mRNAs were particularly concentrated in male urethral lamina propria mesenchyme (Fig.1). This stromal subcompartment, positioned between UGE (labeled by CDH1, red) and urethral muscularis propria (labeled by ACTA2, green) (Fig. 1) (Abler et al.,2011), was previously identified as a key site of AR action during prostate development (Takeda et al.,1987). *Wif1* also exhibited sexually dimorphic expression in UGE. Beginning at E16.5 and continuing until at least P5, *Wif1* was co-expressed with *Nkx3-1* in male UGS and prostatic bud epithelium (Fig.1). *Wif1* was also detected in male Wolffian duct, seminal vesicle and ejaculatory duct mesenchyme between E14.5-P5 and in urethral smooth muscle between stages E16-P5. In female UGS, *Wif1* localized to lower vagina (sinus vagina) mesenchyme and to a discrete population of ventral urethral basal epithelium (Fig. 1 F, K, P). The latter is an anatomical region where small *Nkx3-1* positive epithelial buds have been documented in female C57Bl/6J mouse fetuses (Allgeier et al.,2010). Collectively, these results reveal localized *Wif1* expression in androgen-responsive male UGS stroma and in epithelial bud structures of male and female UGS.

To confirm ISH results showing that *Wif1* is more abundant in male versus female UGS, cDNA from microdissected E17.5 male and female UGE and UGM was analyzed by real-time PCR. We confirmed the purity of each cDNA preparation by demonstrating selective expression of *Acta2* mRNA in the UGS mesenchymal cDNA preparation and *Krt14* in the UGS epithelial cDNA preparation (Supplemental Fig. 1C). We then demonstrated that *Wif1* mRNA was more abundant in male compared to female UGM and UGE (Supplementary Fig. 1C). Taken together, these results reveal that *Wif1* is present in the fetal mouse UGS where it is more abundant in male compared to female fetuses.

Androgens are necessary and sufficient for *Wif1* mRNA expression in fetal mouse prostate mesenchyme

Prostate development requires AR activation in UGM (4, 5). The observation that *Wif1* is more abundant in male compared to female UGM, coupled with the fact that *Wif1* expression overlaps spatially and temporally with androgen-responsive *Srd5a2*, raised the hypothesis that *Wif1* mRNA expression in UGM is regulated by androgens. We assessed whether androgens are necessary for *Wif1* mRNA expression in UGM prior to prostatic bud formation (E14.5), remain necessary after prostatic bud initiation (E17.5) and are sufficient for *de novo* *Wif1* expression in E17.5 female UGM. E14.5 male and E17.5 male and female UGSs were incubated for 24h in organ culture media containing DHT and either vehicle alone or vehicle containing the AR antagonist OHF. Following the culture period, UGSs were stained by ISH to visualize *Wif1* mRNA (pseudocolored green) and by IHC with anti-CDH1 (red, all epithelium) and DAPI (blue)

to localize *Wif1* mRNA expression. Androgen-responsive *Srd5a2* mRNA (Abler et al., 2011) was also visualized to confirm pharmacological sufficiency of AR agonist and antagonist treatments. In male UGSs, OHF treatment noticeably reduced *Srd5a2* and *Wif1* abundance in UGM but not in prostatic bud epithelium (Fig. 2A-J insets). In female UGSs, DHT treatment activated *Srd5a2* and *Wif1* expression specifically in UGM and this action was blocked by OHF (Fig. 2E,F,K,L insets). To independently confirm the requirement of androgens for *Wif1* expression in UGM, timed pregnant dams were dosed with AR antagonist flutamide prior to prostatic bud formation (E13.5 and E14.5) or during prostatic bud formation (E17.5 and E18.5). Male UGSs were collected on E15.5 and P0 and stained by ISH to visualize *Wif1* and *Srd5a2* mRNA (purple) and by IHC with anti-CDH1 (red, all epithelium) and anti-ACTA2 (green, smooth muscle) (Fig. 2M-T). Flutamide treatment noticeably reduced *Srd5a2* and *Wif1* abundance in male UGM (Fig. 2M-T insets) but not *Wif1* abundance in male prostatic bud epithelium (Fig. 2S,T arrowheads). These results support the hypothesis that androgens and active AR signaling are necessary and sufficient for *Wif1* mRNA expression in fetal UGM but not UGE.

Androgens are necessary and sufficient for *Wif1* expression in the adult mouse prostate stroma

We next tested whether androgens are necessary for *Wif1* expression in adult prostate stroma. Male mice were either sham castrated (control) or castrated to reduce circulating androgens and induce prostate involution. There was a significant reduction in relative LUT wet weight among 3d castrated males (3dC) compared to 3d sham-

castrated controls (3dS, Supplemental Fig. 2). Tissues were stained by ISH to visualize *Srd5a2* and *Wif1* mRNA (purple) and by IHC with anti-CDH1 (red), anti-ACTA2 (green) and DAPI (blue) to localize mRNA expression. *Srd5a2* and *Wif1* mRNAs were detected in ventral prostate stroma of both experimental groups but both mRNAs were visibly less abundant in 3dC versus 3dS (Fig. 3 A-B, E-F). We observed a similar trend in other prostate lobes and in seminal vesicle (Supplemental Fig. 3). These results suggest a testicular androgen requirement for normal *Wif1* mRNA expression in adult mouse prostate stroma.

We next tested whether exogenous androgen would restore *Wif1* expression in castrated mouse prostate stroma. 14d castrated mice were implanted with empty or testosterone-filled Silastic® capsules and 3d later were euthanized. Relative LUT wet weight was significantly greater among 14d castrated males implanted with testosterone capsules (14dC+3dT) than among males implanted with empty capsules (14dC+3dE, Supplemental Fig. 2). *Wif1* and *Srd5a2* mRNAs were weakly expressed in 14dC+3dE ventral prostate stroma and both were noticeably more abundant in 14dC+3dT ventral prostate stroma (Fig. 3 C-D, G-H). The same trend existed in the other prostate lobes and seminal vesicle (Supplemental Fig. 3). Collectively these results indicate that androgens are required for *Wif1* expression in the intact adult mouse prostate and are sufficient for reactivating *Wif1* expression during regeneration of the castrated adult mouse prostate.

Exogenous WIF1 protein enhances androgen-dependent prostatic bud formation and basal epithelial cell proliferation

Formation and continued development of *Nkx3-1* positive prostatic buds requires ARs in UGM (4, 5) but AR-responsive genes mediating this action are largely unknown. We hypothesized that WIF1 enhances AR action by stimulating prostatic bud formation. To test this hypothesis, E14.5 male UGSs were grown in UGS organ culture media containing DHT and graded concentrations of exogenous recombinant human WIF1 protein (Fig. 4 A-F). Recombinant human WIF1 protein is 93% homologous to the mouse ortholog and its ability to impair WNT/ β -catenin signaling was demonstrated previously in an array of species and tissues including the *Xenopus* embryo, chicken heart, and mouse thymus (Hsieh et al.,1999-Mathieu,2006). Whole mount ISH was performed as described previously to visualize and quantify *Nkx3-1* positive prostatic buds formed per UGS (24). Exogenous recombinant WIF1 protein elicited a graded increase in *Nkx3-1* positive buds (Fig. 4 G) which supports the hypothesis that WIF1 enhances androgen-dependent prostatic bud formation.

Since WIF1 enhances prostatic bud formation and developing prostatic buds are comprised of basal epithelium, we next tested the hypothesis that WIF1 enhances androgen-induced UGS basal epithelial cell proliferation. UGS explants were incubated in organ culture media as described above, cut into sagittal sections and immunofluorescently stained to reveal mitotically active cells (phospho-histoneH3-positive, PH3+, red) and basal epithelial cells (keratin 14-positive, KRT14+, green) (Fig. 5 A-D). Exogenous WIF1 concentrations that significantly increased *Nkx3-1* positive prostatic bud number (500ng/mL, 1000ng/mL; Fig. 4) also significantly increased the mean fraction of PH3⁺KRT14⁺/total KRT14⁺ cells (Fig. 5 E). These results show a pro-proliferative action of WIF1 on UGS basal epithelium.

Exogenous WIF1 does not visibly change WNT/ β -catenin target gene abundance in UGS explants

The most widely accepted *Wif1* mechanism of action is functional antagonism of WNT/ β -catenin signaling (Hsieh et al.,1999, 36, Surmann-Schmitt et al.,2009). Yet, in developing mouse prostate, *Wif1* is expressed in UGM and UGE and overlaps WNT/ β -catenin-responsive *Axin2* and *Lef1* expression (Pritchard et al.,2008, Zhang et al.,2006, Mehta et al.,2011). Co-expression of *Wif1* with abundant WNT/ β -catenin-responsive mRNA expression would not be predicted if WIF1 functioned as a strong WNT/ β -catenin inhibitor in control UGS. We tested whether prostatic bud-enhancing concentrations of exogenous WIF1 protein visibly reduced WNT/ β -catenin responsive *Axin2* and *Lef1* mRNA abundance within and immediately surrounding prostatic bud tips where both mRNAs were detected previously (19). E14.5 male UGSs were grown for 4d in UGS organ culture media containing DHT and graded recombinant WIF1 protein concentrations. ISH was used to visualize *Axin2* and *Lef1* mRNAs. *Axin2* and *Lef1* were detected in prostatic bud epithelium in all experimental groups and exogenous recombinant WIF1 did not visibly alter their abundance (Fig. 6). Presence of WNT/ β -catenin target genes and *Wif1* at the same time and location during embryonic prostatic development suggests at least two possible mechanisms for prostatic bud enhancement by WIF1: a subtle β -catenin-dependent action we are unable to detect or a β -catenin-independent action.

Exogenous WIF1 is not sufficient to induce prostatic budding in the absence of androgens

To determine whether WIF1 is the only androgen-induced signal needed for prostatic bud formation, E14.5 female UGSs, naïve to high levels of androgens, were cultured for 4d in androgen-free media containing recombinant WIF1. As a positive control, female UGSs were cultured in media containing 10nM DHT and no recombinant WIF1. Following the culture period, ISH was used to visualize three different prostatic bud-marking mRNAs: *Nkx3-1*, *Wnt10b* and *Edar* (19, 24, Abler et al., 2011). While *Nkx3-1* is considered the best marker of prostatic buds, *Wnt10b* and *Edar* are additional mRNAs restricted to distal tips of prostatic buds and are not detected in urethral gland buds (19, 24, 26, 32). As expected, DHT induced the formation of *Nkx3-1*, *Wnt10b* and *Edar* positive prostatic buds in female UGS explants (Fig. 7 D, H, L solid arrowheads). The only *Nkx3-1* positive buds formed in androgen-free medium were in the ventral UGS region where small buds were previously shown to form by an androgen-independent mechanism (Fig. 7 A-C open arrowheads) (34). Exogenous WIF1 protein concentrations greater or equal to those that increased prostatic bud number in the presence of androgens (Fig. 4; $\geq 500\text{ng/mL}$) did not increase *Nkx3-1* (Fig. 7 A-C), *Wnt10b* (Fig. 7 E-G) or *Edar* (Fig. 7 I-K) positive prostatic bud number in the absence of androgens. Therefore, the formation of large prostatic buds that normally occur in male UGSs likely requires the actions of other androgen-induced factors in collaboration with WIF1.

Prostatic bud number is unaltered in *Wif1* mutant mice

We next assessed whether endogenous *Wif1* is required for androgen-dependent prostatic bud formation *in vivo*. To test this hypothesis, we used a *Wif1* targeted mutant mouse containing a knock-in *lacZ* reporter cassette that disrupts the *Wif1* coding sequence (Kansara et al., 2009). E18.5 male *Wif1* wild type (*Wif1*^{+/+}) and mutant (*Wif1*^{lacZ/lacZ}) whole mount UGSs were stained by ISH to visualize and quantify *Nkx3-1* positive prostatic buds. There were no significant genotypic differences in the pattern or number of ventral, anterior, dorsolateral or total number of prostatic buds formed (Fig. 8 A-C). Therefore, *Wif1* is not required for normal prostatic bud formation. We investigated the possibility that WIF1 acts by a functionally-redundant mechanism (potentially in concert with other WNT antagonists) to promote prostatic bud formation. Specifically, we tested whether *Wif1* mutant mice exhibited a visible compensatory increase in *Sfrp* mRNAs. SFRPs have been identified in the developing prostate and like WIF1 are capable of binding and sequestering WNT proteins (14, 15, 20). E18.5 *Wif1* mutant and wild type UGSs were stained by ISH to visualize the mRNA expression of *Sfrp1*, *Sfrp2*, *Sfrp3* (also known as *Frzb*) and *Sfrp4* mRNAs. There was no noticeable genotypic difference in *Sfrp1* or *Sfrp4* abundance (results not shown) but there was noticeably more *Sfrp2* and *Sfrp3* mRNAs in *Wif1* mutant UGSs compared to wild type UGSs (Fig. 8 D-G). Increased *Sfrp2* and 3 mRNA abundance in *Wif1* mutant UGSs suggests that balanced WNT signaling is likely to be important in prostatic bud formation.

Androgens may regulate *Wif1* expression by decreasing DNA methylation

Wif1 is known to be regulated by DNA methylation (Costa et al 2010). We next examined whether androgens are capable of increasing *Wif1* expression by decreasing DNA methylation. Methylated DNA immunoprecipitation (MeDIP) followed by QPCR for *Wif1* was conducted on E17.5 male and female UGSs. There was a trend for less *Wif1* DNA methylation in male versus female UGSs (Fig. 9A). We also examined if androgens themselves are at least partially responsible for the observed trend in *Wif1* DNA methylation differences between male and female UGSs. E14.5 male UGSs were incubated for 4 days in organ culture media containing vehicle alone and the AR antagonist OHF or DHT alone. There was a trend for reduced *Wif1* DNA methylation in the presence of androgen versus AR antagonist (Fig. 9B). We next tested whether changes in DNA methylation are sufficient to induce *Wif1* mRNA expression in the absence of androgen. E14.5 female UGSs (where *Wif1* expression is low) were incubated for 4 days in the absence of androgen and with or without DNA methylation inhibitor (5uM, 5-aza-2-deoxycytidine), followed by ISH for *Wif1* mRNA. Inhibition of DNA methylation alone was sufficient to induce *Wif1* mRNA expression in female UGS in a pattern similar in distribution to that induced by androgens in male UGS (Fig. 9C). Together these results suggest androgens may regulate *Wif1* expression by preventing or decreasing *Wif1* DNA methylation.

Discussion

Fetal mouse urethral lamina propria mesenchyme is revealed as a key site of *Wif1* expression and AR action

It has long been known that AR action in UGS stroma is necessary for prostate development (Sugimura et al.,1986, Cunha et al.,1980Cunha,1973-Lasnitzki et al.,1980). Yet, neither the cellular composition of UGS stroma nor AR activity within UGS stroma is homogeneous (Abler et al.,2011). This study, in conjunction with other recent studies, refines the subanatomical location of AR action in the developing mouse prostate. Fetal urethral lamina propria mesenchyme, positioned between urethral urothelium and muscularis propria was hypothesized long ago to be an important site of AR action (Takeda et al.,1987). More recent studies identified this subanatomical region as the predominant site of AR-responsive *Srd5a2* expression in mouse (Abler et al.,2011) and rat (Berman et al.,1995) and of male-enriched *Sfrp2* expression in mouse (Zhang et al.,2006). We have now identified *Wif1* as an androgen-responsive mRNA in fetal mouse urethral lamina propria mesenchyme. As the prostate development field moves forward, a focus on this particular fetal prostate subcompartment, which accounts for only about 10% of total UGS stroma, will likely yield information about AR-mediated mechanisms of prostate morphogenesis.

We know very little about prostate stromal cell fate but identification of fetal mouse urethral lamina propria mesenchymal cells as a key site of AR action may help to close this knowledge gap. For example, epithelial-mesenchymal interactions are crucial for fetal prostate mesenchyme differentiation (Cunha,2008). Whether urethral lamina propria mesenchyme gives rise to smooth muscle or other UGS stromal cell populations or participates in the instructive patterning of other UGS stromal constituents (blood vessels, nerves, urethral sphincter skeletal muscle) is unknown. Furthermore, whether urethral lamina propria cells expand clonally during prostatic bud

elongation and branching to produce AR-positive peri-ductal mesenchyme cells or whether AR activity is stimulated *de novo* in these cells is unknown. A transgenic mouse that expresses an inducible tag would allow for lineage tracing in urethral lamina propria mesenchyme and would resolve some of these questions.

***Wif1* appears to be differently regulated in UGS mesenchyme compared to UGS epithelium**

A better understanding of *Wif1* transcriptional regulation during prostate development will shed light on the mechanism of AR-induced prostatic bud formation. We identified *Wif1* in UGM and UGE. Differences in onset, duration, pattern and androgen-dependence of *Wif1* expression in UGM and UGE suggest distinct *Wif1* transcriptional regulatory mechanisms in each of these fetal prostate tissue compartments.

In fetal UGE, WNT/ β -catenin signaling is more likely than AR signaling to activate *Wif1* transcription. Although AR protein is present in UGE (Abler et al.,2011, Takeda et al.,1987), its presence there does not lead to appreciable binding of ligand (Takeda et al.,1987), is expendable for prostatic bud formation (Cunha et al.,1987) and is not needed for *Wif1* expression (Fig. 2). In contrast, β -catenin activity associates with *Wif1* expression in other tissues (Reguart et al.,2004Zirn et al.,2006-Martinez et al.,2009), is required for prostatic bud formation (Vezina,, Schmitz et al.,2011) and positively regulates the target genes *Axin2* and *Lef1*, which associate spatially and temporally with *Wif1* in UGE (Mehta et al.,2011, www.gudmap.org).

In fetal UGM, AR signaling is more likely than WNT/ β -catenin signaling to activate *Wif1* transcription. AR protein is present in UGM, binds ligand and is required for prostatic bud formation (1, 3Cunha,1973-5). Although WNT/ β -catenin–responsive *Axin2* and *Lef1* are present in UGM, both transcripts are detectable at an earlier developmental stage and in a different pattern than *Wif1* (Mehta et al.,2011, www.gudmap.org), do not persist in UGM as long as *Wif1* and are not reliant on a high level of androgen signaling in the UGM like *Wif1*. An important future direction of our research is to determine whether *Wif1* transcription in UGM and UGE is regulated directly or indirectly by AR and β -catenin activities. Further, whether androgens regulate *Wif1* expression by preventing or decreasing DNA methylation is an intriguing avenue of future study. The implications of androgens being capable of eliciting their actions by altering the epigenome could help explain unresolved questions related to prostate development and disease mechanisms.

***Wif1* promotes prostatic bud formation and fetal prostate basal epithelial cell proliferation**

A preponderance of evidence supports the idea that WIF1 acts as a suppressor of adult prostate epithelial cell proliferation (Yee et al.,2010Costa et al.,2010Ohigashi et al.,2005-Wissmann et al.,2003). While few studies have examined WIF1 expression and function during prostate development, Li *et al.* (Li et al.,2009) overexpressed WIF1 in UGM, combined the UGM with adult bladder epithelium and grew the tissue recombinants under the kidney capsule of immunocompromised mice. They found that WIF1 overexpression in UGM impaired its ability to reprogram adult bladder epithelium

into prostate epithelium, which suggests that abnormally high *Wif1* expression in UGM impairs the instructive signals needed for prostate development (Li et al.,2009).

The results of the current study differ from those described above. We found exogenous recombinant WIF1 protein, at media concentrations of 500-1000 ng/mL, enhanced androgen-dependent mouse prostatic bud formation and basal epithelial cell proliferation (Fig. 4-5). Discrepancies in WIF1 action reported in our study versus those described above are likely a function of prostate cell populations present, prostate developmental stages examined and WIF1 concentrations used. Studies conducted in cell lines (Yee et al.,2010, Ohigashi et al.,2005) or human prostate carcinomas (Wissmann et al.,2003) vary in their AR responsiveness, exhibit a cancerous phenotype and do not fully recapitulate the mesenchymal-epithelial interactions present during prostate development. Additionally, interactions between fetal UGM and adult bladder epithelium under the kidney capsule of an immunocompromised mouse (Li et al.,2009) may not be the same as the interactions between intact fetal UGM and UGE in UGS culture medium.

While we found that exogenous WIF1 protein stimulated prostatic bud formation and UGS basal epithelial cell proliferation, *Wif1* was expendable for normal prostate development (Fig. 8). These results are consistent with observations made by others that *Wif1* is not required for morphogenesis of most mouse tissues (Kansara et al.,2009). A likely possibility is that other *Wnt* antagonists such as *Sfrps* are upregulated to compensate for deficient WIF1 activity in *Wif1* mutant UGSs. In support of this possibility, we observed noticeably more *Sfrp2* and 3 in *Wif1* mutant UGM compared to

wild type UGM. These results suggest a possible functional redundancy among SFRPs and WIF1 during prostate development.

Possible mechanisms by which WIF1 could enhance fetal prostatic bud formation

One of the most important aspects of the current study is that it reveals WIF1 as one of the first androgen-regulated genes that mediates an androgen action during prostate development. An intriguing question that was raised as a result of our study is how WIF1 enhances prostatic morphogenesis. Though we did not directly answer this question, at least two possible mechanisms should be considered for future investigation. Even though exogenous WIF1 concentrations that induced prostatic bud formation did not elicit noticeable changes in WNT/ β -catenin-responsive *Axin2* and *Lef1* mRNA expression (Fig. 6), we cannot exclude the possibility that it causes small changes that we were unable to detect. We do not fully understand the concentration-response relationship between β -catenin activity and prostatic bud formation and minor changes in this signaling pathway could account for biologically significant changes in prostate development. In chicken proepicardial explant cultures, for example, subtle changes in β -catenin activity in either the positive or negative direction elicit the same response: cardiomyocyte proliferation (Buermans et al., 2010). Therefore, although WNT/ β -catenin-responsive *Axin2* and *Lef1* mRNA abundance were not noticeably changed in UGS explants exposed to WIF1, it is possible that we failed to detect subtle or finely localized WIF1-mediated changes in WNT signaling that were important in shaping the pattern and number of prostatic buds formed in the developing mouse UGS.

Finally, it is possible that WIF1 enhances prostatic bud formation by a WNT-independent mode of action. Addition of exogenous recombinant WIF1 did not appear to alter WNT/ β -catenin target genes *Axin2* and *Lef1* (Fig. 6). Consistent with this finding, abundance of most WNT target genes was unaltered by inappropriately high levels of *Wif1* reported previously in UGM where *Tgfrb2* was conditionally deleted (Li et al.,2009). Vertebrate WIF1 typically functions as an inhibitor of WNT signaling, but *Drosophila* ortholog *shifted* modulates Hedgehog signaling (Glise et al.,2005). While it is thought that vertebrate WIF1 does not bind hedgehog, recent evidence suggests that it may be capable of weakly modulating Hedgehog activity (Avanesov et al.,2012). WIF1 is also capable of binding connective tissue growth factor *in vitro* independently of its inhibitory action on WNT signaling (Surmann-Schmitt et al.,2012). Whether WIF1 is capable of modulating Hedgehog signaling or whether WIF1 binds other signaling molecules during prostate development is unknown.

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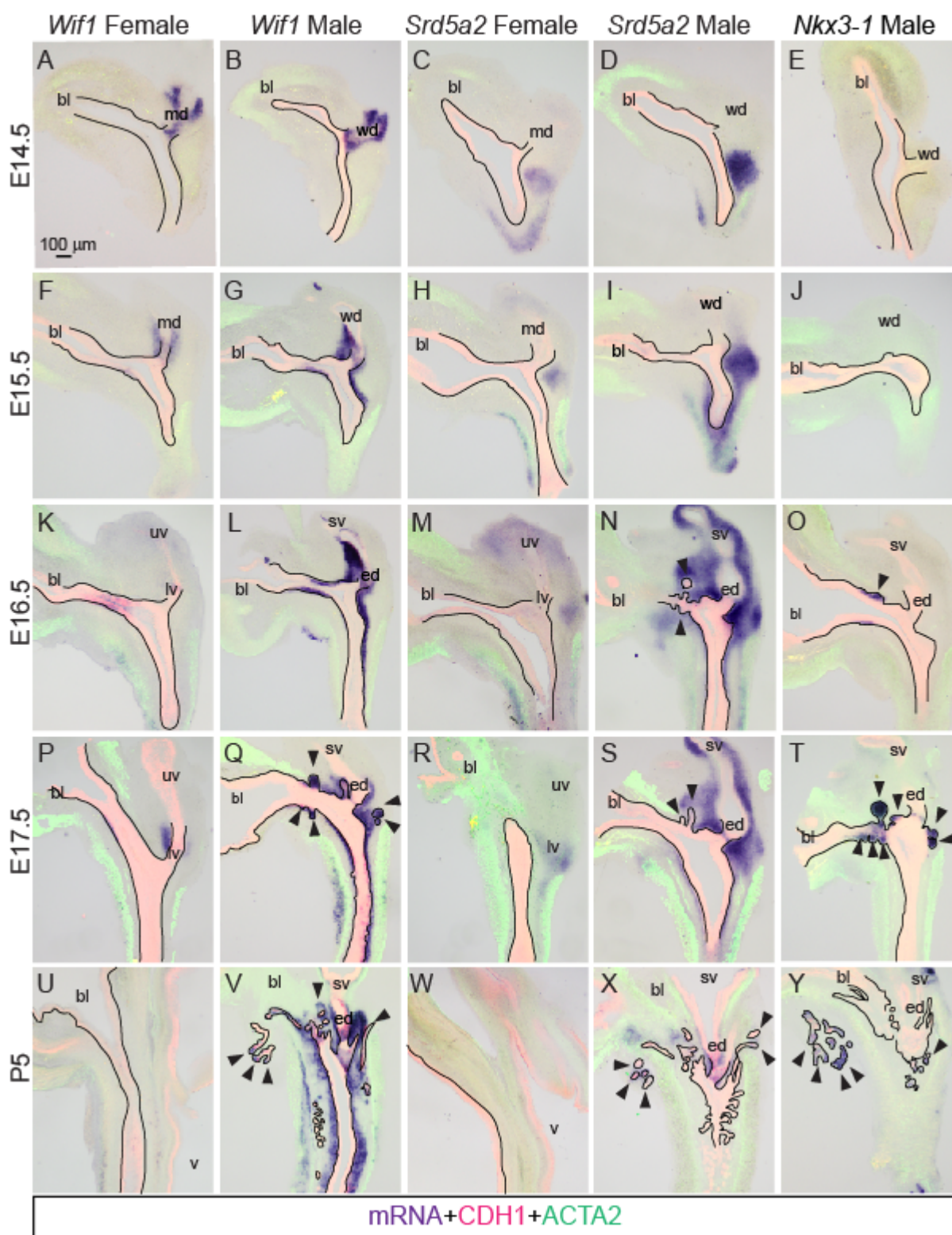


Fig. 1. *Wif1*, *Srd5a2* and *Nkx3-1* mRNA expression in developing male and female mouse urogenital sinus (UGS). Near mid-sagittal sections (50µm) of E14.5 – P5 male and female UGSs were stained by ISH to visualize mRNA expression (purple) of *Wif1*, *Srd5a2* (marker of prostatic stromal AR activity), and *Nkx3-1* (marker of prostatic buds). Sections were then immunofluorescently stained with antibodies against cadherin 1 (CDH1, red) to label all epithelium, and smooth muscle actin alpha 2 (ACTA2, green) present in mesenchyme. Results are representative of three litter-independent samples for each gender and stage. bl, bladder; ed, ejaculatory duct; lv, lower vagina; md, Müllerian duct; sv, seminal vesicle; uv, upper vagina; v, vagina; wd, Wolffian duct. Arrowheads indicate prostatic buds. A black line marks the interface between UGS epithelium and mesenchyme.

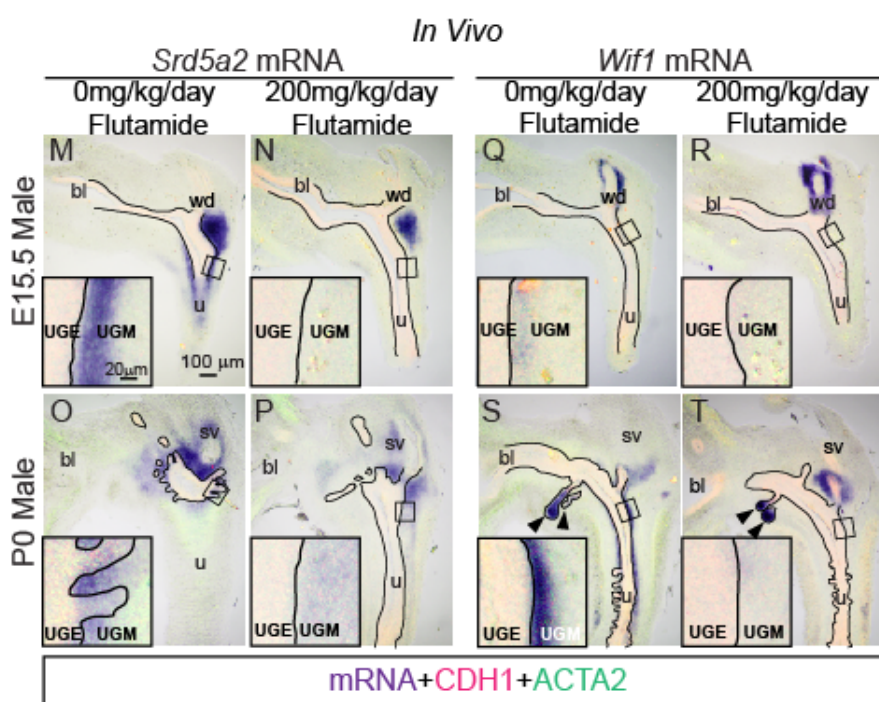
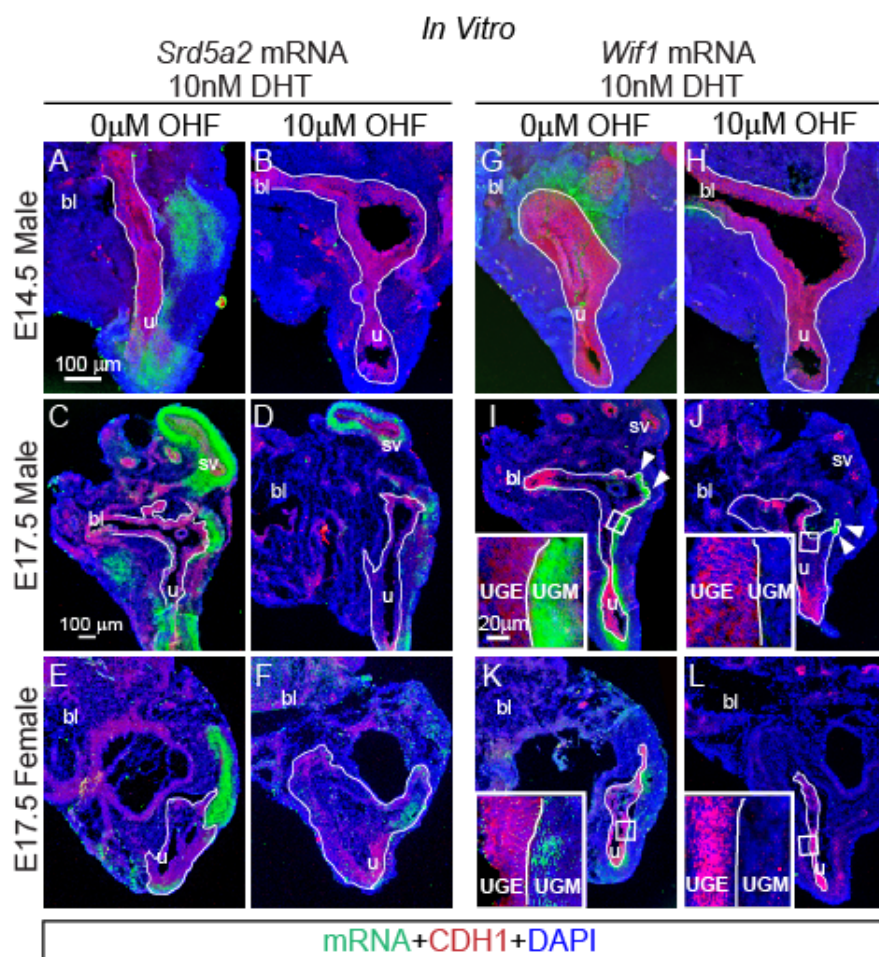


Fig. 2. AR signaling is necessary and sufficient for *Wif1* mRNA expression in fetal mouse prostate stroma. (A-L) E14.5 male or E17.5 male and female UGS explants were cultured for 24h in media containing 5 α dihydrotestosterone (DHT, 10nM) or DHT and the AR antagonist hydroxyflutamide (OHF, 10nM). Near mid-sagittal frozen sections (10 μ m) were stained by ISH to visualize (A-F) *Srd5a2* and (G-L) *Wif1* mRNAs (green). Sections were then immunofluorescently stained with anti-cadherin 1 (CDH1, red) antibody to label all epithelium and DAPI (blue). (M-T) Timed pregnant dams were dosed with corn oil alone or corn oil containing flutamide on E13.4 and E14.5 and male UGSs collected on E15.5 or dosed on E17.5 and E18.5 and male UGSs collected on P0. Near mid-sagittal sections (50 μ m) were stained by ISH to visualize (M-P) *Srd5a2* and (Q-T) *Wif1* mRNAs (purple). Sections were then immunofluorescently stained with anti-cadherin 1 (CDH1, red) antibody to label all epithelium and anti-ACTA2 (green) to label smooth muscle. Insets represent magnified images. Results are representative of three litter-independent samples per treatment group and were processed as a single experimental unit. bl, bladder; sv, seminal vesicle; u, urethra; UGE, UGS epithelium; UGM, UGS mesenchyme; wd, Wolffian duct. Arrowheads indicate presence of epithelial *Wif1* in prostatic buds. A white or black line marks the interface between UGS epithelium and mesenchyme.

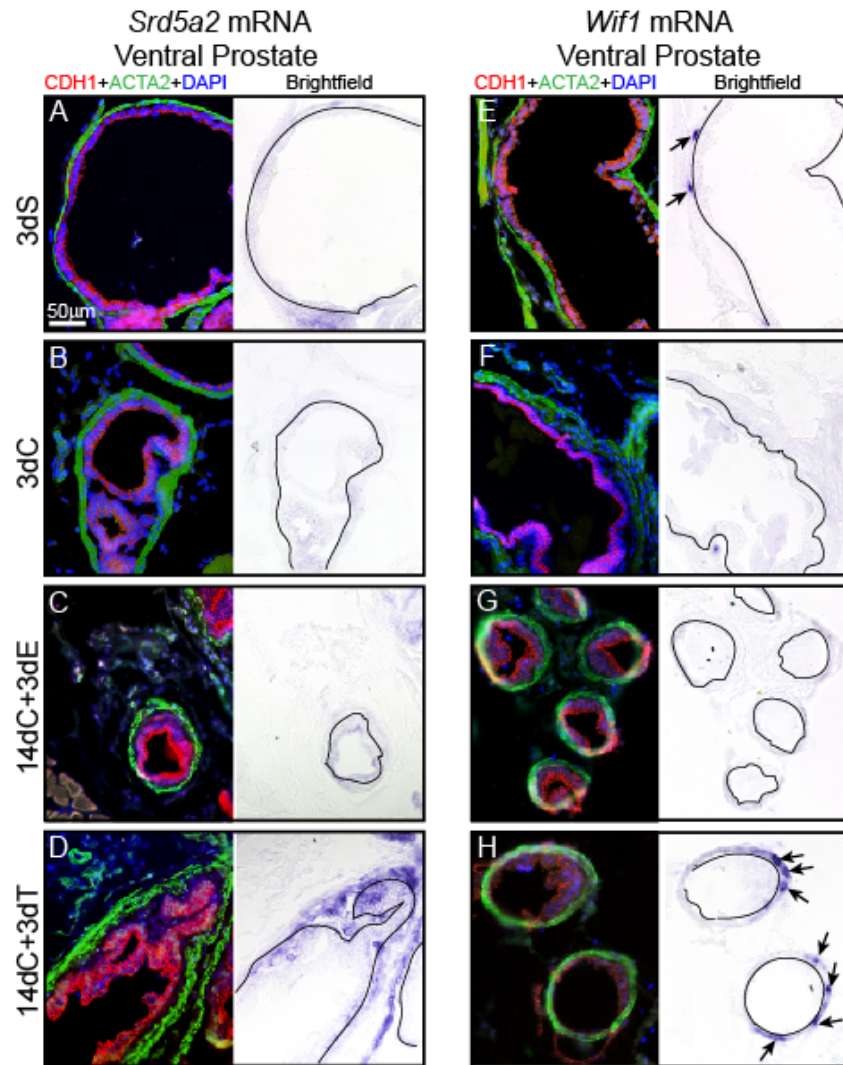


Fig. 3. AR signaling is necessary for *Wif1* mRNA expression in mature prostate stroma. Adult male mice (48-50d) were castrated or sham castrated. Prostate tissue was obtained from some mice 3d post-sham-castration (3dS) or castration (3dC), while other mice received empty (14dC+3dE) or testosterone-filled (14dC+3dT) Silastic® capsule implants 14d post-castration and prostate tissue was obtained 3d later. ISH was used to visualize (A-D) *Srd5a2* and (E-H) *Wif1* mRNA expression in frozen ventral prostate sections (10µm). ISH sections were then immunofluorescently labeled with antibodies against cadherin 1 (CDH1, red) to label all epithelium, smooth muscle actin alpha 2

(ACTA2, green) to label smooth muscle and DAPI (blue). Results are representative of three litter-independent samples per treatment group. Arrows represent *Wif1* mRNA positive muscle cells. A black line marks the interface between UGS epithelium and stroma.

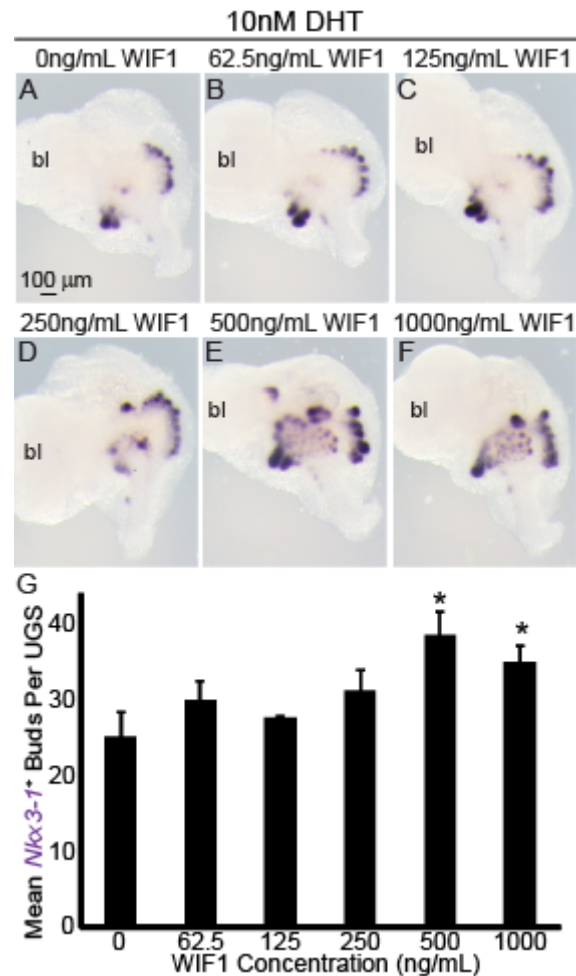


Fig. 4. Exogenous recombinant WIF1 enhances androgen-dependent prostatic bud formation *in vitro*. (A-F) E14.5 WT male UGSs were cultured for 4d in the presence of 10nM DHT and graded concentrations (0-1000ng/mL) of exogenous recombinant WIF1 protein. Whole mount tissues were then stained by ISH to visualize and quantify *Nkx3-1* mRNA-marked prostatic buds (purple). (G) Results are mean $\pm$ SEM, n = four litter-independent samples per group. Asterisks indicate a significant difference compared to 0ng/mL WIF1 ($p < 0.05$). bl, bladder.

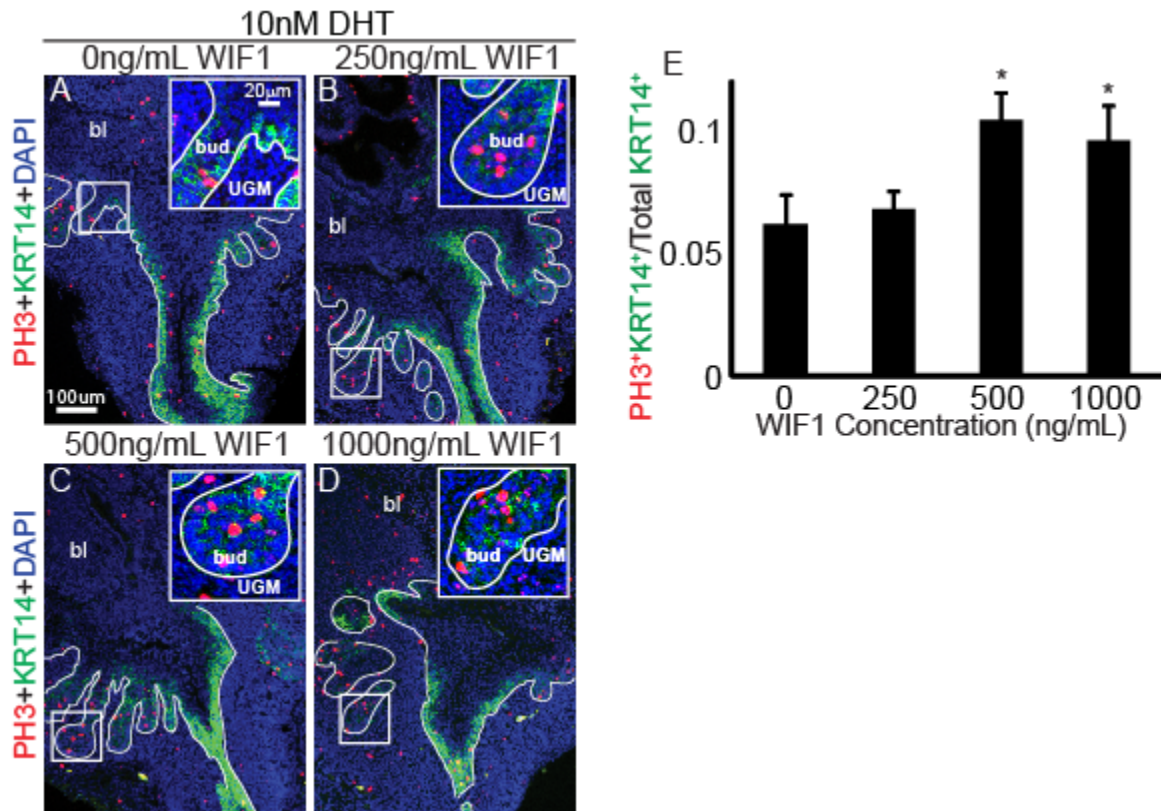


Fig. 5. Exogenous recombinant WIF1 enhances UGS basal epithelial cell proliferation *in vitro*. (A-D) E14.5 WT male UGSs were cultured for 4d in the presence of 10nM DHT and graded concentrations (0-1000ng/mL) of exogenous recombinant WIF1 protein. Near mid-sagittal sections (5 μ m) were immunofluorescently stained to visualize and count the percentage of keratin 14 (KRT14, green) positive basal epithelial cells with detectable nuclear phospho-histone H3 (PH3, red). Cell nuclei were visualized with DAPI (blue). (E) Results are mean $\pm$ SEM, n = three litter-independent samples per group. Asterisks indicate a significant difference compared to 0ng/mL WIF1 ($p < 0.05$). Square insets represent magnified images. bl, bladder; bud, prostatic bud; UGM, UGS mesenchyme. A white line marks the interface between UGS epithelium and mesenchyme.

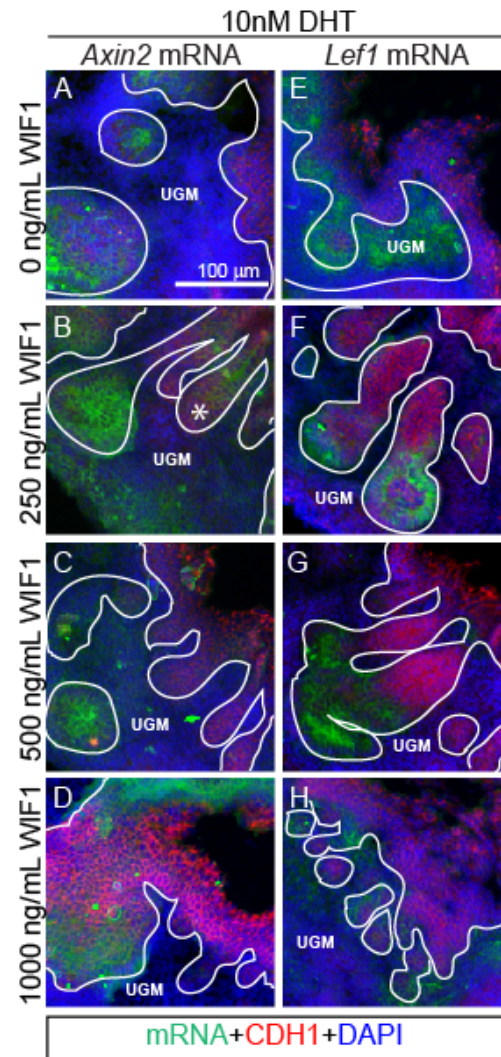


Fig. 6. Exogenous recombinant WIF1 does not visibly alter WNT/ β -catenin-responsive *Axin2* and *Lef1* mRNA expression. E14.5 WT male UGSs were cultured for 4d in the presence of 10nM DHT and exogenous recombinant WIF1 protein (0-1000ng/mL). Near mid-sagittal UGS sections (10μm) were stained by ISH to visualize (A-D) *Axin2* and (E-H) *Lef1* mRNAs (green). Sections were immunofluorescently stained with an antibody against cadherin 1 (CDH1, red) to label all epithelium, and with DAPI (blue). Results are n=3 litter-independent samples/group. UGM: UGS mesenchyme. A white line marks the interface between UGS epithelium and mesenchyme.

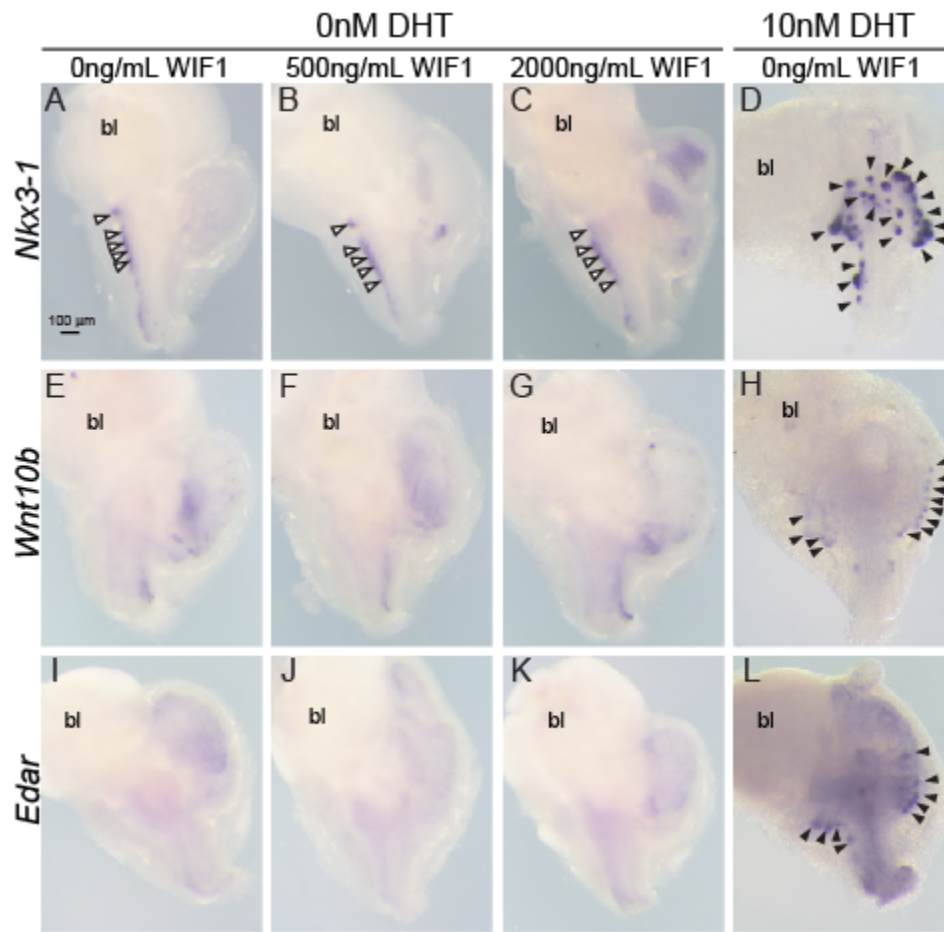


Fig. 7. Exogenous recombinant WIF1 does not stimulate androgen-independent prostatic bud formation *in vitro*. E14.5 WT female UGSs were cultured for 4d in media containing 10nM DHT (positive control) or in androgen-free media containing exogenous recombinant WIF1 protein. Whole mount tissues were then stained by ISH to visualize (A-D) *Nkx3-1*, (E-H) *Wnt10b*, (I-L) *Edar* mRNA-marked prostatic buds. Results are representative of five litter-independent samples per treatment group. bl, bladder. (A-C) Open arrowheads indicate *Nkx3-1* positive epithelial buds observed in females. (D, H, J) Solid arrowheads indicate prostatic and urethral gland buds.

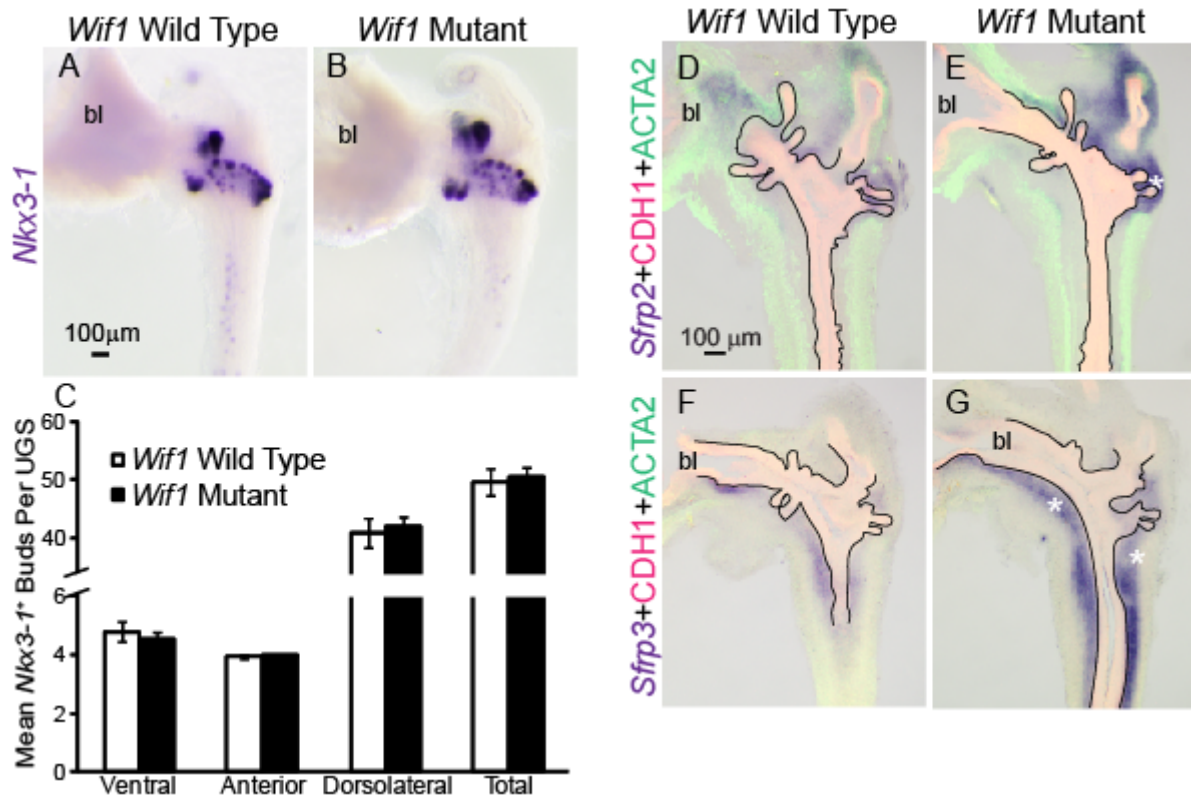


Fig. 8. *Wif1* is not required for normal prostatic bud formation *in vivo*. (A) E18.5 wild type control (*Wif1*^{+/+}) and (B) *Wif1* mutant (*Wif1*^{lacZ/lacZ}) male mouse UGSs were stained by ISH to visualize and quantify the number of *Nkx3-1* mRNA-marked prostatic buds per UGS. (C) Results are mean ± SEM, *n* = five litter-independent samples per group. (D-G) Near mid-sagittal sections (50 μm) of E18.5 *Wif1*^{+/+} and *Wif1*^{lacZ/lacZ} male UGSs were stained by ISH to visualize mRNA expression (purple) of secreted frizzled related proteins (*Sfrps*) 2 and 3. Sections were then stained by IHC with antibodies against cadherin 1 (CDH1, red) to label all epithelium and smooth muscle actin alpha 2 (ACTA2, green). Results are representative of two litter-independent samples per genotype. Asterisks indicate regions of differential mRNA expression between *Wif1*^{+/+} and *Wif1*^{lacZ/lacZ} male UGSs. bl, bladder. A black line marks the interface between UGS epithelium and mesenchyme.

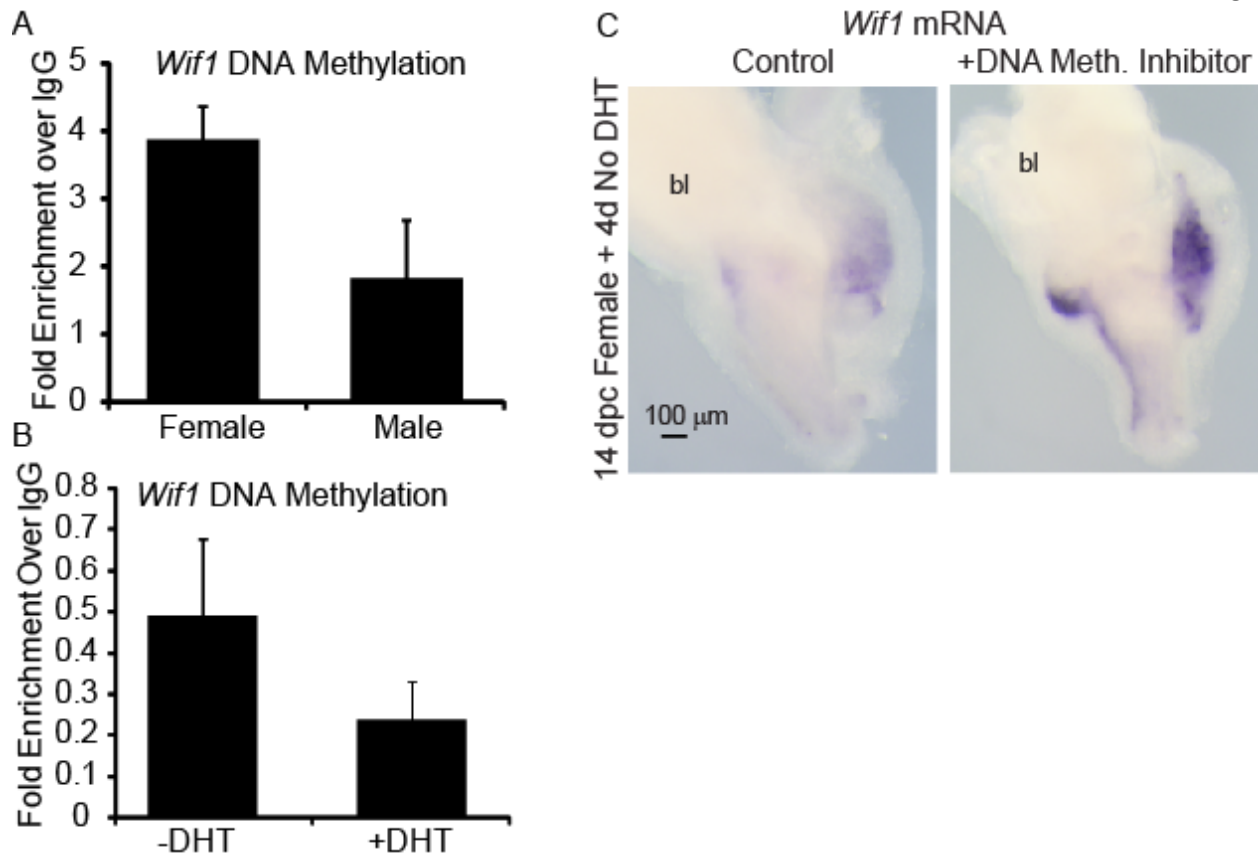
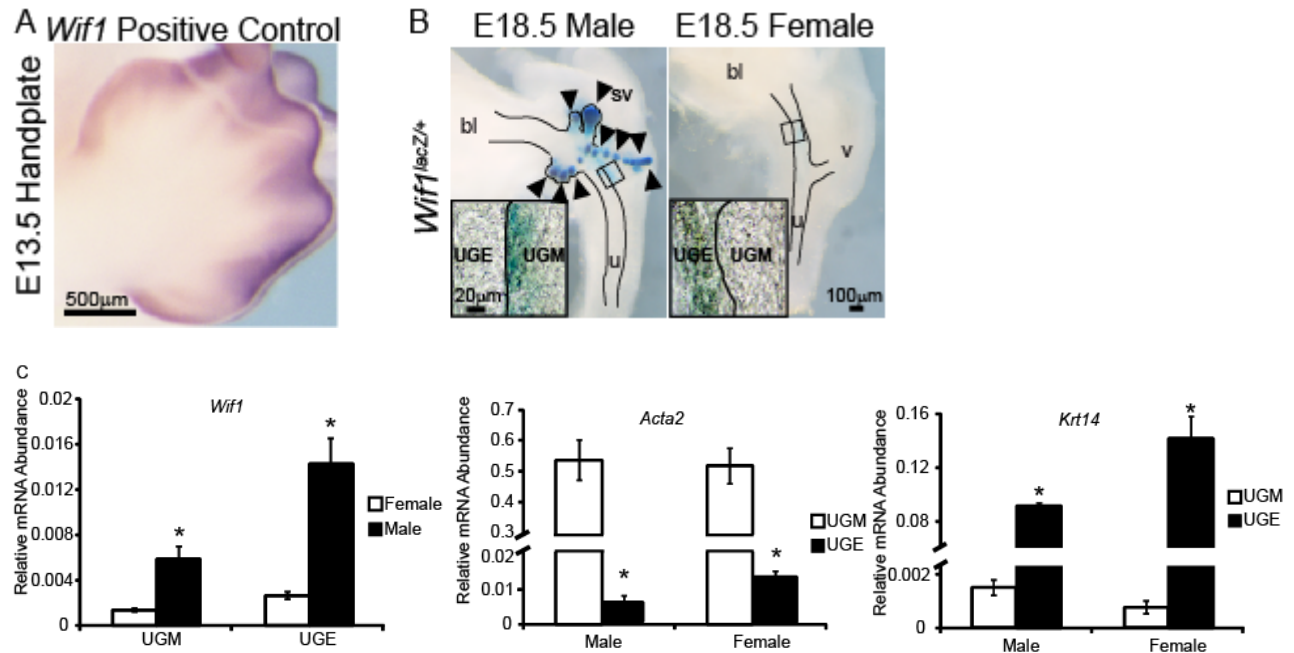
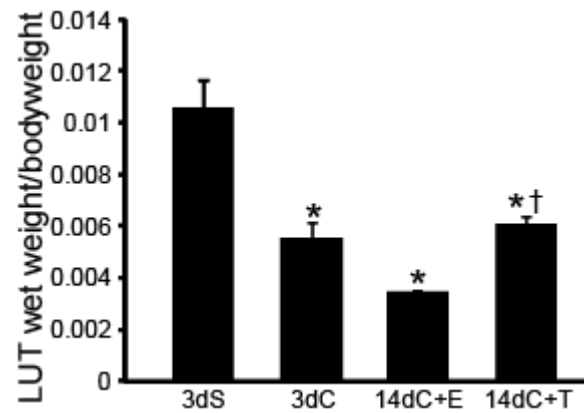


Fig. 9. Androgens may regulate *Wif1* expression by decreasing *Wif1* DNA methylation.

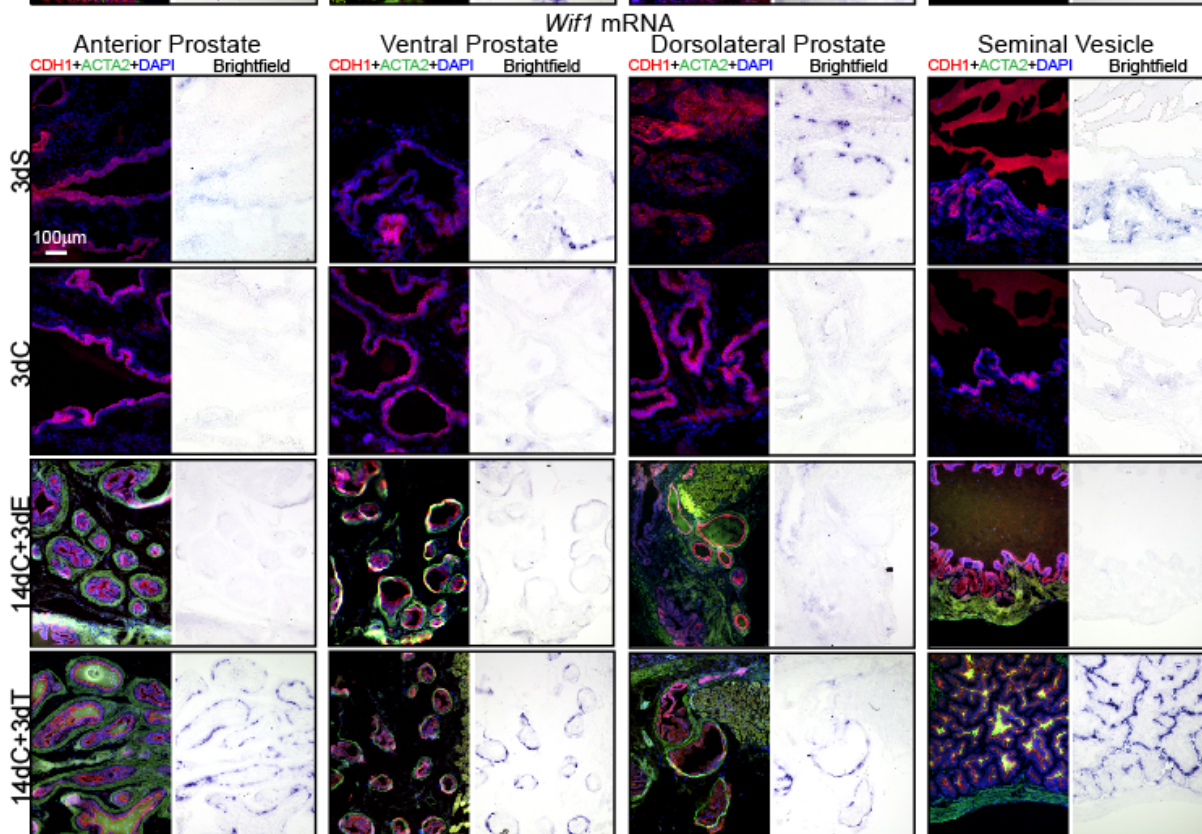
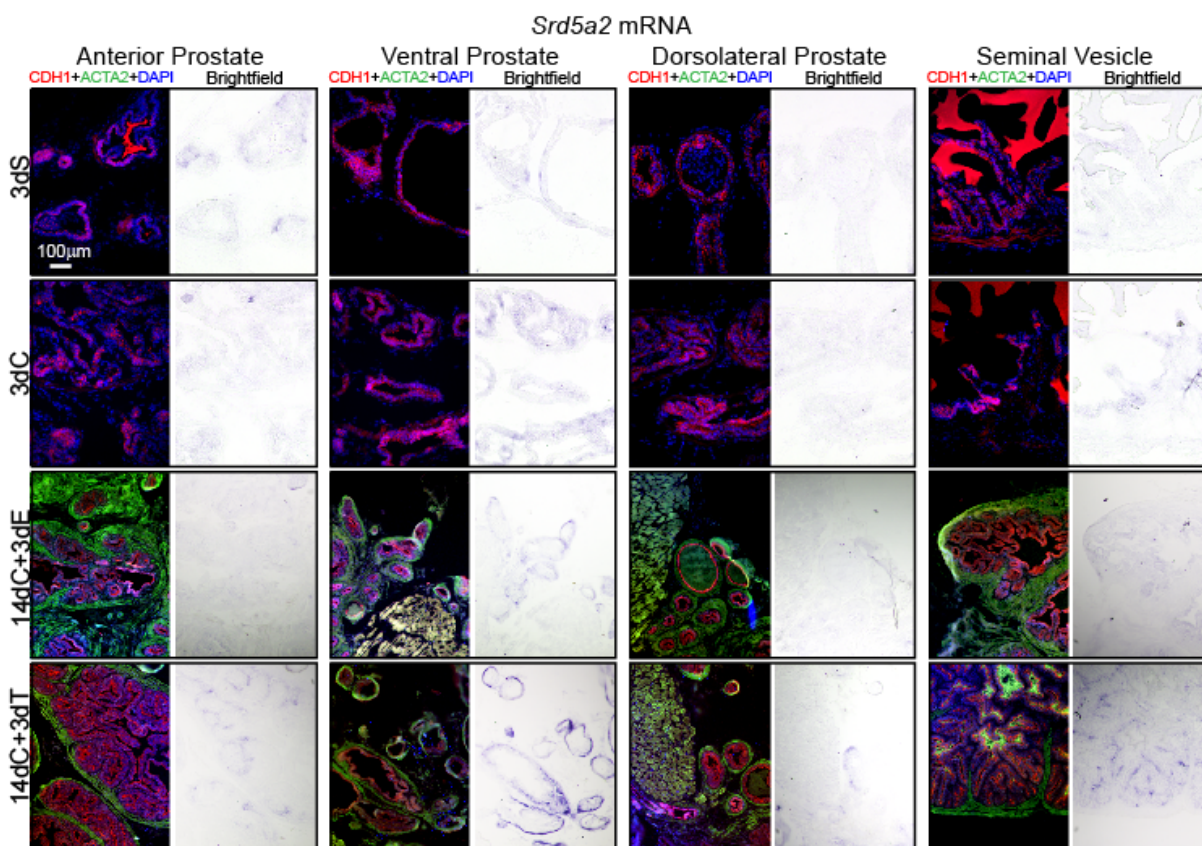
Methylated DNA immunoprecipitation (MeDIP) was performed on (A) E17.5 male and female UGSs and (B) E14.5 male UGSs cultured for 4 days in the absence or presence of androgen (10nM, dihydrotestosterone, DHT) to determine levels of *Wif1* DNA methylation relative to control IgG. (C) In situ hybridization for *Wif1* expression (purple) in E14.5 female UGSs cultured for 4d in androgen free media containing vehicle or DNA methylation inhibitor (5uM 5-aza-2-deoxycytidine). Results are mean $\pm$ SEM, $n \geq 3$ per group.



Supplemental Fig. 1. *Wif1* is more abundant in male compared to female UGM and UGE. (A) Whole-mount ISH was used to identify the expected pattern of *Wif1* mRNA in E13.5 handplate mesenchyme and thus verify *Wif1* riboprobe specificity. (B) β -galactosidase activity (blue) was assessed in E18.5 whole-mount male and female *Wif1^{lacZ/+}* UGS and (inset) tissues were cut into 50 micron sagittal sections to show staining in UGS epithelium and mesenchyme. bl, bladder; sv, seminal vesicle; UGE, UGS epithelium; UGM, UGS mesenchyme; v, vagina; u urethra. Arrowheads indicate prostatic buds. A black line marks the interface between UGS epithelium and mesenchyme. (C) *Wif1*, *Acta2* and *Krt14* mRNA abundance was determined by real-time RT-PCR on E17.5 male and female UGM and UGE and normalized to *Ppia* mRNA abundance. Results are mean $\pm$ SE of $n=3$ independent samples per group from at least 3 separate litters. Asterisks indicate a significant difference of $p \leq 0.05$.



Supplemental Fig. 2. Castration reduces relative prostatic wet weight which is restored upon addition of testosterone. Adult male mice (48-50d) were castrated or sham castrated. Relative prostate wet weight (bladder, prostate, urethra, seminal vesicle)/bodyweight was obtained from some mice 3d post sham-castration (3dS) or castration (3dC), while other mice received empty (14dC+3dE) or testosterone-filled (14dC+3dT) Silastic® capsule implants 14 days post castration and relative prostate wet weight/bodyweight was obtained 3d later. Asterisks indicate a significant difference from 3dS, a dagger indicates a significant difference from 14dC+3dE ($p < 0.05$, $n=5$).



Supplemental Fig. 3. AR signaling is necessary for *Srd5a2* and *Wif1* expression in mature prostate and seminal vesicle stroma. Adult male mice (48-50d) were castrated or sham castrated. Prostate tissue was obtained from some mice 3d post sham-castration (3dS) or castration (3dC), while other mice received empty (14dC+3dE) or testosterone-filled (14dC+3dT) Silastic® capsule implants 14d post-castration and prostate tissue was obtained 3d later. ISH was used to visualize *Srd5a2* and *Wif1* mRNA expression in frozen anterior, ventral, dorsolateral prostate and seminal vesicle sections (10µm). ISH sections were then immunofluorescently labeled with antibodies against cadherin 1 (CDH1, red) and DAPI (blue) or CDH1 (red) and smooth muscle actin alpha 2 (ACTA2, green). Results are representative of three litter-independent samples per treatment group.

Chapter 4: Catalog of mRNA expression patterns for DNA methylating and demethylating genes in developing mouse lower urinary tract

Keil et al., Gene Expr Patterns. (2013) 13:413-424

Introduction

The fetal mouse prostate develops from the definitive urogenital sinus (UGS), a sub-compartment of the lower urinary tract (LUT) located between the bladder neck and caudal urethra. Prostate buds arise from UGS epithelium and require active androgen signaling in UGS mesenchyme (Cunha,1973, Hayashi et al., 1993, Lasnitzki and Mizuno,1980). In C57Bl/6J mice, prostate buds emerge and elongate between 16-18 days post coitus (dpc) (Lin et al., 2003, Vezina et al., 2008). The prostatic buds undergo branching morphogenesis which peaks around postnatal day (P) 5 (Sugimura et al., 1986). While many questions remain about the mechanisms of prostate development, emerging evidence suggests that DNA methylation may play a role (Anway et al., 2006, Dolinoy et al., 2007, 2006, Gupta, 2000, Ho et al., 2006, Sebag et al., 2011, Tang et al., 2012, Timms et al., 2005, Walker, 2011).

DNA methylation is an epigenetic event required for appropriate regulation of gene expression during embryonic development and throughout life (Laird and Jaenisch,1996). We are only beginning to understand how methylation marks are established and remodeled during development and how these events contribute to morphogenesis. Evidence of a role for DNA methylation in androgen-dependent prostate development has emerged from the field of toxicology. The developing prostate is exquisitely sensitive to endocrine disrupting chemicals. Embryonic exposure to the estrogenic endocrine disruptor bisphenol A (BPA) changes DNA methylation patterns and permanently reprograms prostate gene expression (Anway et al., 2006, Dolinoy et al., 2007, 2006, Gupta, 2000, Ho et al., 2006, Tang et al., 2012, Walker, 2011). Further,

embryonic exposure to BPA and other estrogenic chemicals disturbs normal prostate budding morphogenesis and gene expression (Ho et al., 2006, Timms et al., 2005).

The prostate's requirement of active androgen signaling (Cunha,1973, Hayashi et al., 1993, Lasnitzki and Mizuno,1980) and its sensitivity to endocrine disrupting chemicals capable of altering DNA methylation raise the possibility that hormone action via DNA methylation may play a role in prostate morphogenesis. There are several reports of DNA methylation being affected by hormone action. Glucocorticoids influence DNA methylation patterns during brain development (Crudo et al., 2013). Gender differences exist in patterns of mouse left ventricle DNA methylation (Sebag et al 2011). These patterns are changed by gonadectomy and associated with changes in gene expression, structure and function (Sebag et al 2011). In hormone responsive endometrium, DNA methyltransferase abundance changes during the menstrual cycle in synchrony with systemic hormone abundance (van Kaam et al., 2011, Vincent et al., 2011, Yamagata et al., 2009). Further, several genes display altered promoter methylation and mRNA abundance during the menstrual cycle, implantation and in endometriosis (Ding et al., 2012, Gao et al., 2012, Ghabreau et al., 2004, Wang et al., 2012, Yamagata et al., 2009, Zelenko et al., 2012). Changes in DNA methylation associated with polycystic ovary syndrome have been linked to androgens (Xu et al., 2011) and the androgen receptor (AR) interacts with and regulates epigenetic chromatin modifiers in adult prostate (Cai et al., 2011, Duan et al., 2012, Metzger et al., 2005, Wissmann et al., 2007). Together, these results suggest that hormone signaling can influence expression of DNA methylating proteins and model the DNA methylation landscape, raising the possibility that this could be a means of androgen action in the

developing prostate. These results also raise the possibility that there are periods during which appropriate DNA methylation and demethylation are critical for normal prostate morphogenesis.

We cannot begin to sort out the role of DNA methylation in normal prostate development and in aberrant development in response to endocrine disrupting chemicals and other teratogens until we know which players are involved and where they are expressed. The purpose of this study is to characterize the mRNA expression pattern of genes involved in DNA methylation and demethylation during murine prostate development. We use *in situ* hybridization (ISH) to characterize these expression patterns in the male LUT prior to prostate development (14.5dpc), during prostate bud formation (17.5dpc) and during branching morphogenesis (P5) and in the female LUT which does not form appreciable prostatic ducts in the absence of androgens (Allgeier et al., 2010). Our results reveal mRNA pattern changes across developmental time and between males and females, revealing the importance of characterizing these genes and their function during development. In future studies this characterization will enable us to test whether DNA methylation genes are regulated by steroid hormones, whether they mediate the actions of steroid hormones or whether they are needed for other reasons during prostate development.

Results

An ISH screen was conducted on 14.5 dpc, 17.5 dpc and P5 male and female 50-micron sagittal LUT sections as described previously (Abler et al., 2011a,b). The anatomical focus of this screen is the developing prostate and cranial aspect of the pelvic urethra from which it derives. The reason for analyzing mRNA expression in both

sexes in late embryogenesis and early neonatal periods is because androgen signaling is more abundant in male than female LUT at these stages (Cooke et al., 1991, Takeda et al., 1991, 1987). We therefore seek to reveal mRNA patterns that possibly correspond with androgen signaling and prostate development in males during these stages. Age- and gender-matched samples were processed in the same tube and stained and imaged as a single experimental unit consisting of at least three samples per group. Immunohistochemistry (IHC) was used to label epithelium (anti-CDH1, red) and smooth muscle (anti-ACTA2, green) in each ISH-stained sample. Labeled epithelium and smooth muscle define boundaries between cell populations to facilitate high-resolution and consistent pattern mapping for each mRNA within the LUT subcompartments illustrated in Figure 1 and described previously (Abler et al., 2011b). The anatomical descriptors are based on Edinburgh Mouse Atlas Project and GUDMAP ontologies (Burger et al., 2004, Little et al., 2007, www.gudmap.org). In the results that follow, we only highlight noteworthy temporal, spatial or sex differences in mRNA expression within the prostate and cranial aspect of the pelvic urethra. Expression patterns for additional urethra-associated tissue compartments (bladder neck, Wolffian duct, ejaculatory duct, seminal vesicle, caudal Müllerian duct, upper vagina and lower vagina) are summarized in Supplemental Table 1 but are not discussed further.

DNA methyltransferase mRNAs are dynamic during embryonic and postnatal LUT development

DNA methyltransferase (*Dnmt*) enzymes catalyze methylation of the 5' carbon in cytosine. Three mammalian DNMTs are catalytically active: *Dnmt1*, *Dnmt3a* and

Dnmt3b (Li et al., 1992, Okano et al., 1999). Although their patterns at each developmental stage are different, *Dnmt1*, *3a* and *3b* mRNAs show some degree of overlap in how their urethral expression pattern changes during the period spanning 14.5 dpc to P5 (Fig. 2). The trend is that *Dnmt* expression predominates in urethral mesenchyme during early development but gradually shifts to urethral epithelium and eventually prostatic bud tips in P5 male neonates (Fig. 2). The specific timing of this shift in expression is not the same for *Dnmt* family members. *Dnmt1* begins to localize to basal epithelium and prostatic buds by 17.5 dpc (Fig. 2B), while *Dnmt3a* and *Dnmt3b* localize to prostatic buds by P5 (Fig. 2I, O). Sex differences in *Dnmt1* staining are evident at P5 when *Dnmt1* is detected in a spotty pattern in periurethral mesenchyme (lamina propria and submucosa) of male but not female LUT sections (Fig. 2C, F). Sex differences for *Dnmt3a* and *Dnmt3b* are not apparent at the stages examined.

DNA demethylation pathway members are present in developing LUT before, during and after prostatic bud formation

DNA demethylation is less well understood than DNA methylation. It can occur passively by inhibition of *Dnmt1*, such that with each successive DNA synthesis event the level of methylation is reduced. DNA demethylation can also occur by an active process and several genes are capable of modifying and removing methyl groups from DNA. Active DNA demethylation is thought to occur through events ranging from base modifications to base excision repair pathways.

Hydroxymethylation is a base modification known to occur throughout the genome and functions as a stable epigenetic mark and as an intermediate in the

demethylation process (Guo et al., 2011, Ito et al., 2010, Tahiliani et al., 2009, Tan et al., 2012). Hydroxymethylation is catalyzed in part by members of the Ten-eleven translocation (Tet) family (*Tet1*, *Tet2* and *Tet3*) which convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) (Tahiliani et al., 2009).

The *Tets* are unlike the *Dnmts* in that there is no apparent trend within this family in how mRNA expression patterns change during LUT development from 14.5 dpc to P5. *Tet1* and *Tet2* predominate in urethral mesenchyme at 14.5 dpc and staining intensity therein increases from 14.5 dpc to 17.5 dpc (Fig. 3A-L). *Tet3* predominates in urethral epithelium at 14.5 dpc and staining intensity decreases therein from 14.5 dpc to P5 (Fig. 3M-O). The three *Tet* genes also differ in the stage and pattern in which they are first detected in urethral epithelium. *Tet1* staining is first visible in male urethral epithelium at P5 when it localizes to prostatic buds (Fig. 3C, solid arrowheads). *Tet2* staining is detectable in urethral epithelium at 14.5 dpc and thereafter is restricted to intermediate and superficial urethral epithelium and is not apparent in prostatic buds (Fig. 3H; inset, solid arrowheads). *Tet3* staining is present in urethral and prostatic bud epithelium at 14.5 dpc, 17.5 dpc and P5 (Fig. 3M-O, solid arrowheads).

There are some noteworthy sex differences in *Tet* staining patterns within the LUT. At 17.5 dpc *Tet1* is not detected in male epithelium but is present in a spotted pattern in female urethral epithelium (Fig. 3B, E; insets). At 17.5 dpc *Tet2* staining is weaker throughout urethral epithelium in male compared to female LUT (Fig. 3 H, K; insets). A sex difference for *Tet3* staining is observed at 17.5 dpc when light staining exists in male but not female urethral smooth muscle mesenchyme (Fig. 3N, Q).

Another proposed mechanism of DNA demethylation is through cytidine deamination, which converts 5mC to thymine to create a T:G mismatch which activates DNA repair pathways (Bhutani et al., 2010, Muramatsu et al., 2000, Morgan et al., 2004, Popp et al., 2010, Rai et al., 2008). Cytidine deamination is mediated by activation induced cytidine deaminase (*Aicda*, or *Aid*) and apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1-3 (*Apobec1*, 2 and 3), which are collectively referred to as AID/APOBEC family members.

AID/APOBEC family members exhibit unique spatiotemporal mRNA expression patterns in the mouse LUT. *Aicda* mRNA staining is detected in 14.5 dpc fetal urethral mesenchyme and epithelium where it gradually decreases until at least P5 (Fig. 4A-C). *Apobec1* mRNA staining was absent from urethral mesenchyme and epithelium of the male at the time points examined (Fig. 4G-I). *Apobec2* staining in urethral and prostatic bud epithelium persists from 14.5 dpc to P5 while staining in urethral mesenchyme is restricted to smooth muscle by 17.5 dpc (Fig. 4M-O). Weak *Apobec3* staining is present in urethral mesenchyme and epithelium at 14.5 dpc which gradually increases until at least 17.5 dpc before staining is again reduced and no longer detected in urethral mesenchyme by P5 (Fig. 4S-U). There are no noteworthy sex differences in AID/APOBEC staining within the LUT at 14.5 dpc, 17.5 dpc or P5.

As part of DNA damage repair, DNA glycosylases remove damaged DNA bases which are then repaired through the base excision repair pathway (Jost et al., 1995, Jost, 1993). There are several DNA glycosylases including: thymine DNA glycosylase (*Tdg*), uracil DNA glycosylase (*Ung*), single-strand selective monofunctional uracil DNA glycosylase (*Smug1*) and methyl-CpG binding domain protein 4 (*Mbd4*) that have been

identified to play specific roles in DNA demethylation. *Tdg* glycosylase activity is necessary for murine development and establishing proper DNA methylation patterns (Cortellino et al., 2011). While there is evidence that *Tdg* and *Mbd4* can recognize and bind 5mC:G *in vitro* (Zhu, 2009, Zhu et al., 2000a, b), all of the DNA glycosylases are capable of participating in DNA demethylation by recognizing and removing T:G or U:G mismatches generated from the AID/APOBEC family or from deamination of a *Tet* mediated 5hmC (Cortellino et al., 2011, Guo et al., 2011a, b).

The DNA glycosylases predominate in epithelium at 14.5 dpc and 17.5 dpc but their expression subsequently dissipates by P5 (Fig. 5, 6A-F). Surprisingly, these mRNAs also differ in their location within the epithelium prior to P5. *Tdg* is found in all urethral epithelium layers including prostatic buds (Fig. 5A-C, solid arrowheads), *Ung* epithelial staining becomes restricted to prostatic bud epithelium by 17.5 dpc (Fig. 5G-I), *Smug1* epithelial staining is localized to intermediate and superficial epithelium (Fig. 5M-O; insets) and *Mbd4* staining is found in all epithelium including prostatic buds until at least 17.5 dpc (Fig. 6A-C). A few DNA glycosylases also display light mesenchymal staining which also dissipates by P5 (Fig. 5, 6A-F). *Tdg* and *Smug1* staining is detected in urethral smooth muscle mesenchyme at 17.5 dpc while *Mbd4* staining in urethral mesenchyme is uniquely increased until at least 17.5 dpc before it is no longer detected at P5 (Fig. 6A-C). Differences between sexes are observed for *Tdg* at 17.5 dpc and for *Ung* and *Mbd4* at P5. At 17.5 dpc *Tdg* staining is present in male but absent in female urethral mesenchyme (Fig. 5B, E). At P5 *Ung* staining is absent from the male but present in female urethral epithelium (Fig. 5I, L). Also at P5, *Mbd4* staining is present in male but not female urethral epithelium (Fig. 6C, F).

Mbd4 is the only MBD family member with glycosylase activity but *Mbd2* may also contribute to DNA demethylation. *Mbd2* is reported to have demethylase activity, though this is controversial and doesn't exclude the possibility that *Mbd2* acts in concert with other factors in the demethylation process (Bhattacharya et al., 1999, Hamm et al., 2008, McGowan et al., 1997, Ng et al., 1999). Unlike *Mbd4*, *Mbd2* predominates in epithelium especially within intermediate and superficial urethral epithelial layers (Fig. 6H; insets). By P5 *Mbd2* staining in urethral epithelium is diminished and only detected in tips of prostatic buds (Fig. 6I, solid arrowheads) and a small portion of the pelvic urethra. A similar dissipation of *Mbd2* staining during the 14.5 dpc to P5 period is observed for female urethral epithelium (Fig. 6J-L) and there are no apparent sex differences at the time points examined.

Mismatches generated by AID/APOBEC family members are also recognized by the DNA mismatch repair pathway. The DNA mismatch repair proteins excise and replace an entire region surrounding a mismatch; therefore one CpG mismatch site could lead to demethylation of surrounding sites (Franchini et al., 2012, Modrich, 2006). MutS homolog 2 (*Msh2*), is the common subunit of the MutS alpha and MutS beta heterodimer which recognizes and binds mismatches in DNA (Acharya et al., 1996, Iaccharino et al., 1998, Gradia et al., 1999, Su et al., 1986, de Wind et al., 2005, Varlet et al., 1994, Prolla et al 1994, Reenan et al., 1992, Modrich et al 1991). Binding of the MSH2 complex to DNA mismatches initiates the repair process by recruiting the MutL alpha complex consisting of MutL homolog 1 (*Mlh1*) and postmeiotic segregation increased 2 (*Pms2*) which possess endonuclease activity (Kadyrov et al., 2006, Prolla

et al 1994). *Msh2*, *Mlh1* and *Pms2* are also often decreased in prostate cancer (Barrow et al., 2013, Chen et al., 2003, Soni et al., 2011, Wagner et al., 2010).

The mismatch repair pathway members *Msh2*, *Mlh1* and *Pms2* predominate in epithelium at 14.5 dpc, 17.5 dpc and P5 but their expression patterns in urethral mesenchyme vary (Fig. 7A-R). While *Msh2* is not detected in urethral mesenchyme at the time points examined (Fig. 7A-F), *Mlh1* is detected in urethral mesenchyme (Fig. 7G-L). Interestingly *Mlh1* staining in peri-prostatic mesenchyme diminishes from 17.5 dpc to P5 (Fig. 7G-L). *Pms2* staining is present in urethral mesenchyme at 14.5 dpc but dissipates by P5 (Fig. 7M-R). No apparent sex differences are observed at the time points examined.

Chromatin modifiers also participate in the DNA damage response. Chromodomain helicase DNA binding protein 2 (*Chd2*), a chromatin remodeling gene, is required for repair of damaged DNA (Nagarajan et al., 2009, Rajagopalan et al., 2012) and is necessary for normal kidney development and function (Marfella et al., 2008, 2006). *Chd2* staining is detected in mesenchyme and epithelium at 14.5 dpc (Fig. 7S). Mesenchymal staining is reduced after 14.5 dpc and epithelial staining is strongest in intermediate urethral epithelium (Fig. 7T; inset). A sex difference for *Chd2* staining exists at 17.5 dpc where basal urethral epithelial staining is less apparent in male compared to female urethra (Fig. 7T, W; insets).

Discussion

The focus of this study was to identify spatial and temporal changes in the mRNA staining patterns of genes implicated in adding, maintaining or removing DNA

methylation marks and to determine whether their expression patterns are different in male and female LUT. Whether corresponding proteins are present, active or lead to biologically significant changes in DNA methylation was not determined and is beyond the scope of this study. Though ISH has some limitations, including the inability to quantify transcript level, ISH is uniquely suited for determining when and where genes involved in DNA methylation are expressed in the developing prostate. We have discussed previously measures taken to ensure the sensitivity and specificity of our ISH method in terms of riboprobe design, sample processing and validation (Abler et al., 2011a,b, Keil et al., 2012, Mehta et al., 2011). Additionally, all gender- and age-matched samples were run in the same tube during ISH and color development.

We describe for the first time a comprehensive mRNA expression atlas for genes involved or suspected to be involved in DNA methylation and demethylation over the course of early mouse prostate development. These gene products are hypothesized to be involved in remodeling the epigenome by establishing, erasing or maintaining methylation so the proper DNA methylation patterns exist for normal development, cellular plasticity and differentiation. One of the interesting findings was that the LUT cell populations expressing these mRNAs changed during the course of early prostate development in males. While the focus of this study was DNA methylation, the temporal shift in mRNA expression we observed in several genes is consistent with what others have observed for epigenetic chromatin modifiers. Enhancer of zeste homolog 2 (*Ezh2*), a subunit of the polycomb repressor complex 2 involved in chromatin remodeling (H3K27 methylation), changes temporally during mouse UGS development, with embryonic epithelial and mesenchymal expression that becomes restricted to luminal

epithelium by P10 (Duan et al., 2012). How these changes in DNA methylation and chromatin remodeling gene expression patterns relate to prostate morphogenesis and prostate cell differentiation remains to be determined. Our results provide information which will enable future studies to specifically target these genes for deletion or assess their activity in the appropriate tissue and during the appropriate time to see whether they participate in prostate development.

A change in mRNA staining pattern commonly observed over the course of prostate development is urethral mesenchymal expression as early as 14.5 dpc which becomes less predominant or shifts to epithelium by P5. The significance of this change in staining pattern is that it occurs during the critical window when androgens are eliciting their actions in urethral mesenchyme to direct prostate development (14.5-17.5 dpc) (Lasnitzki and Mizuno 1977). Future studies will be necessary to determine if these genes play a role in the instructive potential of urethral mesenchyme (Cunha, 2008, Cunha et al., 1983). These temporal changes may also be significant in understanding how developmental pathways are inappropriately reawakened during disease. A recent study of the chromatin remodeling gene high mobility group AT-hook 2 (*Hmga2*) found that it is also more abundant in embryonic urethral mesenchyme compared to adult stroma (Zong et al., 2012). When *Hmga2* is overexpressed in immortalized prostate mesenchymal cells and these cells are combined with non-tumorigenic adult mouse prostate epithelium and grafted into a host mouse, the resultant graft forms high-grade prostate intraepithelial neoplasia (Zong et al., 2012), a hypothesized precursor lesion to prostate cancer (Bostwick and Qian 2004, Lee et al. 2010). This result is evidence that inappropriate epigenetic alterations in prostate stroma can change the behavior of

prostate cells associated with it (Zong et al., 2012). Together these results highlight the need for additional studies into how DNA methylation patterns are established, maintained and remodeled during the course of embryonic prostatic bud formation and specifically if and how the actions of other DNA methylation modifiers in prostate stroma influence prostate epithelial morphogenesis, differentiation and proliferation.

The androgen receptor is capable of interacting with or regulating the epigenome via chromatin modifiers including lysine specific demethylase 1 (*Lsd1* or *Kdm1a*), enhancer of zeste homolog 2 (*Ezh2*) and mixed lineage leukemia 1 (*Mll1*) (Cai et al., 2011, Duan et al., 2012, Metzger et al., 2005, Wissmann et al., 2007). Whether the AR is involved with other epigenetic modifications such as DNA methylation and whether this occurs during prostate development has never been determined. *Dnmt* expression changes during the human female menstrual cycle and *Dnmt3a* and *3b* are regulated by female sex hormones *in vitro* (van Kaam et al., 2011, Yamagata et al., 2009). Though we identified a few mRNAs which were noticeably different in pattern or abundance between male and female LUT, none of these displayed a pattern similar to the known androgen responsive genes steroid 5 alpha reductase 2 (*Srd5a2*) and WNT inhibitory factor 1 (*Wif1*) (Abler et al., 2011b, Keil et al., 2012, Matsui et al., 2002). While we did not look into the regulation of these genes by sex hormones, these observations suggest that these genes are likely not regulated by androgens in the male LUT, at least not directly or in the same manner as the androgen responsive genes mentioned above. This does not preclude the possibility that some DNA methylation modifying genes are post-transcriptionally modified or involved with AR signaling in some other capacity. Whether these genes act upstream, in concert with or independently of androgen

signaling remains to be determined. Future investigation into the mechanism controlling these genes during prostate development and how their downstream actions direct prostatic bud formation will be useful tools in prostate research.

Experimental Procedures

Animals

Wild type C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were maintained on a 12 hr light and dark cycle at 25±5°C and 20-50% humidity. Feed (Diet 2019 for males and 7002 for females, Harlan Teklad, Madison, WI) and water were available *ad libitum*. All procedures were approved by the University of Wisconsin Animal Care and Use Committee and conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals. To obtain timed pregnant dams, female mice were paired overnight with males and the next morning was considered 0.5 dpc. Dams were euthanized by CO₂ asphyxiation and fetuses were maintained in phosphate-buffered saline prior to dissection.

***In Situ* Hybridization (ISH)**

Tissue collection, storage, sectioning and ISH were conducted as described previously (Abler et al., 2011a,b). Primer sequences used to generate probe templates are provided in Table 1. We used the Primer Blast Program (Ye et al., 2012) to ensure specificity of PCR primers for the target sequence. We selected primer sequences that uniquely matched the target sequence and no other sequence in the mouse reference genome. We used the MegaBLAST program (Zhang et al., 2000) to ensure specificity of

the riboprobe sequence. The riboprobe sequence was considered specific for its target when, using an EXPECT threshold of 0.01 and a word size of 128, it did not align with other members of the mouse RefSeq RNA database. A positive control gene (uroplakin 1b) as well as a no probe negative control was used to ensure run quality. The run was considered high quality when: 1) uroplakin 1b staining emerged within the expected window of staining development time (1-3 hours) and was uniquely localized to intermediate and superficial urothelium and 2) when no staining was detected in no probe control tissues with up to 100 hours of development time. The staining pattern for each riboprobe was assessed in at least two LUT sections/mouse and at least three litter-independent LUTs. Age- and gender-matched samples were processed together in a single tube for ISH and color development to allow for qualitative comparisons among biological replicates and between male and female for each stage examined. All stage- and gender-differences in staining patterns reported in this manuscript were observed across all three litter independent samples per group. Additional images of some ISH-stained LUT sections are available at www.gudmap.org.

Immunohistochemistry (IHC)

IHC was conducted on ISH-stained sections as described previously (Abler et al., 2011b). Primary antibodies used were: rabbit anti-CDH1 (1:300, Cell Signaling Technologies, Beverly, MA) and mouse anti-ACTA2 (1:300, Leica Microsystems, Bannockburn, IL). Secondary antibodies used were: Dylight 488-conjugated goat anti-mouse IgG (1:500) and Dylight 546-conjugated goat anti-rabbit IgG (1:500) (Jackson ImmunoResearch, West Grove, PA). Sections were mounted in anti-fade media

(phosphate-buffered saline containing 80% glycerol and 0.2% n-propyl gallate).

Sections were imaged on an Eclipse E600 compound microscope (Nikon Instruments Inc., Melville, NY). Brightfield (ISH, purple) and fluorescent (red, green) images were merged using NIS elements imaging software (Nikon Instruments Inc). No modifications were made to brightfield ISH images. Fluorescent channels were adjusted to optimize brightness and contrast.

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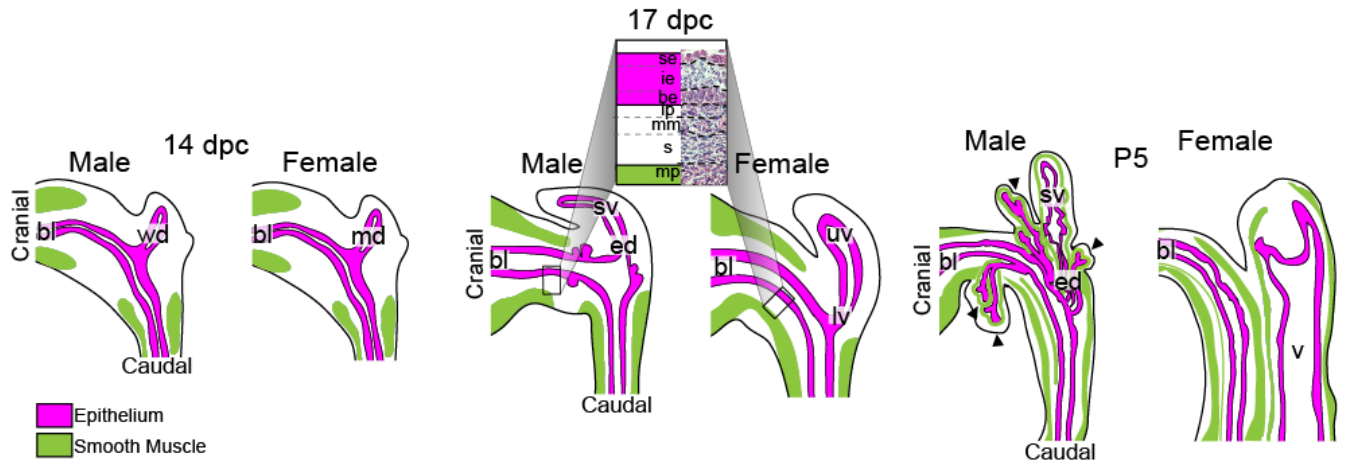


Fig. 1. Schematic representation of near mid-sagittal 14.5 dpc, 17.5 dpc and P5 male and female mouse LUT. Smooth muscle (muscularis propria) is indicated in green and epithelium in pink. These same tissue compartments were fluorescently labeled to serve as anatomical landmarks in the mapping of mRNA expression patterns in subsequent figures. The inset consists of a schematic representation as well as a 20x hematoxylin and eosin stained 5µm mid-sagittal section of the layers present in the dorsal and ventral pelvic urethra. Three epithelial and four mesenchymal urethral sub-compartments are present at 17.5 dpc as indicated in the inset with the following abbreviations; se, superficial epithelium; ie, intermediate epithelium; be, basal epithelium; lp, lamina propria; mm, muscularis mucosa; s, submucosa; mp, muscularis propria. Other abbreviations bl, bladder; wd, Wolffian duct; md, Mullerian duct; sv, seminal vesicle; ed, ejaculatory duct; lv, lower vagina; uv, upper vagina. Closed arrowheads indicate prostatic buds.

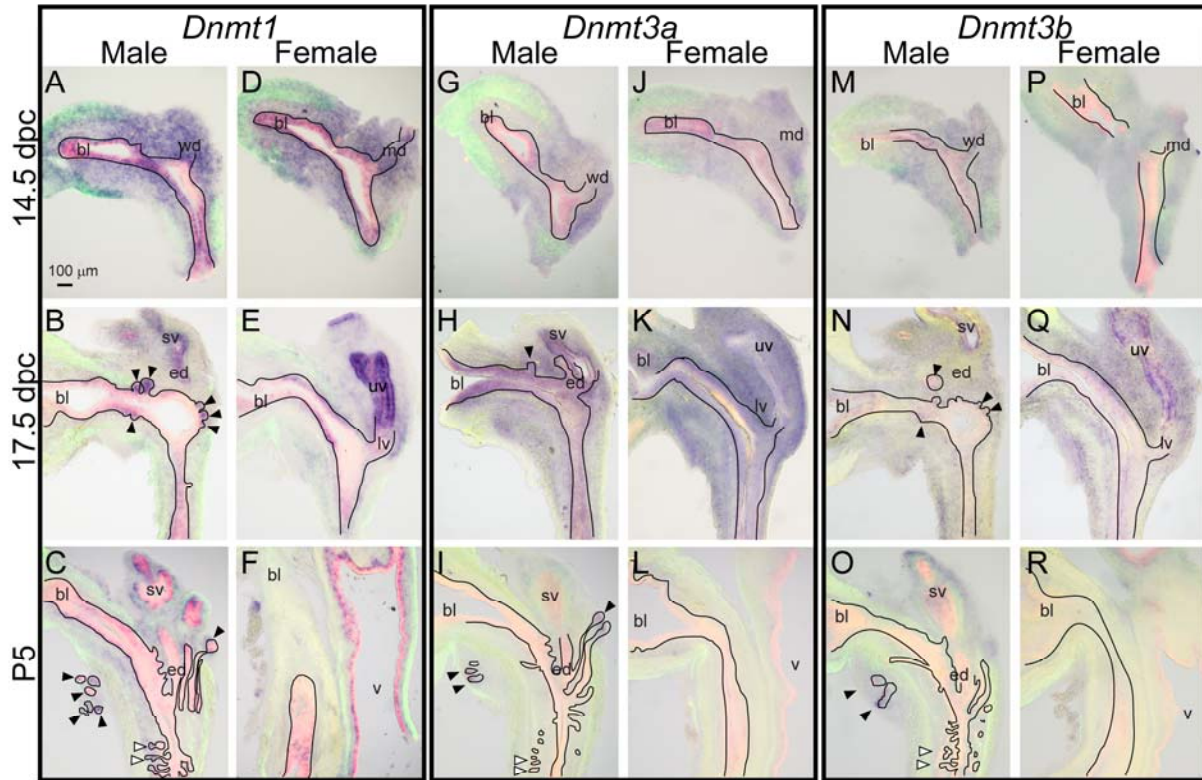


Fig 2. *Dnmt1*, *Dnmt3a* and *Dnmt3b* mRNA expression in developing male and female mouse LUT. ISH was used to visualize mRNA expression (purple) of (A-F) *Dnmt1*, (G-L) *Dnmt3a*, and (M-R) *Dnmt3b* in near midsagittal sections (50µm) of 14.5 dpc, 17.5 dpc and P5 male and female LUTs. Sections were then immunofluorescently stained with antibodies against CDH1 (red) to label all epithelium and ACTA2 (green) to label smooth muscle present in mesenchyme. Results are representative of three litter-independent samples per gender and age. bl, bladder; ed, ejaculatory duct; lv, lower vagina; md, Müllerian duct; sv, seminal vesicle; uv, upper vagina; v, vagina; wd, Wolffian duct. Closed arrowheads indicate prostatic buds; open arrowheads indicate urethral gland buds. A black line separates mesenchyme from epithelium within the pelvic urethra, prostatic buds and urethral gland buds.

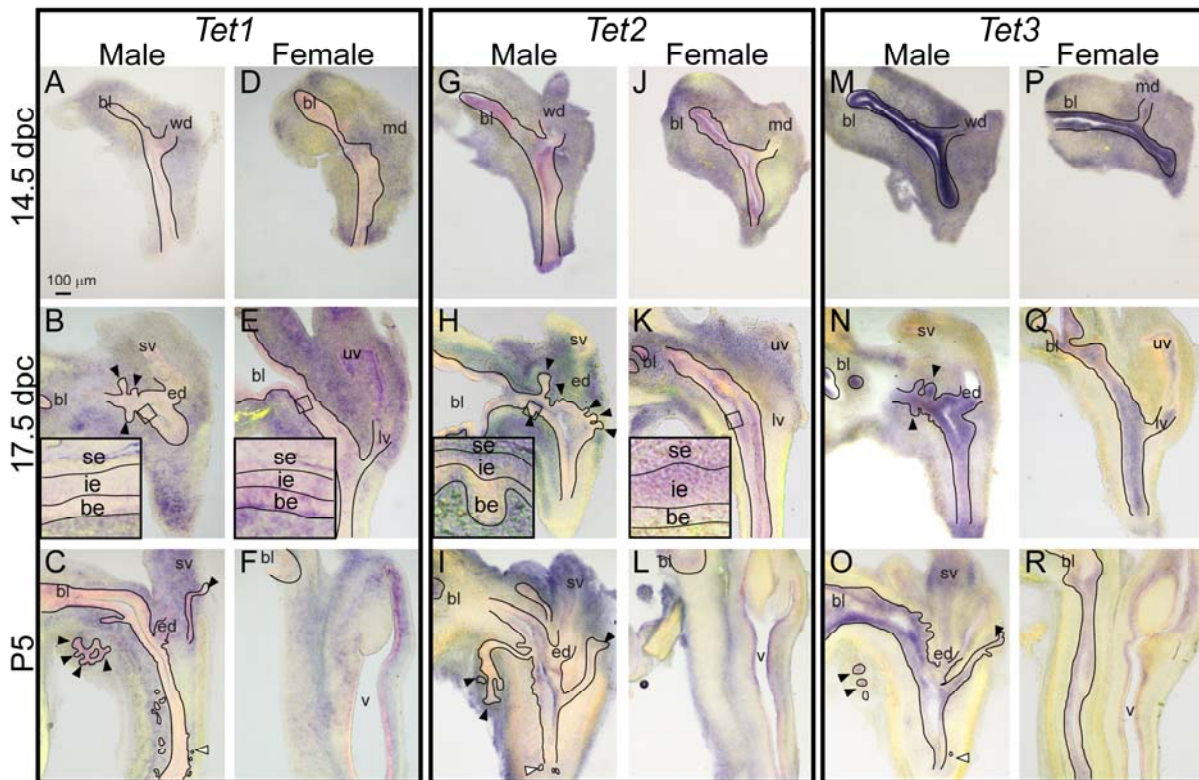


Fig 3. *Tet1*, *Tet2* and *Tet3* mRNA expression in developing male and female mouse LUT. ISH was used to visualize mRNA expression (purple) of (A-F) *Tet1*, (G-L) *Tet2*, and (M-R) *Tet3* in near midsagittal sections (50µm) of 14.5 dpc, 17.5 dpc and P5 male and female LUTs. Sections were immunofluorescently stained with antibodies against CDH1 (red) to label all epithelium and ACTA2 (green) to label smooth muscle mesenchyme. Results are representative of three litter-independent samples per gender and age. be, basal epithelium; bl, bladder; ed, ejaculatory duct; ie, intermediate epithelium; lv, lower vagina; md, Müllerian duct; se, superficial epithelium; sv, seminal vesicle; uv, upper vagina; v, vagina; wd, Wolffian duct. Closed arrowheads indicate prostatic buds; open arrowheads indicate urethral gland buds. A black line separates mesenchyme from epithelium within the pelvic urethra, prostatic buds and urethral gland buds. Insets are enlarged from the same image in the region indicated by the black box.

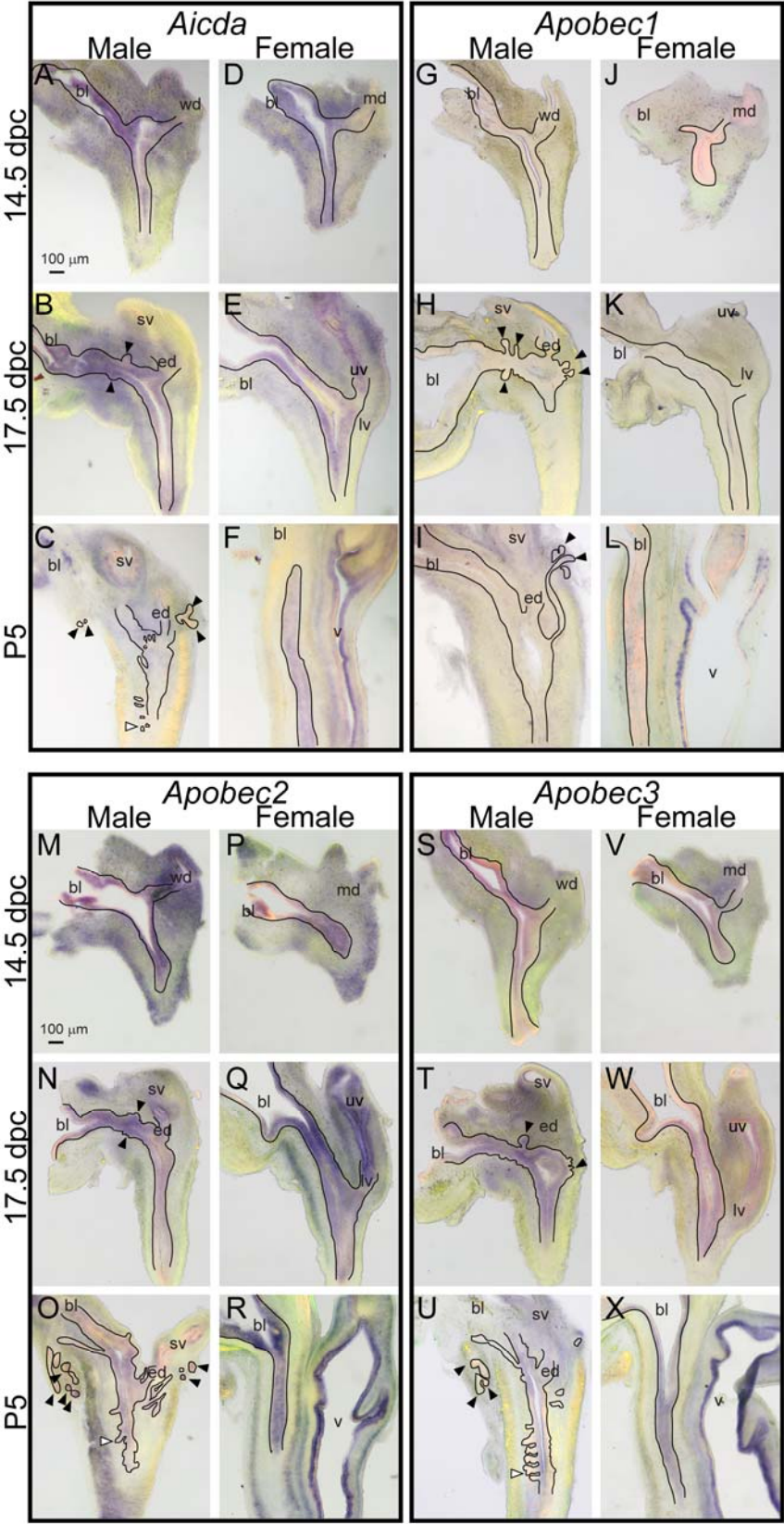


Fig 4. *Aicda*, *Apobec1*, *Apobec 2* and *Apobec3* mRNA expression in developing male and female mouse LUT. ISH was used to visualize mRNA expression (purple) of (A-F) *Aicda*, (G-L) *Apobec1*, (M-R) *Apobec2* and (S-X) *Apobec3* in near midsagittal sections (50µm) of 14.5 dpc, 17.5 dpc and P5 male and female LUTs. Sections were then immunofluorescently stained with antibodies against CDH1 (red) to label all epithelium and ACTA2 (green) to label smooth muscle present in mesenchyme. Results are representative of three litter-independent samples per gender and age. bl, bladder; ed, ejaculatory duct; lv, lower vagina; md, Müllerian duct; sv, seminal vesicle; uv, upper vagina; v, vagina; wd, Wolffian duct. Closed arrowheads indicate prostatic buds; open arrowheads indicate urethral gland buds. A black line separates mesenchyme from epithelium within the pelvic urethra, prostatic buds and urethral gland buds.

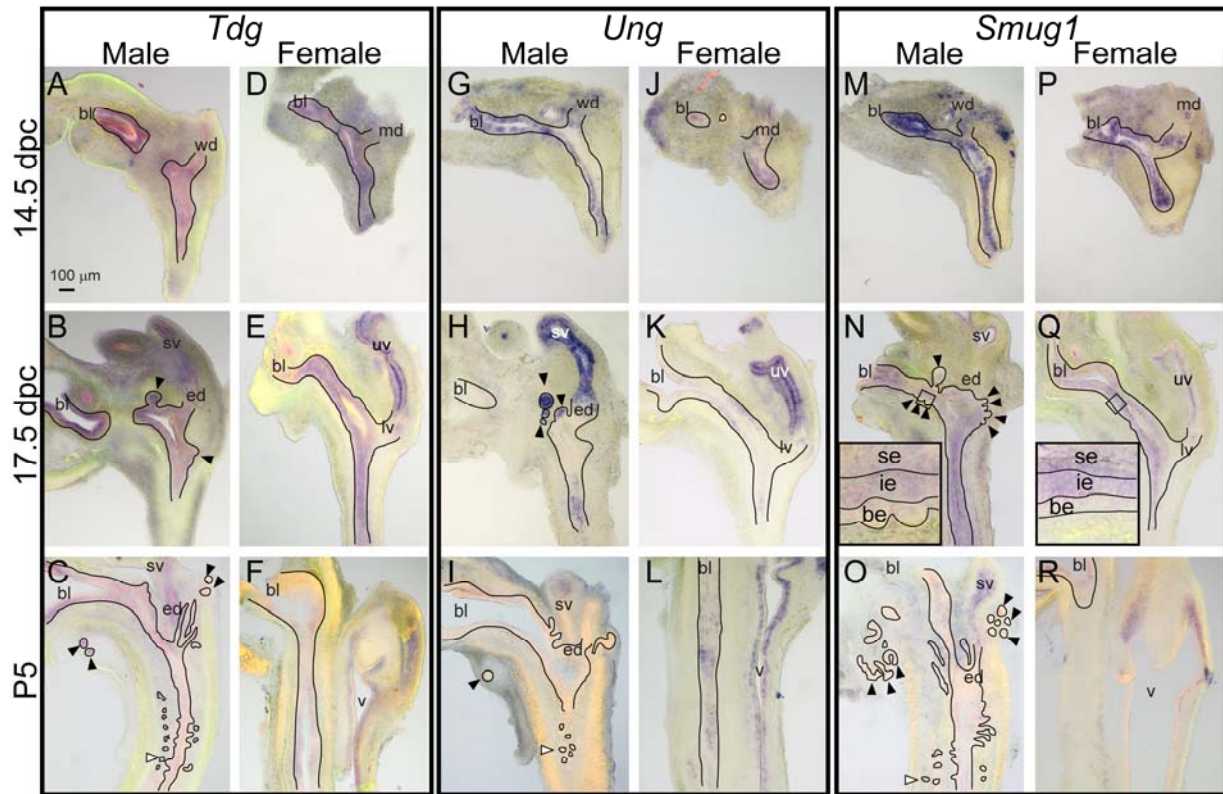


Fig 5. *Tdg*, *Ung*, and *Smug1* mRNA expression in developing male and female mouse LUT. ISH was used to visualize mRNA expression (purple) of (A-F) *Tdg*, (G-L) *Ung*, and (M-R) *Smug1* in near midsagittal sections (50 μ m) of 14.5 dpc, 17.5 dpc and P5 male and female LUTs. Sections were immunofluorescently stained with antibodies against CDH1 (red) to label all epithelium and ACTA2 (green) to label smooth muscle present in mesenchyme. Results are representative of three litter-independent samples per gender and age. be, basal epithelium; bl, bladder; ed, ejaculatory duct; ie, intermediate epithelium; lv, lower vagina; md, Müllerian duct; se, superficial epithelium; sv, seminal vesicle; uv, upper vagina; v, vagina; wd, Wolffian duct. Closed arrowheads indicate prostatic buds; open arrowheads indicate urethral gland buds. A black line separates mesenchyme from epithelium within the pelvic urethra, prostatic buds and urethral gland buds. Insets are enlarged from the same image in the region indicated by the black box.

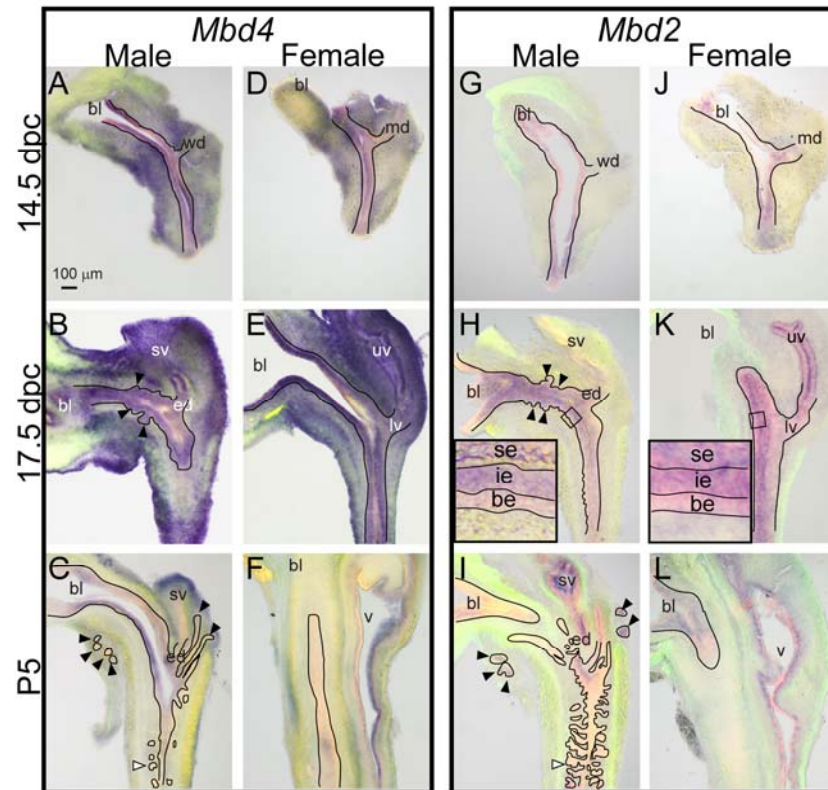


Fig 6. *Mbd4* and *Mbd2* mRNA expression in developing male and female mouse LUT. ISH was used to visualize mRNA expression (purple) of (A-F) *Mbd4*, and (G-L) *Mbd2* in near midsagittal sections (50μm) of 14.5 dpc, 17.5 dpc and P5 male and female LUTs. Sections were stained by IHC with anti-CDH1 (red) to label epithelium and anti-ACTA2 (green) to label smooth muscle present in mesenchyme. Results represent three litter-independent samples per gender and age. be, basal epithelium; bl, bladder; ed, ejaculatory duct; ie, intermediate epithelium; lv, lower vagina; md, Müllerian duct; se, superficial epithelium; sv, seminal vesicle; uv, upper vagina; v, vagina; wd, Wolffian duct. Closed arrowheads indicate prostatic buds; open arrowheads indicate urethral gland buds. A black line separates mesenchyme from epithelium within the pelvic urethra, prostatic buds and urethral gland buds.

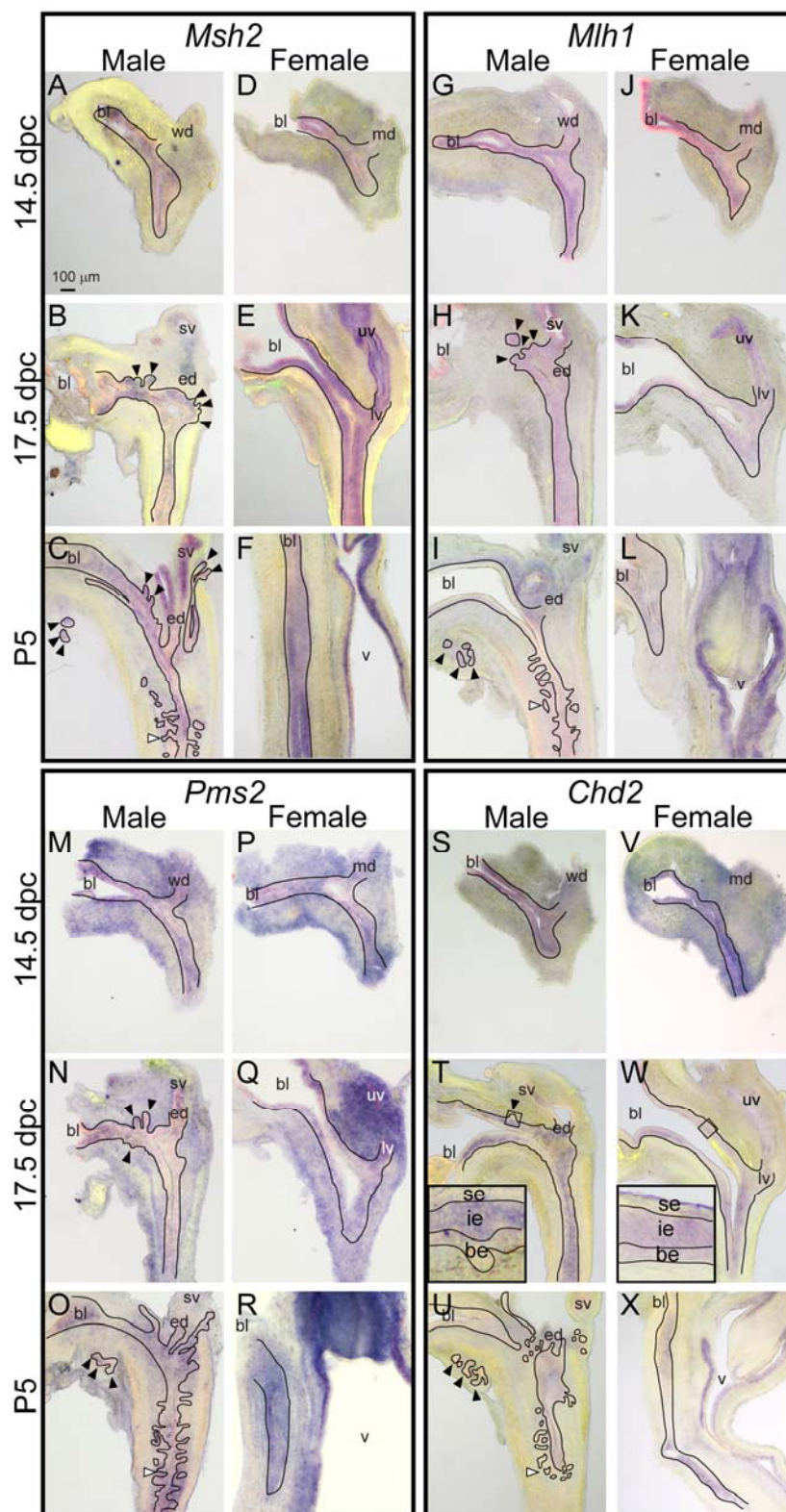


Fig 7. *Msh2*, *Mlh1*, *Pms2* and *Chd2* mRNA expression in developing male and female mouse LUT. ISH was used to visualize mRNA expression (purple) of (A-F) *Msh2*, (G-L) *Mlh1*, (M-R) *Pms2* and (S-X) *Chd2* in near midsagittal sections (50µm) of 14.5 dpc, 17.5 dpc and P5 male and female LUTs. Sections were then immunofluorescently stained with antibodies against CDH1 (red) to label all epithelium and ACTA2 (green) to label smooth muscle present in mesenchyme. Results are representative of three litter-independent samples per gender and age. be, basal epithelium; bl, bladder; ed, ejaculatory duct; ie, intermediate epithelium; lv, lower vagina; md, Müllerian duct; se, superficial epithelium; sv, seminal vesicle; uv, upper vagina; v, vagina; wd, Wolffian duct. Closed arrowheads indicate prostatic buds; open arrowheads indicate urethral gland buds. A black line separates mesenchyme from epithelium within the pelvic urethra, prostatic buds and urethral gland buds. Insets are enlarged from the same image in the region indicated by the black box.

**Chapter 5: DNA methylation of E-cadherin is a priming mechanism for prostate
development**

Keil et al., Dev Biol. (2014) 387:142-153

Introduction

E-cadherin (*Cdh1*) fine tunes cell adhesion, migration, and intracellular signaling dynamics during epithelial development and is a defined mediator of breast, salivary gland, hair follicle, lung and intestinal epithelial morphogenesis (Boussadia et al., 2002; Ewald et al., 2012; Hermiston et al., 1996; Hirai et al., 1989; Hsu et al., 2013; Jamora et al., 2003; Nanba et al., 2001; Reardon et al., 2012; Tinkle et al., 2008; Walker et al., 2008; Young et al., 2003). Though *Cdh1* is specifically required for early embryogenesis (Larue et al., 1994), the need for localized control of its activity persists throughout development and adulthood (Boussadia et al., 2002; Hermiston et al., 1996; Reardon et al., 2012). CDH1 also maintains mature epithelial homeostasis and its down-regulation associates with an invasive phenotype in some cancers (van Horssen et al., 2012).

Regulators of CDH1 expression have been identified in developing tissues, including growth factors like TGF- β and FGF, transcriptional repressors such as *Snail* and *Slug* and other signaling pathways like NOTCH and WNT (Carraro et al., 2010; Herfs et al., 2008; Jamora et al., 2003; Jamora et al., 2005; Sirour et al., 2011; Wang et al., 2012). These same pathways regulate *Cdh1* in epithelial cancers, as do other regulators. One example is DNA methylation, which frequently down-regulates *Cdh1* in epithelial tumors to create an invasive phenotype (Graff et al., 1995; Yoshiura et al., 1995). Whether DNA methylation also controls *Cdh1* expression and epithelial behavior during organ morphogenesis has not been investigated. Here, for the first time we reveal a novel mechanism whereby DNA methylation of *Cdh1* is necessary for prostate morphogenesis.

The prostate develops under the control of androgens in a region of the pelvic urethra known as the urogenital sinus (UGS). Prostate buds initiate as small epithelial projections that elongate into surrounding stroma, undergo branching morphogenesis, and arborize into the mature ductal network. How developing prostate ducts elongate into surrounding stroma during normal development is a particularly intriguing question because it may bear mechanistic similarities to the invasive behavior of prostate cancer. We describe an entirely new mechanistic link between DNA methylation and prostate ductal morphogenesis and the role of *Cdh1* in this process. *Cdh1* promoter methylation increases and its mRNA and protein abundance decrease in basal epithelial cells giving rise to prostate, and these events are necessary for ductal outgrowth and specification. We propose a priming mechanism whereby DNA methylation restricts *Cdh1* abundance in cells that give rise to the prostate, thereby creating a permissive environment for continued development and morphogenesis of prostatic ducts. Our results are the first to reveal a requirement for DNA methylation and CDH1 in prostate development, and describe how these events are mechanistically linked to orchestrate prostate ductal morphogenesis.

Materials and Methods

Animals

C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME), housed in polysulfone cages containing corn cob bedding and maintained on a 12 hour light and dark cycle at 25±5°C and 20-50% relative humidity. Feed (Diet 2019 for males and Diet 7002 for pregnant females, Harlan Teklad, Madison, WI. USA) and water were

available ad libitum. All procedures were approved by the University of Wisconsin Animal Care and Use Committee and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Females were paired overnight with males to obtain timed-pregnant dams. The next morning was considered 0 days post coitus (dpc). Dams were euthanized by CO₂ asphyxiation.

***In situ* hybridization (ISH)**

ISH was conducted on paraformaldehyde (PFA) fixed urogenital sinus (UGS) which were cut into sections with a cryotome or vibrating microtome prior to staining, or stained in whole-mount as described previously (Abler et al., 2011a; Abler et al., 2011b; Keil et al., 2012a; Keil et al., 2012b). Detailed protocols for PCR-based riboprobe synthesis are available at www.gudmap.org. The staining pattern for all riboprobes was assessed in two sections from at least three litter-independent UGSs. Tissue sections were processed in the same tube as a single experimental unit to allow for qualitative comparisons among biological replicates and among treatment groups.

Organ Culture

Male or female UGSs were removed from 14 dpc embryos and cultured for four to seven days as described previously (Vezina et al., 2008). Media was supplemented with 10 nM 5 α -dihydrotestosterone (DHT) and one or more of the following treatments; vehicle control (0.1% dimethyl sulfoxide, DMSO), 5 μ M 5-aza-2'-deoxycytidine (5AzadC, A3635, Sigma-Aldrich, St. Louis, MO. USA), 100 μ g/mL anti-CDH1 function blocking antibody targeting the fifth ectodomain repeat (EC5) of CDH1 (U3254, DECMA-1,

Sigma-Aldrich). Media and supplements were changed every 2 days. Sodium azide, which was found to be toxic to explant tissue cultures (results not shown), was removed from the CDH1 blocking antibody prior to culture using a 10,000 molecular weight cutoff dialysis cassette (Pierce, Rockford, IL. USA).

Immunohistochemistry (IHC)

Immunofluorescent staining of ISH-stained and paraffin sections was performed as described previously (Abler et al., 2011a). Primary antibodies were diluted as follows: 1:200 rabbit anti-CDH1 (3195, Cell Signaling Technology, Beverly, MA. USA), 1:200 rabbit anti-KI67 (Ab15580, Abcam, Cambridge, MA. USA), 1:50 mouse anti-KRT14 (ms-115-p0, Thermo Fisher Scientific, Waltham, MA. USA), 1:200 rabbit anti-cleaved caspase 3 (Asp 175) (9661S, Cell Signaling Technology), 1:250 rabbit anti-AR (sc-816, Santa Cruz Biotechnology, Santa Cruz, CA. USA), 1:50 mouse anti-TRP63 (Sc-8431, Santa Cruz Biotechnology), 1:250 rabbit anti-pan cytokeratin (18-0059, Life Technologies, Grand Island, NY. USA). Secondary antibodies were diluted as follows: 1:250 Dylight 549-conjugated goat anti-rabbit IgG (111-507-003, Jackson ImmunoResearch, West Grove, PA. USA), 1:250 AlexaFluor 594-conjugated goat anti-rabbit IgG (A11012, Life Technologies), 1:250 Dylight 488-conjugated goat anti-mouse IgG (115-487-003, Jackson ImmunoResearch), 1:250 Dylight 488-conjugated goat anti-rat IgG (112-486-003, Jackson ImmonoResearch), 1:250 Dylight 488-conjugated goat anti-rabbit IgG (111-487-003, Jackson ImmunoResearch). Labeled tissues were counterstained with 4',6-diamidino-2-phenylindole, dilactate (DAPI), to label cell nuclei or wheat germ agglutinin (W21505, Life Technologies) to label cell membranes and

mounted in anti-fade media (phosphate buffered saline containing 80% glycerol and 0.2% n-propyl gallate). Whole mount immunohistochemistry on ISH stained tissue was performed as described previously (Keil et al., 2012a). Primary antibody was rabbit anti-CDH1 diluted 1:750 and secondary antibody was biotin conjugated goat anti-rabbit IgG diluted at 1:500 (BA-1000, Vector, Burlingame, CA. USA). Following ISH and IHC, some tissues were paraffin embedded and sectioned as described previously (Keil et al., 2012a) and counterstained with nuclear fast red. Brightfield images were captured using an Eclipse 80i compound microscope for sections and a SMZ1000 microscope for whole mount, fluorescent images were captured using an Eclipse E600 compound microscope (Nikon Instruments Inc., Melville, NY. USA) and merged using NIS elements imaging software (Nikon Instruments Inc.). All treatment groups were imaged using the same settings and captured at the same exposure.

Methylated DNA Immunoprecipitation (MeDIP)

Genomic DNA was isolated using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA. USA) according to manufacturer's instructions. Two micrograms of genomic DNA was diluted into a 50 μ l volume with phosphate buffered saline + 0.1% Tween 20 (PBS) and was fragmented by 30 cycles of 30 seconds on, 30 seconds off in an ice cold sonicating water bath (Chicago Electric 120v/60Hz/35W, model 3305). Agarose gel electrophoresis was used to confirm efficient DNA fragmentation to an average size of 200-1000 base pairs. Fragmented DNA was boiled for 10 minutes and cooled on ice for 10 minutes. Each sample was divided into two fractions with 10% of the divided fraction saved as input control. 2 μ g anti-mouse IgG antibody (ab18413,

Abcam) was added to one fraction, 2 µg anti-5-methyl cytidine antibody (33D3 ab10805, Abcam) was added to the other fraction and both fractions were incubated overnight at 4°C. The conjugated antibodies were captured during a 2 hour incubation at 4°C with 30 µL/mL of Protein A Agarose/Salmon Sperm DNA Beads (#16-157, Millipore, Billerica, MA. USA). Beads were washed with PBS, resuspended in 100 µL of digestion buffer (50 mM Tris pH8, 10 mM EDTA, 0.5% SDS) containing 0.4 mg/mL Proteinase K and incubated overnight on a rotating platform at 50°C to release the DNA. Samples were washed twice with digestion buffer and supernatant collected. MeDIP DNA was purified using the QIAquick PCR Purification Kit (Qiagen, #28104). Real time quantitative PCR (QPCR) was performed as described previously (Keil et al., 2012b) using gene specific primers for *Cdh1*: 5'-CACGGAGGGAGAACAATGTAAG-3' and 5'-TATGCATCCCACATCCATCAG-3' (Entrez gene ID 12550, NC_000074.6). Results were analyzed using the ΔC_t method and expressed as enrichment over IgG (Livak and Schmittgen, 2001). Results are representative of at least three litter independent pools of DNA with at least five tissues per pool.

Flow Cytometry

Single cell suspensions from pooled intact male 17 dpc UGSs (3-5 UGSs/pool) were generated as described previously (Lukacs et al., 2010). UGSs were dissected to remove seminal vesicle rudiments and the bladder was trimmed at the bladder neck. Cells were labeled with the following fluorescently conjugated primary antibodies: rat anti-CD49f-PE, rat anti-CD31-FITC, rat anti-CD45-FITC and rat anti-TER119-FITC (clones: GoH3, 390, 30-F11, and TER-119 respectively, eBioscience, San Diego, CA.

USA) as described previously (Lukacs et al., 2010), washed in 0.5% BSA/PBS, fixed in 2% PFA for 10 minutes and permeabilized with PBS + 0.5% Tween20 at room temperature for 15 minutes. Cells were then incubated with unconjugated rabbit anti-CDH1 primary antibody diluted at 1:200 (3195, Cell Signaling Technology) at room temperature for 30 minutes, followed by incubation with APC-conjugated goat anti-rabbit secondary antibody diluted at 1:100 (Sc-3846, Santa Cruz) at room temperature for 30 minutes. Cells were washed in PBS and stored in 2% PFA protected from light until analyzed on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA. USA). Single antibody staining controls were used for each sample. Flow cytometry data were analyzed using FlowJo software (TreeStar Inc. Ashland, OR. USA).

Renal Grafting

Female UGSs removed at 14 dpc were cultured for seven days as described above in media containing 10 nM DHT and either vehicle control (0.1% DMSO) or 5 μ M 5AzadC. Tissues were then placed under the renal capsule of intact male syngeneic (C57BL/6J) host mice and grown for 1 month. Graft volume was calculated using the modified ellipsoid formula of $\frac{1}{2}$ (length x width²) (Euhus et al., 1986; Tomayko and Reynolds, 1989).

Statistical analyses

Prostatic buds were counted as described previously (Keil et al., 2012a). Prostatic bud length, from the base to the distal tip, was measured for at least 6 buds per UGS in at least three UGSs per treatment group. The frequency of immunolabeled

Ki-67 positive, KRT14 positive and double-positive cells was determined in at least two sections per UGS in at least three UGSs per treatment group. Image J software was used to determine the relative integrated density, a measure of the sum of converted grayscale values of the pixels in a field, for red and green channels captured from 3 basal epithelial fields per UGS in at least three litter independent samples per group. Statistical analysis was performed using R version 2.13.1. Homogeneity of variance was determined using Levene's test. Student's *t*-test, ANOVA or Kruskal-Wallis test followed by Tukey's Honestly Significant Difference (HSD) were used to identify significant differences ($p \leq 0.05$) between or among treatment groups.

Results

***Cdh1* mRNA and protein expression are diminished in a prostate epithelial cell subpopulation during male prostatic bud initiation.**

In situ hybridization (ISH) and immunohistochemistry (IHC) were used to visualize *Cdh1* mRNA and protein expression patterns within the male and female UGS prior to (14 dpc) and during (17 dpc) prostatic bud outgrowth in males. Prostate develops only in male C57BL/6J mice under normal hormonal conditions (Price and Williams-Ashman, 1961), though both sexes are competent to form prostatic buds in response to androgens during this time period (Bard et al., 1979; Price and Williams-Ashman, 1961; Lasnitzki and Mizuno, 1980). IHC sections were counterstained with the cell membrane marker wheat germ agglutinin to visualize the epithelial-mesenchymal boundary and to evaluate epithelial membrane density. *Cdh1* is present in all epithelial cells at the stages examined, but differs in its relative abundance among epithelial

layers (Fig. 1). *Cdh1* mRNA and protein staining is abundant and ubiquitously expressed across male and female UGS epithelium at 14 dpc, but a graded pattern emerges in both sexes by 17 dpc when prostatic buds are forming in C57BL/6J male mice (Fig. 1A,B). The graded staining pattern features strong *Cdh1* staining in epithelial cells closest to the urethral lumen and weak staining in cells closest to the basement membrane (Fig. 1C). The weakest *Cdh1* mRNA and protein staining is in prostatic bud tips (Fig. 1). These results reveal a change in *Cdh1* expression during the window when the UGS becomes competent to form prostate. Additional staining was used to determine that CDH1 diminishes specifically in KRT14+ basal epithelium, the epithelial cell type present in developing prostatic buds (Fig. 2A,B), and that is required for prostatic bud formation (Kurita et al., 2004; Signoretti et al., 2000). Because this change is observed across male and female UGS, it is not likely to be reliant on the presence of testicular androgens.

We used independent markers and methodology to further investigate the relationship between basal epithelial cell identity and CDH1 abundance. CD49f (also known as integrin alpha 6, *Itga6*) marks mature prostate basal epithelium (Lukacs et al., 2008; Lukacs et al., 2010; Lawson et al., 2007) and we confirmed it also marks fetal prostate basal epithelium within the urethra and developing prostate buds (Fig. 2C, inset). We conducted flow cytometry on lineage (Ter119, CD45, CD31) negative intact 17 dpc male UGS cells to exclude erythrocytes (TER-119), hematopoietic (CD45) and endothelial (CD31) cells. The cells were then sorted on the basis of CD49f and CDH1 abundance. The CD49f^{High}, CDH1^{Low} (quadrant 1) illustrate that basal epithelial cells have low CDH1 expression (Fig. 2D), consistent with our results in Figure 1. The

CD49f^{High}, CDH1^{High} (quadrant 2) cells likely represent proximal rudiments of ejaculatory duct which remain even after removal of seminal vesicle and maintain high CDH1 and CD49f expression at this stage (Fig. 2C). The CD49f^{Low}, CDH1^{Low} cells (quadrant 3) represent stromal cells and the CD49f^{Low}, CDH1^{High} cells (quadrant 4) represent the non-basal cells within the epithelium. These results in conjunction with those in Figure 1 support the hypothesis that during prostate ductal initiation, *Cdh1* mRNA and protein expression are decreased in basal epithelium giving rise to prostatic buds.

***Cdh1* promoter methylation increases in UGS epithelium during the period of prostatic bud outgrowth**

Our next objective was to determine why *Cdh1* abundance diminishes in UGS basal epithelium during prostate development. Two factors led us to consider DNA methylation as a possible mechanism: DNA methylation represses *Cdh1* expression in prostate and other cancers (Yoshiura et al., 1995, Graff et al., 1995) and the enzymes responsible for catalyzing DNA methylation, the DNA methyltransferases, increase in abundance at the same time and in the same basal epithelial cell population where *Cdh1* abundance diminishes during prostate development (Keil et al., 2013). We compared *Cdh1* methylation prior to (14 dpc) and during (17 dpc) prostatic bud formation. UGSs at both stages were treated with trypsin, microdissected to separate epithelium from stroma (Fig. 3A) and the purified epithelium was analyzed by MeDIP-QPCR. *Cdh1* DNA methylation significantly increased in UGS epithelium between 14 and 17 dpc (Fig. 3B). Therefore, at the same time *Cdh1* abundance decreases in UGS epithelium, *Cdh1* methylation increases.

DNA methylation is required for prostatic bud specification and outgrowth of prostate epithelium

In prostate cancer, CDH1 abundance and activity are inversely associated with cell invasiveness and motility (Graff et al., 1995; Yoshiura et al., 1995; van Horsen et al., 2012). We surmised that these same cell behaviors guide prostatic bud outgrowth into surrounding stroma. To test this hypothesis, we examined prostate development in the presence of a DNA methylation inhibitor, which we expected to increase CDH1 abundance and impair prostatic bud outgrowth.

The DNA methylation inhibitor 5-aza-2'-deoxycytidine (5AzadC) interferes with testicular development (Cisneros and Branch, 2003; Choi et al., 2013). Since associated changes in testicular androgen synthesis would confound our study, we used an in vitro approach that recapitulates many features of in vivo prostate development (Doles et al., 2005) but isolates the UGS from influences of testes and other tissues. Female 14 dpc UGSs were used since they are naïve to high levels of circulating androgen prior to the culture period (vom Saal, 1989), are capable of forming prostate in the presence of androgens (Lasnitzki and Mizuno, 1980), and form the same number of prostatic buds as male UGSs grown under equivalent conditions in androgen-containing medium (Keil et al., 2012a). UGS explants were grown for seven days in a defined serum-free culture medium containing androgen, 5 α -dihydrotestosterone (DHT, 10 nM) and graded concentrations of 5AzadC.

Tissue explants were stained to visualize CDH1 and the earliest marker of prostate identity expressed by prostatic buds, NK-3 transcription factor, locus 1 (*Nkx3-1*)

(Bhatia-Gaur et al., 1999; Bieberich et al., 1996; Keil et al., 2012a; Sciavolino et al., 1997) (Fig. 4A-E). This dual staining approach enabled us to objectively assess whether buds expressed *Nkx3-1* and were therefore appropriately specified as prostate, to quantify the number and length of buds formed in culture, and to determine whether buds underwent branching morphogenesis, a normal aspect of continuing ductal development. 5AzadC decreases prostatic bud length (Fig. 4F) and reduces the number of buds with detectable *Nkx3-1* staining (Fig. 4G) without changing overall number of buds formed per UGS (Fig. 4H). 5AzadC also blocks branching morphogenesis (Fig. 4A-E). We used an independent marker of developing prostate identity, ectodysplasin A receptor (*Edar*) (Keil et al 2012a) to confirm the results observed with 5AzadC and *Nkx3-1*. 5AzadC also reduces the number of buds with detectable *Edar* staining (Fig. S1). Together these results indicate that the DNA methylation inhibitor, interferes with prostatic bud specification (expression of *Nkx3-1* and *Edar*), elongation and branching morphogenesis.

High concentrations of 5AzadC are capable of decreasing proliferation, inducing apoptosis, and altering differentiation status of cells grown in vitro (Konieczny and Emerson, 1984; Patra et al., 2011). We determined that 5AzadC impaired bud outgrowth without causing obvious changes in formation of basal epithelium (Fig. S2A,B), percentage of proliferating cells within basal epithelium, stroma or the distal most tips of buds (Fig. S2C,D), cell death (Fig. S3A-C), androgen receptor (AR) protein expression, or distribution of androgen responsive steroid 5 alpha reductase 2 (*Srd5a2*) (Abler et al., 2011a; Matsui et al., 2002) (Fig. S3D-G).

To determine if inhibition of DNA methylation during prostatic outgrowth impairs continued glandular development, we grew UGS explants for seven days in a defined serum-free culture medium containing androgen (10 nM DHT) and either 5 μ M 5AzadC or its vehicle (control), and then grew them for one month under the renal capsule of intact adult male syngeneic (C57BL/6J) host mice. The host mice were untreated during this period. Control grafts develop extensive, organized prostate ductal structures with evident secretory products (Fig. 4I). 5AzadC treated tissues are significantly smaller (Fig. 4J) and feature smaller, disorganized ducts with few secretions (Fig. 4K). These results suggest that DNA methylation during the initial phases of prostate outgrowth is required for continued prostate ductal maturation.

DNA methylation is required for appropriate CDH1 expression in basal epithelium

We next tested if 5AzadC reduced *Cdh1* DNA methylation and increased *Cdh1* abundance in UGS epithelium. To assess *Cdh1* DNA methylation, MeDIP-QPCR was conducted on isolated UGS epithelium from 14 dpc female mouse UGS tissues cultured in the presence of androgen $\pm$ 5AzadC (5 μ M). 5AzadC significantly reduces *Cdh1* promoter methylation (Fig. 5A) and increases *Cdh1* mRNA staining intensity in basal epithelium (Fig. 5B,C). Also within basal epithelium, 5AzadC increases relative integrated density (sum of converted grayscale pixel values) of CDH1 protein (green) when normalized to cell membrane density (red, unaltered by treatment) in 5AzadC versus control tissues (Fig. 5D). These results reveal that 5AzadC reduces CDH1 promoter methylation and increases relative CDH1 abundance in basal epithelium.

A CDH1 function-blocking antibody bypasses the requirement for DNA methylation in prostatic bud elongation

Because 5AzadC is a non-specific DNA methylation inhibitor and likely to reduce DNA methylation at many sites across the genome, we next wanted to pinpoint whether *Cdh1* hypomethylation and an associated increase in *Cdh1* abundance are responsible for its impairment of prostatic bud morphogenesis. Female 14 dpc UGS explants were grown in culture media containing androgen and either 5AzadC, a CDH1 function-blocking antibody or both 5AzadC and antibody (Fig. 6A-D). This antibody is directed against the extracellular fifth ectodomain repeat (EC5) of CDH1 which disrupts homotypic interactions (Ozawa et al., 1990; Vestweber and Kemler, 1985) and has been used successfully to impair CDH1 function in salivary gland and lung explant cultures (Tsao et al., 2008; Walker et al., 2008). CDH1 function-blocking antibody penetrates UGS stroma and localizes to its intended target in UGS epithelium (Fig. S4). 5AzadC reduces prostatic bud length by 31% when added to culture medium without the CDH1 function blocking antibody (Fig. 6E). Though the antibody does not independently change prostatic bud number, length or number of branched tips per bud in UGSs grown without 5AzadC (Fig. 6), it restores prostatic bud outgrowth in 5AzadC-treated UGSs (compare Fig. 6B to 6D) and increases *Nkx3-1*-positive bud percentage in tissues treated with or without 5AzadC, without changing total bud number (Fig. 6E-G). These results show that 5AzadC impairs prostatic bud specification and outgrowth through a CDH1 dependent mechanism. This mechanism is specific to the initial phases of prostatic development because the CDH1 function-blocking antibody does not restore branching morphogenesis in 5AzadC-treated UGSs (Fig. 6H-J).

***Cdh1* controls the rate of prostate ductal maturation**

In control UGS tissues, the CDH1 function-blocking antibody increases the number of buds undergoing branching morphogenesis (Fig. 6H-J), suggesting additional roles for CDH1 activity in later stages of prostate development. The CDH1 function-blocking antibody accelerates several hallmarks of prostate ductal maturation. It causes precocious bud lumen formation (Fig. 7A, asterisks). Prostatic ducts do not typically form lumens until several days after birth and are not typically observed in 14 dpc explants cultured for seven days (Doles et al., 2005; Keil et al., 2012a; Sugimura et al., 1986). The CDH1 function-blocking antibody also changes the pattern of basal epithelium from a uniform distribution around the bud and extending into the urethra, to a localized concentration in prostatic bud tips and discontinuous single cell layer extending into the urethra (Fig. 7B,C; arrowheads). The formation of a discontinuous basal epithelium is especially striking since this pattern typically occurs postnatally in normal prostate (Fig. 7D) at a stage much older than that achieved by the six day organ culture. A schematic representation of how the CDH1 function-blocking antibody alters prostate bud epithelial organization is illustrated in Fig. 7E. Together these results suggest that CDH1 not only impacts prostate bud specification and outgrowth but also the maturation and differentiation of prostatic bud epithelium. Therefore *Cdh1* DNA methylation not only primes UGS epithelium for prostatic bud specification and outgrowth but, permits continued development and morphogenesis of prostatic ducts.

Discussion

Our results are the first to establish a mechanistic link between DNA methylation and prostate morphogenesis in addition to providing evidence for the role of CDH1 during prostate ductal development and maturation. We reveal *Cdh1* DNA methylation during prostate initiation is a priming event that creates a permissive environment for outgrowth and continued morphogenesis of prostatic ducts during later stages of development (Fig. 8). Our results suggest that a temporal reduction of *Cdh1* activity in basal epithelium is necessary for prostatic bud maturation. Prostatic bud outgrowth is impaired under conditions of elevated CDH1 abundance (the presence of a DNA methylation inhibitor) and restored under conditions that neutralized CDH1 activity (the presence of a blocking antibody). Prostatic buds can form without *Cdh1* methylation. However, we show that the specific role of *Cdh1* methylation is to promote bud elongation, maintenance and acquisition of prostate identity through the reduction of CDH1 activity. We also show that CDH1 activity influences prostatic bud lumen formation and arrangement of ductal epithelium. These events are needed to establish the architecture of mature prostate glandular epithelium. Thus, fine tuning of *Cdh1* expression by DNA methylation during development, at least in part, controls the timing and sequence of several prostate ductal maturation events.

We also provide evidence that appropriate DNA methylation patterns are required for prostatic ductal branching morphogenesis. Approximately 20% of buds form at least one branch in UGSs cultured for seven days in basal medium containing androgen, but branching formation is completely inhibited by a DNA methylation inhibitor and cannot be restored by neutralizing CDH1 activity (Fig. 4,6). These results indicate a CDH1-independent role of DNA methylation in branching morphogenesis.

Focused studies on the identity of methylated genes during prostatic branching morphogenesis, and the developmental timeframe in which methylation is required, will improve understanding of how DNA methylation functions to coordinate organogenesis of the prostate and other developing organs.

A question raised by our results is whether DNA methylation intersects or functions in parallel with other known *Cdh1* transcriptional regulators. *Cdh1* has previously been shown to be down-regulated by localized actions of beta-catenin (CTNNB1) and the Snail family of genes (Jamora et al., 2003, Jamora et al., 2005). Though *Ctnnb1* is required for formation of UGS basal epithelium and prostatic buds, conditional deletion or activation of *Ctnnb1* signaling during prostatic bud formation does not appear to alter *Cdh1* abundance in basal epithelium (Francis et al., 2013; Mehta et al., 2013). These results indicate that down-regulation of *Cdh1* expression in fetal prostate epithelium is likely to happen independently of CTNNB1 function. Similarly, the Snail family of transcriptional repressors do not localize exclusively to developing prostate basal epithelium (gudmap.org), however some are present in this cell population and elsewhere during prostatic bud formation. One function of Snail family members is to down-regulate *Cdh1* through chromatin modifications (Espada et al., 2011; Herranz et al., 2008; Hou et al., 2008; Lin et al., 2010; Peinado et al., 2004). An intriguing avenue of future study will be to identify whether factors such as the Snail family members recruit chromatin modifiers to the *Cdh1* promoter during prostate development, to fine tune epithelial morphogenesis by acting in concert with or upstream of DNA methylation.

Cdh1 promoter methylation occurs inappropriately in several cancers, including prostate cancer, where it is associated with tumor invasiveness (Behrens and Birchmeier, 1994; Costa et al., 2010; Takeichi, 1993; van Horsen et al., 2012). Why CDH1 becomes methylated in cancer is not known and difficult to determine in part because of the need to capture tumor cells precisely as DNA methylation occurs. We have revealed a specific window during mouse prostate development (14 dpc to 17 dpc) during which *Cdh1* methylation increases and *Cdh1* mRNA and protein become less abundant in cells giving rise to the prostate. Having defined the window, we are now poised to uncover mechanisms controlling *Cdh1* methylation, which may inform future studies of how CDH1 methylation occurs inappropriately in prostate and other cancers.

Though *Cdh1* abundance diminishes in early tumor progression, it often increases in remote sites of metastases to re-establish cell adhesion (Graff et al., 2000). This raises the question of whether methyl marks are removed from CDH1 once prostate development is completed. Determining if there is plasticity in *Cdh1* methylation during all stages of prostate development will be critical in determining regulatory mechanisms for *Cdh1* DNA methylation.

The identified role of DNA methylation in priming prostate development may also shed light on mechanisms by which endocrine disrupting chemicals perturb prostate development in utero. Bisphenol A and others are capable of altering prostate development (Anway et al., 2006; Dolinoy et al., 2007; Gupta, 2000; Ho et al., 2006; Timms et al., 2005; Walker, 2011) and have been shown to alter DNA methylation in prostate and elsewhere (Ho et al., 2006; Tang et al., 2012; Walker, 2011). The critical window during which CDH1 methylation occurs overlaps the known window of action for

some of these chemicals (Dolinoy et al., 2007; Gupta, 2000; Ho et al., 2006; Tang et al., 2012), raising questions about whether their teratogenic actions are mediated in part through DNA methylation.

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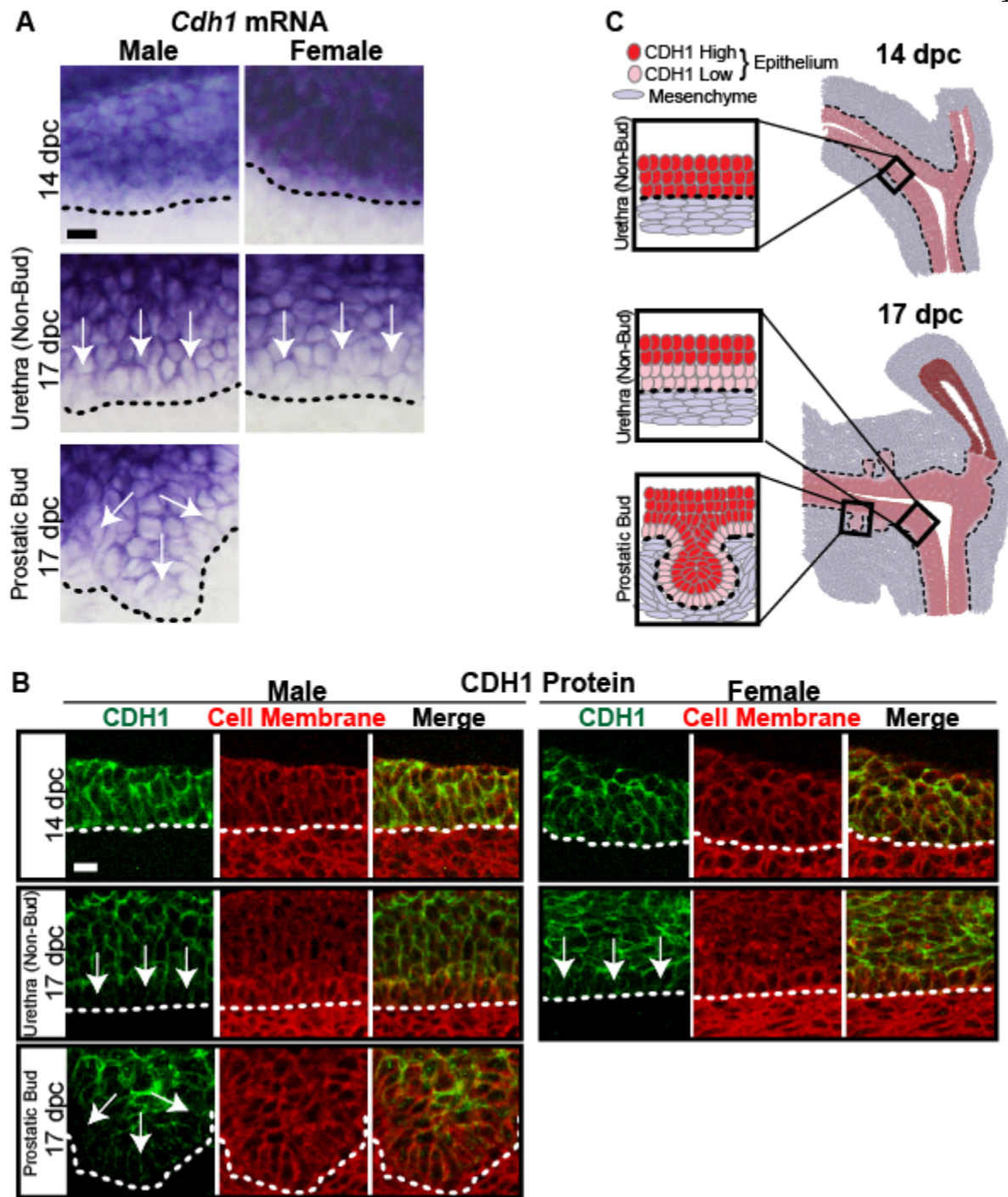


Fig. 1. E-cadherin (*Cdh1*) mRNA and protein expression are diminished in a specific UGS epithelial cell subpopulation during the period of prostatic bud formation. UGS sections were stained to visualize *Cdh1* abundance within urethra and prostatic bud

epithelium. (A) Male and female sagittal sections (50 μm) were stained by ISH to visualize *Cdh1* mRNA expression (purple). (B) Male and female sagittal sections (5 μm) were immunofluorescently labeled to detect CDH1 (green) and cell membranes (wheat germ agglutinin, red). (C) Schematic representation of E-cadherin mRNA and protein expression within UGS epithelium prior to (14 dpc) and during (17 dpc) prostatic bud formation. A dotted line indicates the interface between epithelium and mesenchyme, as determined by cell morphology. Arrows indicates areas of diminished E-cadherin mRNA and protein abundance. Scalebar represents 10 μm .

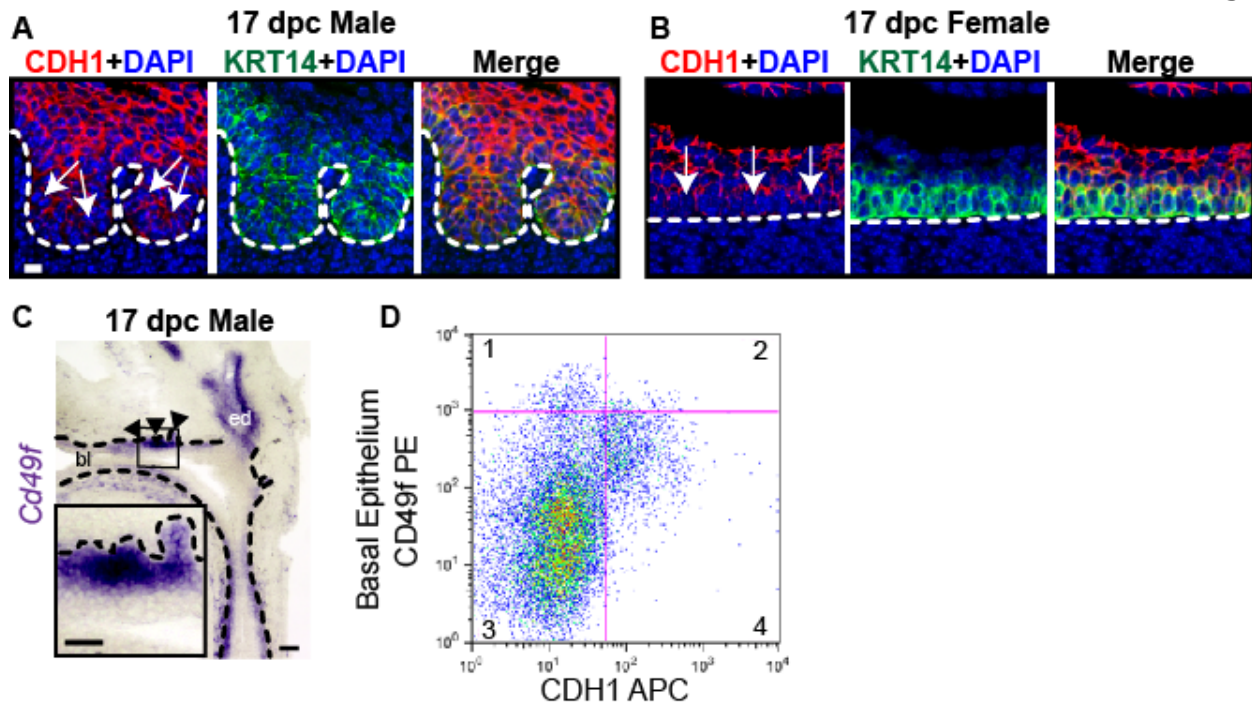


Fig. 2. Diminished E-cadherin (*Cdh1*) mRNA and protein abundance occurs in the subpopulation of basal epithelial cells during prostatic bud outgrowth. (A-B) 17 dpc male and female UGS sagittal sections (5 μm) were immunofluorescently labeled to detect E-cadherin (CDH1, red) and basal epithelial cell marker keratin 14 (KRT14, green). Cell nuclei were stained with DAPI (blue). Scalebar 10 μm. (C) 17 dpc male UGS sagittal sections (50 μm) were stained by ISH to confirm localization of *Cd49f* mRNA (purple) in basal epithelium. Scalebar 100 μm, inset 50 μm. (D) Flow cytometry plot of 17 dpc UGS single cell suspensions labeled with CD49f-PE/CDH1-APC in the Lin (CD31, TER-119, CD45) negative population. A dotted line indicates the interface between epithelium and mesenchyme, as determined by cell morphology. Arrows indicate areas of diminished CDH1 abundance. Closed arrowheads indicate prostatic buds. Abbreviations: bl, bladder; ed, ejaculatory duct.

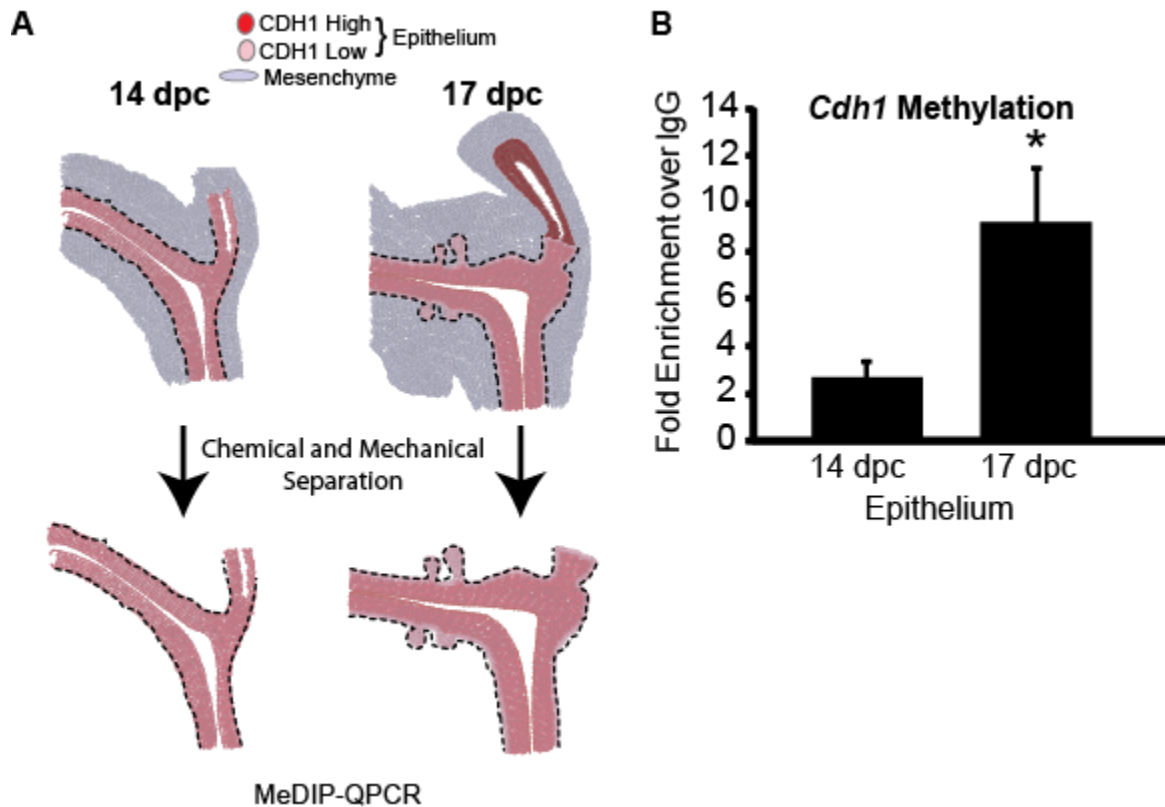


Fig. 3. *Cdh1* promoter methylation increases in UGS epithelium during the period of prostatic bud outgrowth. (A) Schematic representation of isolating UGS epithelium. (B) MeDIP-QPCR was conducted to quantify E-cadherin (*Cdh1*) methylation in isolated UGS epithelium prior to (14 dpc) and during (17 dpc) prostatic bud initiation. Results are mean $\pm$ s.e.m, $n \geq 3$ litter independent UGS tissue pools per group. Asterisk indicates a significant difference between 17 dpc and 14 dpc UGS epithelium ($p < 0.05$).

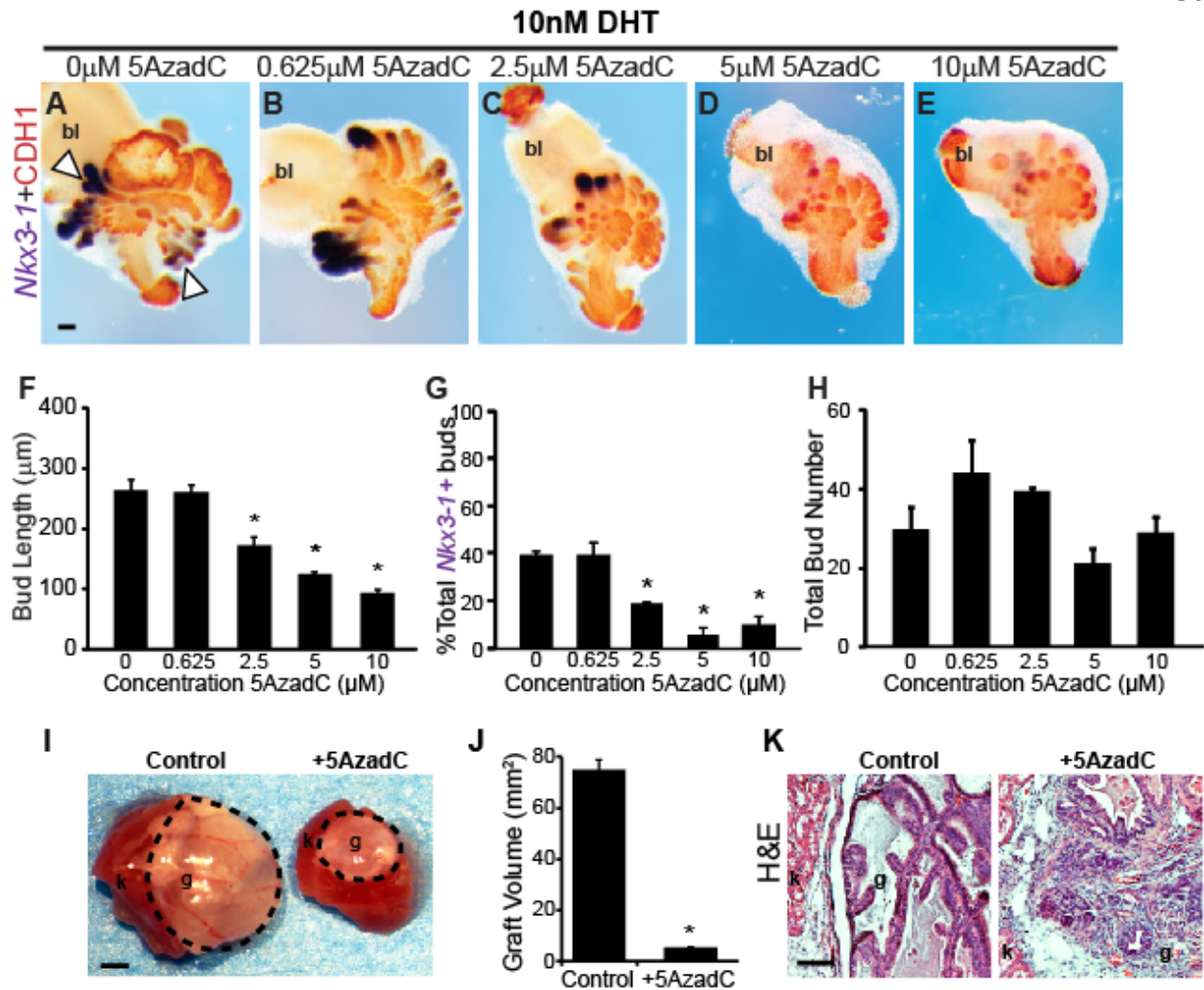


Fig. 4. DNA methylation is required for proper prostate bud specification, outgrowth, and branching in UGS explant cultures. (A-E) 14 dpc female UGSs were cultured for 7 days in media containing 5 α -dihydrotestosterone (DHT, 10 nM) and increasing concentrations of the DNA methylation inhibitor 5-aza-2'-deoxycytidine (5AzadC, 0-10 μ M). At the end of the culture period, tissues were stained by ISH to visualize the prostate marker NK-3 transcription factor, locus 1 (*Nkx3-1*, purple), counterstained by IHC with CDH1 (red) to visualize all epithelium and imaged in a sagittal orientation. Scalebar 100 μ m. Quantification of (F) prostate bud length from tip to base, (G) percent

total buds expressing the prostate marker *Nkx3-1* and (H) total prostatic bud number. (I) Following 7 day organ culture control and tissues treated with 5 μ M 5AzadC were grafted under the renal capsule of an intact male syngeneic host mouse for 1 month. Scalebar 10 mm. (J) Quantification of graft volume in control and 5AzadC treated tissues after 1 month under the renal capsule. (K) Renal capsule grafts were paraffin embedded and sectioned into 5 μ m sagittal sections and stained with hematoxylin and eosin. Scalebar 100 μ m. Results are presented as mean $\pm$ s.e.m, $n \geq 3$ litter independent UGSs per group. Asterisks indicate a significant difference from control ($p < 0.05$). Arrowheads indicate branching buds. A dotted line surrounds graft tissue. Abbreviations; bl, bladder; g, graft; k, kidney.

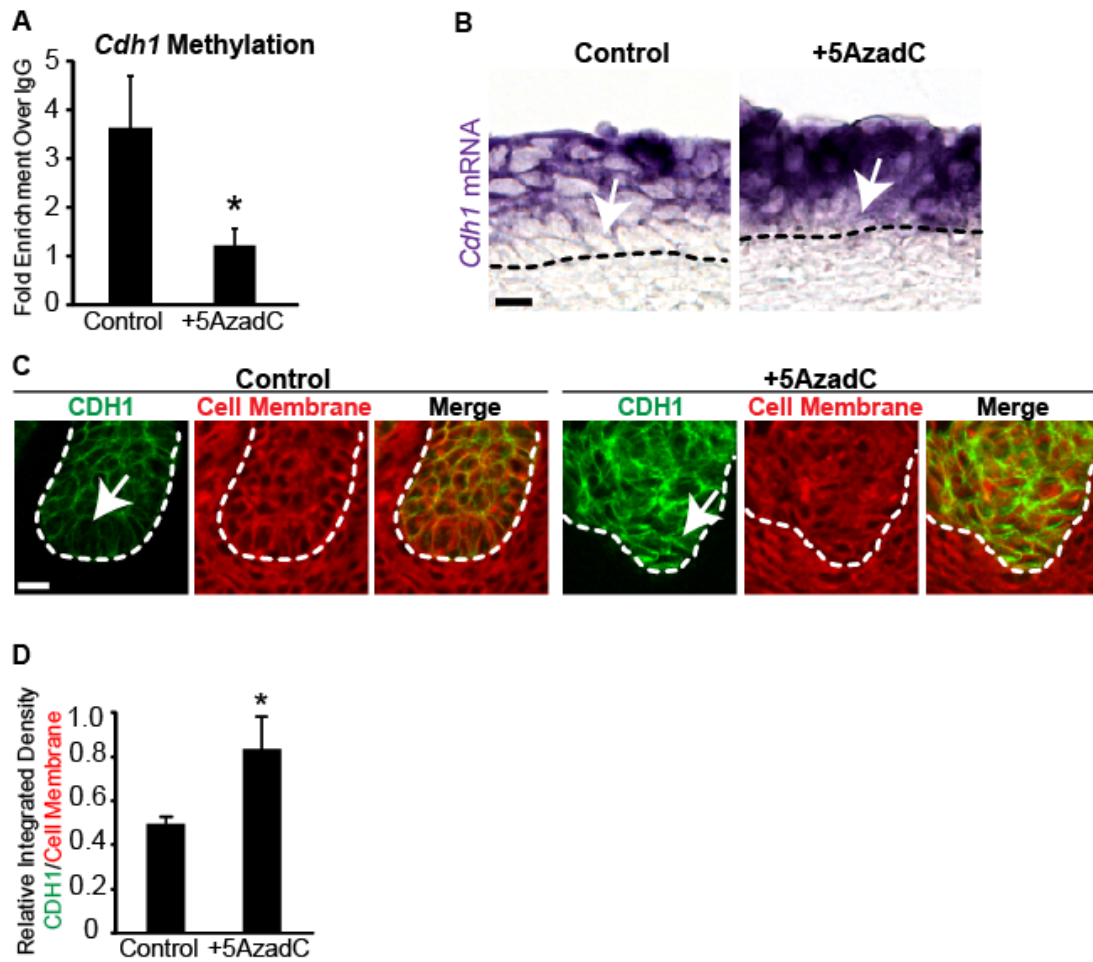


Fig. 5. DNA methylation is required for appropriate E-cadherin mRNA and protein expression in basal epithelium of UGS explant cultures. (A) MeDIP-QPCR was used to quantify E-cadherin (*Cdh1*) methylation in epithelium from 14 dpc UGSs cultured for 4 days in media containing 5 α -dihydrotestosterone (DHT, 10 nM) alone or DHT plus the DNA methylation inhibitor 5-aza-2'-deoxycytidine (5AzadC, 5 μ M). Female 14 dpc UGSs cultured for 7 days in media containing DHT (10 nM) alone or DHT plus 5AzadC were cut into 5 μ m sagittal sections and (B) stained by ISH to visualize *Cdh1* mRNA expression (purple) and (C) IHC to visualize CDH1 expression (green) and cell membranes (wheat germ agglutinin, red). (D) Quantification of relative integrated

density for CDH1 (green) versus cell membrane (wheat germ agglutinin, red) within the basal-most layer in control and 5AzadC treated UGSs. Results are presented as mean $\pm$ s.e.m, $n \geq 3$ litter independent UGS sections and tissues per group. Asterisks indicate a significant difference from control ($p < 0.05$). A dotted line indicates the interface between epithelium and mesenchyme, as determined by cell morphology. Arrows indicate cell layers with altered E-cadherin mRNA and protein abundance. Scalebar 10 μm .

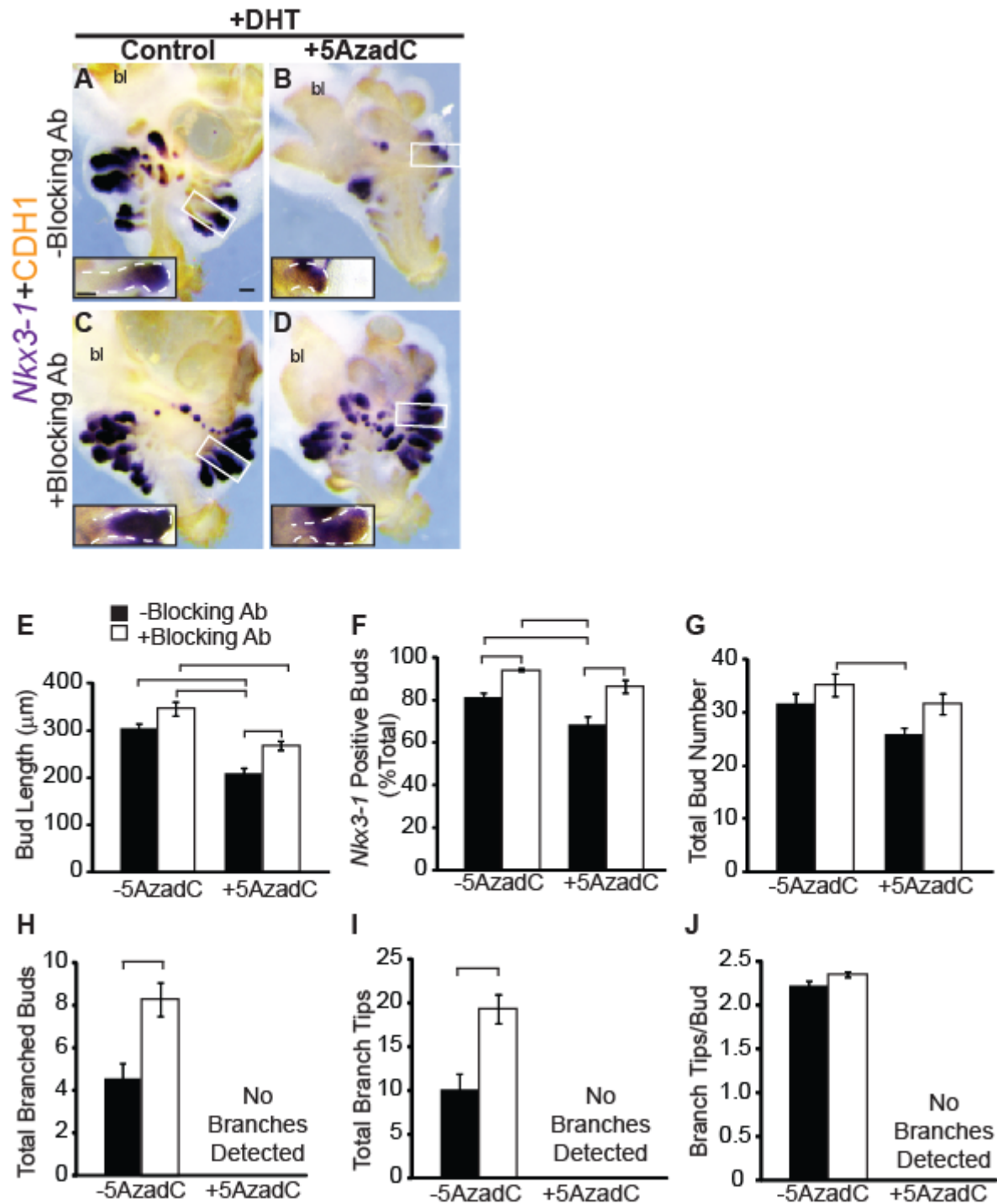


Fig. 6. A CDH1 function-blocking antibody restores prostatic bud specification and outgrowth in UGS explant cultures treated with a DNA methylation inhibitor. (A-D) Female 14 dpc UGSs were cultured for 6 days in media containing 5 α -

dihydrotestosterone (DHT, 10 nM) and either vehicle, the DNA methylation inhibitor 5-aza-2'-deoxycytidine (5AzadC, 5 μ M), an E-cadherin (CDH1) function-blocking antibody (Blocking Ab, 100 μ g/mL) or both 5AzadC and Blocking Ab. Following culture, tissues were stained by ISH for the prostatic bud marker NK-3 transcription factor, locus 1 (*Nkx3-1*, purple), immunohistochemically stained for CDH1 (red) to label all epithelium and imaged in a sagittal orientation. Scalebars 100 μ m. Quantification of (E) prostatic bud length, (F) percent total *Nkx3-1* positive buds (G) total bud number, (H) total number of buds with branches, (I) total number of branch tips and (J) branch tips per bud was conducted on cultured UGSs. Results are presented as mean $\pm$ s.e.m, $n \geq 6$ litter independent UGS tissues per group. Insets are magnified images across the length of a representative prostatic bud, a white dotted line outlines the epithelium. Lines above bar graphs indicate a significant difference between values ($p < 0.05$). Abbreviations; bl, bladder.

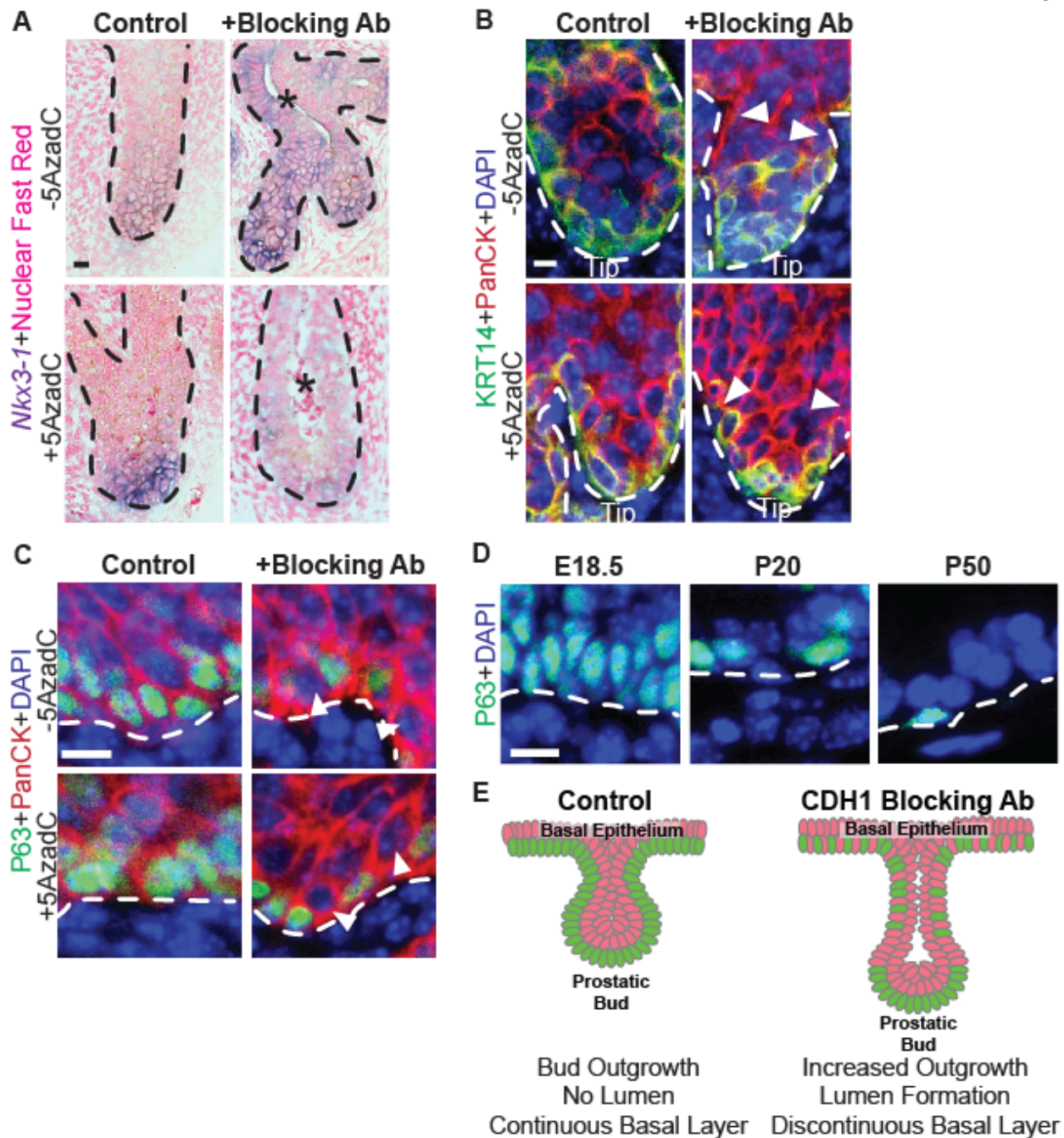


Fig. 7. E-cadherin (CDH1) controls the rate of prostate ductal maturation. (A-D) Female 14 dpc UGSs were cultured for 6 days in media containing 5 α -dihydrotestosterone (DHT, 10 nM) and either vehicle, the DNA methylation inhibitor 5-aza-2'-deoxycytidine (5AzadC, 5 μ M), a CDH1 function-blocking antibody (Blocking Ab, 100 μ g/mL) or both

5AzadC and Blocking Ab. (A) Whole mount ISH was used to visualize NK-3 transcription factor, locus 1 (*Nkx3-1*, purple) tissues were then cut into sagittal sections (5 μm) and counterstained with nuclear fast red. (B-D) IHC was used to detect basal epithelial markers keratin 14 (KRT14, green) or transformation-related protein, 63 (TRP63, green), and general epithelial marker Pan-cytokeratin (PanCK, red) in urethra and developing prostatic buds in sagittal sections (5 μm). Cell nuclei were stained with DAPI (blue) (D) 18.5 dpc, postnatal day P20 and P50 mouse prostate sagittal sections (5 μm) were immunofluorescently labeled to visualize the basal epithelial marker TRP63 (green). (E) Schematic representation of observed changes in basal epithelial organization in UGSs cultured in the presence of CDH1 function blocking antibody. Asterisks indicate the presence of lumens. A dotted line indicates the interface between epithelium and mesenchyme, as determined by cell morphology and epithelial staining. Closed arrowheads indicate areas of discontinuous basal epithelium. Scalebar 10 μm .

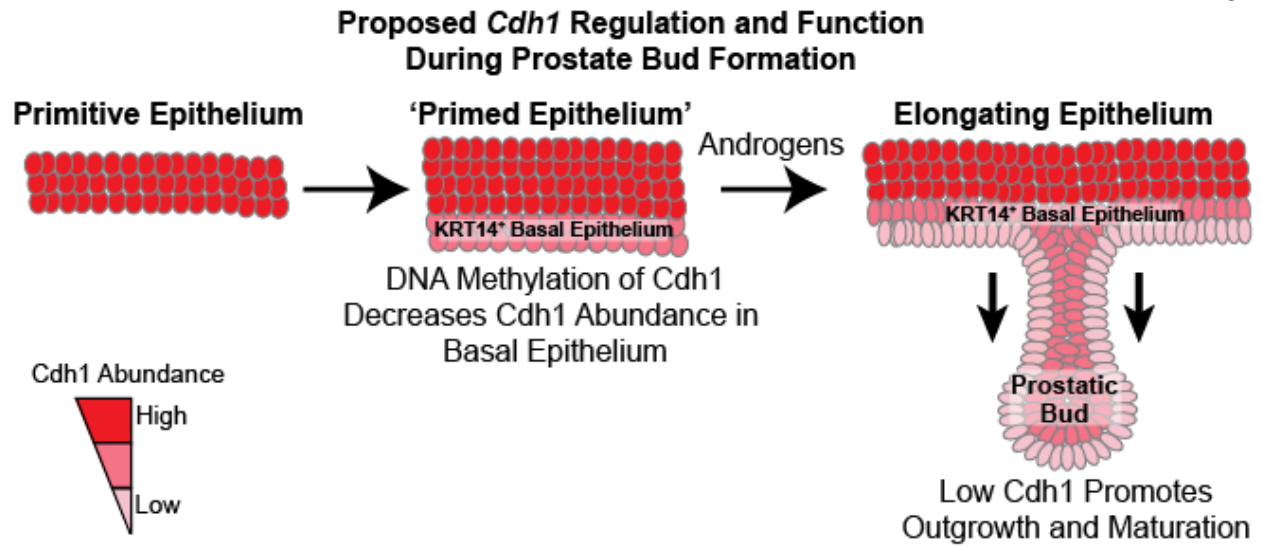
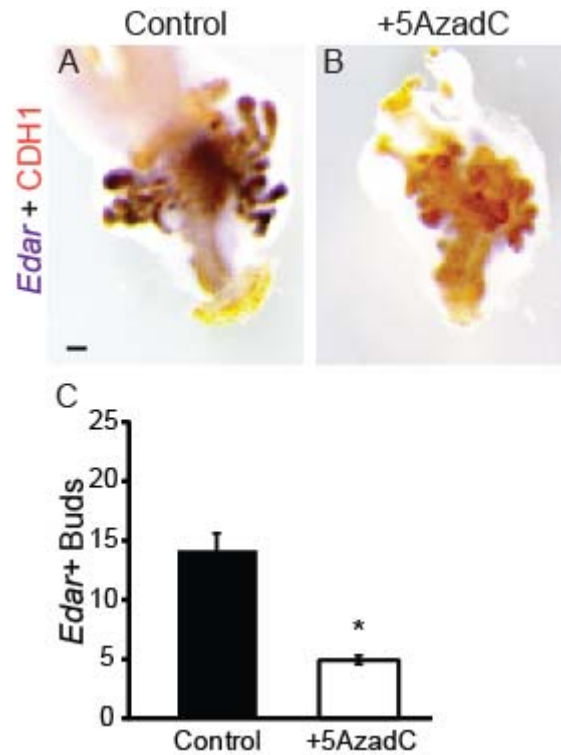
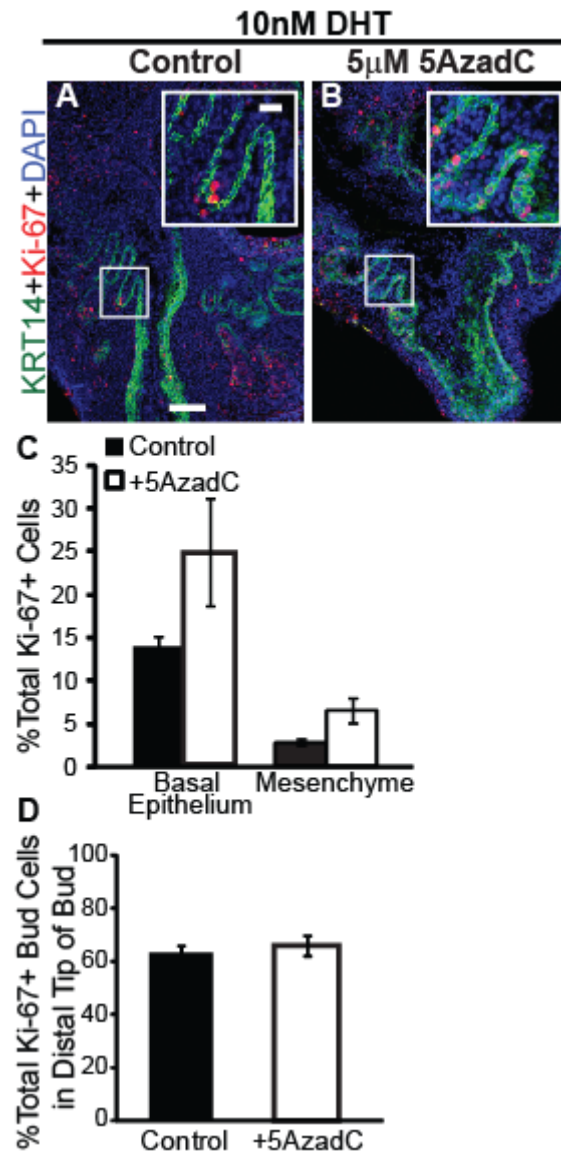


Fig. 8. Proposed mechanism of E-cadherin regulation and function during mouse prostatic bud formation. Methylation of E-cadherin (*Cdh1*) decreases mRNA and protein abundance in basal epithelial cells that give rise to prostatic buds. This event primes prostatic buds for the subsequent actions of androgens and other factors that are needed for outgrowth, differentiation and maturation of prostatic buds.

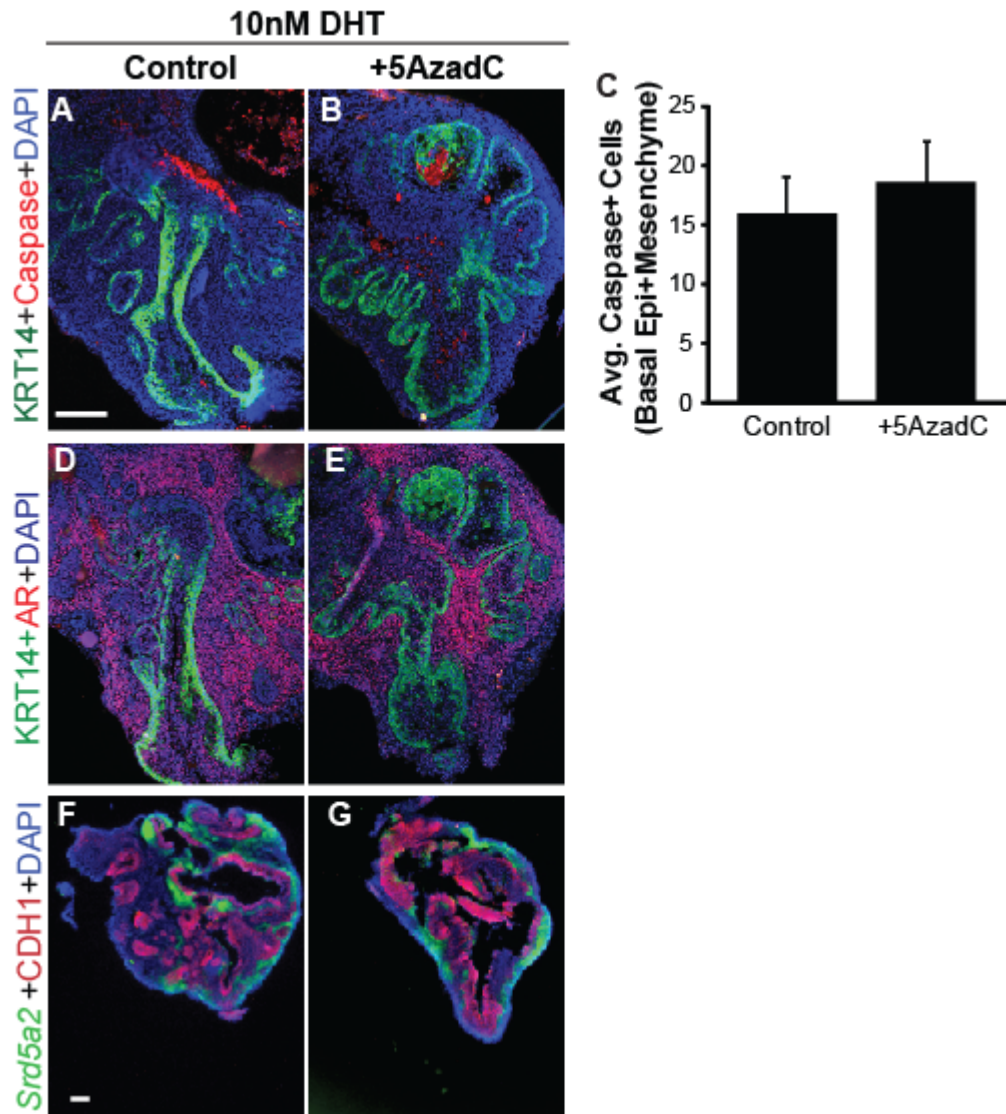


Supplemental Fig. 1. DNA methylation is required for proper prostate bud specification, outgrowth, and branching in UGS explant cultures. (A-B) 14 dpc female UGSs were cultured for 7 days in media containing 5 α -dihydrotestosterone (DHT, 10 nM) and DNA methylation inhibitor 5-aza-2'-deoxycytidine (5AzadC, 5 μ M). At the end of the culture period, tissues were stained by ISH to visualize the prostate marker ectodysplasin A receptor (*Edar*, purple), counterstained by IHC with CDH1 (red) to visualize all epithelium and imaged in a sagittal orientation. (C) Quantification of total buds expressing prostate marker, *Edar*. Scalebar 100 μ m. Results are presented as mean $\pm$ s.e.m, $n \geq 3$ litter independent UGS tissues per group. Asterisk indicates a significant difference from control ($p \leq 0.05$).



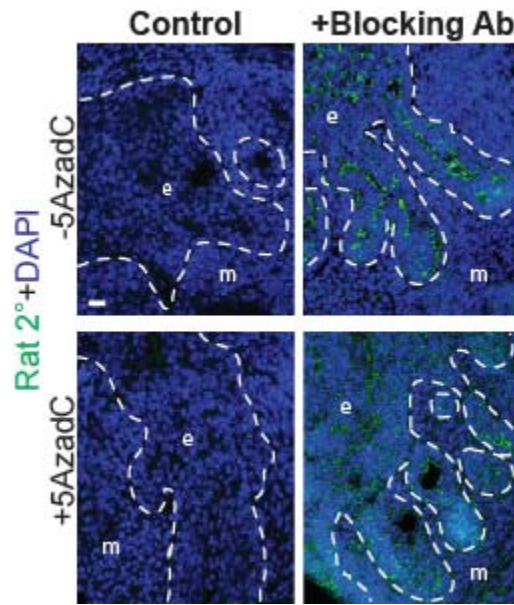
Supplemental Fig. 2. A DNA methylation inhibitor does not significantly change cell proliferation in cultured UGSs. Female 14 dpc UGSs were cultured for 7 days in media containing 5 α -dihydrtestosterone (DHT, 10 nM) alone or DHT and the DNA methylation inhibitor 5-aza-2'-deoxycytidine (5AzadC, 5 μ M). (A-B) Following culture, sagittal tissue sections (5 μ m) were immunofluorescently labeled to visualize cell proliferation marker Ki-67 (red) and basal epithelial marker keratin 14 (KRT14, green). Cell nuclei were labeled with DAPI (blue). Inset represents a magnified image. Scalebar 100 μ m, inset

20 μm . Quantification of (C) percent total Ki-67 positive cells within basal epithelium and mesenchyme and (D) percentage of prostatic bud Ki67 positive cells residing within distal prostatic bud tips in control and 5AzadC treated UGSs. Results are presented as mean $\pm$ s.e.m, $n \geq 3$ litter independent UGS tissues per group. There was no significant difference between control and 5AzadC treated tissues in basal epithelium ($p = 0.1$) or mesenchyme ($p = 0.06$).



Supplemental Fig. 3. A DNA methylation inhibitor does not alter cell death and causes no change in expression of androgen receptor or an androgen responsive gene. Female 14 dpc UGSs were cultured for 7 days in media containing 5 α -dihydrotestosterone (DHT, 10 nM) alone or DHT and the DNA methylation inhibitor 5-aza-2'-deoxycytidine (5AzadC, 5-10 μ M). (A-E) Following culture, sagittal sections (5 μ m) were immunofluorescently labeled to visualize cell death marker cleaved-caspase 3 (Caspase, red) or androgen receptor (AR, red) and basal epithelial marker keratin 14

(KRT14, green). Cell nuclei were labeled with DAPI (blue). (C) Graph represents total caspase positive cells within the KRT14 positive basal epithelium and mesenchyme. (F-G) Cultured tissues were frozen, sectioned (10 μm) and stained by ISH to visualize expression of the androgen responsive gene steroid 5 alpha reductase type 2 (*Srd5a2*, pseudo-colored green) and counterstained by immunofluorescence with E-cadherin (CDH1, red) to label all epithelium. Cell nuclei were labeled with DAPI (blue). Scalebar 100 μm . Results are presented as mean $\pm$ s.e.m, $n \geq 3$ litter independent UGS tissues per group.



Supplemental Fig. 4. CDH1 function-blocking antibody is detected in the epithelium of cultured UGSs. Female 14 dpc UGSs were cultured for 6 days in media containing 5 α -dihydrotestosterone (DHT, 10 nM) and either vehicle, the DNA methylation inhibitor 5-aza-2'-deoxycytidine (5AzadC, 5 μ M), an E-cadherin (CDH1) function-blocking antibody (Blocking Ab, 100 μ g/mL) or both 5AzadC and Blocking Ab. Cultured tissues were sectioned sagittally (5 μ m) and immunofluorescently labeled with anti-rat secondary (green) to detect the Blocking Ab. Cell nuclei were labeled with DAPI (blue). A dotted line indicates the interface between epithelium and mesenchyme. Abbreviations: e, epithelium; m, mesenchyme. Scalebar 20 μ m.

**Chapter 6: Androgen receptor DNA methylation regulates the timing and
androgen sensitivity of mouse prostate ductal development**

Keil et al., Dev Biol. (2014) In press

Introduction

Precisely timed hormonal cues mediate several crucial developmental transitions such as metamorphosis in invertebrates and amphibians (Kulkarni et al.,2014, Di Cara et al.,2013), molting in insects (Ono,2014, Suzuki et al.,2013), and growth and puberty in mammals (Di Cara et al.,2013, Kurian et al.,2012, Walker et al.,2013). During mouse prostate ductal development, precisely timed androgenic cues guide a series of morphogenetic events that include prostatic bud specification (15-16 days post coitus, dpc), initiation (16-18 dpc), elongation (18+ dpc), and branching morphogenesis (birth-postnatal day 15) (Vezina et al.,2008, Sugimura et al.,1986). Our research has been focused on elucidating mechanisms of prostatic bud formation.

Androgens initiate prostatic bud formation by binding and activating androgen receptors (ARs) in urogenital sinus (UGS) mesenchyme during prostatic bud specification (Allgeier et al.,2010, Lasnitzki et al.,1980). Interestingly, mouse prostate development lags behind the onset of gonadal testosterone synthesis by several days, a feature shared by other mammalian species (Wilson et al.,2003). Mouse testicular androgen synthesis begins at 13 dpc but prostate bud outgrowth does not begin until three days later, at 16 dpc (Lin et al.,2003). The lag is not due to the time it takes nascent testicular androgens to reach the UGS, or to accumulate in the UGS at sufficient concentrations, because it cannot be accelerated *in vitro* by growing UGSs in media containing physiological or supra-physiological androgen concentrations (Lasnitzki et al.,1977).

We hypothesized that DNA methylation may function as a molecular switch controlling the transition from prostatic bud specification to initiation. DNA methyltransferases (*Dnmts*) catalyze addition of a methyl group to the 5' position of deoxycytidine, which can influence chromatin conformation and gene expression (Jaenisch et al.,2003). The *Dnmt* mRNA expression pattern is reorganized between prostatic bud specification and initiation. While *Dnmts* predominate in UGS mesenchyme during prostatic bud specification, they are more noticeable in UGS epithelium during bud initiation and early branching morphogenesis (Keil et al.,2013). This temporal change in *Dnmt* expression coincides with an increase in UGS mesenchymal *Ar* mRNA expression (Takeda et al.,1991, Keil et al.,2012). These previous observations led us to hypothesize that DNMT actions may converge on the *Ar* to regulate its expression, thereby modulating UGS androgen responsiveness and the onset of prostatic bud formation.

In this study, we found that DNA methylation regulates UGS androgen sensitivity and the timing of prostatic bud formation. The abundance of *Ar* promoter DNA methylation in UGS mesenchyme decreases during prostatic outgrowth *in vivo*, coinciding with decreased *Dnmt* expression over the same period. Administration of a DNA methylation inhibitor during prostatic bud formation *in vitro* accelerates prostatic bud formation and increases bud number. The DNA methylation inhibitor also acts on the UGS *in vitro* by reducing *Ar* DNA methylation, increasing *Ar* mRNA and protein abundance, and increasing sensitivity of prostatic bud formation to androgens. These results led us to conclude that a regulatory region of the *Ar* gene is likely methylated prior to prostate bud formation to dampen AR expression and protect against

precocious development; DNA methylation is then decreased allowing for androgen-dependent onset of prostatic bud formation. Our findings are important because they provide new testable mechanisms for how prostate development is temporally regulated and how environmental and estrogenic chemicals may perturb prostate development.

Materials and Methods

Animals

Wild type C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed in polysulfone cages containing corn cob bedding and were maintained on a 12 hour light and dark cycle at $25\pm 5^{\circ}\text{C}$ and 20-50% relative humidity. Feed (Diet 2019 for males and Diet 7002 for pregnant females, Harlan Teklad, Madison, WI) and water were available *ad libitum*. All procedures were approved by the University of Wisconsin Animal Care and Use Committee and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. To obtain timed-pregnant dams, females were paired overnight with males. The next morning was considered 0 days post coitus (dpc). Pregnant dams were euthanized by CO_2 asphyxiation and UGS tissue collected from resulting fetuses. For tissue separations 14 and 17 dpc UGS mesenchyme was enzymatically and mechanically separated from UGS epithelium and homogenized as described previously (Vezina et al.,2008).

***In situ* hybridization (ISH)**

ISH was conducted on whole mount tissues as described previously (Abler et al.,2011, Keil et al.,2012). Detailed protocols for PCR-based riboprobe synthesis are

available at www.gudmap.org. Staining patterns were assessed in at least three litter independent tissues per group. Tissues were processed as a single experimental unit to allow for qualitative comparisons among biological replicates and treatment groups.

Organ Culture

Male or female 14 dpc mouse urogenital sinus (UGS) explants were placed on 0.4- μ m Millicell-CM filters (Millipore, Billerica, MA) and cultured as described previously (Vezina et al.,2008). Media was supplemented with one or all of the following: 0.01-10 nM 5 α -dihydrotestosterone (DHT), 0.1% dimethyl sulfoxide (DMSO, vehicle control) or DMSO containing 5 μ M 5-aza-2'-deoxycytidine (5AzadC, A3635, Sigma-Aldrich, St. Louis, MO). Media and supplements were changed every 2 days.

Immunohistochemistry (IHC)

Immunofluorescent staining of ISH-stained tissues and paraffin sections was performed as described previously (Abler et al.,2011, Keil et al.,2012). Primary antibodies were diluted as follows: 1:200 rabbit anti-CDH1 (3195, Cell Signaling Technology, Beverly, MA), 1:250 mouse anti-CDH1 (610181, BD Transduction Laboratories, San Jose, CA) 1:50 mouse anti-KRT14 (ms-115-p0, Thermo Fisher Scientific), 1:250 rabbit anti-AR (sc-816, Santa Cruz Biotechnology, Santa Cruz, CA), 1:200 rabbit anti-DNMT1 (5032, Cell Signaling Technology). Secondary antibodies were diluted as follows: 1:250 Dylight 549-conjugated goat anti-rabbit IgG (111-507-003, Jackson ImmunoResearch, West Grove, PA), 1:250 Dylight 488-conjugated goat anti-rabbit IgG (111-487-003, Jackson ImmunoResearch) and 1:250 Dylight 488-conjugated

goat anti-mouse IgG (115-487-003, Jackson ImmunoResearch). Immunofluorescently labeled tissues were counterstained with 4',6-diamidino-2-phenylindole, dilactate (DAPI), and mounted in anti-fade media (phosphate buffered saline containing 80% glycerol and 0.2% n-propyl gallate). Whole mount immunohistochemistry was performed as described previously (Keil et al.,2012). Primary antibody was diluted 1:750 rabbit anti-CDH1 and secondary antibody was diluted 1:500 biotin conjugated goat anti-rabbit IgG (BA-1000, Vector, Burlingame, CA).

Methylated DNA Immunoprecipitation (MeDIP)

MeDIP was conducted as described previously (Keil et al.,2014). UGS explants were pooled (5-6 tissues/pool) and each experimental group consisted of five pools. Real time quantitative PCR (QPCR) was performed as described previously (Keil et al.,2012) using gene specific primers for mouse *Ar*: 5'-AGAGACGAGGAGGCAGGATAAG-3' and 5'-CGCTCCTCGATAGGTCTTGG-3' (Entrez gene ID 11835) spanning the region +58bp to -84bp of the transcription start site (TSS).

Pyrosequencing of Bisulfite Converted DNA

Genomic DNA was isolated using the Qiagen DNeasey Blood and Tissue Kit according to manufacturer's instructions. Bisulfite conversion of DNA was performed using the Qiagen Epiect Bisulfite Kit per manufacturer's instructions. Calibration standards of highly methylated DNA, prepared as described previously (Tost et al.,2007), and unmethylated DNA, prepared using the Qiagen REPLI-g Mini Kit according to manufacturer's instructions, were used as controls. One microgram of

bisulfite treated DNA was used to generate PCR amplified templates for pyrosequencing. Fifty microliter PCR reactions consisted of bisulfite DNA, 1x GoTaq Buffer, 0.4mM dNTP, 0.3uM primers and 0.05U/uL GoTaq (Promega, Madison, WI) and the following primers generated by PyroMark 2.0 software: *Ar* (NC_000086.7) (+209 to + 230 of *Ar* TSS) forward 5'-GTTTAAGGATGGAGGTGTAGTTAG-3', and biotinylated reverse 5'-AAACCAAATAACCTATAAAACCTCTAAT-3'. PCR conditions were as follows: 95°C x 5min, followed by 45 cycles of 95°C x 30s, 50°C x 30s, and 72°C x 1min followed by 72°C for 5 min. Ten microliters of biotinylated PCR product was used for each sequencing assay, which included the following sequencing primer: 5'-ATAGTAGAGGTAGGAGATTAGTTT-3'. Pyrosequencing was performed using PyroMark Gold Q96 reagents (Biotage, Uppsala, Sweden) and streptavidin sepharose beads (GE, Cat# 17-5113-01) with the PSQ HS 96 Pyrosequencer. Percent methylation of each CpG was determined using Pyro Q-CpG Software (Biotage, Uppsala, Sweden). Average methylation at each CpG was calculated from three technical replicates with at least three litter independent sample pools per group.

Real Time Quantitative PCR (QPCR)

QPCR was conducted as described previously (Keil et al.,2012) on UGS explant pools (5-6 tissues/pool) with five pools per experimental treatment group using the following gene specific primers: *Ar*, 5'-GATGGTATTTGCCATGGGTTG-3' and 5'-GGCTGTACATCCGAGACTTGTG-3' and peptidyl prolyl isomerase a (*Ppia*), 5'-TCTCTCCGTAGATGGACCTG-3' and 5'-ATCACGGCCGATGACGAGCC-3'. Relative

mRNA abundance was determined by the $\Delta\Delta\text{Ct}$ method as described previously (Livak et al.,2001) and normalized to *Ppia* abundance.

Statistical analyses

For prostatic bud counting, UGSs were stained by ISH for *Nkx3-1* mRNA and counted as described previously (Keil et al.,2012). For immunolabeled cell counting, AR positive cells were counted in at least two sections from three litter independent tissues per treatment group. Statistical analysis was performed using R version 2.13.1. Homogeneity of variance was determined using Bartlett's test. Student's T-test, one way analysis of variance (ANOVA), followed by Tukey's Honest Significant Difference (HSD) or analysis of covariance (ANCOVA) tests were used to identify significant differences ($p \leq 0.05$) between or among treatment groups.

Results

DNA Methylation Regulates Prostatic Ductal Development by Restricting Bud Formation

We previously reported that the spatial pattern of DNA methyltransferase 1 (*Dnmt1*) mRNA changes during early prostate development (Keil et al.,2013). Here, we demonstrate that DNMT1 protein follows the same spatiotemporal expression pattern as its mRNA. During prostatic bud specification, DNMT1 protein predominates in UGS mesenchyme (Fig. 1A). During prostatic bud initiation and outgrowth, DNMT1 protein is diminished in UGS mesenchyme and is abundant in UGS epithelium and prostatic buds (Fig. 1B). This change in DNMT1 protein expression pattern over the course of prostatic

bud formation led us to hypothesize DNA methylation elicits different actions at each stage of prostatic bud formation. To elucidate the early stage actions of DNA methylation, we pulse-treated UGSs with a DNA methylation inhibitor during specification (when DNMT1 predominates in mesenchyme) and examined subsequent bud formation. UGSs were collected from 14 dpc male mouse fetuses (prior to prostatic formation), grown for two days in androgen-free media containing DNA methylation inhibitor 5'-aza-2'-deoxycytidine (5 μ M, 5AzadC) or vehicle (0.1% DMSO) alone, then grown for an additional five days in media containing androgen (10nM dihydrotestosterone, DHT) but no DNA methylation inhibitor (Fig. 2A). UGS explants were stained by ISH to visualize the prostate marker, NK-3 transcription factor, locus 1 (*Nkx3-1*) (Keil et al.,2012, Bieberich et al.,1996, Sciavolino et al.,1997) and by IHC to visualize UGS epithelium so that prostatic buds could be counted. UGS explants cultured in the presence of DNA methylation inhibitor during the first two days of a seven day culture period formed a greater number of buds compared to controls (Fig. 2B-C).

We next tested whether inhibition of DNA methylation augments prostatic bud formation specifically in male UGS, or whether this action is generalizable to both male and female UGS. Although male and female UGS development differs *in vivo*, female UGSs can be induced by exogenous androgens to form prostatic buds in a pattern that approximates bud formation in males (Cunha,1975). Female UGS explants grown in the presence of 5AzadC during the first two days of a seven day culture period also formed a greater number of buds compared to controls (Supplemental Figure 1). Together these results are consistent with DNA methylation functioning as a general mechanism

to refine androgen dependent prostatic development by restricting the number of prostatic buds formed.

DNA Methylation Acts During Early Development to Control Prostatic Bud Formation

The DNMT1 protein expression pattern changes from UGS mesenchyme dominant to UGS epithelium dominant during the period spanning mouse prostatic bud specification, initiation and outgrowth (Fig. 1). To test whether inhibition of DNA methylation augments prostatic bud formation during all stages of prostatic bud formation, or exclusively during the specification stage when DNMT1 predominates in UGS mesenchyme, male 14 dpc UGS explants were pulse-treated with 5AzadC during the first two days (prior to bud outgrowth, during specification), middle two days (during bud initiation) or last two days (during bud elongation) of a seven day culture period in media containing androgen (10nM DHT) (Fig. 3A). UGS explants were then stained and prostatic buds counted as described above (Fig. 3B). 5AzadC significantly increased bud number if administered during the first two days of culture but not during the middle or last two days (Fig. 3C). These results indicate that DNA methylation functions during prostatic bud specification, but not during bud initiation and elongation, to control the number of prostatic buds formed.

DNA Methylation Controls the Rate of Androgen Dependent Prostatic Bud Formation

We hypothesized inhibition of DNA methylation may augment prostatic bud number by allowing an increased number of prostatic buds to form during the earliest stages of prostatic ductal development. To test this hypothesis, we exposed 14 dpc male UGSs to a single two day pulse of 5AzadC or vehicle alone, and then examined prostatic bud formation after one, three, or five additional days of growth in media containing androgens but no 5AzadC. UGS explants were stained and total prostatic bud number quantified as described above (Fig. 4B). UGSs that were pulse treated with 5AzadC showed significantly more prostatic buds at all three time points than control-treated UGSs (Fig. 4C). Linear regression analysis revealed that 5AzadC-treated UGSs had a significantly greater rate of prostatic bud formation than control-treated UGSs (Fig. 4D). These results suggest that DNA methylation acts to control the timing and rate of androgen-induced prostatic bud formation from the initial stages of prostate development.

DNA Methylation Controls Androgen Sensitivity During Androgen Dependent Prostate Development

The number of prostatic buds formed in control C57BL/6J fetuses is highly reproducible between individual mice (8). Our results support the hypothesis that DNA methylation is one factor that restricts the quantity of prostatic buds formed. One possible mechanism by which this restriction occurs is that DNA methylation regulates UGS sensitivity to androgens. To examine this mechanism, we tested whether inhibition of DNA methylation augments prostatic bud formation in a low androgen environment. Male UGSs already exhibit androgen-induced gene expression at 14dpc (Abler et

al.,2011). To ensure that UGSs used in this experiment were naïve to high androgen levels prior to the culture period, we used female UGSs, which also form prostatic buds in response to androgens (21, Cunha,1975). Female UGSs were grown for two days in media containing 5AzadC (5 μ M) or vehicle alone, followed by five additional days in media containing androgen alone. The androgen concentration in this study (0.01nM DHT) was 1000x lower than the concentration traditionally used to support prostate morphogenesis in mouse UGS organ culture (Allgeier et al.,2010, Vezina et al.,2008). While female UGSs are capable of forming small androgen independent buds (7), the addition of 5AzadC caused female UGSs to form significantly more buds (by at least two fold) versus control UGSs (Fig. 5). These results indicate that DNA methylation regulates prostatic bud formation at least in part by regulating androgen sensitivity of the budding response.

DNA Methylation of the Androgen Receptor Controls Prostate Bud Formation

We have shown that inhibition of DNA methylation via 5AzadC enhances prostatic bud number and increases androgen sensitivity. One potential mechanism to explain both of these results is that 5AzadC reduces *Ar* DNA methylation, thereby increasing *Ar* mRNA and protein expression. The *Ar* gene locus contains a 1.5kb CpG island that encompasses the transcription start site and exon 1, a region that is actively methylated in humans and rodents (Jarrard et al.,1998,Takahashi et al.,2002-Kim et al.,2013). DNA methylation changes within this region have been demonstrated in cell lines (Tian et al.,2012) and C57Bl/6 mouse tissues (Kim et al.,2013). We used this known region of *Ar* DNA methylation to examine whether 5AzadC is capable of

decreasing *Ar* DNA methylation in mouse UGS explants, and to validate analytical tools for assessing *Ar* DNA methylation. 14 dpc female UGS explants were cultured in the presence of androgen alone or androgen and 5AzadC, then pyrosequencing of bisulfite converted DNA was performed on isolated UGS tissues. DNA methylation was detected at all sites (Supplemental Figure 2) and 5AzadC significantly reduced the percentage of DNA methylation at five of five CpG sites in the region analyzed (Supplemental Figure 2).

While pyrosequencing demonstrated decreased DNA methylation of the *Ar* CpG region following 5AzadC treatment, this method cannot discriminate between 5-methylcytosine and 5-hydroxymethylcytosine, a base modification that can mediate DNA demethylation (Mohr 2011) and that could potentially confound our results. Therefore, we used methylated DNA immunoprecipitation followed by QPCR (MeDIP-QPCR), a method specific to 5-methylcytosine-mediated DNA methylation, to independently confirm our pyrosequencing results. Using QPCR primers designed to amplify the same region examined by pyrosequencing, the MeDIP-QPCR results corroborated our pyrosequencing results, showing that 5AzadC significantly reduces *Ar* DNA methylation in mouse UGS (Supplemental Figure 2) and validating use of MeDIP-QPCR for analysis of *Ar* DNA methylation in this study.

We next used MeDIP-QPCR, along with RT-QPCR and immunostaining, to test whether 5AzadC treatment influences *Ar* DNA methylation status and mRNA and protein expression during prostatic bud specification. 14 dpc male UGS explants were cultured for two days in the presence of androgens and either 5AzadC or vehicle, after which *Ar* promoter DNA methylation was measured by MeDIP-QPCR. 5AzadC

significantly decreased *Ar* promoter DNA methylation (Fig. 6A) and also increased *Ar* mRNA (Fig. 6B) and protein abundance (Fig. 6C-F). 5AzadC treatment increased the frequency of detectable AR staining in UGS epithelial cells by almost 20%, detectable AR staining in KRT14+ basal epithelial cells by nearly 10%, and detectable AR staining in UGS mesenchyme cells by 10% (Fig. 6D-F). These results reveal that 5AzadC decreases *Ar* promoter DNA methylation and increases AR expression, suggesting a possible mechanism for the increased androgen sensitivity of the prostatic budding response we observed.

Based on the above *in vitro* findings and the diminished expression of *Dnmts* in mesenchyme from 14 dpc to 17 dpc (Fig. 1) (Keil et al., 2013), we tested the hypothesis that *Ar* DNA methylation also decreases in UGS mesenchyme over the course of prostate bud formation. 14 and 17 dpc male UGS mesenchyme was isolated and *Ar* promoter DNA methylation measured by MeDIP-QPCR. The *Ar* promoter was more highly methylated in male UGS mesenchyme at 14 dpc compared to 17 dpc (Fig. 7A). This temporal change in *Ar* DNA methylation coincides with an increase in UGS mesenchymal *Ar* mRNA (Takeda et al., 1991) and protein expression from 14 dpc to 17 dpc (Fig. 7B-C). These results provide evidence *in vivo* for the potential mechanism we identified with inhibiting DNA methylation *in vitro*: *Ar* DNA methylation is diminished during initiation of prostate development to trigger an increase in AR expression and androgen-dependent prostatic bud formation.

Discussion

Our results suggest a link between DNA methylation and androgen responsive prostatic bud growth through a mechanism likely involving the AR. Inhibiting DNA methylation in the UGS prior to prostatic bud formation decreases *Ar* promoter DNA methylation, increases *Ar* mRNA and protein abundance, increases UGS sensitivity to androgens, and causes the UGS to form prostatic buds at an earlier stage and in greater number. These results suggest that DNA methylation establishes a developmental checkpoint that restricts UGS androgen sensitivity and responsiveness prior to the onset of prostate development.

The tools for investigating mechanistic roles of DNA methylation at specific CpG loci are limited. We used a pharmacological approach to identify prostate developmental phenotypes associated with carefully timed exposure to a global DNA methylation inhibitor, 5AzadC. While 5AzadC is capable of inhibiting DNA methylation across the genome, other studies using this drug reveal its scope of action on gene expression is surprisingly narrow - often involving only a few hundred genes (Yamashita et al.,2006). We previously used 5AzadC as a chemical probe to demonstrate a crucial role for E-cadherin DNA methylation in prostatic bud outgrowth (Keil et al.,2014). Here, using the same approach but modifying the timing of 5AzadC exposure, we revealed a second prostate development phenotype linked to DNA methylation – the timing and scope of prostatic bud formation. While we acknowledge that many potential DNA methylation sites could contribute to this process, our collective results point toward DNA methylation of the *Ar* promoter as playing a key role in this process. Specifically, we showed that 5AzadC 1) increases the number of prostatic buds formed in culture, 2) accelerates the rate of prostatic bud formation, 3) causes a greater quantity of prostate

buds to form under low androgen concentrations, 4) significantly decreases *Ar* promoter DNA methylation, 5) significantly increases *Ar* mRNA abundance and 6) significantly increases the percentage of AR protein-positive cells in the UGS. We also confirmed *in vivo* that *Ar* promoter DNA methylation decreases from 14 to 17 dpc.

Our results indicate that the DNA methylation status of the male mouse UGS prior to prostatic bud formation influences the pattern and number of prostatic buds it will form. Several environmental chemicals and estrogenic chemicals inappropriately influence prostate budding and our new results may shed light on the mechanisms by which these chemicals act on the UGS to influence prostate development. For example, bisphenol A (BPA), diethylstilbestrol and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin modulate the number and pattern of prostatic buds formed in fetal mice (Vezina et al.,2008, Timms et al.,2005) and have separately been shown to influence DNA methylation patterns in mouse UGS and elsewhere (Tang et al.,2012, Li et al.,2003). BPA also increases AR expression in the UGS, but the responsible mechanisms have not been identified (Richter et al.,2007). Our new results suggest the intriguing hypothesis that BPA, and potentially other estrogenic chemicals, may influence prostatic bud formation by altering *Ar* DNA methylation in the UGS.

Fetal mouse prostatic bud formation trails the onset of testicular testosterone synthesis by about three days. The cause for this delay between onset of androgen synthesis and initiation of androgen-dependent prostate bud formation is not known. We showed that prostate androgen sensitivity and bud formation can be enhanced in the presence of a DNA methylation inhibitor, thereby revealing the possibility that DNA methylation of the *Ar* promoter may account for this delay, safeguarding against

precocious development of prostatic buds. How DNA methylation is regulated in the UGS and whether DNA methylation continues to control prostate androgen sensitivity throughout life is unknown. Further, whether DNA methylation guides checkpoints of hormonal responsiveness in other developing hormone responsive tissues is yet to be determined.

An interesting finding resulting from our studies is that the DNA methylation inhibitor enhanced AR expression not only in UGS mesenchyme, where it is known to be necessary for prostate development (Cunha,1973), but also in UGS epithelium. There was a 20% increase in AR positive cells in the epithelium of UGSs treated with 5AzadC versus control and a 10% increase in mesenchyme. AR is not required in UGS epithelium for prostate development (Cunha,1973), but the consequence of precocious epithelial AR expression during prostatic bud formation has never been examined. Determining the consequence of increased UGS epithelial AR and whether changes in AR DNA methylation in UGS mesenchyme or epithelium are responsible for enhanced budding in response to 5AzadC pose intriguing avenues of future study.

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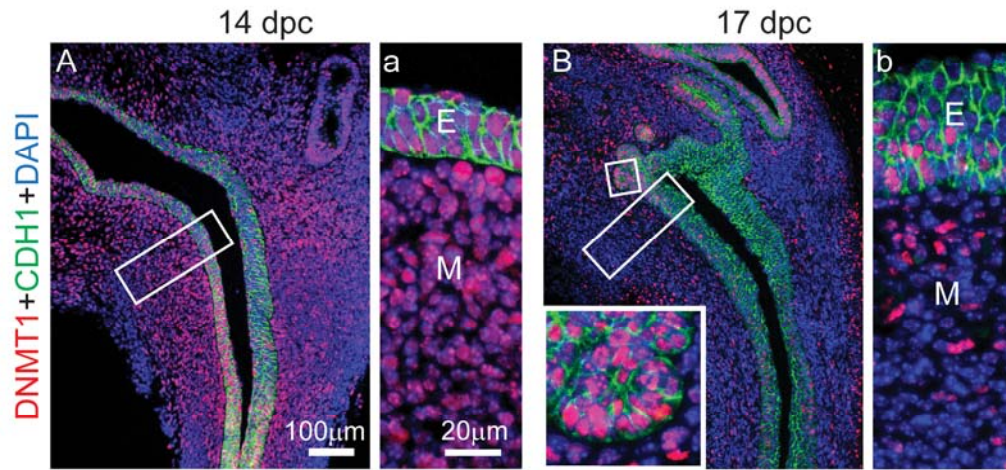


Figure 1. DNMT1 protein abundance diminishes in the mesenchyme during prostate development. (A,a) 14 dpc and (B,b) 17 dpc lower urinary tract sagittal sections were stained by immunohistochemistry to visualize DNMT1 (red) protein and all epithelium E-cadherin (CDH1, green). Cell nuclei were stained with DAPI. Insets represent magnified images. Abbreviations: E, epithelium; M, mesenchyme.

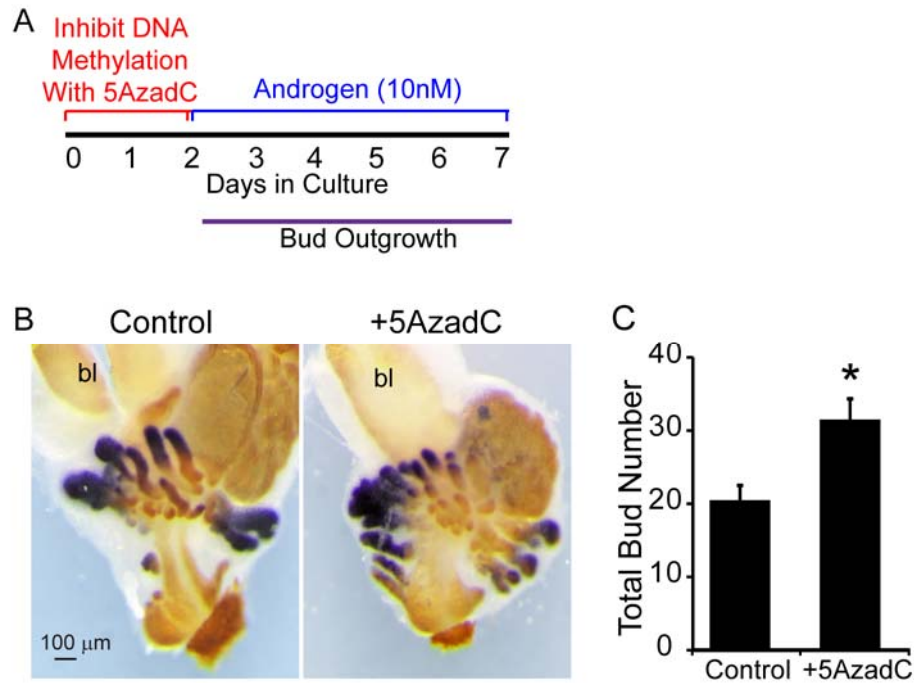


Figure 2. DNA methylation restricts prostate bud number. (A) 14 dpc male UGSs were cultured for 2 days in the presence of DNA methylation inhibitor 5'-aza-2'-deoxycytidine (5 μ M, 5AzadC) alone followed by 5 days in media containing androgen (10nM dihydrotestosterone, DHT) alone. (B) Following culture tissues were stained by ISH to visualize prostate bud marker NK-3 transcription factor locus 1 (*Nkx3-1*) (purple) and IHC to visualize all epithelium (E-cadherin, CDH1, brown). (C) Quantification of total prostatic bud number in control and 5AzadC treated samples. Results are mean $\pm$ SE, n=5/group. Asterisk indicates significant difference from control $p \leq 0.05$. Abbreviations: bl, bladder.

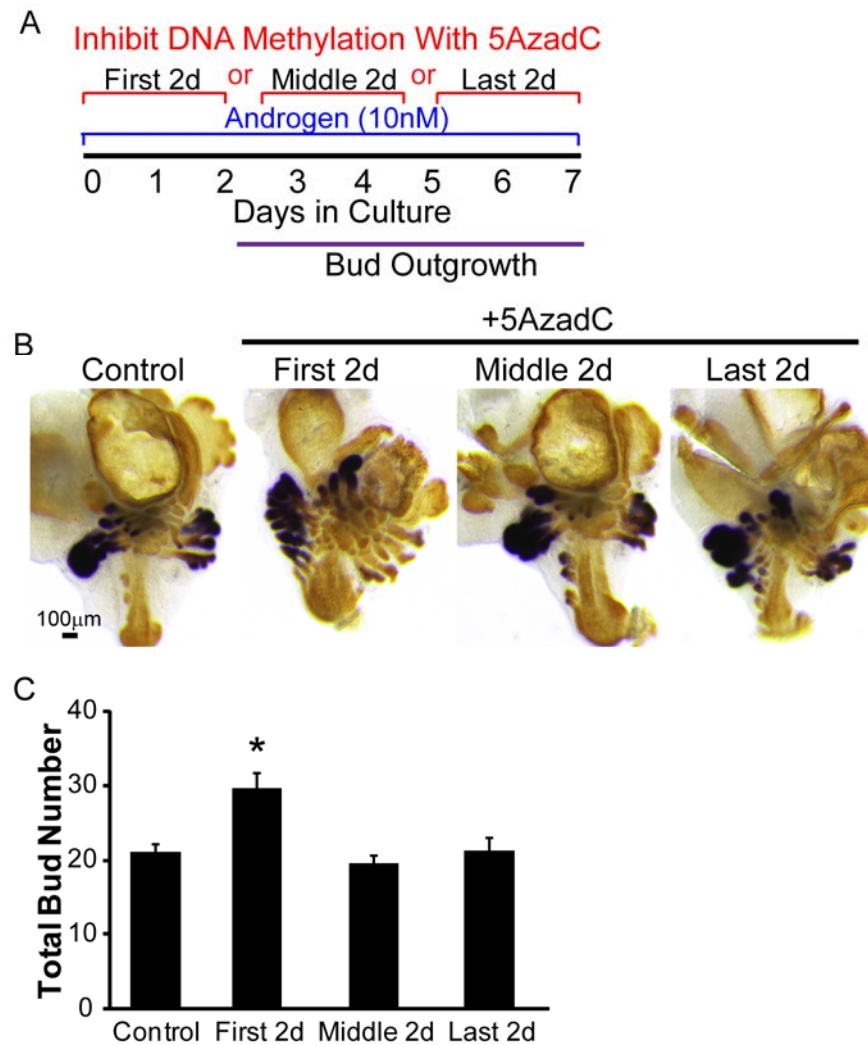


Figure 3. DNA methylation acts during specification to restrict prostate bud number. (A) 14 dpc male UGSs were cultured in media containing androgen (10nM dihydrotestosterone, DHT) and DNA methylation inhibitor 5'-aza-2'-deoxycytidine (5µM, 5AzadC) either on the first, middle or last 2 days of culture. (B) Tissues were stained by ISH for NK-3 transcription factor locus 1 (*Nkx3-1*) (purple) to visualize prostate buds and IHC to visualize all epithelium (E-cadherin, CDH1, brown). (C) Quantification of total prostatic bud number in control and 5AzadC treated samples. Results are mean ± SE, n≥3/group. Asterisk indicates significant difference from control p≤0.05.

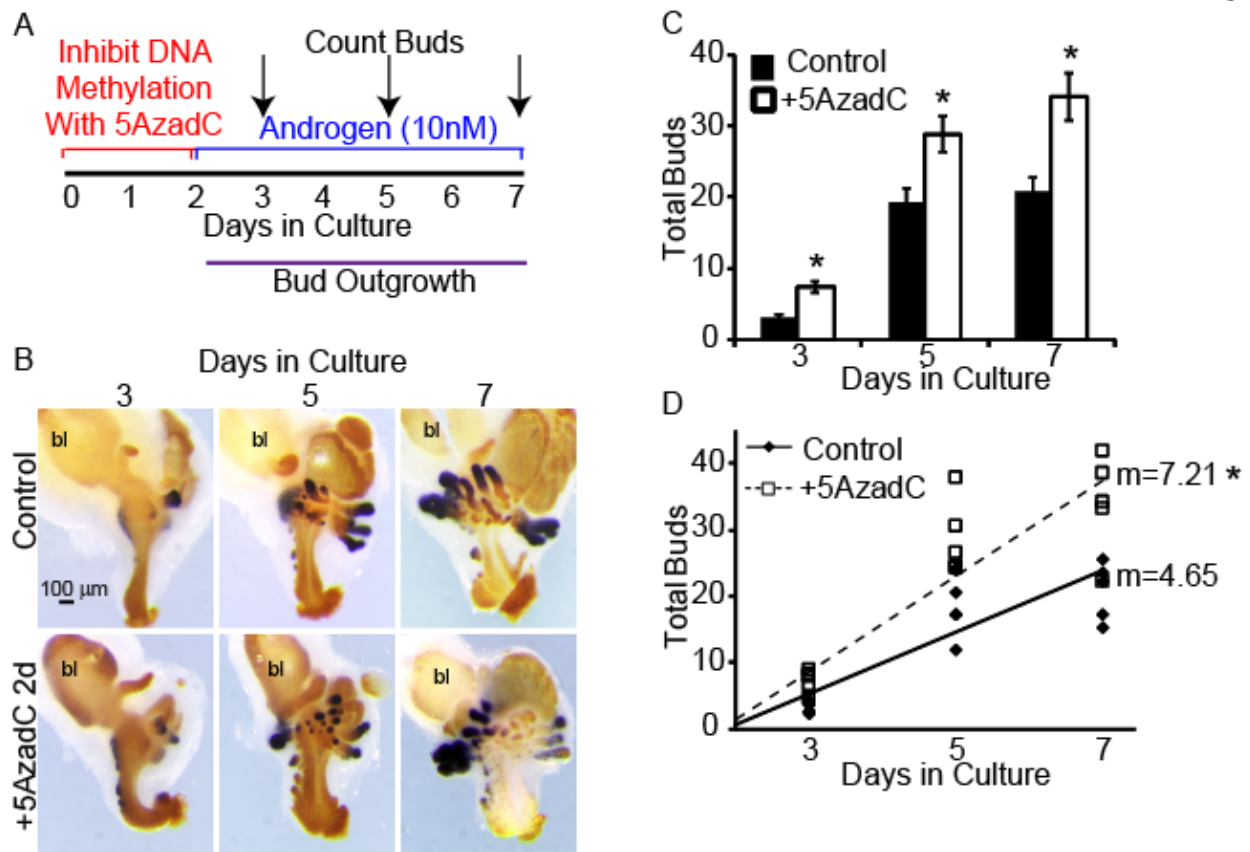


Figure 4. DNA methylation restricts rate of prostate bud formation. (A) 14 dpc male UGSs were cultured for 2 days in the presence of DNA methylation inhibitor 5'-aza-2'-deoxycytidine (5 μ M, 5AzadC) alone followed by 1, 3 or 5 additional days in media containing androgen (10nM dihydrotestosterone, DHT) alone. (B) Tissues were stained by ISH for prostate bud marker NK-3 transcription factor locus 1 (*Nkx3-1*) (purple) and IHC to visualize all epithelium (E-cadherin, CDH1, brown). (C) Quantification of total buds formed at each timepoint. Results are mean $\pm$ SE, n=5/group. Asterisk indicates significant difference from control $p \leq 0.05$. (D) Linear regression of the rate of bud formation, analysis of covariance revealed a significant difference between treatment groups ($p=0.004$). Abbreviations: bl, bladder.

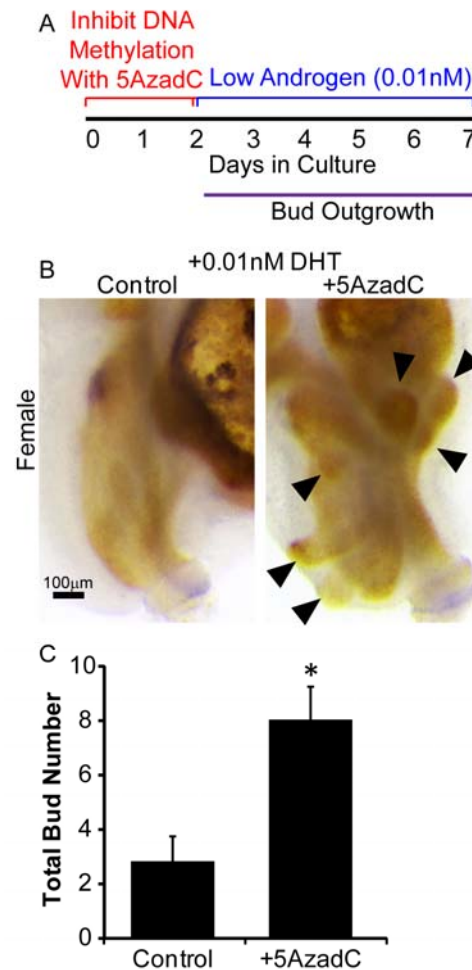


Figure 5. DNA methylation controls developing prostate androgen sensitivity. (A) 14 dpc female UGSs were cultured for 2 days in the presence of DNA methylation inhibitor 5'-aza-2'-deoxycytidine (5µM, 5AzadC) alone followed by 5 days in media containing androgen (0.01nM dihydrotestosterone, DHT) alone. (B) Tissues were stained by ISH for prostate bud marker NK-3 transcription factor locus 1 (*Nkx3-1*) (purple) and IHC to visualize all epithelium (E-cadherin, CDH1, brown). (C) Quantification of total bud number in control and 5AzadC tissues. Results are mean ± SE, n=5/group. Asterisk indicates significant difference from control p≤0.05. Arrowheads indicate epithelial buds.

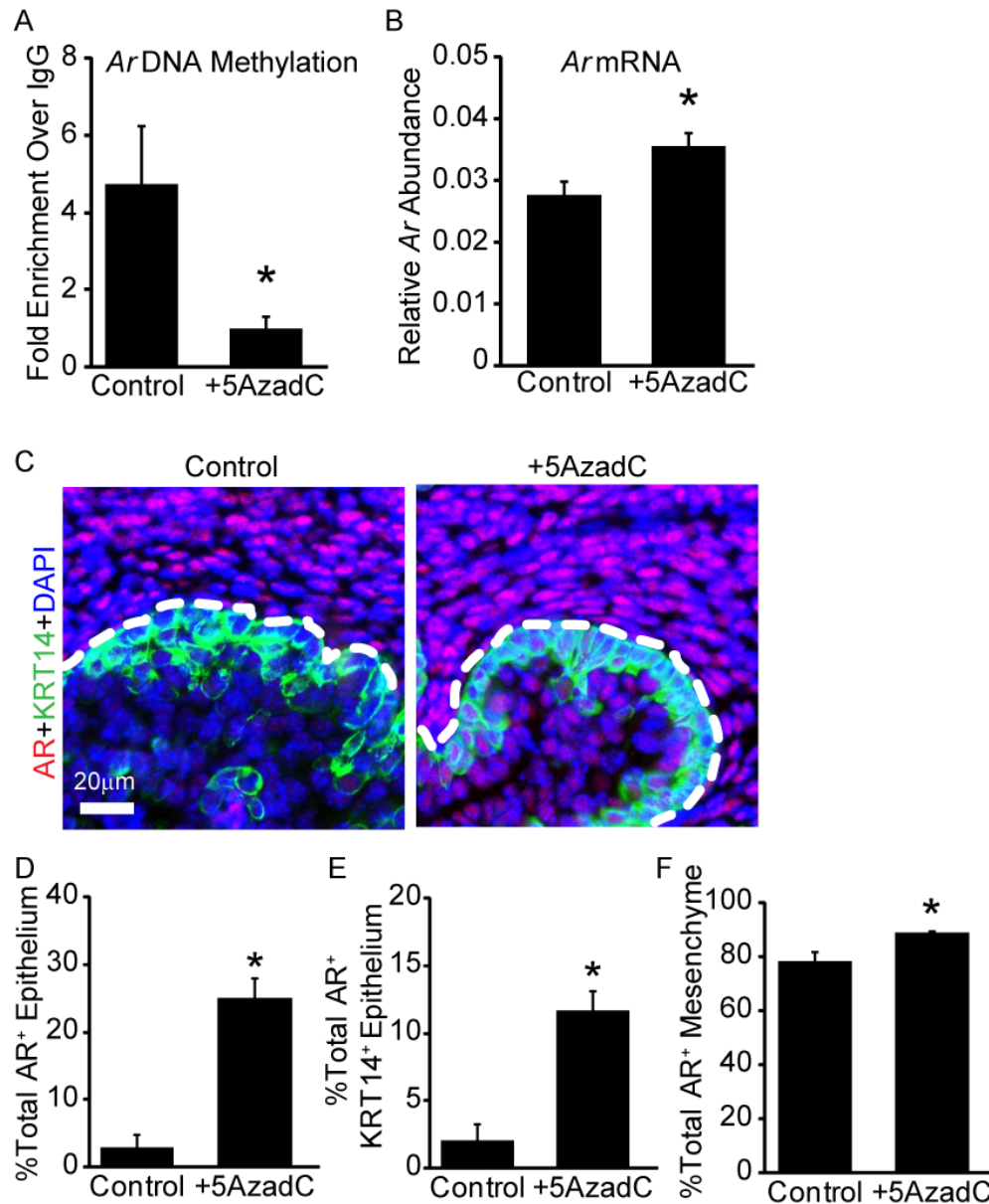


Figure 6. Androgen Receptor (Ar) DNA methylation is correlated with mRNA and protein abundance. 14 dpc male UGSs were cultured for 2 days in the presence of DNA methylation inhibitor 5'-aza-2'-deoxycytidine (5µM, 5AzadC) or vehicle (0.1% DMSO) in medium containing androgen (10nM dihydrotestosterone, DHT). (A) Ar DNA methylation assessed by methylated DNA immunoprecipitation and normalized to IgG

control. (B) Relative Ar mRNA abundance quantified by real time QPCR relative to peptidyl prolyl isomerase a (Ppia). (C) AR protein (red) and basal epithelium (keratin 14, KRT14, green) were visualized using immunohistochemistry. Cell nuclei were counterstained with DAPI (blue). Dotted line indicates the boundary between mesenchyme and epithelium. Quantification of percent total AR positive cells in (D) all epithelium (E) KRT14+ basal epithelium and (F) mesenchyme. Results are mean $\pm$ SE, n=5/group. Asterisk indicates significant difference from control $p \leq 0.05$.

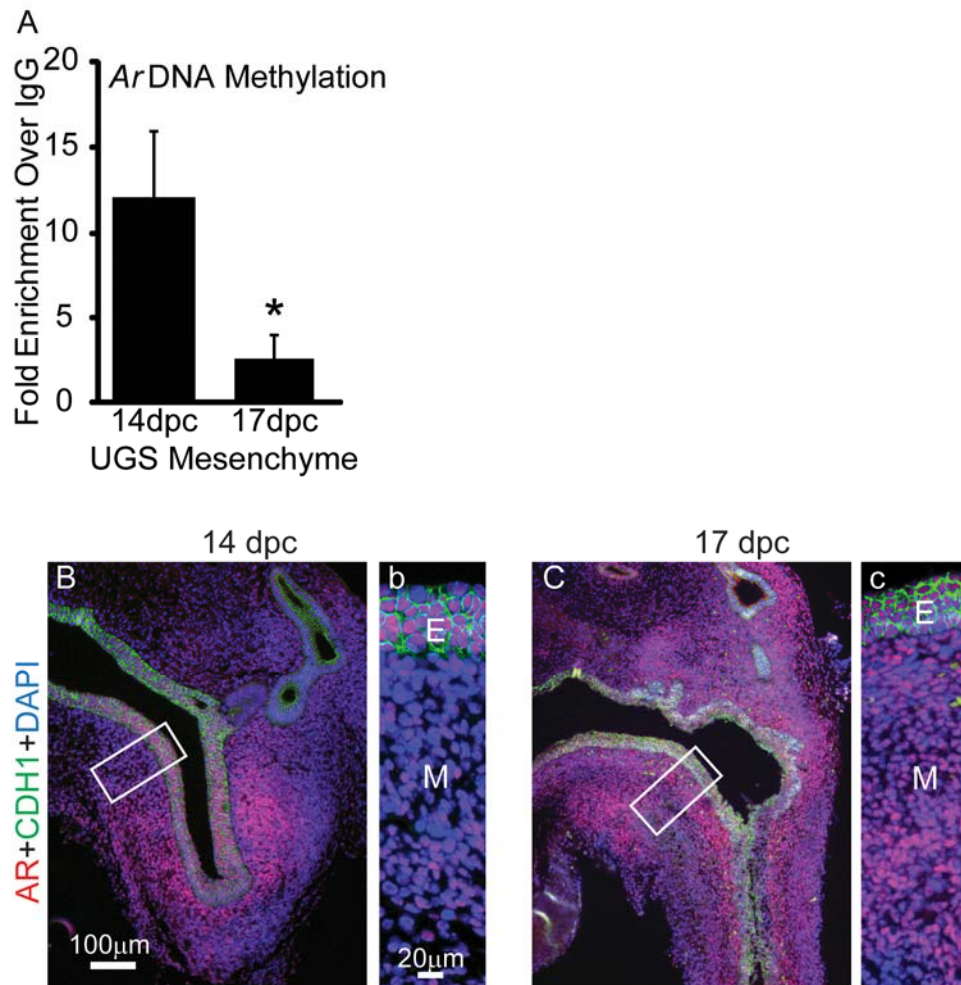
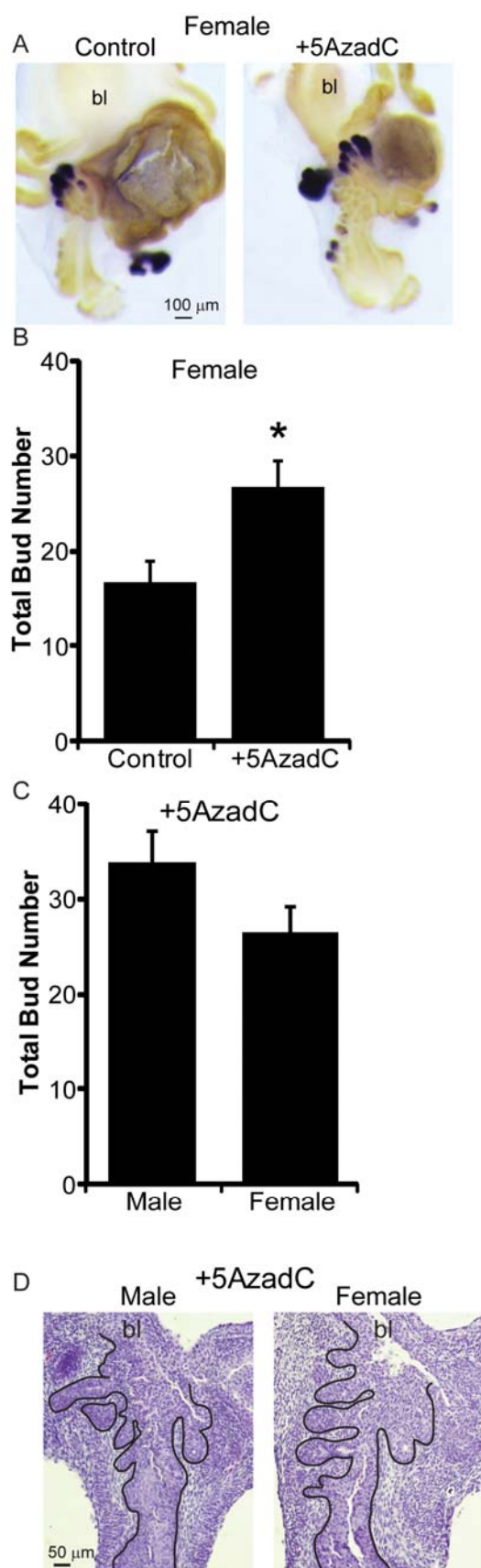
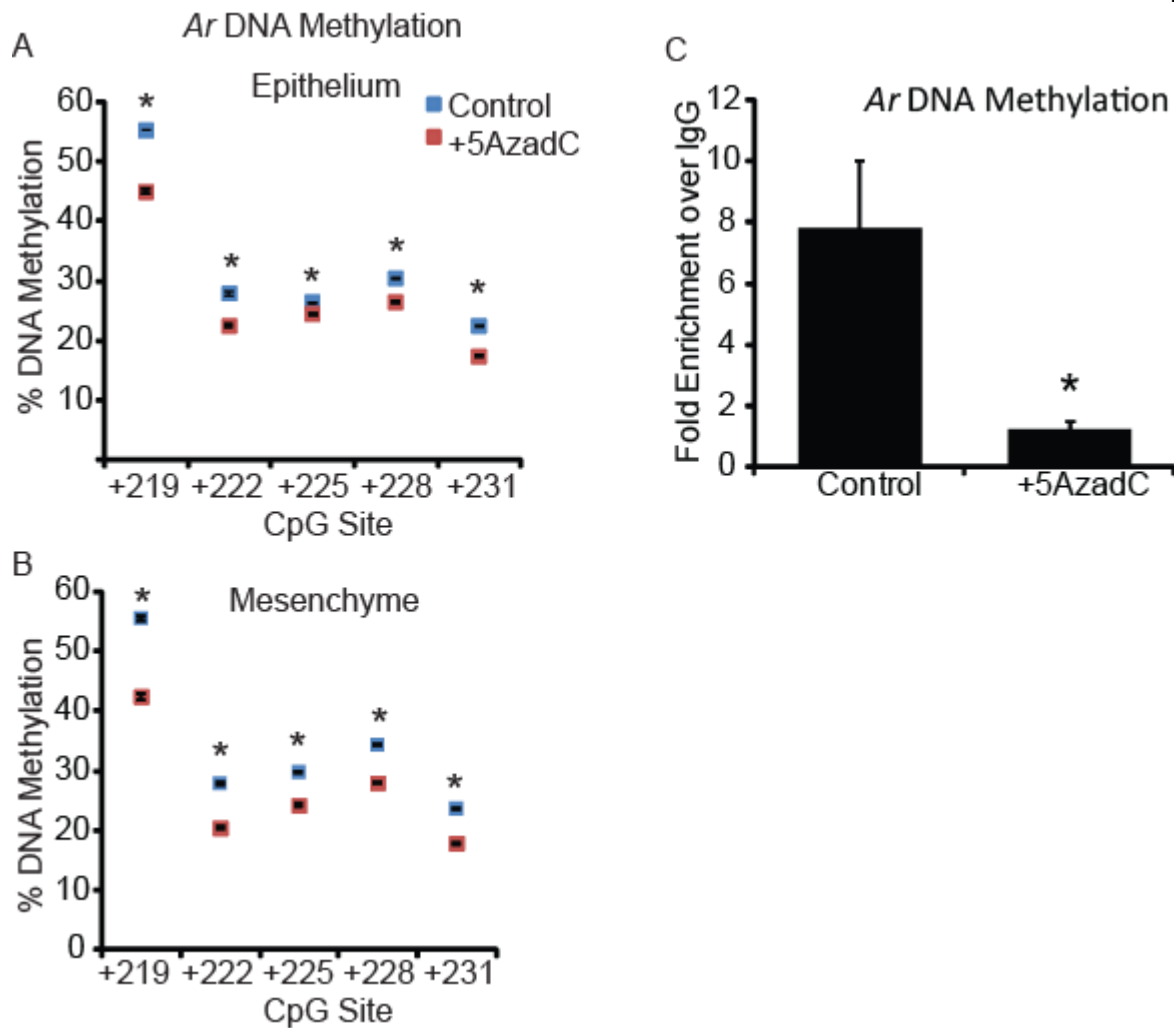


Figure 7. Androgen receptor (Ar) DNA methylation is correlated with AR protein abundance in vivo. (A) UGS mesenchyme was isolated from 14 dpc and 17 dpc male UGS. Ar DNA methylation assessed by methylated DNA immunoprecipitation and normalized to IgG control. (B,b) 14 dpc and (C,c) 17 dpc lower urinary tract sagittal sections were stained by immunohistochemistry to visualize AR (red) E-cadherin protein (CDH1, green). Cell nuclei were stained with DAPI. Insets represent magnified images. Abbreviations: E, epithelium; M, mesenchyme. Results are mean $\pm$ SE, n=5/group. Asterisk indicates significant difference from control $p \leq 0.05$.



Supplemental Figure 1. DNA methylation restricts prostate bud number in female

UGSs. 14 dpc female UGSs were cultured for 2 days in the presence of DNA methylation inhibitor 5'-aza-2'-deoxycytidine (5 μ M, 5AzadC) alone followed by 5 days in media containing androgen (10nM dihydrotestosterone, DHT) alone. (A) Following culture tissues were stained by ISH to visualize prostate bud marker NK-3 transcription factor locus 1 (*Nkx3-1*) (purple) and IHC to visualize all epithelium (E-cadherin, CDH1, brown). (B) Quantification of total prostatic bud number in control and 5AzadC treated samples. Results are mean $\pm$ SE, n=5/group. Asterisk indicates significant difference from control $p \leq 0.05$. Abbreviations: bl, bladder.



Supplemental Figure 2. DNA methylation inhibitor 5AzadC decreases *Ar* DNA methylation. 14 dpc UGSs were cultured for 4 days in the presence of DNA methylation inhibitor 5'-aza-2'-deoxycytidine (5 μ M, 5AzadC) or vehicle (0.1% DMSO) in media containing androgen (10nM dihydrotestosterone, DHT). (A-B) Pyrosequencing of bisulfite converted DNA with primers designed to target a CpG dense region of the *Ar* (+209 to +230 of the TSS). Results reported as percent *Ar* DNA methylation. (C) Methylated DNA immunoprecipitation followed by QPCR (MeDIP-QPCR) was used to confirm pyrosequencing results. Results are mean $\pm$ SE, n=5/group. Asterisk indicates significant difference from control $p \leq 0.05$.

**Chapter 7: Influence of animal husbandry practices on void spot assay outcomes
in C57Bl/6J male mice**

Keil et al., Neurourology and Urodynamics. (2014) In press

Introduction

Lower urinary tract symptoms (LUTS) associated with benign prostatic hyperplasia (BPH) pose a significant burden to the aging population (Saigal et al., 2005). There is a growing need to identify the underlying basis of LUTS to improve diagnosis and develop new noninvasive therapies.

Mice are frequently used to model aspects of urinary dysfunction experienced by humans, including incontinence, overactive bladder, and bladder outlet obstruction (Lee et al., 2013, Kanasaki et al., 2013). However, mice are anatomically and behaviorally distinct from humans, and cannot be assessed by the same urodynamic tests. As the number of mouse models of urinary dysfunction expands, so does the need for assays that quantify physiologically relevant aspects of voiding function while minimizing variables associated with mouse voiding behavior.

Many assays are available for measuring mouse voiding function, including: cystometry for measuring intravesicular pressures associated with bladder filling and emptying, bladder organ baths for characterizing in vitro contractile responses to various stimuli, electromyography for assessing neuromuscular function, and metabolic cages for examining uroflow (Nicholson et al., 2012, Lee et al., 2013, Kanasaki et al., 2013). Many of these methods are invasive, labor intensive, require specialized equipment, or do not allow for repeated analysis of urinary function. To complement these approaches, the void spot assay has been used to study urinary function (Desjardins et al., 1973, Stewart et al., 1991, Stevens et al., 1991, Lundbeck et al., 1993, Sugino et al., 2008, Hodges et al., 2008, Gharaee-Kermani et al., 2013, Daly et al., 2014, Yu et al., 2014). This method is inexpensive, noninvasive and can be used to

track individual mice over time. Mice are housed for a fixed period of time in a cage fitted with filter paper, the paper is removed and urine visualized under UV light. Urinary patterns are graded by blinded reviewers (Gharaee-Kermani et al., 2013) or analyzed by imaging software (Yu et al., 2014).

The void spot assay, like other urinary function assays in mice, is confounded by social and behavioral influences. Mice void to scent mark territory, manifest social behaviors and establish dominance (Hurst et al., 2001). Yu et al., 2014 validated the spontaneous void spot assay in female mice. They examined testing time of day, repeated days of testing, mouse age and strain on several voiding parameters (spot number, total urine area, primary void area and percentage of total urine area within primary void, corner voids and center voids). They found minimal individual and within strain variability. However, variability was observed between strains, revealing a genetic component to micturition.

Though male mice may be uniquely susceptible to behavioral influences on voiding function (Desjardins et al., 1973, Hurst et al., 2001) there has been no study to identify husbandry practices (i.e. caging density) or experimental factors (i.e. animal age, time of day conducted) that influence male voiding behavior using the void spot assay. There is a particular need to understand whether and how husbandry influences voiding in male mice so they can also be used to investigate outcomes of prostate biology and function on voiding behavior. This study utilized the C57Bl/6J male mouse line to identify animal husbandry conditions that impact spontaneous void spot assay outcomes. We found mouse age profoundly influences voiding frequency and 5-10% of male mice develop a frequent spotting pattern after 6 weeks of age. Voiding was stable

across repeated daily measures, not influenced by mating or caging density at weaning. Voiding was altered by time of day, routine cage changes and when caging density was changed in adulthood. Together these results identify common practices that influence male voiding behaviors in the spontaneous void spot assay. These results should be considered when voiding behavior analysis is conducted with male C57Bl/6J mice.

Materials and Methods

Animals

C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) and bred to produce mice used in this study. Mice were housed in polysulfone cages containing corn cob bedding and maintained on a 12 hour light and dark cycle at $25\pm 5^{\circ}\text{C}$ and 20-50% relative humidity. Feed (Diet 2019 for males and Diet 7002 for pregnant females, Harlan Teklad, Madison, WI) and water were available *ad libitum* except during the testing period, when only feed was available. All procedures were approved by the University of Wisconsin Animal Care and Use Committee and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Spontaneous Void Spot Assay

Void assays were conducted and analyzed as described previously (Yu et al., 2014). Single or group housed animals (4-6 per cage) were moved to a clean, empty cage lined with 3MM Whatman filter paper (Fisher Scientific #057163W) with one animal per cage. Assays were conducted over four hours with access to food but not

water in the same quiet location and time of day unless indicated otherwise. Filter papers were imaged using UV light and analyzed using Image J Software (Version 1.46r). Images were analyzed by an individual blinded to treatment conditions. Brightness and contrast were optimized to uniformly visualize urine. Thresholding of images was done in Image J to make urine spots black. Image J particle analysis (Image J 1.46r) was performed on spots greater than 0.02cm^2 (corresponding to $\sim 0.75\mu\text{l}$ urine), reducing non-specific marks potentially deposited by paws and tails that pass through urine spots (Supplemental Figure 1). Overlapping urine spots were defined by the presence of a definitive border outlining each spot in the raw images, these spots were then counted by hand and area determined individually using the freehand selection tool (Supplemental Figure 1). These values were incorporated into the Image J analysis. Similarly if the primary void was in proximity to another distinct void, the primary void area was calculated by hand using the freehand selection tool to only incorporate a single spot.

Statistical analyses

All data are reported as mean $\pm$ standard error of the mean unless indicated otherwise. Statistical analysis was performed using R version 2.13.1. Homogeneity of variance was determined using Levene's test, $p \leq 0.05$ indicating unequal variance and use of nonparametric analysis. For parametric data, student's T-test, or one way ANOVA followed by Tukey's Honest Significant Difference (HSD) test were used to identify significant differences ($p \leq 0.05$) between or among treatment groups. For nonparametric data Welch's T-test or Kruskal Wallis Test followed by Wilcoxon tests

with bonferroni correction were used to identify significant difference ($p \leq 0.05$) between or among treatment groups. For assays conducted on the same animals, repeated measures ANOVA followed by pairwise T-test with Holm correction were used to identify significant differences between or among treatment groups.

Results

Male mouse age influences voiding

Void spot assays can be used to evaluate voiding function over time. Baseline analysis often begins during puberty or shortly after sexual maturation, and experimental cohorts are matched within a defined age range. We examined whether age influences voiding behavior in young mice, which are most likely to be used to initiate a study. Three, six and nine week old male mice were evaluated by the void spot assay (Figure 1A) and relative frequency of spot number determined (Figure 1B). Three week old males had a median void spot number of 4, with 86% voiding less than 8 times within the testing period. Six week old mice had a median void spot number of 15, with 90% voiding less than 30 times within the testing period. Nine week old mice had a median void spot number of 28, with 82% voiding less than 60 times within the testing period (Figure 1B). A subpopulation of six and nine week old male mice display frequent spotting, defined here as 50 and 100+ spots at six and nine weeks respectively, and represent 5 and 10% of the population at these ages.

Mouse urine output and frequency increase with advanced age (Gharaee-Kermani et al., 2013). Whether voiding parameters also change over time in young male mice, and how quickly these changes occur, is unknown. To test whether aging

influences voiding parameters, the same cohort of mice was tested at three, six, and nine weeks of age. Body mass significantly and progressively increased with age (Figure 2A). Urine spot number (index of urinary frequency) significantly increased from three to six or nine weeks of age (Figure 2B). Total urine area (index of total urine output) significantly and progressively increased from three to six and nine weeks (Figure 2C), while individual urine spot size did not change with age (Figure 2D). Primary void area (largest void during the testing period) significantly increased from three to six or nine weeks of age (Figure 2E). Percent total urine contained in the primary void decreased between three and nine weeks of age (Figure 2F). Percent total urine area within corners (each corner defined as 1% of paper area) significantly increased from three to nine and six to nine weeks but not from three to six weeks (Figure 2G) while percentage of total urine within the center (center defined as 9% of paper area) was unchanged at all time points (Figure 2H). These results reveal that voiding parameters rapidly change as C57BL/6J adolescent male mice undergo sexual maturation, particularly during the three to six week age interval.

We next tested whether mouse body mass, which increases with age (Figure 2A), correlates with voiding parameters. There was a significant positive correlation between mouse body mass and urine spot number, total urine area, primary void area and percent total urine area within corners (Supplemental Figure 2). There was no significant correlation between mouse mass and individual urine spot size, percent total urine area in primary void and percent total urine within center (Supplemental Table I). Therefore, it is appropriate to normalize urine spot number, total and primary void area and percent urine within corners to body mass. When this was done on voiding data

from mice at three, six and nine weeks of age, total urine area remained significantly different between all three time points (Figure 2I). Together these results indicate that tightly matched age cohorts should help minimize variability between groups. Initiating studies with baseline assays on six+ week old mice will identify frequent spotting mice to ensure they are equally distributed among experimental groups or excluded from studies when appropriate.

Conventional housing does not alter voiding behavior in young adult mice

If male mice are to be housed with other male mice, it is recommended that males be group housed from time of weaning to reduce fighting and cage costs (Jackson Labs, www.jax.org). We examined if spontaneous voiding is influenced by group versus single housing. Male littermates were void tested prior to weaning and exhibited no significant differences in voiding parameters listed above (results not shown). Mice were weaned with littermates to group housing (up to six mice/cage) or transferred to single housing. Void spot assays were repeated at six weeks of age. There were no significant differences in body mass or measured void spot assay parameters between group or singly housed males (Figure 3A-B). Therefore, male littermates can be group housed up to at least six weeks of age without altering voiding behavior relative to mice that were singly housed from weaning to six weeks of age.

Group to single housing decreases urine area in mature mice

It is often necessary to place group housed animals into single cages once they reach adulthood (i.e. recovery after surgery, monitoring food and water consumption,

etc). A change in caging density can be stressful. It can influence hormone levels, which in turn are capable of influencing voiding (Rasmussen et al., 2011, Mucignat-Caretta et al., 2014). We therefore tested whether changes from group to single housing after six weeks of age alters voiding. Adult virgin males group housed at weaning were void tested at eight weeks of age, individually housed for one week, and void tested again (Figure 3C-D). There was a significant reduction in urine area following the move to single versus group housing (Figure 3D). There were no significant differences in spot number, spot size, primary void area, percent urine area within primary void, corners or center (Supplemental Table II). This suggests a change in caging density after mice reach adulthood alters voiding behavior. Therefore, maintaining animals in the same housing conditions throughout an experiment will decrease variability in void spot assays.

We also examined if single housed males can be used for breeding without influencing voiding behavior. The same singly housed virgin male mice used above were housed with a multiparous female mouse for one week and then void tested (Figure 3C-D). There were no significant differences in void spot assay parameters after males had been housed with female mice versus when males were singly housed (Figure 3D, Supplemental Table II). Body mass significantly increased over the three week testing period; body mass did not alter parameters affected by housing or breeding. However, normalizing to body mass significantly reduced the difference in total urine area between group to singly housed males and between group and breeding males (Figure 3E).

Voiding exhibits stable daily patterns

Female mice display reproducible day to day void spot assay patterns, suggesting a single void assay is representative of their voiding and acclimation to the cage fitted with filter paper does not alter voiding on consecutive days (Yu et al., 2014). To determine whether this is also true for males, nine week old male mice were tested at the same time of day over three consecutive days (Figure 4A). There were no significant differences in body mass, spot number, urine area, spot size, percent area in primary void, percent urine area in corners and percent area in center (Supplemental Table III). There was a significant increase in average urine area in the primary void on day 3 (22.8 cm²) compared to days 1 (15.3 cm²) and 2 (16.2 cm²) (Figure 4B). These results suggest that void assays are relatively stable day to day but male mice exhibit more variation in day to day voiding than females.

Routine cage changes affect voiding parameters

Routine cage changes increase anxiety behaviors in C57Bl/6J mice (Rasmussen et al., 2011). Whether this effects voiding behavior is unknown. Male mice (nine weeks old) were void tested one day prior to cage changes, the following day cages were changed per normal animal husbandry and void assays conducted that same day (Figure 4C). There was no significant change in body mass, spot number, spot size, primary void area or percent area in primary void and center before versus after cage changes (Supplemental Table IV). There was a significant decrease in total urine area and increase in percent urine area in corners after cage changes versus before (Figure 4D), indicating cage changes alone can alter voiding parameters. These results in

conjunction with those observed during testing over three consecutive days suggests day to day changes exist in the male mouse population. Conducting repeated measurements of voiding not on the day of routine cage changes may help reduce variability.

Urine output is affected by time of day

Mice are typically maintained on a 12 hour light/dark cycle and are most active during the dark cycle. Mouse and human voiding behavior change with activity (ie. mice urinate less frequently during the light cycle) and mice are known to have diurnal variation in urine production (Negoro et al., 2013). We next tested if time of day when voiding assays are conducted influences voiding patterns as assessed by the void spot assay. The same cohort of virgin male mice was tested either in the morning (9am-1pm), afternoon (2pm-6pm) or night (10pm-2am) during a one week period (Figure 5A, representative images from same animal). Total urine area was greater at night compared to morning and afternoon, and in afternoon compared to morning (Figure 5B). The primary void area was also greater in afternoon compared to morning (Figure 5C). There were no significant differences in spot number, spot size, percent area in primary void, corners or center (Supplemental Table V). We also observed a significant difference in body mass, lowest in the afternoon and highest at midnight (Figure 5D). The same voiding parameters (urine area and primary void area) were affected when voiding was normalized to body mass (Figure 5E-F, Supplemental Table V). Normalized values show an increase in total urine area from morning to afternoon or night, but not between afternoon and night (Figure 5E) and an increase in primary void area from

morning to afternoon (Figure 5F). These results indicate that time of day influences male mouse urine output and conducting voiding assays at the same time of day should minimize variability.

Discussion

This study addressed animal husbandry and experimental design influences on spontaneous void spot assays for male C57Bl/6J mice. Isolating functional voiding parameters from confounding variables is crucial to accurately assess physiological mechanisms responsible for voiding function and dysfunction.

Our results indicate heterogeneity of voiding in relation to spot number as young adult male C57Bl/6J mice mature. This is likely due to multiple factors including increasing body mass and fluid requirements, establishing dominance and/or age associated changes of the LUT (Daly et al., 2014, Yu et al., 2014). Based on these findings, mice six weeks of age or older should be used to initiate a study. Our results also reveal a subpopulation of young adult male mice which display frequent spotting. These mice can be identified around six to nine weeks of age (coinciding with sexual maturity). Conducting baseline voiding assays prior to experimentation is essential for controlling the mouse population's natural heterogeneity in voiding behavior. The underlying cause for the appearance of frequent spotters within a population is unknown and, in the inbred C57BL/6J mice used in this study, is not genetic. This pattern is typical of dominant male voiding behavior (Desjardins et al., 1973). Whether these characteristics are innate, potentially derived from an epigenetic origin, triggered by housing, stress or simply from some mice being messier than others in regard to urine

placement and/or tracking through their urine or underlying inflammation/infection is an unknown mechanism that requires further study.

Our results indicate that male C57Bl/6J mice display more variability in day to day voiding compared to that of females (Yu et al., 2014). We observed an increase in primary void area on the third consecutive void assay compared to the previous two days. No other voiding parameters were significantly altered. This variation could be due to habituation to the cage/filter paper environment and surroundings, both of which can influence behavior (Arakawa et al., 2008a, Arakawa et al., 2008b). In an effort to reduce these variables, our assays were conducted in the same style of cage used for routine housing and the same rooms where mice are normally housed. These results suggest that void assays collected over multiple days may vary and one or two days of testing may be more accurate than three or more days of consecutive testing. We also found that routine cage changes and time of day are significant factors that need to be controlled in voiding studies using male mice.

Even though male mice scent mark their territory with urine in response to males of a different strain, they do not typically mark in response to their own urine or that of genetically identical mice (Hurst et al., 2001, Arakawa et al., 2008a). Since the C57Bl/6J mice used here are genetically similar it is not surprising that our results indicate male mice can be group housed at weaning without altering voiding parameters by 6 weeks of age-thus saving on housing costs. However, our results did reveal moving from group to single housing once mice reached six weeks of age was sufficient to decrease urine area. These results are consistent with the fact that changes in caging density alone are sufficient to alter testosterone levels, which may subsequently influence voiding

frequency in male mice (Mucignot-Caretta et al., 2014). Therefore, for all experiments requiring single housing for any portion of the experiment, singly housing mice for at least one week prior to and throughout the testing period should reduce variability in the void spot assay.

There is a strong hormonal influence on male mouse marking behavior. Castration decreases marking while addition of testosterone propionate and estradiol benzoate restore marking behavior in castrated males (Ralls et al., 1971, Desjardins et al., 1973, Kimura et al., 1985). Postnatally androgenized female mice also exhibit increased marking behavior (Kimura et al., 1985). Therefore, chemical or genetic studies that alter mouse hormone levels could potentially change void spot assay endpoints independent of physiological changes of the prostate, bladder, urethra or other anatomical sites. Additionally our studies were conducted on wild type mice. Studies using animal models of bladder inflammation, obstruction, diabetes etc. in which voiding is altered may require additional analysis (i.e categorizing spot number by size) (Nicholson et al., 2014) or optimization (i.e animal models of diabetes which void large volumes of urine may not require a four hour testing period).

As with any method there are both advantages and limitations to the void spot assay. Advantages include its ease, requiring just cages and filter papers and a four hour time period. One of the biggest advantages is that it can produce repeated measurements on the same animal. Limitations are that it is a semi-quantitative analysis. Void spots can be overlapped on the paper making quantitation more difficult and mice are able to walk freely raising the possibility that they can leave spots on the paper from their paws or tails. Similarly it is difficult to truly acclimatize mice to filter

papers since changing out filter paper introduces a new surface to mark on which to mark. Adaptations to the void spot assay using wire mesh to suspend mice over the filter paper or video recording of voiding events can be used as complementary approaches to minimize some of these variables. Overall the void spot assay is a valuable tool that can be used in combination with other complementary methods to analyze voiding function in mice.

Conclusion

Several steps can be taken to reduce variability when conducting void spot assays on C57Bl/6J male mice. Animals can be weaned with male littermates to group housing (two to six mice/cage) until at least six weeks of age. Baseline assays around six weeks of age allow for the identification and distribution of frequent spotters into the experimental design. During the study, conducting void spot assays on the same day of the week, at the same time of day, not on the day of a cage change and reducing changes in housing density should reduce variability. These practices will help minimize variability due to handling, housing and behavior and allow for a better representation of physiologically relevant changes in voiding function.

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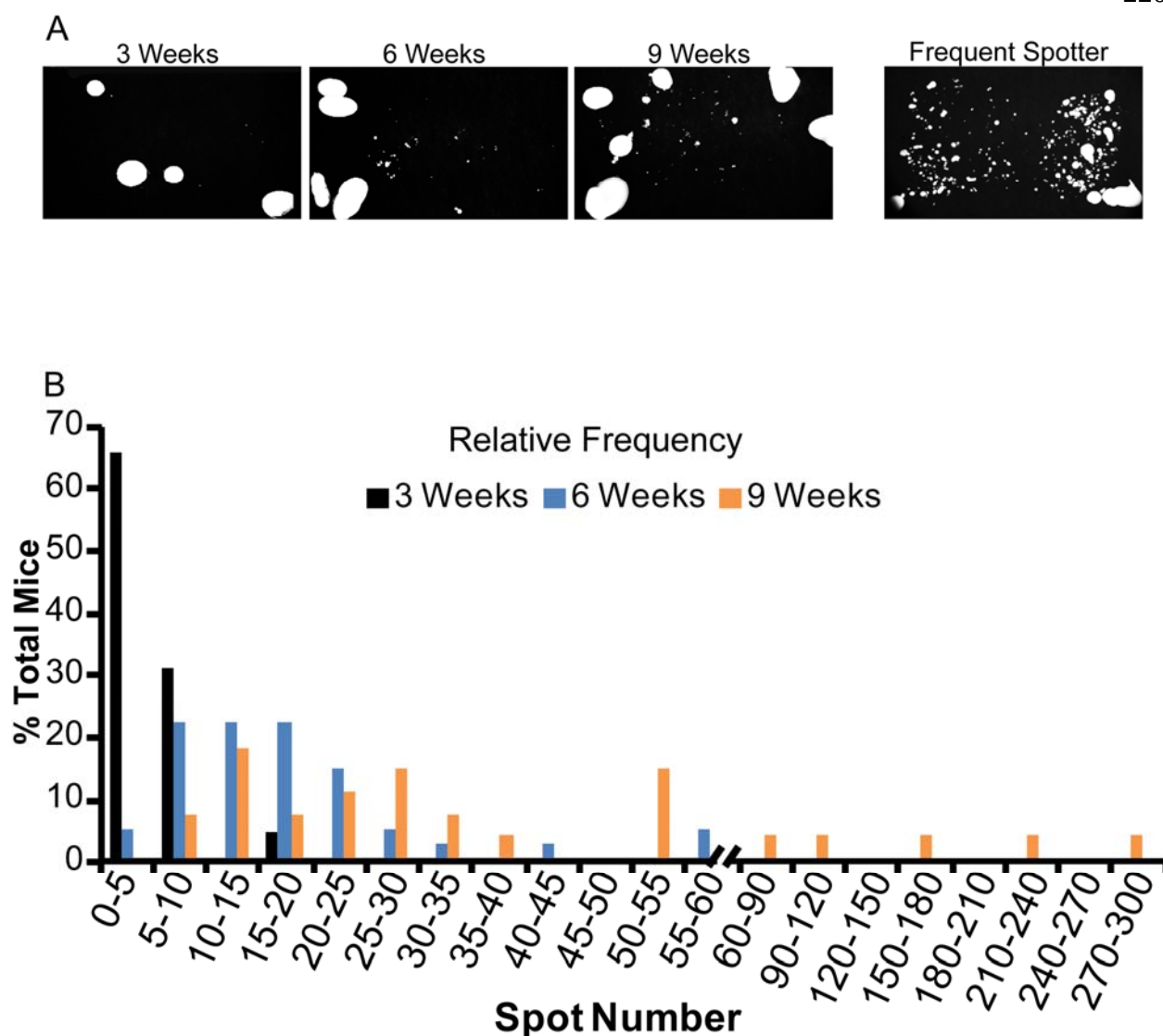


Figure 1. Aging increases spot number in young adult male mice. Void assays were conducted and urine visualized under UV transillumination to quantify spot number. (A) Representative images of spontaneous void spot assay testing in adult virgin C57Bl/6J male mice. (B) Relative frequency of urine spot number in 3-9 week old male C57Bl/6J mice. Results represent n=23, 41 and 28 mice at 3, 6 and 9 weeks of age respectively.

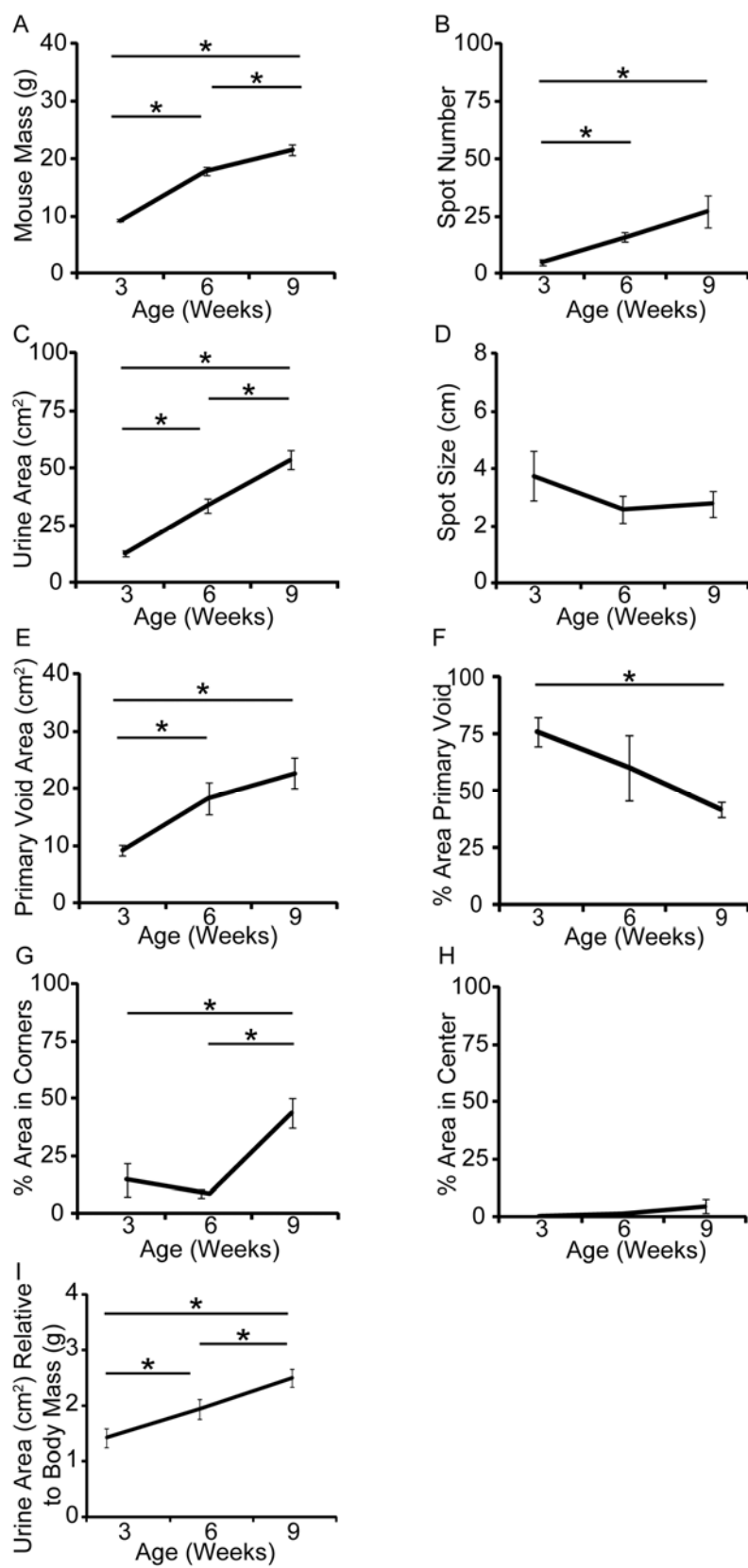


Figure 2. Age influences several voiding parameters in young adult male mice.

Void assays were conducted on the same cohort of mice at 3, 6 and 9 weeks of age. Quantification of (A) mouse mass, (B) spot number, (C) total urine area, (D) spot size, (E) primary void area, (F) percent area within primary void, (G) percent area within corners and (H) percent area within the center. (I) Total urine area normalized to mouse mass at three, six and nine weeks. Results are mean $\pm$ SE, n=11. Asterisk indicates significant differences $p\leq 0.05$.

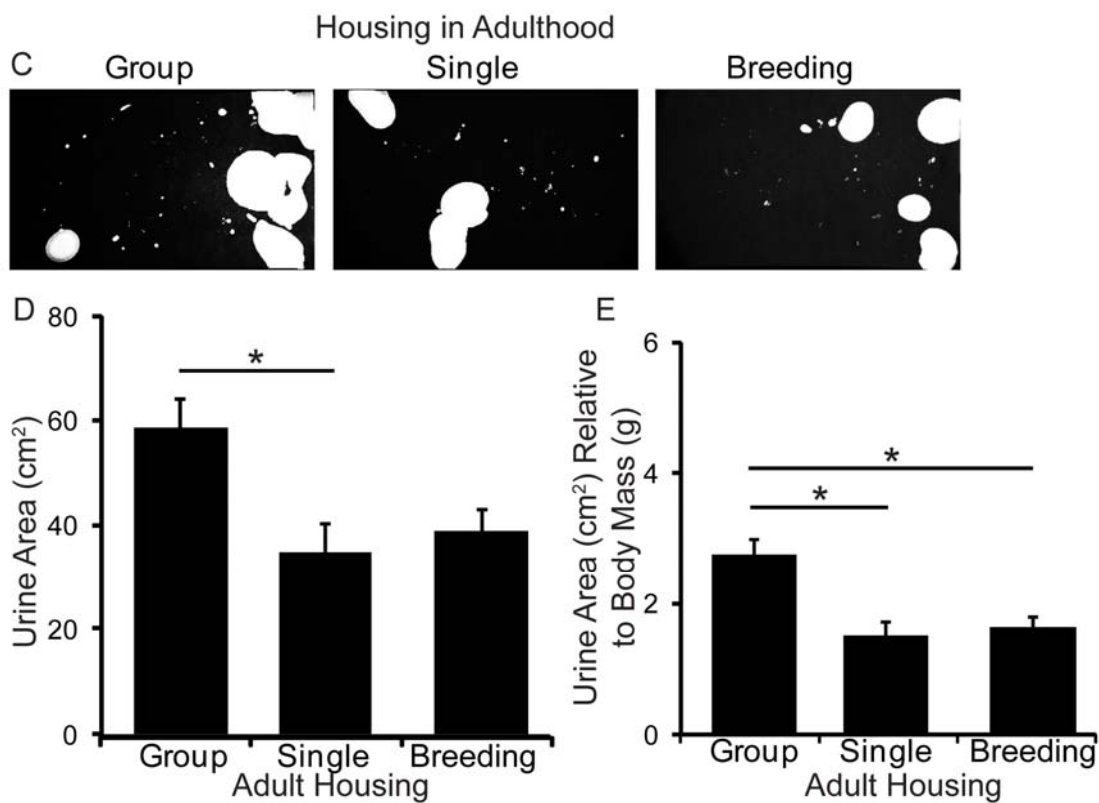
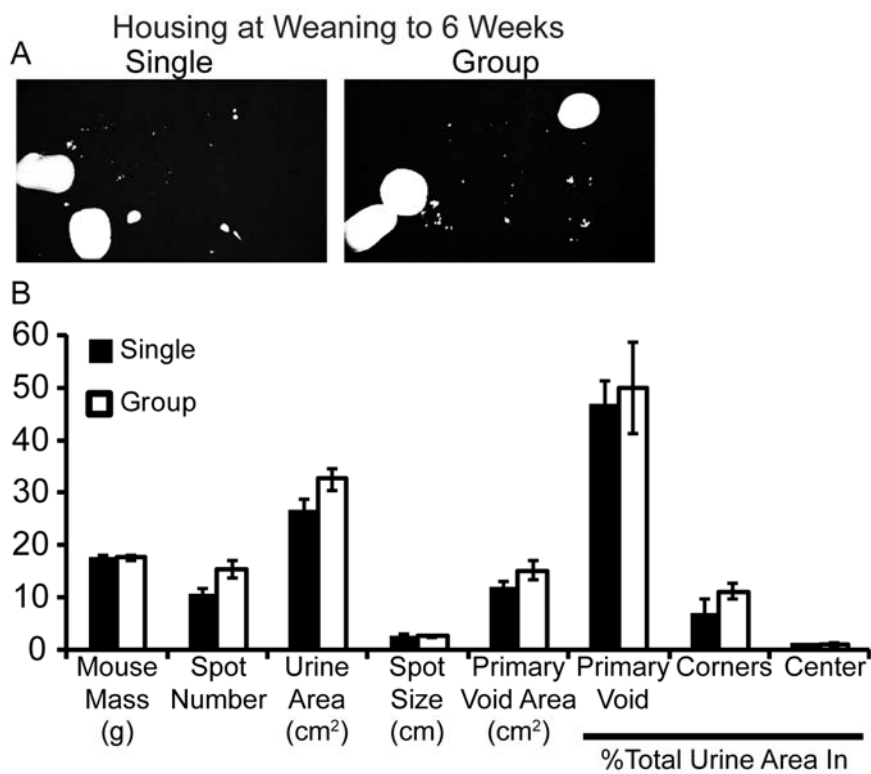


Figure 3. Group vs single housing has no appreciable impact on voiding behavior from weaning to 6 weeks of age but housing changes after 6 weeks of age decreases urine area. (A) Representative images of spontaneous void assay testing in adult virgin C57Bl/6J male mice singly or group housed from time of weaning to 6 weeks of age. (B) Quantification of mouse mass, spot number, total urine area, spot size (diameter), primary void area, percent area in primary void, corners and center. Results are mean $\pm$ SE, n=9 single, n=19 (5 cages) group. (C) Representative images of spontaneous void assay testing in adult virgin C57Bl/6J male mice. Mice group housed from weaning to 8 weeks of age were void tested, separated into single housing for one week and void tested again, then paired with a multiparous female for one week followed by a void assay. (D) Quantification of urine area. (E) Quantification of urine area relative to mouse body mass. Results are mean $\pm$ SE, n=6. Asterisk indicates significant differences $p \leq 0.05$.

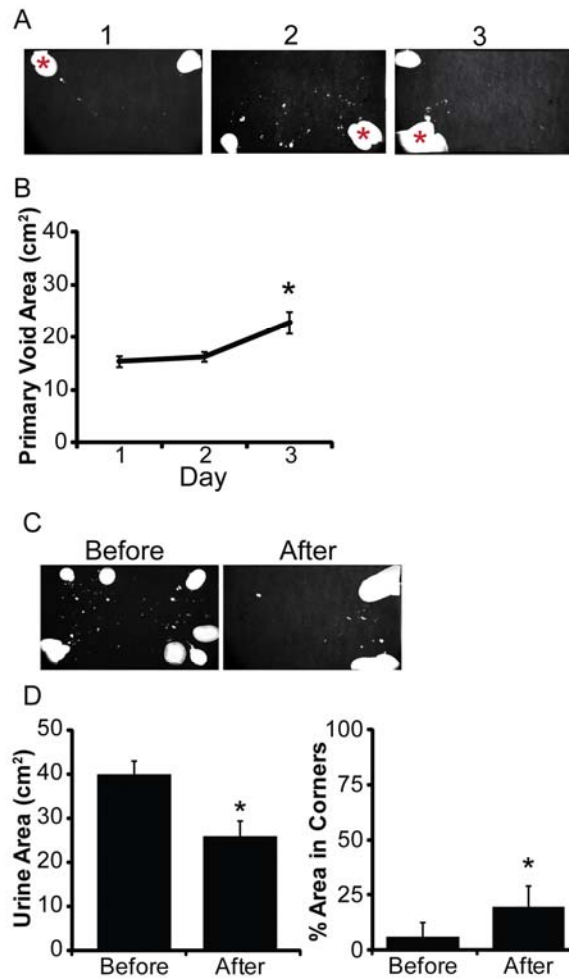


Figure 4. Consecutive daily void testing of mice increases primary void area. (A) Representative images of spontaneous void assay testing in adult virgin C57Bl/6J male mice over three consecutive days. Asterisk indicates the primary void. Mice were tested on three consecutive days at the same time of day. (B) Quantification of primary void area. Results are mean±SE. Asterisk indicates a significant difference $p \leq 0.05$. (C) Representative images of spontaneous void assay testing in adult virgin C57Bl/6J male mice before and after routine cage changes. (D) Quantification of urine area and percent area in corners. Results are mean±SE, $n=10$. Asterisk indicates significant differences $p \leq 0.05$.

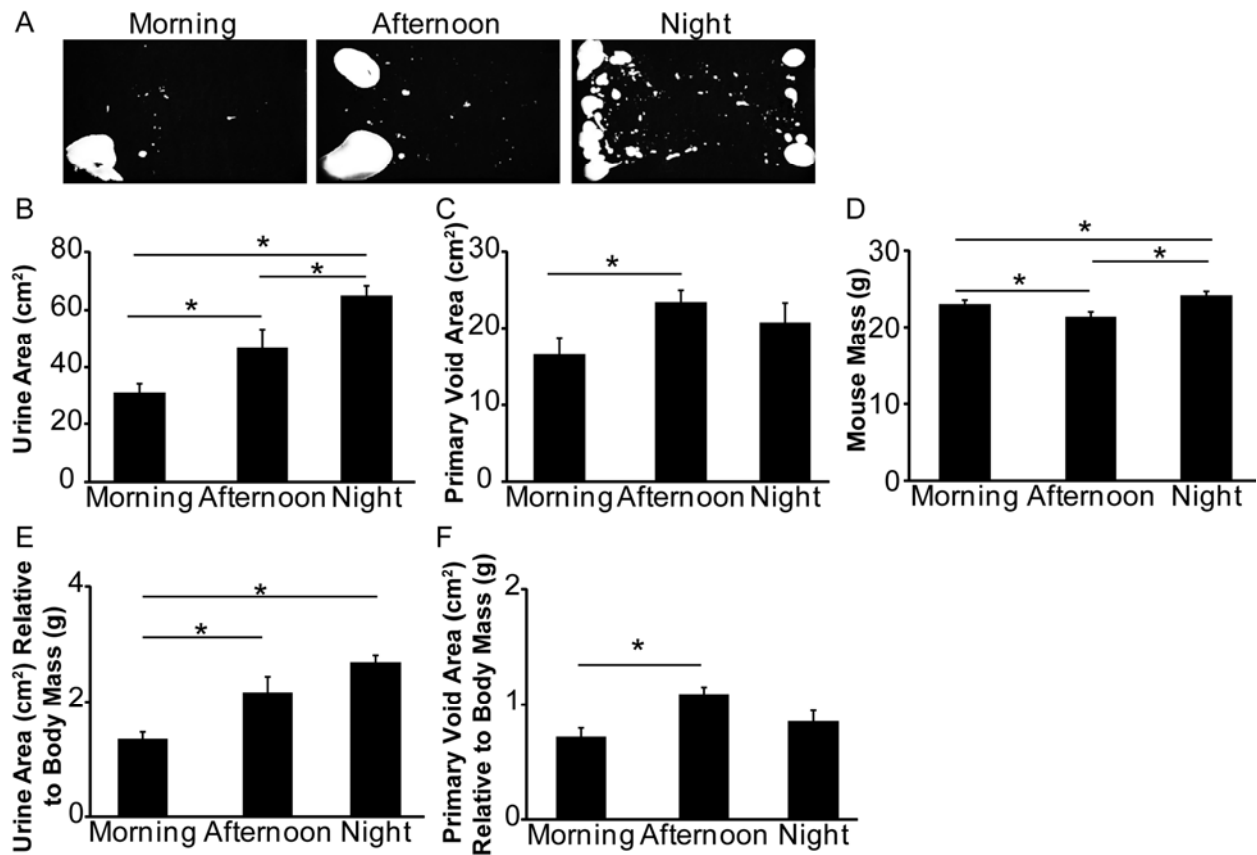
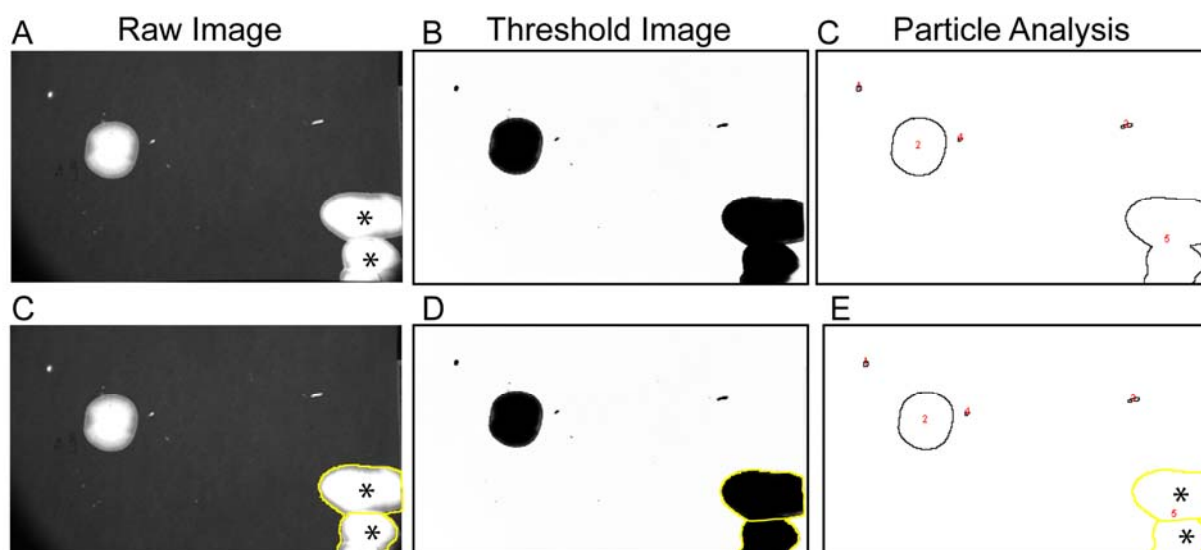
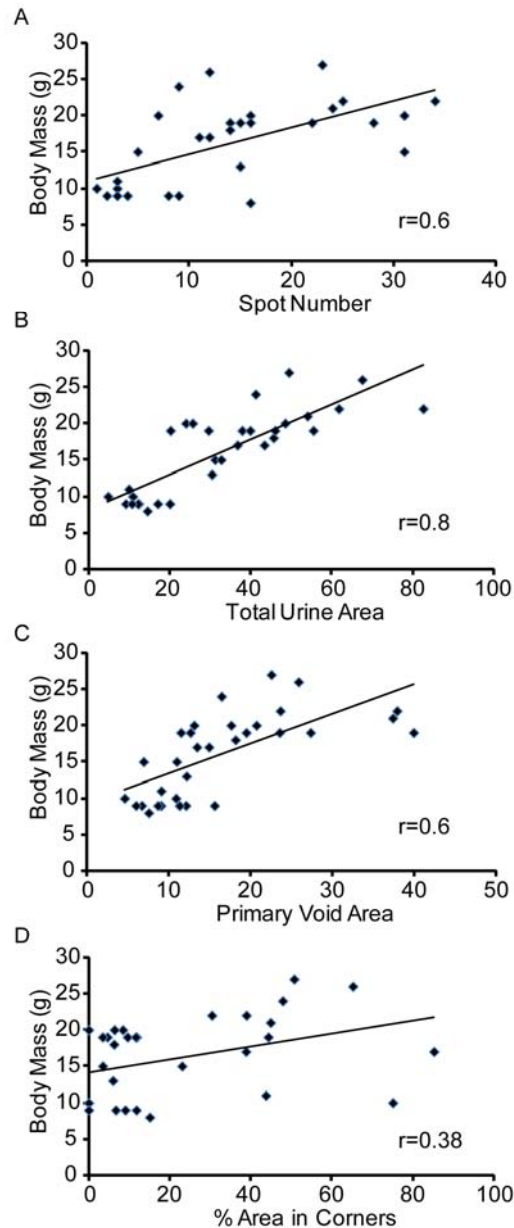


Figure 5. Time of day influences voiding behavior in adult male mice. (A) Representative images of spontaneous void assay testing in the same adult virgin male mice. Mice were tested in the morning (9am-1pm), afternoon (2pm-6pm) or night (10pm-2am) over a one week period. Quantification of (B) urine area, (C) primary void area, (D) body mass, (E) urine area normalized to body mass and (F) primary void area normalized to body mass. Results are mean $\pm$ SE, n=8. Asterisk indicates significant differences $p \leq 0.05$.



Supplemental Figure 1. Analysis of void assay papers. (A) Raw image of filter paper under UV illumination, asterisks indicate overlapping spots. (B) The same image after thresholding. (C) Same image after Image J particle analysis on threshold image reveals overlapping spots as one large spot. (D-F) Same image showing correction for overlapping spots. Some overlapping spots can be identified in the raw image and their area calculated by hand using the freehand selection tool in Image J. Yellow line indicates manual outline of separate spots calculated independently.



Supplemental Figure 2. Mouse body mass positively correlates with urine spot size, area, primary void and percent corner voiding. Body mass was measured and void assays conducted on a cohort of mice age 3-9 weeks of age. Pearson's correlation coefficient was determined for body mass and (A) spot number ($r=0.6$, $p=0.0001$), (B) total urine area ($r=0.8$, $p=1.21 \times 10^{-8}$), (C) primary void area ($r=0.6$, $p=2.22 \times 10^{-5}$) and (D) percent total urine area within corners ($r=0.4$, $p=0.029$). $n=32$.

Supplemental Table I. Relationship between mouse body mass and voiding

	Spot Size (cm)	%Area Primary Void	% Area Center
Pearson's r	-0.14	-0.26	0.14
p value	0.42	0.13	0.43

Parameters significantly correlated with body mass are shown in Supplemental Figure 1. Mean $\pm$ SEM, n=32.

Supplemental Table II. Adult male changes in housing

Average	Spot Number	Median Spot Number	Spot Size (cm)	Area Primary Void (cm ²)	%Area Primary Void	% Area Corners	% Area Center
Group Male Housing	39 ± 10	30	1.87 ± 0.360	26.1 ± 4.13	44.2 ± 5.86	7.17 ± 1.53	1.88 ± 1.27
Single Male Housing	18 ± 4	18	2.49 ± 0.640	20.2 ± 3.05	61.5 ± 10.7	14.8 ± 5.36	4.25 ± 3.21
Breeding (Male+Female)	19 ± 2	20	2.28 ± 0.470	18.8 ± 3.04	50.5 ± 9.68	11.1 ± 4.06	0.460 ± 0.170
p value	0.08	-	0.70	0.20	0.20	0.20	0.40

Parameters significantly altered by housing are shown in Figure 3.

n=6

Supplemental Table III. Male mouse voiding over three consecutive days

Average	Body Mass (g)	Spot Number	Median Spot Number	Total Urine Area (cm ²)	Spot Size (cm)	%Area Primary Void	%Area Corners	%Area Center
Day 1	22 ± 0.57	20 ± 2.8	19	34.1 ± 3.14	1.98 ± 0.260	48.2 ± 5.70	18.7 ± 12.3	1.29 ± 12.3
Day 2	22 ± 0.54	23 ± 6.1	16	34.1 ± 3.72	2.18 ± 0.390	52.9 ± 6.63	15.6 ± 12.4	0.450 ± 7.29
Day 3	22 ± 0.70	15 ± 3.6	12	38.2 ± 3.25	4.47 ± 1.11	62.7 ± 6.83	14.5 ± 8.23	0.630 ± 11.1
p value	0.8	0.1	-	0.6	0.01	0.2	0.4	0.4
Post Hoc Analysis	-	-	-	-		-	-	-
Day 1 vs 2	-	-	-	-	0.57	-	-	-
Day 1 vs 3	-	-	-	-	0.11	-	-	-
Day 2 vs 3	-	-	-	-	0.11	-	-	-

Parameters significantly affected by consecutive day testing are shown in Figure 4. n=10

Supplemental Table IV. Adult male mouse voiding parameters after cage changes

Average	Body Mass (g)	Spot Number	Median Spot Number	Spot Size (cm)	Area Primary Void (cm ²)	% Area Primary Void	%Area Center
Before	22 ± 0.66	43 ± 16	15	1.87 ± 0.390	17.0 ± 1.84	45.4 ± 6.36	1.69 ± 9.45
After	22 ± 0.59	15 ± 3	19	2.83 ± 0.680	15.1 ± 1.40	63.5 ± 6.76	0.930 ± 9.75
p value	0.6	0.1	-	0.2	0.4	0.06	0.3

Parameters significantly altered by cage changes are shown in Figure 4. Mean±SEM, n=10

Supplemental Table V. Adult male mouse voiding parameters at different times of day

Average	Spot Number			Spot Size		% Area Primary Void		%Area Corners		%Area Center	
	Mean	Median	Mean Normalized to Body Mass (g)	Mean	Mean Normalized to Body Mass (g)	Mean	Mean Normalized to Body Mass (g)	Mean	Mean Normalized to Body Mass (g)	Mean	Mean Normalized to Body Mass (g)
Morning	13 ± 2	12	0.58 ± 0.10	2.91 ± 0.660	0.13 ± 0.030	55.4 ± 7.11	2.4 ± 0.28	9.12 ± 2.42	0.40 ± 0.11	0.350 ± 0.150	0.010 ± 0.010
Afternoon	17 ± 3	19	0.77 ± 0.13	3.77 ± 0.980	0.18 ± 0.050	57.7 ± 8.63	2.7 ± 0.45	7.47 ± 1.48	0.35 ± 0.080	1.29 ± 0.700	0.060 ± 0.030
Night	36 ± 10	20	0.58 ± 0.11	3.08 ± 0.790	0.13 ± 0.030	32.2 ± 3.85	2.4 ± 0.28	11.3 ± 1.03	0.40 ± 0.10	0.310 ± 0.110	0.010 ± 0.0040
p value	0.01		0.02	0.5	0.2	0.04	0.02	0.2	0.4	0.1	0.2
Post Hoc Analysis				-	-			-	-	-	-
Morning vs Afternoon	0.28		0.2	-	-	0.84	0.54	-	-	-	-
Morning vs Night	0.11		0.12	-	-	0.1	0.053	-	-	-	-
Afternoon vs Night	0.12		0.16	-	-	0.1	0.053	-	-	-	-

Parameters significantly altered by time of day are shown in Figure 5. Mean ± SEM, CV; coefficient of variation (Standard deviation/mean). n=8

Chapter 8: Impact of DNA methylation and a folic acid enriched diet on prostate and urinary function in hormone treated mice susceptible to urinary dysfunction

Introduction

Benign prostatic hyperplasia (BPH) is characterized by benign growth of the prostate. BPH often associates with lower urinary tract symptoms (LUTS) which include storage or irritative symptoms (increased frequency, urgency, pain, nocturia) and voiding or obstructive symptoms (weak stream, hesitancy, dribbling, incomplete emptying and overflow incontinence) (McVary et al. 2011). Approximately 70% of men over the age of 70 experience BPH and/or LUTS (Berry et al. 1984), which decreases quality of life and poses a significant healthcare burden to the aging population (Saigal and Joyce 2005). Currently available pharmacotherapies include drug therapy with alpha adrenergic receptor blockers, 5-alpha reductase inhibitors, phosphodiesterase 5 inhibitors or a combination to alleviate symptoms and reduce prostate volume (Radomski 2014). Some men respond to these treatments while others require invasive surgery to remove prostatic tissue and alleviate severe LUTS. Why some men respond to therapy better than others is unknown, largely because the molecular underpinnings of BPH are not known.

Changes in the hormonal milieu caused by aging have been hypothesized as a driving factor in onset and progression of BPH and LUTS. As men age, their plasma testosterone concentration declines while their estradiol concentration remains the same or even rises (Harman et al. 2001). Rodents treated with testosterone (T) and estradiol (E2) have circulating plasma hormone concentrations similar to aging men (Ricke et al. 2008) and also exhibit increased prostate wet weight, decreased prostatic urethral diameter, and urinary retention consistent with bladder outlet obstruction

(Nicholson et al. 2012). Inappropriate expression of genes and signaling pathways that mediate prostate development have been hypothesized as a BPH driving factor (McNeal 1978). Many signaling pathways are activated in developing and benign hyperplastic prostate. A reliance on hormone action is also shared between proliferative growth in the developing prostate and in BPH. Testosterone drives prostatic bud initiation, outgrowth and prostatic cellular differentiation (Cunha et al. 1987). Estrogens can change early prostate development by influencing DNA methylation, androgen receptor (*Ar*) expression and the number of developing prostatic ductal bud progenitors that form (Timms et al. 2005; Richter et al. 2007). Recently, we showed that DNA methylation is itself an important regulator of prostate development (Keil et al. 2014). Specifically, DNA methylation regulates *Ar* mRNA and protein abundance to determine prostatic responsiveness to androgens and onset and rate of prostatic bud formation (Keil et al., 2014a). Together these findings support the hypothesis that DNA methylation functions as a regulatory axis for hormone action and growth restriction during prostate development. Whether this regulatory action is perturbed later in life to change prostatic hormone responsiveness or stimulate benign growth is not known, but recent studies support this possibility.

Global 5-methylcytosine levels are reduced (Bedford and van Helden 1987) and tumor suppressor genes are often hypermethylated in BPH compared to histologically normal prostate tissue (Henrique et al. 2005). DNA methylation has also been shown to decrease prostatic steroid 5 alpha reductase type 2 (*Srd5a2*) expression (Niu et al. 2011), raising the possibility that men with highly methylated *SRD5A2* may be resistant to 5 alpha reductase inhibitor therapy (Bechis et al. 2014). Age, one of the best known

risk factors for BPH/LUTS, is also a predictor of DNA methylation status (Damaschke et al. 2013; Teschendorff et al. 2013; West et al. 2013). Epigenetic drift, or age associated changes in the DNA methylation landscape, occurs in prostate as it does in other organs (Damaschke et al. 2013). Typically, a global loss of DNA methylation is associated with aging (Gonzalo 2010). However, gene specific changes in DNA methylation (including hypo- and hyper-methylation) as well as prostate tissue region specific changes in DNA methylation have also been observed (Damaschke et al. 2013). Whether changes in DNA methylation contribute to BPH/LUTS onset or progression and whether interventions to enhance DNA methylation will be therapeutically beneficial has not been examined.

One reason to examine the role of DNA methylation in BPH pathobiology is that DNA methylation is malleable and influenced by a variety of environmental and dietary factors that could be risk factors or therapies for BPH. Diet can further modify DNA methylation status, which is relevant because several dietary factors have been associated with BPH symptoms (Espinosa 2013). Folic acid acts as a methyl donor for DNA methylation events (Wolff et al. 1998). This is of particular interest because folic acid is included as a supplement in most one-a-day vitamins, prenatal vitamins and occurs naturally in some foods and is supplemented in others. Folic acid is used widely as a dietary supplement among pregnant women during a sensitive period of prostate development in male offspring. Though this supplement plays an important role in protecting against neural tube closure defects, it has never been considered that it may also impact development of the prostate and urinary tract. Excessive folate intake is also a concern for aging men. Data from the National Health and Nutrition Examination

Survey (NHANES) indicate that serum folate concentrations have risen ~2.5-fold in the US population since cereal grain fortification was initiated in 1998 (Pfeiffer et al 2012). Additionally, over 40% of American men over age 60 take dietary supplements containing folic acid. These factors are likely responsible for the observation that over 34% of American men over age 60 have biomarkers consistent with folic acid excess (Bailey et al. 2010). This is precisely the population of men that is at risk for BPH/LUTS but we have no idea how excessive folic acid in adulthood impacts urinary function. Therefore, our studies to investigate folic acid effects on urinary function are warranted.

Here we tested the hypothesis that dietary folic acid enrichment and T+E2 treatment change DNA methylation in prostate, bladder and dorsal root ganglia that project to these tissues. We also characterize influences of T+E2 treatment and dietary folic acid enrichment on urinary physiology, and test the hypothesis that a folic acid enriched diet delays or decreases severity of urinary dysfunction in T+E2 mice. Our results reveal that a folic acid enriched diet increases dorsal prostate, ventral prostate, and dorsal root ganglia global DNA methylation. We found that a folic acid enriched diet protects against T+E2 mediated increases in urine output and intervoid interval. These benefits do not appear to be mediated by changes in prostate wet weight, as the folic acid enriched diet did not impact these endpoints in T+E2 mice. Changes in neural control of micturition may underlie the beneficial effects of a folic acid enriched diet. An intravesicular infusion of capsaicin into the bladders of T+E2 mice uniquely increases voiding frequency in mice consuming a folic acid enriched diet but not in mice consuming a control diet. Capsaicin is a selective agonist of transient receptor potential cation channel, subfamily V, member 1 (*Trpv1*), a nociceptor expressed by afferent

neurons which contributes to the bladder's response to filling and emptying. Our results suggest that bladders from T+E2 mice maintained on a folic acid enriched diet are more sensitive to afferent stimulation than bladders from T+E2 mice maintained on a control diet. Together our results provide evidence that changes in DNA methylation exist in T+E2 mice maintained on control or a folic acid enriched diet and urinary dysfunction is improved with folic acid diet independent of changes in prostate mass. These results are consistent with folic acid acting to reverse or slow the age/disease associated changes seen in mice treated with T+E2 in part by preserving afferent neuronal function.

Materials and Methods

Mice

Sexually mature C57BL/6J nulliparous female mice were housed in polysulfone cages containing corn cob bedding and maintained on a 12hr light and dark cycle at 25±5°C and 20-50% relative humidity. At least two weeks prior to their first mating, female mice were placed on a base diet (control diet, Harlan Diet 2019, Harlan Teklad, Madison, WI) containing 4mg/kg folic acid or fed the same base diet supplemented with folic acid to achieve a final concentration of 24mg/kg folic acid (folic acid enriched diet, Harlan Diet #120256). This two week loading period was previously shown to be sufficient to increase maternal serum folic acid concentrations (Kyung Hee Oh 2009) and induce changes in DNA methylation in their offspring (Wolff et al. 1998). Food and water were available ad libitum. Dams were maintained on diets throughout lactation and their offspring placed on the same diets after weaning and for the remainder of the

experiment. Six week old male mice underwent sham surgery or surgery to subcutaneously implant testosterone (25mg) and estradiol (2.5mg) pellets. All procedures were approved by the University of Wisconsin Animal Care and Use Committee and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Mice.

Morphometric analysis

Litter size, mass and crown to rump length were measured in E18.5 fetuses from control or folic acid diet dams. Crown to rump length was determined using a digital caliper and was measured from the crown of the head to the base of the tail. Prostatic buds were visualized and quantified as described previously (Keil et al. 2012). E18.5 urogenital sinuses (UGSs) were stained by in situ hybridization (ISH) to visualize NK3 transcription factor locus 1 (*Nkx3-1*) mRNA, which marks prostatic buds and then co-stained by IHC to visualize E-cadherin, which marks UGS epithelium. Prostate buds and UGS epithelium were visualized using a compound microscope and bud number for three samples per treatment group was quantified by three blinded individuals.

Serum and urine measurements

Blood glucose concentrations were measured by analyzing tail vein blood collected after a 4 hr fasting period with the AlphaTRAK 2 veterinary monitoring system (canine setting). Blood was collected by cardiac puncture post euthanasia, allowed to clot for 30min and centrifuged at 6000g x 10 min to collect serum. Serum homocysteine concentrations were determined using the Mouse Homocysteine (Hcy) ELISA Kit

(#MBS260152, MyBioSource, San Diego, CA) according to manufacturer's instructions. Serum anti-diuretic hormone concentration was determined using the arginine vasopressin EIA kit (#583951, Cayman Chemical, Ann Arbor MI) according to manufacturer's instructions. Serum creatinine concentration was determined using serum creatinine colorimetric assay kit (#700460, Cayman Chemical) according to manufacturer's instructions. Free catch urine was collected from mice, centrifuged at 8000g x 8 min and supernatant collected for specific gravity and protein analysis. Urine specific gravity was determined by refractometry (Vet360-Check digital refractometer, feline setting, Reichert Technologies, Depew NY). Urine protein was quantified using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Madison WI).

DNA methylation determination

Global DNA methylation levels were determined using the colorimetric MethylFlash Methylated DNA Quantification kit (#p-1034-96, Epigentek, Farmingdale, NY) according to manufacturer's instructions. DNA was isolated using the Qiagen DNeasy Blood and Tissue kit (#69506, Qiagen, Frederick, MD).

Spontaneous void assay

Void assays were conducted and analyzed as described previously (Keil 2014b; Yu et al. 2014). Single or group housed mice were removed from their cages and singly housed in a clean, empty cage lined with 3MM Whatman filter paper (Fisher Scientific #057163W). Assays were conducted over four hours with access to food but not water. Assays were conducted in the same quiet location and time of day unless indicated

otherwise. Filter papers were imaged using UV light on a transilluminator and analyzed using Image J Software (Version 1.46r) as described previously (Keil 2014b).

Bladder bath assay

In vitro bladder bath studies were conducted essentially as described previously (Tengowski et al. 1997). Excised bladders were divided longitudinally from apex to bladder neck and weighed. 4-0 sutures were used to secure the bladder neck of each hemisection to the specimen arm and the bladder apex to a force displacement transducer (FT-03, Grass Instruments, Quincy MA). Bladder hemisections were suspended in a 37°C water-jacketed tissue chamber filled with Krebs solution (133mM NaCl, 16mM NaHCO₃, 5mM KCl, 1mM MgCl₂, 1.4mM NaH₂PO₄, 2.5mM CaCl₂ 2H₂O, 7.8mM d glucose, pH 7.2) and aerated with 95% O₂/5% CO₂. Tissues were maintained at a tension of 1g for 75 min prior to experimentation. Tension was recorded using the AxoScope Application of pCLAMP™ Software (Molecular Devices, Sunnyvale, CA). Bladder hemisections were equilibrated for approximately 1 hr in Krebs solution and increasing concentrations (0-10μM) of the cholinergic agonist carbachol were added stepwise thereafter. Tissues were then washed with carbachol-free Krebs solution to return the tension to baseline and a maximal response was generated by adding 60mM KCl to the baths. The average bladder hemisection response was determined for each animal and normalized to mass of the bladder section for 5 mice per treatment group.

Cystometry

Mice were anesthetized with urethane (1.25g/kg, ip). A midline incision was made in the abdominal wall to expose the bladder. A purse string suture using 6-0 thread was inserted near the dome of the bladder. Catheters were created from PE50 tubing, cut 2cm shorter than the length of a 26G syringe needle, and one end of the tubing was melted to create a cuff. The needle was run through the tubing and inserted through the apex and across the bladder wall. The needle was then removed and the purse string suture tightened, followed by two circumferential sutures, to fix the catheter in place. The body wall and skin were sutured and the animal was allowed to recover for 45 min prior to saline infusion. During experimentation, the animal was maintained in a supine position and the cystometry catheter was connected to three way stopcock connected to an infusion pump and pressure transducer. Sterile saline was perfused at a rate of 1.6 ml/hr and voiding was recorded for at least one hour for each animal until a stable pattern was achieved. In some mice, capsaicin (30uM diluted in saline) was instilled at a rate of 1.6ml/hr after mice reached steady state voiding under saline conditions. Measurements were analyzed from at least six voiding events per animal once a consistent pattern was achieved. Parameters analyzed include intercontractile interval (ICI, defined as the length of time between voids), maximal intravesical pressure associated with each voiding or nonvoiding contraction (peak to baseline pressure), void duration (time to return to baseline after a voiding contraction), threshold pressure (pressure to elicit a voiding contraction), and leak point pressure (pressure required to induce a voiding contraction by applying pressure to the bladder filled to half capacity). Voiding events were subdivided into voids, defined here as events where two or more droplets of urine were produced, and drips, where a single drop of urine was produced.

Statistics

Results are reported as mean $\pm$ SEM. Statistical analysis was performed using R version 2.13.1. Homogeneity of variance was determined using Levene's test. For parametric data with two treatment groups, Student's T-test was used to identify significant differences ($p \leq 0.05$) between treatment groups. For parametric data with more than two treatment groups one way analysis of variance (ANOVA), followed by Tukey's honest significant difference (HSD) or a repeated measures ANOVA followed by pairwise T-tests using holm correction were used to identify significant differences ($p \leq 0.05$) between or among treatment groups. For nonparametric data with more than two treatment groups the Kruskal Wallis test was conducted. Contingency tables were analyzed using Fisher's exact test.

Results

We used a 2x2 design to test the variables of a folic acid enriched diet and T+E2 treatment on DNA methylation, prostate growth, and urinary function in mice. Adult nulliparous C57Bl/6J dams were fed a control (4mg/kg folic acid) or folic acid enriched (24mg/kg) diet for two weeks prior to mating and through pregnancy and lactation. This provides 6 times more folic acid than the recommended daily requirement for mice. The magnitude of folic acid enrichment is clinically relevant because it approximates the magnitude of folic acid recommended for a subset of pregnant women. The recommended folic acid intake for most pregnant women is 0.4mg/day, but 10 times more is recommended for women who have had a previous pregnancy involving neural

tube defects, who are diabetic, or who take medications known to interfere with folate metabolism (1992). Timed matings were performed and the resulting offspring either collected at E18.5 or matured to adulthood. Male weanlings were maintained on the maternal diet and at 6 weeks of age received either sham surgery or surgery to implant T+E2 pellets. Mice were maintained on diets for the two months following surgery. We then assessed prostate histology, molecular biology, and urinary physiology (Fig. 1).

Folic acid alone does not alter fetal growth

Because prostate anatomy may influence urinary physiology, our first objective was to determine whether a folic acid enriched diet given to dams prior to and during pregnancy influences fetal growth rate and prostate ductal development in male offspring. Male embryos from timed pregnant dams from both diet groups were collected at E18.5. A folic acid enriched diet increases litter size by three pups per litter compared to the control diet (Fig. 2A), while embryo weight and fetal crown to rump length are unaffected (Fig. 2B). E18.5 fetal lower urinary tracts were stained with the prostatic bud marker *Nkx3-1* and an antibody against E-cadherin to visualize epithelium and enable quantification of prostatic bud number. Diet did not significantly change prostatic bud number (Fig. 2C,D). These results suggest that while a folic acid enriched diet increases litter size, it does not alter fetal growth or prostatic bud formation.

Folic acid enriched diet increases growth in adulthood

We next determined that maternal consumption of the folic acid enriched diet does not significantly change male offspring mass at weaning (3 weeks of age, Fig. 3A).

However, male offspring that continue on this diet exhibit a greater mass at four, five and six weeks of age (Fig. 3A). Food consumption increases equally over time in both diet groups (Fig. 3B) and there is no difference in water consumption between diet groups at three, four or five weeks of age (Fig. 3C). These results suggest that a maternal folic acid enriched diet combined with the same diet given during early adulthood may augment male mouse growth.

A folic acid enriched diet decreases serum homocysteine concentration

We next tested whether the folic acid enriched diet was sufficient to act as a methyl donor in mice. Since folic acid measurements in plasma can fluctuate based on daily eating habits, we used a more stable endpoint - homocysteine serum concentration. Folic acid acts as a methyl donor in several metabolic pathways including DNA methylation, one carbon metabolism and in the conversion of homocysteine to methionine (Bhargava and Tyagi 2014). Serum homocysteine concentration is therefore inversely related to folic acid concentration. Serum homocysteine concentrations are significantly lower in 14 week old adult mice exposed to a folic acid enriched diet *in utero* through adulthood compared to mice on the control diet (Figure 4A). These data indicate the folic acid enriched diet used in this study is sufficient to increase methyl groups available for one carbon metabolism and DNA methylation.

Testosterone and estradiol treated mice maintained on control and folic acid diets exhibit changes in urine physiology

We next determined the effects of a folic acid enriched diet in adult mice treated with T+E2. This treatment produces changes in the hormonal milieu similar to that seen in aging men and induces prostatic hyperplasia and urinary dysfunction (Ricke et al. 2008; Nicholson et al. 2012). Adult male mice maintained on a folic acid enriched or control diet *in utero* through adulthood received either sham surgery or surgery to implant T+E2 pellets. Mouse mass, food and water consumption were monitored for the entire eight week period following surgery. Body mass is significantly greater in folic acid diet mice compared to control diet mice on week one through four, and is greater in T+E2 mice compared to sham mice on week two and three (Figure 5A, Supplemental Table 1). There were no statistically significant differences in body mass at 6-8 weeks post-surgery (Figure 5A,B, Supplemental Table 1). Repeated measures analysis of variance (ANOVA) revealed a statistically significant interaction between treatment group and change in body mass over time; the rate of body mass increase for mice on a folic acid enriched diet is greater than mice on control diet for sham and T+E2 mice (Figure 5A, Supplemental Table 2).

Food consumption measured at weeks one and two post surgery is significantly higher in control diet sham mice versus folic acid diet sham mice (Figure 5C, Supplemental Table 1). This difference is no longer observed by week three through eight and there was no interaction between treatment group and food consumption over time (Figure 5C,D, Supplemental Table 2).

Unlike mouse mass and food consumption, water consumption did not become equal between treatment groups by eight weeks post surgery. Overall, a folic acid enriched diet alone decreases water intake versus control diet sham mice (Figure 5E,

Supplemental Table 1) while T+E2 treatment increases water intake compared to sham mice. Interestingly, a folic acid enriched diet delays the T+E2 induced increase in water consumption until five weeks post-surgery (Figure 5E-G, Supplemental Table 1). There was also a significant interaction in water consumption by treatment group over time (Supplemental Table 2). Overall these results indicate that while mass and food consumption normalize between treatment groups by the end of the eight week treatment period, water consumption is affected by diet and rapidly (within one week post T+E2 surgery in control diet mice) increases with T+E2 treatment. Thus, T+E2 treatment induces polydipsia (increased fluid intake) in mice, and a folic acid enriched diet delays this response in T+E2 treated mice.

A folic acid enriched diet and T+E2 treatment change urinary physiology

We next tested whether increases in water consumption in T+E2 mice was accompanied by dilute urine production. Urine specific gravity was measured at baseline, 1 and 2 months post surgery. There were no statistically significant differences in urine specific gravity between treatment groups at baseline (results not shown). The folic acid enriched diet did not affect urine specific gravity in sham mice (Fig. 6). T+E2 treatment significantly decreases urine specific gravity at the one (Fig. 6A) and two month time points (Figure 6B). Diet further affects this hormone action. A folic acid enriched diet further reduces T+E2 mediated decrease in urine specific gravity at the two month time point (Fig. 6B). These results align with increases in water intake in these treatment groups (Fig 5E). Urine protein concentration is also reduced in T+E2

treated mice from both diet groups (Fig. 6C). Together these results indicate that T+E2 treatment causes hyposthenuria (dilute urine production).

We next investigated the underlying basis for T+E2 induced polydipsia and hyposthenuria. These actions are not likely caused by diabetes mellitus since fasting blood glucose concentrations are unchanged by T+E2 treatment (Fig. 6D). Diabetes insipidus is associated with excessive thirst and dilute urine production and is characterized by defects in anti-diuretic hormone (ADH) production (primary diabetes insipidus) or kidney responsiveness (nephrogenic diabetes insipidus). T+E2 treatment increases serum ADH and decreases serum creatinine in mice maintained on a control diet and the folic acid enriched diet protects against these defects (Fig. 6E-F). Together these results indicate that T+E2 treatment affects water balance by increasing water intake and causing dilute urine production. Feeding these mice a folic acid enriched diet protects against T+E2 induced changes in serum ADH and creatinine concentrations, without affecting water consumption and urine concentration.

A folic acid enriched diet reduces T+E2 induced abnormalities in voiding function

T+E2 mice exhibit abnormal voiding function by one month post-surgery (Nicholson et al. 2014) characterized by increased voiding frequency and reduced volume per void (Nicholson et al. 2012; Nicholson et al. 2014). We conducted the spontaneous void spot assay at defined intervals (baseline, 1 and 2 months post-surgery) to test whether the folic acid enriched diet influences adult control mouse voiding function and protects against T+E2 induced urinary abnormalities. Spot number, total urine area, spot size, area within the primary (largest) void, % total urine area in

primary void, % total area in corner of paper, and % total area in center of paper were analyzed as described previously (Keil 2014b; Yu et al. 2014). The specific variables of urine spot number, urine area, primary void area and % area in corners were shown previously to correlate with body mass (Keil 2014b) and were normalized accordingly.

The total urine area voided by mice fed the folic acid enriched diet is less at baseline compared to that of mice fed the control diet, however this difference recedes with age (Fig. 7A, Supplemental Table 3). We next tested whether the folic acid enriched diet protects against T+E2-induced voiding abnormalities. There were no significant differences in spot number, spot size, percent area within primary void and percent area urine in center of paper among the four experimental mouse groups at one and two months post-surgery (Supplemental Table 3). Diet had no impact on any of the examined voiding parameters in sham mice at one and two months post-surgery (Supplemental Table 3). T+E2 treatment in both diet groups increases total urine area and primary void area versus sham mice at one month post-surgery (Fig. 7B,C, Supplemental Table 3). T+E2 treatment in control diet mice decreases percent total urine area within corners versus control diet sham mice and the folic acid enriched diet protects against this endpoint (Fig. 7D). Voiding parameters were then normalized to body mass, revealing additional differences between groups. T+E2 treatment in both diet groups increases relative total urine area and primary void area versus sham mice at one month post-surgery however the folic acid enriched diet diminishes this effect (Fig. 7E,F).

We next examined how spontaneous voiding parameters change over time among treatment groups. There was a significant effect of time, treatment group and an

interaction of these two variables on total urine area and relative urine area from baseline to two months post-surgery (Figure 7G,H, Supplemental Table 4,5). These differences in urine area observed at one month are the only differences which persist to two months post-surgery (Supplemental Table 3). These results suggest that by one month of age there are T+E2 induced changes in voiding parameters and a folic acid enriched diet is capable of reducing these T+E2 effects on void spot assay outcomes.

Changes in prostate DNA methylation accompany folic acid enriched diet and T+E2 treatment

Changes in prostate and/or bladder growth and physiology could contribute to the observed protective effects of a folic acid enriched diet on T+E2 induced urinary dysfunction. We first focused on the prostate. T+E2 treatment increases prostate wet weight, prostatic ductal quantity within the periurethral region, and narrows the urethral lumen within the prostatic region (Nicholson et al. 2012). This abnormal prostatic growth pattern in T+E2 mice has been hypothesized as a contributing factor to urinary dysfunction. We have found that DNA methylation is capable of regulating prostatic bud growth during development (Keil 2014a). We tested whether T+E2 treatment or folic acid enriched diet induce changes in global DNA methylation levels in the prostate, and whether these changes associate with changes in prostatic wet weight. Diet and T+E2 treatment cause prostate lobe specific changes in global DNA methylation. The folic acid enriched diet decreases anterior prostate global DNA methylation compared to control diet in sham and T+E2 mice (Fig. 8A). Conversely, a folic acid enriched diet increases dorsal prostate global DNA methylation independent of T+E2 treatment (Fig.

8B). Diet or T+E2 treatment did not alter lateral prostate global DNA methylation (Fig. 8C). The folic acid enriched diet given to T+E2 mice uniquely increases ventral prostate global DNA methylation compared to T+E2 mice fed the control diet (Fig. 8D). These results indicate that folic acid diet is capable of increasing (dorsal and ventral prostate) or decreasing (anterior) prostate lobe global DNA methylation and can alter the response to T+E2 treatment (as seen in ventral prostate). We next evaluated whether diet- and treatment-induced changes in DNA methylation were accompanied by changes in prostate lobe, seminal vesicle, or bladder wet weight. Diet alone had no impact on wet weights (Fig. 9). T+E2 treatment of mice in both diet groups significantly increases bladder volume, and wet weight of anterior, dorsal, lateral, and ventral prostate, and of seminal vesicle and urethra compared to sham mice (Fig. 9). Bladder wet weight only increases by T+E2 treatment in folic acid enriched diet mice (Fig. 9). The same differences were observed when these parameters were normalized to mouse body mass except in lateral prostate. T+E2 treatment in both diet groups increases relative lateral prostate wet weight compared to sham treatment, but this effect is muted by the folic acid enriched diet (Fig. 9I). Together, these results indicate that folic acid enriched diet effects on voiding function are likely not due to differences in prostate growth. Though relative lateral prostate wet weight was lower in T+E2 mice fed the folic acid enriched diet, the fact that no other lobes are changed indicates there are likely other mechanisms involved.

A folic acid enriched diet and T+E2 treatment alter bladder physiology

We next tested whether the beneficial effects of the folic acid enriched diet on T+E2 treated mouse voiding function potentially occur through mechanisms involving the bladder and neural control of micturition. We examined global DNA methylation in bladder as well as L5 and L6 dorsal root ganglia (DRG) which innervate bladder and prostate. Neither the folic acid enriched diet nor T+E2 treatment change bladder global DNA methylation (Fig. 10A). However, the folic acid enriched diet increases DRG global DNA methylation in T+E2 mice compared to control diet fed T+E2 mice (Fig. 10). These results indicate that a mechanism by which the folic acid enriched diet influences T+E2 treated mouse voiding function may be mediated in part by influencing bladder function through neuronal control of micturition.

To determine whether the folic acid enriched diet impacts T+E2 treated mouse bladder function, we first conducted *in vitro* bladder bath studies to assess contractile response to graded concentrations of the cholinergic agonist carbachol and a fixed concentration of potassium chloride. Responses were normalized to bladder strip wet weight to account for differences in bladder size between sham and T+E2 mice. T+E2 treatment decreases the maximum contractile response elicited by carbachol without changing the potency of carbachol (EC₅₀) in eliciting bladder contraction (Fig. 11).

We next used anesthetized cystometry to analyze bladder response to filling and emptying, endpoints which require appropriate neural function. Cystometry was performed by placing a catheter in the dome of the bladder of anesthetized mice, infusing saline at a constant rate and monitoring voiding pressure. Parameters analyzed include, intervoid interval (IVI), peak to baseline pressure of voiding and nonvoiding contractions, time to return to baseline pressure after a void, number of nonvoiding

contractions, leak point pressure (applied pressure required to elicit a void when bladder is at half capacity), and the threshold pressure (pressure to elicit a void). We observed that the folic acid enriched diet changes T+E2 treated mouse cystometric profiles. One factor modified by the diet was whether mice were capable of initiating a void during the monitoring period, an endpoint previously shown to be affected by T+E2 treatment (Nicholson et al. 2012). The folic acid enriched diet significantly increases the proportion of T+E2 mice capable of eliciting a voiding contraction (Table 1). In mice that could elicit a voiding contraction, T+E2 treatment increases intervoid interval in mice fed a control diet (Fig. 12A). However, the folic acid enriched diet protects against the T+E2 induced increase in intervoid interval (Fig. 12A). The number of drips and peak to baseline pressures were similar among all treatment groups (Fig. 12B,C). T+E2 treatment increases the time to return to baseline after a void regardless of diet (Fig. 12D). The number and pressure of nonvoiding contractions was unaltered by diet or T+E2 treatment (Fig. 12E,F). The folic acid enriched diet alone decreases leak point pressure versus control sham mice, no differences were observed with T+E2 treatment (Fig. 12G). A folic acid enriched diet in T+E2 treated mice increases threshold pressure compared to folic acid diet sham mice (Fig. 12H). Together these results indicate that T+E2 treatment alters voiding parameters and a folic acid enriched diet has beneficial effects in T+E2 treated mice on intervoid interval.

The inability of some control diet T+E2 mice to elicit bladder contractions, and the rescue of this ability with a folic acid enriched diet (Table 1) could arise through changes in bladder decompensation/ function or the bladder's ability to respond to afferent stimulation. We next tested whether bladders from T+E2 treated mice were capable of

responding to afferent stimulation. The detection of bladder filling is mediated in part by transient receptor potential vanilloid subfamily member 1 (TRPV1) receptors (Daly et al. 2007). TRPV1 receptors are activated by capsaicin and approximately 60% of bladder afferent neurons are capsaicin sensitive (Hu-Tsai et al. 1992). To further investigate changes in bladder and voiding responses in T+E2 mice and the beneficial actions of a folic acid enriched diet we administered intravesicular capsaicin to anesthetized mice undergoing cystometry. The capsaicin concentration (30uM) was sufficient to decrease intervoid interval (Fig 13A) as reported previously (Schroder et al. 2003a). The folic acid enriched diet increased the number of voiding events (drips during a 6 void interval) in T+E2 treated mice (Fig. 13B). In both diet groups, capsaicin treatment prevents the T+E2 induced increase in time to return to baseline after a void seen under saline infusion (Fig. 13C). Additional changes in voiding function before versus after infusion of capsaicin as assessed by repeated measures ANOVA are shown in Supplemental Figure 1. Among these changes we observed a folic acid enriched diet among sham mice, increases peak to baseline pressure of voiding contractions after capsaicin versus before (Supplemental Fig. 1B). These results indicate that the folic acid enriched diet in T+E2 mice may confer a higher sensitivity to afferent stimulation, leading to enhanced ability to detect bladder filling and eliminate urine. These findings have potential clinical relevance, since men who suffer from urinary obstruction experience an inability void and empty the bladder.

Together our results indicate that the folic acid enriched diet and T+E2 treatment induce changes in urinary function and global prostate and DRG DNA methylation, with folic acid diet providing beneficial effects on urine output and voiding function. Folic acid

diet may slow or prevent the deleterious effects of T+E2 treatment on kidney function, urinary physiology and response to afferent stimulation. Our results indicate that folic acid diet benefits do not appear to be a result of changes in prostate growth, but rather through changes in bladder afferent innervation and sensitivity.

Discussion

In this study, we revealed new dimensions of voiding dysfunction in T+E2 treated mice and showed that a folic acid enriched diet modifies voiding function in sham animals as well as in T+E2 mice. T+E2 treatment increases water consumption, serum ADH concentrations and urine output and decreases serum creatinine concentration, urine specific gravity, and deteriorates several aspects of voiding function. A folic acid enriched diet protects against T+E2 mediated changes in serum ADH and creatinine, urine output, intervoid interval and response to afferent stimulation. These benefits do not appear to be mediated by blocking T+E2 mediated prostate hypertrophy as assessed by prostatic wet weight. However, we did find that the folic acid enriched diet increases global DNA methylation in dorsal prostate, ventral prostate, and dorsal root ganglia and some of these changes may underlie physiological changes to voiding function that are exerted by folic acid.

Previous research focused on T+E2 mediated prostatic growth as a basis for urinary dysfunction (Nicholson et al. 2012). Our results implicate an additional contributor: kidney ADH resistance leading to excessive urine production. The following T+E2 mouse endpoints are consistent with nephrogenic diabetes insipidus: increased water consumption (Fig. 5E-G) and urine production (Fig. 7B), decreased urine specific

gravity (Fig. 6A,B) and urine protein concentration (Fig. 6C), decreased serum creatinine (Fig. 6F) and increased serum ADH (Fig. 6E). Consumption of the folic acid enriched diet protects against the T+E2 induced changes in serum creatinine and ADH (Fig. 6E,F). While the influence of dietary folic acid on nephrogenic diabetes insipidus is not fully known, it does protect against mouse hypertension induced renal damage by improving cortical blood flow, vascular density and reducing fibrotic collagen deposition (Pushpakumar et al. 2013). Further investigation into how T+E2 influences ADH production or responsiveness is warranted. Aquaporin 2 (*Aqp2*) is a transepithelial water channel expressed by collecting duct epithelial cells to regulate urine concentration by promoting water absorption. Mouse *Aqp2* genetic deletion causes nephrogenic diabetes and increases water consumption, voiding frequency and urine production (Yang et al. 2006). ADH positively impacts AQP2 function by increasing its localization in the epithelial cell membrane and by increasing its mRNA abundance. Impairment of either process by T+E2 would provide additional evidence for impairment of ADH function at the level of the kidney. Additional studies, including the fluid deprivation test followed by administration of vasopressin and measuring of serum and urine osmolality, would distinguish between T+E2-induced primary polydipsia, central diabetes insipidus, and nephrogenic diabetes insipidus. Whether mice with diabetes insipidus also exhibit increases in bladder volume, prostate wet weight and alterations in afferent stimulation as do T+E2 treated mice remains to be determined.

The beneficial actions of the folic acid enriched diet on T+E2 mouse urinary function may involve DNA methylation dependent and independent mechanisms. While folic acid is known to be a methyl donor capable of enhancing DNA methylation (Barua

et al. 2014) it is also the main regulator of plasma homocysteine levels, which in turn impact DNA methylation. The homocysteine precursor S-adenosylhomocysteine is a competitive inhibitor of S-adenosylmethionine, the methyl donor in DNA methylation events (Ingrosso and Perna 2009). Hyperhomocysteinemia, or high homocysteine levels, are prevalent in patients with chronic kidney disease and uremia, DNA hypomethylation is also higher in these men compared to healthy controls (Ingrosso et al. 2003). Folic acid decreases homocysteine levels and restores DNA methylation in these men (Ingrosso et al. 2003). These results reveal a need for continued study in the role of T+E2 and folic acid enriched diet in kidney function. Specifically in relation to changes in homocysteine levels and DNA methylation and how these changes associate with lower urinary tract dysfunction.

T+E2 treatment was shown in a previous study to increase mouse prostate wet weight and prostatic ductal density within the periurethral region, to narrow the urethral lumen and decrease voiding function in a manner consistent with bladder outlet obstruction (Nicholson et al. 2012). The hallmark of bladder outlet obstruction in men is high detrusor pressure and low flow rate (Nitti 2005). These endpoints are also seen in mice with surgically induced urethral obstruction. Mice with urethral obstruction have increased bladder wet weight, and volume, and exhibit decreased pressure generation and sensitivity to stimulation (Austin et al. 2004). These mice also have increased threshold pressure, bladder capacity and post void residual urine (Schroder et al. 2003b). Increases in threshold pressure, micturition volume, residual volume and frequency of nonvoiding contractions is also seen in T+E2 treated rats (Tatemichi et al. 2006). Our cystometry results are consistent with bladder outlet obstruction and urethral

resistance in T+E2 treated mice from both diet groups. T+E2 treatment increases bladder volume, prostate mass (Fig. 9), and threshold pressure, the latter of which reached significance among mice fed the folic acid enriched diet (Fig. 12). Since the folic acid enriched diet fails to moderate the prostatic wet weight and threshold pressure increases mediated by T+E2 (Fig. 9,12), its beneficial actions likely occur through alternative mechanisms.

A previous report revealed that T+E2 treatment for four months thins the detrusor and bladder wall and increases bladder collagen content, potentially impacting bladder compliance leading to decompensation (Nicholson et al. 2012). Our results indicate that after two months of T+E2 treatment, despite T+E2 induced increase in bladder mass, bladder volume and residual urine, the bladder itself remains sensitive to cholinergic and afferent stimulation. Further, we show that intravesicular capsaicin infusion into bladders of T+E2 treated mice uniquely increases void events in mice consuming a folic acid enriched diet but not in mice consuming a control diet (Fig. 13). This result potentially indicates a folic acid-induced increase or maintenance of bladder sensitivity to afferent stimulation and reveals a new potential target for augmenting urinary function in mice with bladder outlet obstruction. It remains unclear whether the folic acid enriched diet augmented bladder filling sensitivity in T+E2 treated mice, or whether it delays the T+E2-mediated decline of bladder function.

Our results reveal T+E2 and folic acid diet dependent changes in global DNA methylation in DRG neurons which innervate the prostate and bladder. Which genes are involved and whether T+E2 or folic acid diet induced changes in voiding function are mediated by changes in DNA methylation remains to be determined. DNA methylation

and histone acetylation have been shown to regulate gene expression in nociceptive DRG neurons innervating the lower extremities of rats (Hong et al. 2014).

Environmental stressors increase *Dnmt1* abundance uniquely in L6-S2 DRG neurons, increase promoter DNA methylation of cannabinoid receptor 1, and thereby increase TRPV1 expression and visceral sensation to pain (Hong et al. 2014). Cannabinoid receptors and TRPV1 play important roles in bladder afferent signaling, nociception and voiding function (Wang et al. 2014). Our results raise the hypothesis that cannabinoid and *Trpv1* receptors may be targets of DNA methylation in response to T+E2 and folic acid enriched diet.

In addition to changes in DRG global DNA methylation we also observed changes in prostate global DNA methylation with T+E2 and folic acid enriched diet. While we did not observe changes in prostate wet weight, indicating that DNA methylation events may not lead to increased prostatic growth, it does not preclude the possibility that differential prostate gene expression exists and contributes to pathologies later in life. In rats prolonged exposure (75-92 weeks) to T+E2 results in prostatic adenocarcinoma (Bosland et al. 1995). T+E2 treatment also drives malignant transformation of human epithelial cells combined with mouse urogenital sinus mesenchyme xenografts (Ricke et al. 2006). Steroid hormones have the capacity to shape the epigenome. Androgens and estrogens influence DNA methylation in brain and other organs (Sebag et al. 2011; Ghahramani et al. 2014). Whether T+E2 induced changes in prostate DNA methylation contribute to carcinogenesis later in life and whether a folic acid diet can mitigate the effects of T+E2 warrants further investigation.

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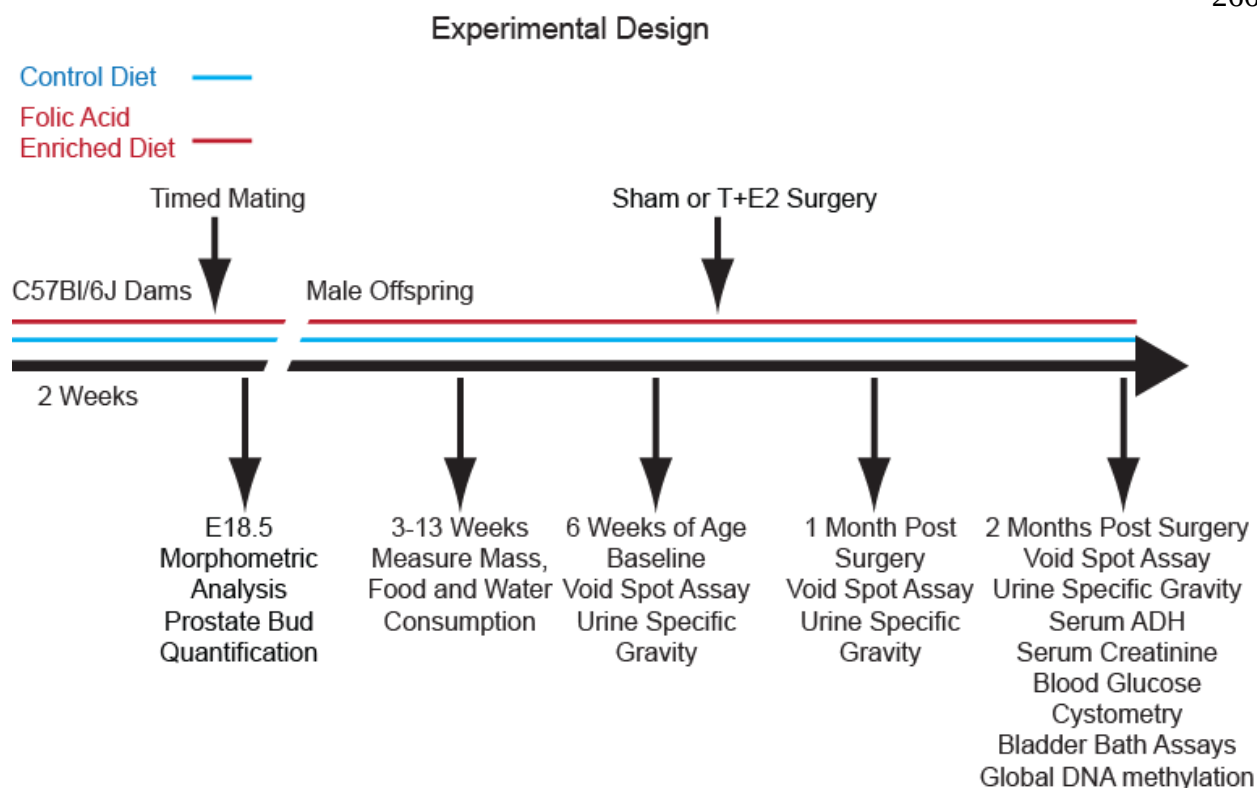


Figure 1. Experimental design. Schematic representation of the experimental design used in this study. C57BL/6J dams were maintained on a control (4mg/kg folic acid) or control diet supplemented with folic acid (24mg/kg folic acid) for two weeks prior to timed mating. Some dams were sacrificed and embryos collected at embryonic day (E) 18.5 for morphometric analysis and prostate bud quantification. Other dams were allowed to drop and resulting male offspring were maintained on maternal diets through lactation, weaning and adulthood. Body mass, food and water consumption were measured from three weeks of age through the end of the experiment. At six weeks of age adult male mice underwent subcutaneous implantation of testosterone or estradiol pellets. Following surgery physiology and voiding function were determined at one month and two months post surgery.

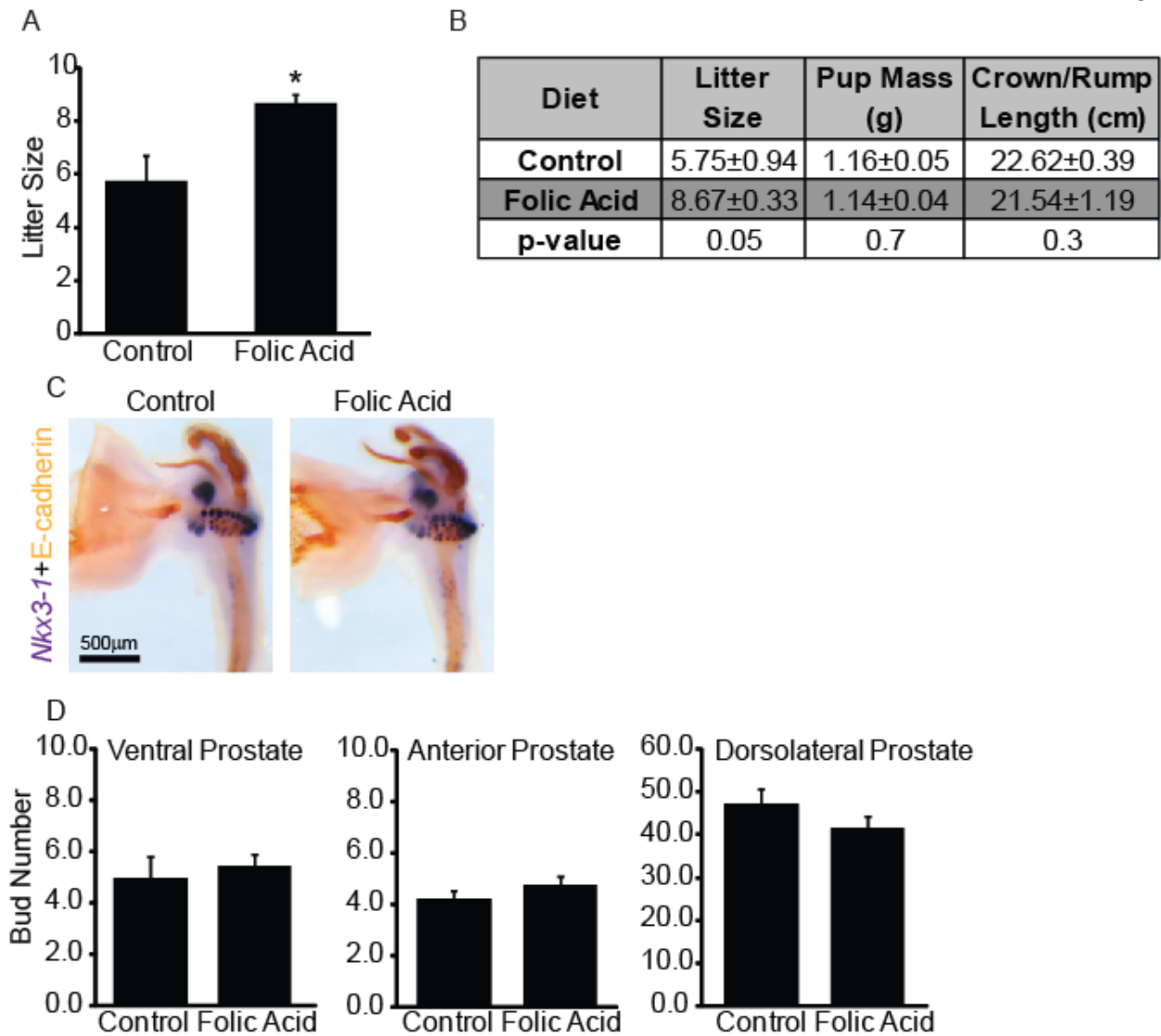


Figure 2. Maternal folic acid enriched diet increases litter size. Embryos were collected from dams maintained on control or folic acid enriched diet at embryonic day (E) 18.5. (A-B) Quantification of litter size, embryo mass and embryo crown to rump length. (C-D) Urogenital sinus (UGS) stained by ISH for the prostate selective marker, NK transcription factor locus 3 (*Nkx3-1*, purple) and an antibody to E-cadherin (brown) to visualize and quantify ventral, anterior and dorsolateral prostatic bud number. Results are mean $\pm$ SEM, asterisk indicates significant difference $p \leq 0.05$.

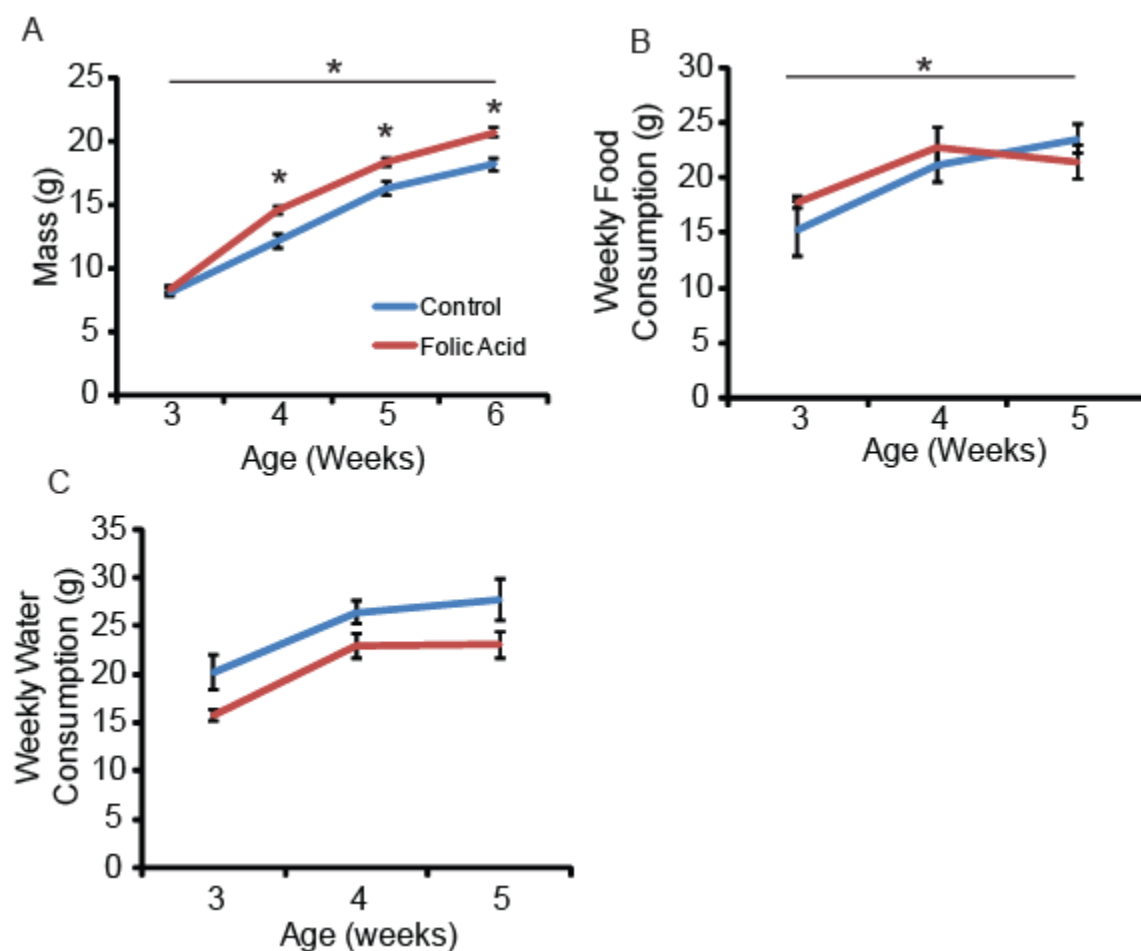


Figure 3. Folic acid enriched diet increases weanling male mouse mass. Male mice were maintained on the maternal control or folic acid enriched diet through lactation and weaning. Quantification of (A) body mass, (B) weekly food consumption, (C) weekly water consumption from three to six weeks of age. Results are mean $\pm$ SEM, asterisk indicates significant difference $p \leq 0.05$.

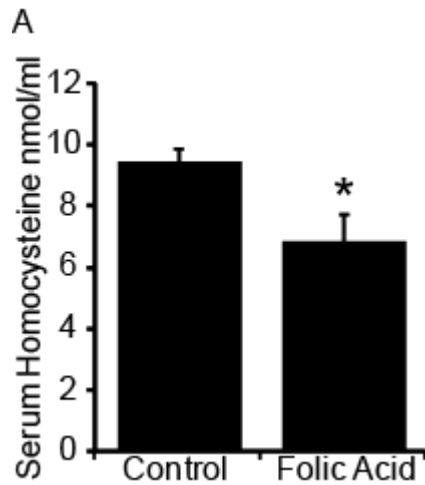


Figure 4. A folic acid diet decreases serum homocysteine levels. Male mice maintained on control or folic acid enriched diet *in utero*, lactation and adulthood. (A) Quantification of serum homocysteine concentration. Results are mean $\pm$ SEM, asterisk indicates significant difference $p \leq 0.05$.

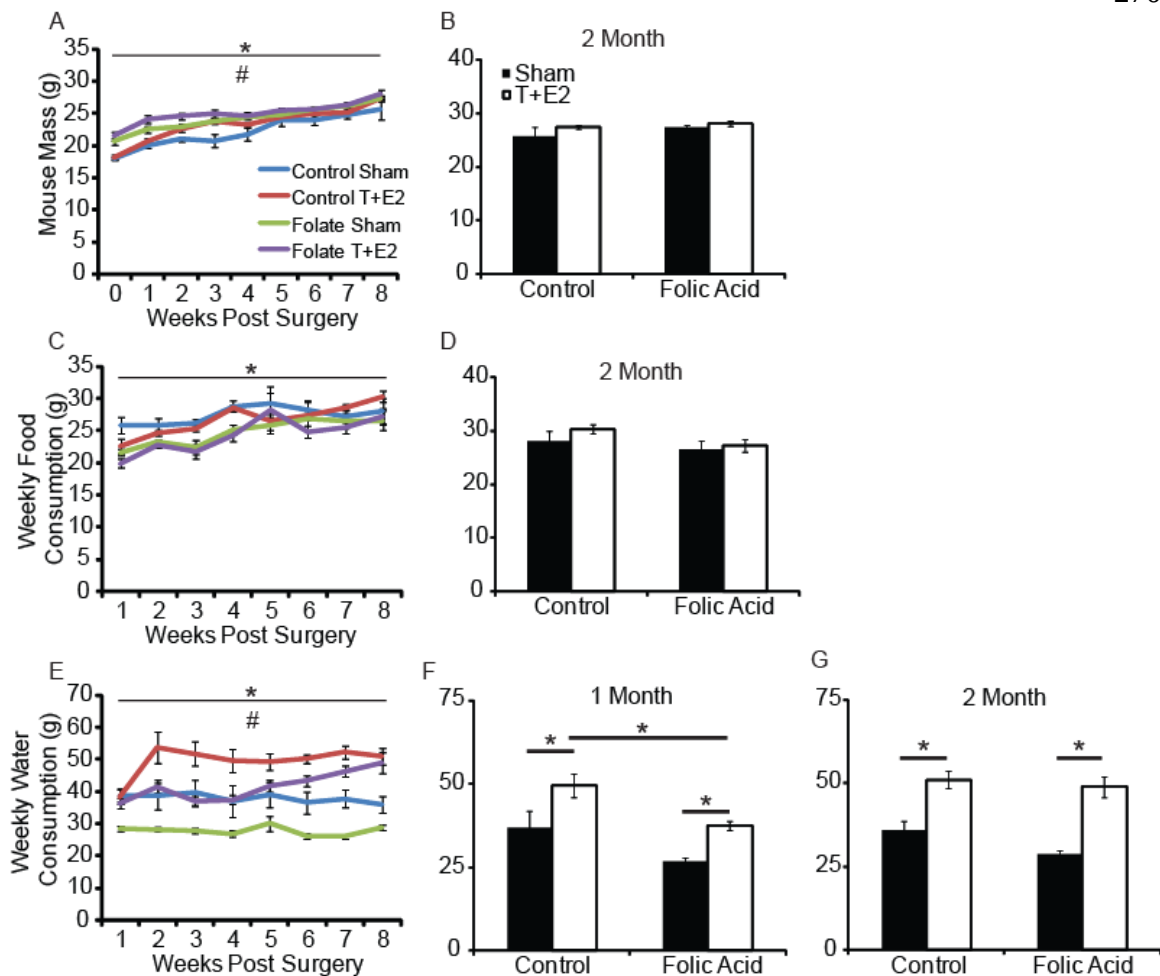


Figure 5. Folic acid enriched diet slows T+E2 induced increase in water

consumption. Adult male mice maintained on control or folic acid enriched diet *in utero* through adulthood underwent sham surgery or surgery to implant testosterone (T) and estradiol (E2). (A-B) Mouse body mass, (C-D) weekly food consumption, (E-G) weekly water consumption were quantified from zero to eight weeks post surgery. Results are mean $\pm$ SEM, asterisk indicates significant difference $p \leq 0.05$. Pound sign indicates a significant interaction between time and treatment group.

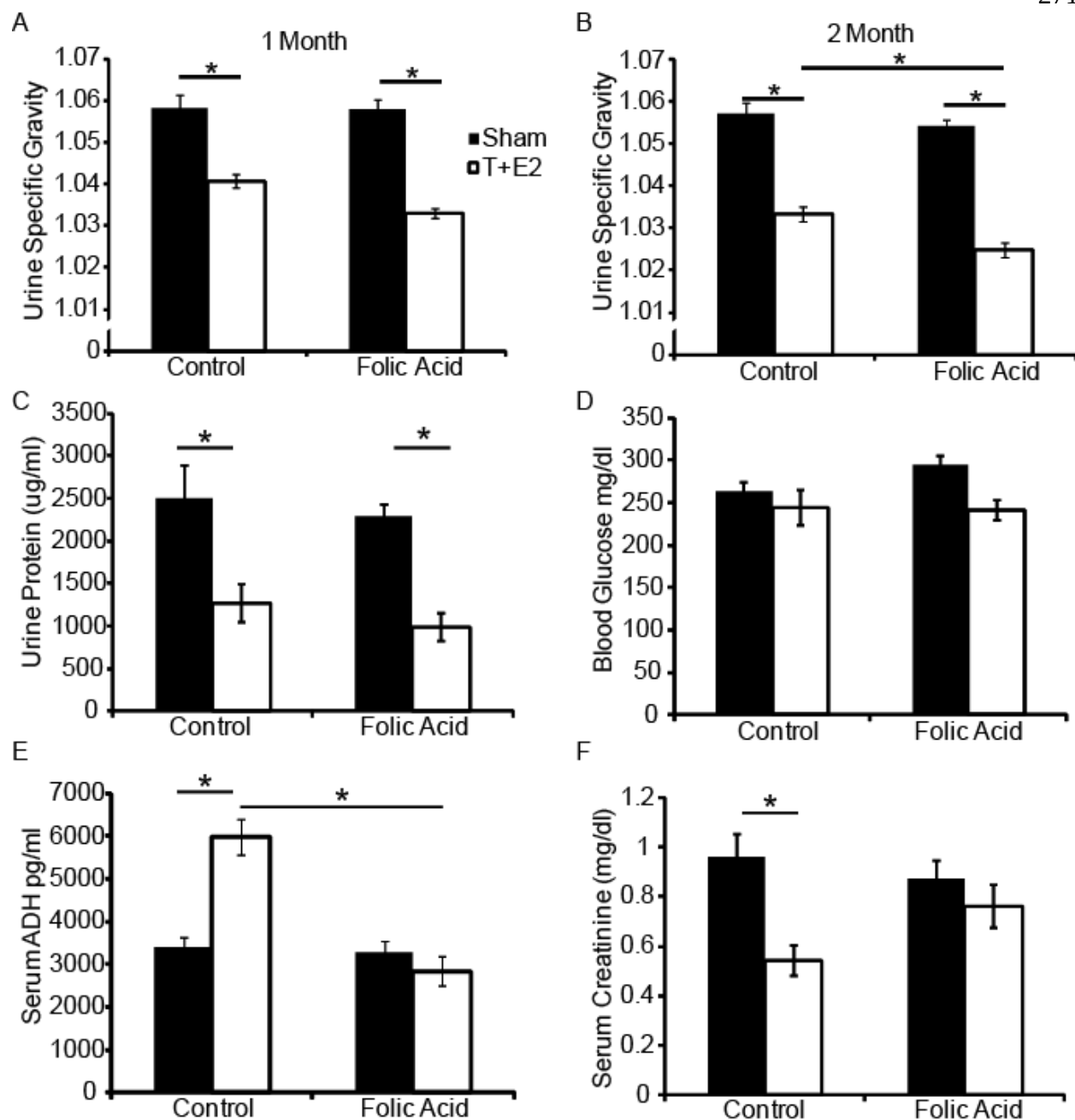


Figure 6. Folic acid enriched diet protects against T+E2 induced increases in serum ADH levels. Adult male mice maintained on control or folic acid enriched diet *in utero* through adulthood underwent sham surgery or surgery to implant testosterone (T) and estradiol (E2). (A-B) Urine specific gravity measurements at one and two months post surgery. Quantification of (C) urine protein concentration, (D) blood glucose

concentration, (E) serum anti-diuretic hormone (ADH) concentration and (F) serum creatinine concentration at two months post surgery. Results are mean $\pm$ SEM, asterisk indicates significant difference $p \leq 0.05$.

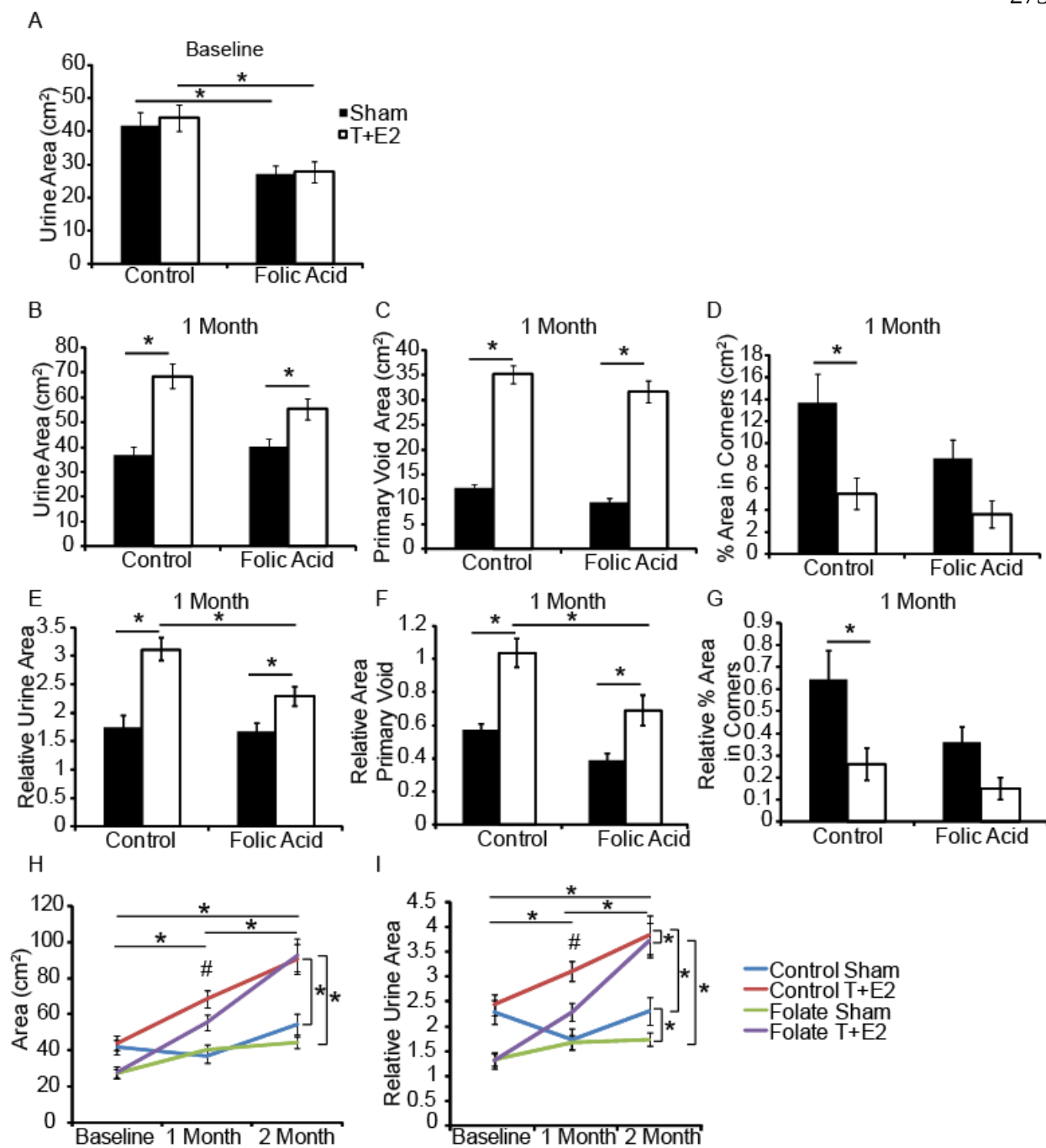


Figure 7. Folic acid enriched diet protects against some T+E2 induced changes in void spot assay parameters. Adult male mice maintained on control or folic acid enriched diet *in utero* through adulthood underwent sham surgery or surgery to implant testosterone (T) and estradiol (E2). Void spot assays were conducted at baseline, one

month and two months post surgery. Quantification of (A) baseline differences in total urine area. Quantification of (B) urine area, (C) primary void area, (D) percent area in corners at one month post surgery. Void spot assay parameters normalized to mouse body mass include (E) relative urine area, (F) relative area in primary void and (G) relative percent area in corners. Change in (H) urine area and (I) relative urine area over time. Results are mean $\pm$ SEM, asterisk indicates significant difference $p \leq 0.05$. For repeated measures analysis vertical bars indicate differences between treatment groups, horizontal bars indicate differences over time, pound sign indicates a significant interaction between time and treatment group $p \leq 0.05$.

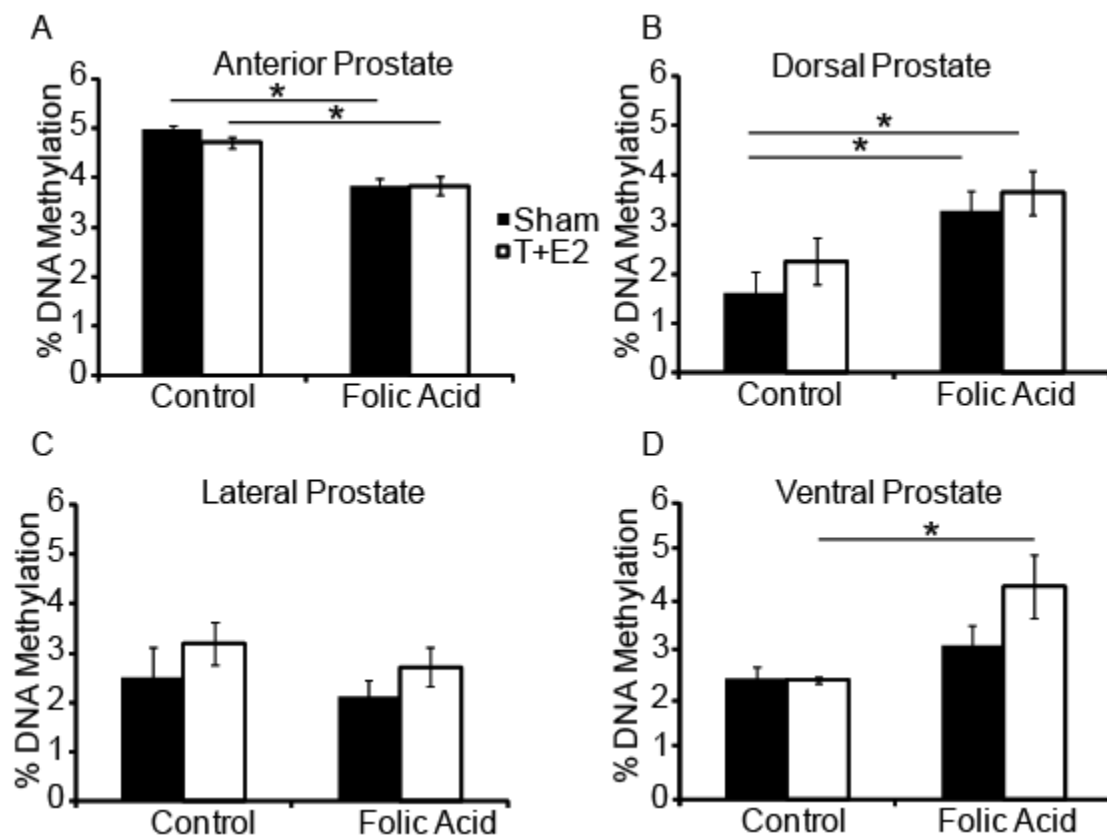


Figure 8. Folic acid enriched diet and T+E2 induce lobe specific changes in prostate global DNA methylation. Adult male mice maintained on control or folic acid enriched diet *in utero* through adulthood underwent sham surgery or surgery to implant testosterone (T) and estradiol (E2). Percent global DNA methylation in (A) anterior, (B) dorsal, (C) lateral and (D) ventral prostate two months after surgery. Results are mean $\pm$ SEM, asterisk indicates significant difference $p \leq 0.05$.

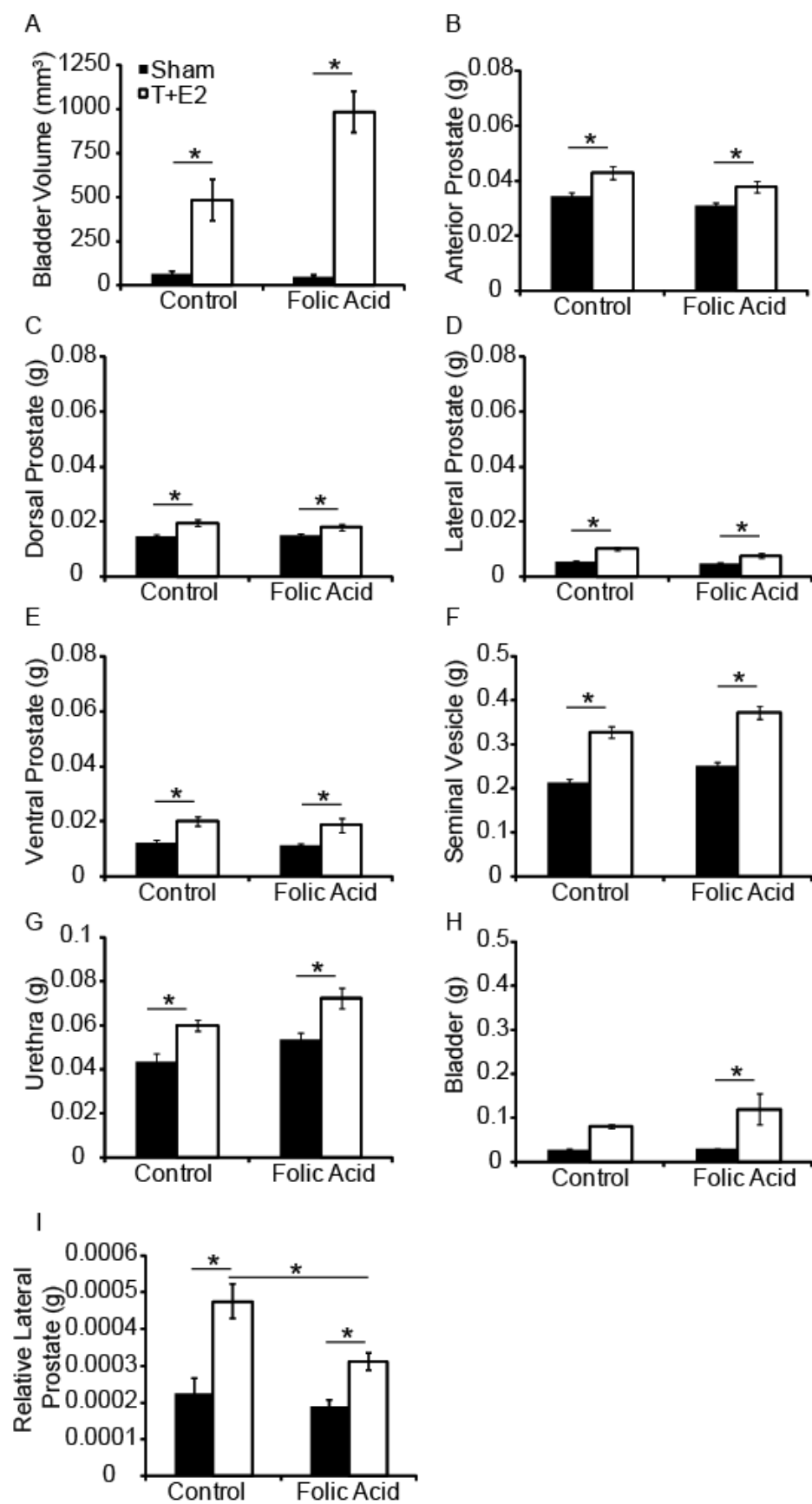


Figure 9. Folic acid enriched diet does not protect against T+E2 increases in

bladder volume and prostate wet weight. Adult male mice maintained on control or folic acid enriched diet *in utero* through adulthood underwent sham surgery or surgery to implant testosterone (T) and estradiol (E2). Quantification of (A) bladder volume, (B) anterior prostate, (C) dorsal prostate, (D) lateral prostate, (E) ventral prostate, (F) seminal vesicle, (G) urethra, (H) bladder and (I) relative lateral prostate wet weights. Results are mean $\pm$ SEM, asterisk indicates significant difference $p \leq 0.05$.

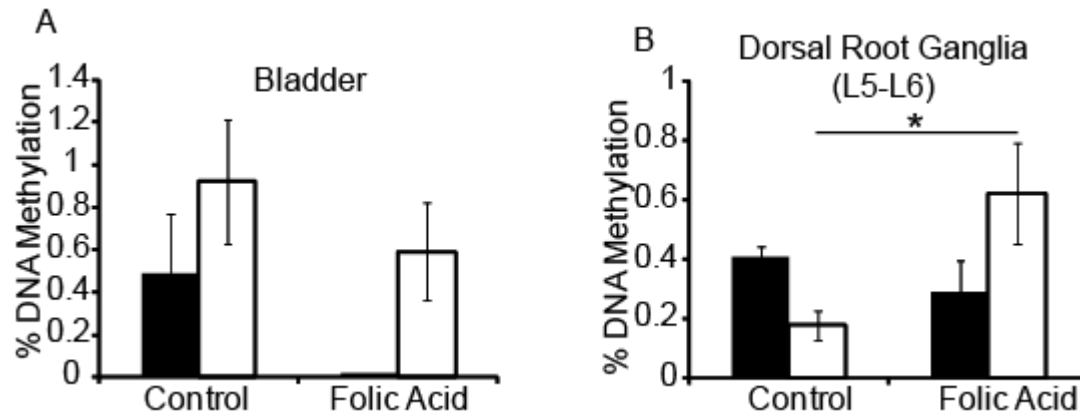


Figure 10. Folic acid enriched diet increases T+E2 induced dorsal root ganglia global DNA methylation. Adult male mice maintained on control or folic acid enriched diet *in utero* through adulthood underwent sham surgery or surgery to implant testosterone (T) and estradiol (E2). Quantification of global DNA methylation in (A) bladder and (B) dorsal root ganglia (DRG) at two months post surgery. Results are mean $\pm$ SEM, asterisk indicates significant difference $p \leq 0.05$.

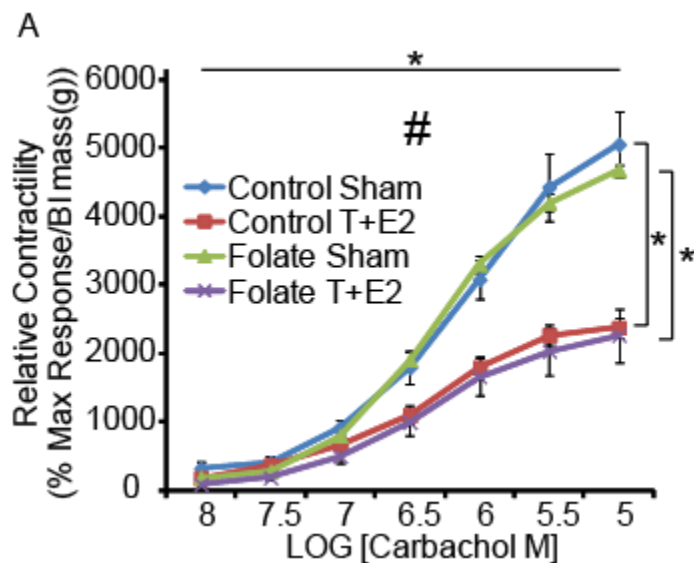


Figure 11. T+E2 diminishes bladder contractility. Adult male mice maintained on control or folic acid enriched diet *in utero* through adulthood underwent sham surgery or surgery to implant testosterone (T) and estradiol (E2). (A) Relative bladder contractility (percent max response to KCl normalized to bladder mass) in isolated bladder strips exposed to graded concentrations of the cholinergic agonist, carbachol. Results are mean $\pm$ SEM, asterisk indicates significant difference $p \leq 0.05$. Vertical bars indicate differences between treatment groups, horizontal bars indicate differences over time, pound sign indicates a significant interaction between change in carbachol concentration and treatment group $p \leq 0.05$.

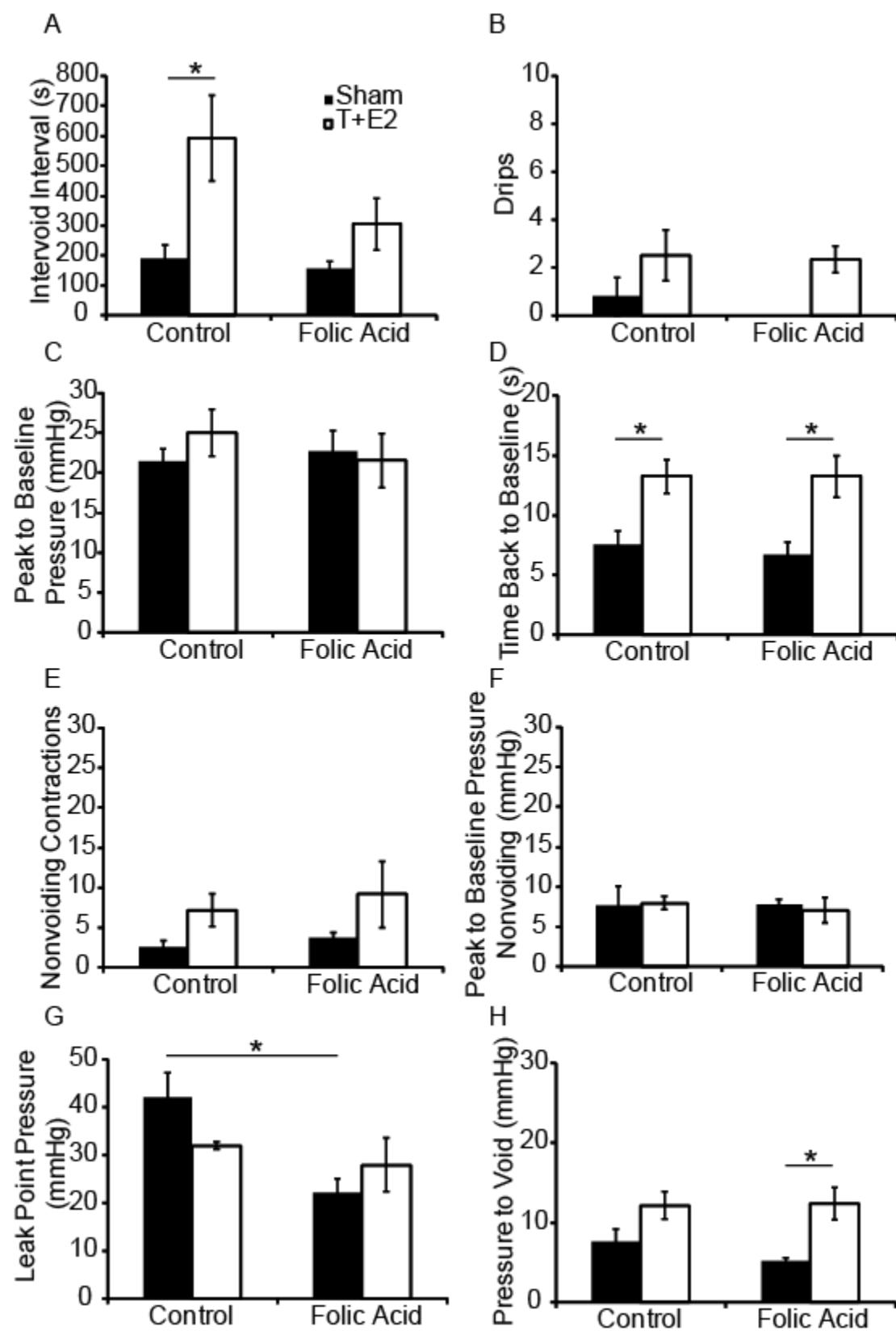


Figure 12. Folic acid enriched diet protects against T+E2 induced increases in intervoid interval. Adult male mice maintained on control or folic acid enriched diet *in utero* through adulthood underwent sham surgery or surgery to implant testosterone (T) and estradiol (E2). At two months post surgery mice underwent anesthetized cystometry. Quantification of (A) intervoid interval, (B) number of drips, (C) peak to baseline pressure of voiding contractions, (D) time to return to baseline after voiding contraction, (E) number of nonvoiding contractions, (F) peak to baseline pressure of nonvoiding contractions (G) leak point pressure, (H) threshold pressure to elicit a void. Results are mean $\pm$ SEM, asterisk indicates significant difference $p \leq 0.05$.

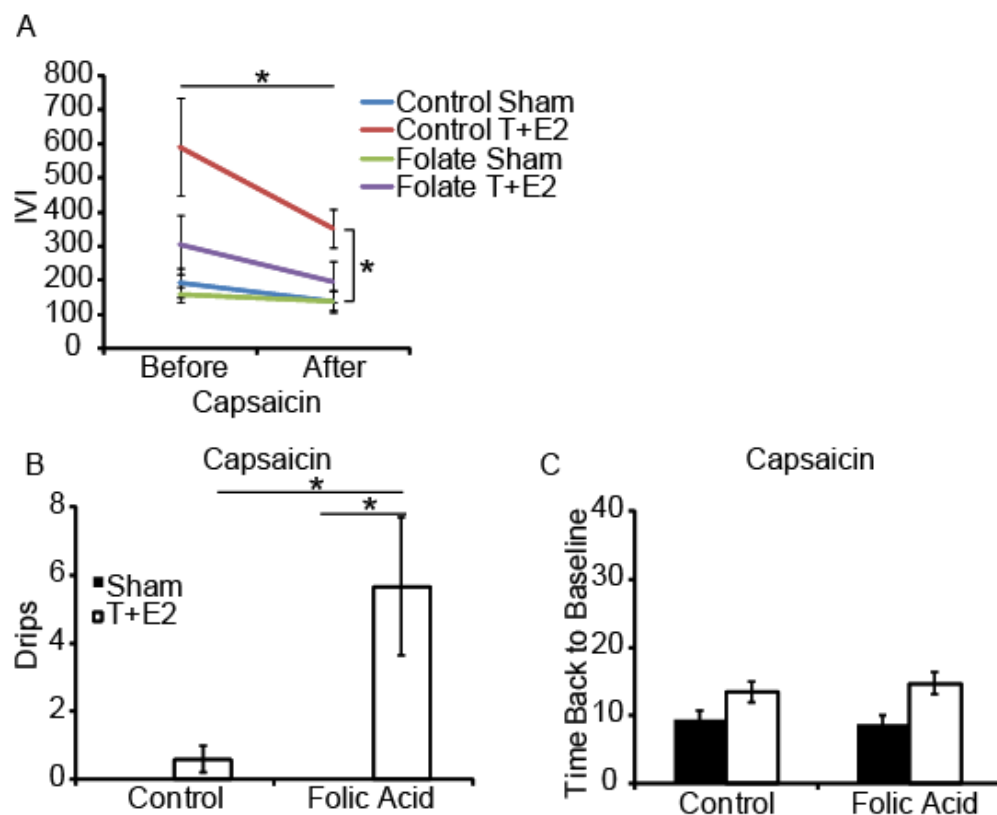
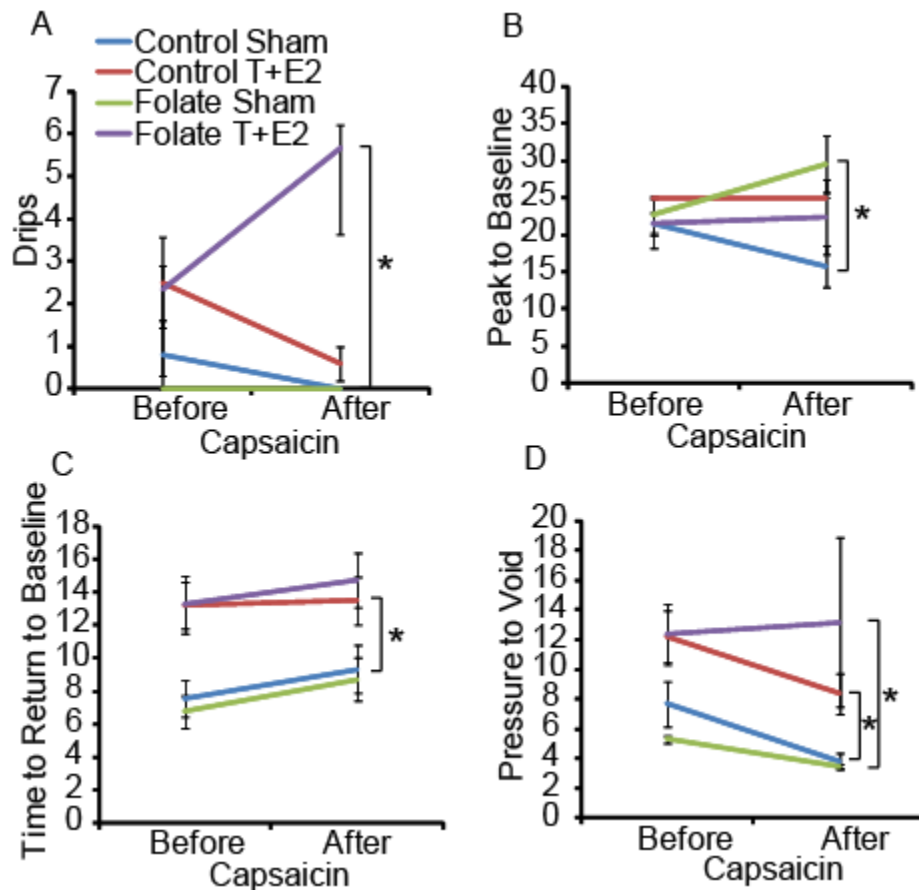


Figure 13. Folic acid enriched diet enhances T+E2 bladder sensitivity to afferent stimulation. Adult male mice maintained on control or folic acid enriched diet *in utero* through adulthood underwent sham surgery or surgery to implant testosterone (T) and estradiol (E2). At two months post surgery intravesicular instillation of afferent agonist, capsaicin, was infused in anesthetized mice after undergoing cystometry. Quantification of (A) intervoid interval before and after capsaicin instillation. Quantification of (B) number of drips occurring over the course of six voids and (C) time to return to baseline after void in mice treated with capsaicin. Results are mean $\pm$ SEM, asterisk indicates significant difference $p \leq 0.05$. Vertical bars indicate differences between treatment groups, horizontal bars indicate differences over time, pound sign indicates a significant interaction between time and treatment group $p \leq 0.05$.

Table 1. Proportion of mice able to elicit voiding contraction

	%Total Mice Able To Elicit Voiding Contraction
Control Sham	100
Control T+E2	70
Folate Sham	100
Folate T+E2	100
p-value	0.01



Supplemental Figure 1. Folic acid enriched diet and T+E2 induced changes in voiding after capsaicin instillation. Adult male mice maintained on control or folic acid enriched diet *in utero* through adulthood underwent sham surgery or surgery to implant testosterone (T) and estradiol (E2). Quantification of change in (A) drips occurring over six voids, (B) peak to baseline pressure, (C) time to return to baseline after voiding, and (D) pressure to elicit a void before versus after capsaicin in mice two months post surgery. Results are mean $\pm$ SEM, asterisk indicates significant difference $p \leq 0.05$. Vertical bars indicate differences between treatment groups, horizontal bars indicate differences over time, pound sign indicates a significant interaction between time and treatment group $p \leq 0.05$.

Supplemental Table 1: p-values for weekly differences in mass, food and water consumption between treatment groups.

	Week							
Mass	1	2	3	4	5	6	7	8
cs - ct	ns	ns	0.04	ns	ns	ns	ns	ns
cs - fs	0.05	0.04	0.008	0.03	ns	ns	ns	ns
cs - ft	0.0007	0.0001	0.0002	0.01	ns	ns	ns	ns
ct - fs	ns	ns	ns	ns	ns	ns	ns	ns
ct - ft	0.007	0.05	ns	ns	ns	ns	ns	ns
fs - ft	ns	0.02	ns	ns	ns	ns	ns	ns
Food Consumption								
cs - ct	ns	ns	ns	ns	ns	ns	ns	ns
cs - fs	0.01	0.03	ns	ns	ns	ns	ns	ns
cs - ft	0.0002	0.01	0.08	0.03	ns	ns	ns	ns
ct - fs	ns	ns	ns	ns	ns	ns	ns	ns
ct - ft	ns	ns	ns	0.02	ns	ns	ns	ns
fs - ft	ns	ns	ns	ns	ns	ns	ns	ns
Water Consumption								
cs - ct	ns	ns	ns	0.01	ns	0.001	0.0001	0.03
cs - fs	0.0008	ns	0.02	0.01	ns	0.003	0.0001	ns
cs - ft	ns	ns	1	ns	ns	ns	0.007	0.02
ct - fs	0.001	0.008	0.008	1E-07	0.00017	0	0	0.0001
ct - ft	ns	ns	0.02	0.002	ns	ns	ns	ns
fs - ft	0.0006	0.0001	0.0006	0.0002	0.001	0	0	0.00001

cs, control sham; ct, control T+E2; fs, folic acid sham; ft, folic acid T+E2; ns, not significant $p > 0.05$

Supplemental Table 2: p-values for repeated measures ANOVA on weekly mass, food and water consumption

	Mass	Food Consumption	Water Consumption
Treatment Group	2x10 ⁻¹⁶	0.0002	2.2x10 ⁻¹⁶
Time	2x10 ⁻¹⁶	7x10 ⁻⁶	3x10 ⁻⁶
Interaction	0.02	ns	4.6x10 ⁻⁶
cs - ct	ns	ns	0.0003
cs - fs	0.0005	2.6x10 ⁻⁶	1.1x10 ⁻¹²
cs - ft	2.9x10 ⁻⁶	1.4x10 ⁻⁶	ns
ct - fs	ns	0.001	4.3x10 ⁻¹⁵
ct - ft	0.003	0.0005	1.1x10 ⁻⁵
fs - ft	0.02	ns	2x10 ⁻¹⁶

cs, control sham; ct, control T+E2; fs, folic acid sham; ft, folic acid T+E2; ns, not significant p>0.05

Supplemental Table 3: p-values for void spot assays.

Void Spot Assay	Timepoint			Void Spot Assay Normalized to mass	Timepoint		
	Baseline	1 Month	2 Month		Baseline	1 Month	2 Month
Mass	9x10 ⁻⁷	4.9x10 ⁻⁶	0.01				
cs - ct	ns	ns	ns				
cs - fs	0.01	0.0005	ns				
cs - ft	0.0001	0.0001	ns				
ct - fs	0.0009	0.001	0.02				
ct - ft	0.000006	0.0005	ns				
fs - ft	ns	ns	ns				
Spot Number	ns	ns	ns	Spot Number	ns	ns	ns
Urine Area	0.0006	1x10 ⁻⁵	1x10 ⁻⁶	Urine Area	1x10 ⁻⁵	4x10 ⁻⁶	7x10 ⁻⁷
cs - ct	ns	0.0002	0.02	cs - ct	ns	0.0002	0.02
cs - fs	0.02	ns	ns	cs - fs	0.005	ns	ns
cs - ft	0.04	0.02	0.007	cs - ft	0.005	ns	0.02
ct - fs	0.003	0.00008	0.0002	ct - fs	0.0003	0.000004	0.00004
ct - ft	0.007	ns	ns	ct - ft	0.0003	0.01	ns
fs - ft	ns	0.01	0.000007	fs - ft	ns	0.03	0.000005
Spot Size	ns	0.05	ns				
cs - ct	ns	ns	ns				
cs - fs	ns	ns	ns				
cs - ft	ns	ns	ns				
ct - fs	ns	0.04	ns				
ct - ft	ns	ns	ns				
fs - ft	ns	ns	ns				
Primary Void Area	0.01	1x10 ⁻⁵	ns	Primary Void Area	0.0006	1x10 ⁻⁶	ns
cs - ct	ns	0.004	ns	cs - ct	ns	0.003	ns
cs - fs	ns	ns	ns	cs - fs	ns	ns	ns
cs - ft	ns	ns	ns	cs - ft	ns	ns	ns
ct - fs	0.01	0.000008	ns	ct - fs	0.0005	0.0000006	ns
ct - ft	ns	ns	ns	ct - ft	0.01	0.01	ns
fs - ft	ns	0.004	ns	fs - ft	ns	0.007	ns
% Area in Primary Void	0.03	ns	ns				
cs - ct	ns	ns	ns				
cs - fs	ns	ns	ns				
cs - ft	0.03	ns	ns				
ct - fs	ns	ns	ns				
ct - ft	ns	ns	ns				
fs - ft	ns	ns	ns				
% Area in Corners	ns	0.002	ns	% Area in Corners	ns	0.001	ns
cs - ct	ns	0.03	ns	cs - ct	ns	0.02	ns
cs - fs	ns	ns	ns	cs - fs	ns	ns	ns
cs - ft	ns	0.002	ns	cs - ft	ns	0.005	ns
ct - fs	ns	ns	ns	ct - fs	ns	ns	ns
ct - ft	ns	ns	ns	ct - ft	ns	ns	ns
fs - ft	ns	ns	ns	fs - ft	ns	ns	ns
% Area in Center	ns	0.03	ns				
cs - ct	ns	ns	ns				
cs - fs	ns	ns	ns				
cs - ft	ns	0.01	ns				
ct - fs	ns	ns	ns				
ct - ft	ns	ns	ns				
fs - ft	ns	ns	ns				

cs, control sham; ct, control T+E2; fs, folic acid sham; ft, folic acid T+E2; ns, not significant p>0.05

Supplemental Table 4: p-values for repeated measures ANOVA for void spot assays

Void Spot Assay	Spot Number	Total Area	Spot Size	Area Primary Void	% Area Primary Void	% Area Corners	% Area Center
Treatment Group	ns	1x10 ⁻⁹	0.09	8x10 ⁻⁵	ns	0.02	0.06
Time	3x10 ⁻⁹	2x10 ⁻¹²	5x10 ⁻⁶	ns	2x10 ⁻⁷	0.0001	0.0002
Interaction	ns	6x10 ⁻⁵	ns	ns	0.005	ns	ns
cs - ct	ns	0.002	ns	0.01	ns	ns	ns
cs - fs	ns	ns	0.03	0.02	ns	ns	0.05
cs - ft	ns	ns	ns	ns	ns	0.0009	0.008
ct - fs	ns	1x10 ⁻⁶	0.004	1x10 ⁻⁶	ns	ns	ns
ct - ft	ns	ns	ns	ns	ns	ns	ns
fs - ft	ns	0.004	ns	0.007	ns	ns	ns
baseline - one month	1.5x10 ⁻⁹	2.2x10 ⁻⁶	1.4x10 ⁻⁷	ns	1x10 ⁻⁵	ns	2.6x10 ⁻⁶
baseline - two month	1.9x10 ⁻⁸	1x10 ⁻⁹	0.002	ns	4x10 ⁻⁶	0.0005	2.3x10 ⁻⁵
one month - two month	ns	1x10 ⁻⁵	0.005	ns	ns	0.0004	ns

cs, control sham; ct, control T+E2; fs, folic acid sham; ft, folic acid T+E2; ns, not significant p>0.05

Supplemental Table 5: p-values for repeated measures ANOVA for void spot assays normalized to body mass.

Void Spot Assay Normalized to Mass	Spot Number	Total Area	Area Primary Void	% Area in Corners
Treatment Group	ns	1x10 ⁻¹⁰	5x10 ⁻⁶	0.005
Time	7x10 ⁻⁹	6x10 ⁻⁸	0.07	0.0001
Interaction	ns	7x10 ⁻⁵	0.03	ns
cs - ct	ns	0.0007	0.02	ns
cs - fs	ns	0.01	0.02	ns
cs - ft	ns	ns	ns	0.0006
ct - fs	ns	1x10 ⁻⁹	1x10 ⁻⁷	ns
ct - ft	ns	0.02	0.02	ns
fs - ft	ns	0.002	0.01	ns
baseline - one month	4x10 ⁻⁹	0.002	0.01	ns
baseline - two month	1x10 ⁻⁷	8x10 ⁻⁷	ns	0.0001
one month - two month	ns	0.002	ns	0.0001

Chapter 9: Conclusions and Future Research Opportunities

Adapted from Keil et al., (2014). Epigenomics. Under review

Interactions between DNA methylation and hormones- a future research opportunity in developing prostate

DNA methylation regulates estrogen receptor (ER) and androgen receptor (AR) expression in adult prostate and other tissues (Jarrard et al. 1998; Kerdivel et al. 2013). ER and AR signaling both influence prostate development (reviewed in (McPherson et al. 2008)), but it is not yet known if and how DNA methylation acts in concert with these hormones during prostate morphogenesis. The next section reviews links between hormone action and DNA methylation in other organs as potential mechanisms by which these factors could interact in developing prostate.

There is growing evidence that hormone action can alter *Dnmt* expression, activity and DNA methylation levels. For example, changes in estrogen and progesterone levels during the female menstrual cycle alter endometrial *Dnmt* expression (Yamagata et al. 2009; van Kaam et al. 2011; Vincent et al. 2011). *In vitro* estrogen and progesterone administration to human endometrial stromal cells decreases *Dnmt3a* and *3b* transcript abundance (Yamagata et al. 2009), while administration to human proliferative phase endometrium increases *Dnmt1* levels (van Kaam et al. 2011). Estradiol or dihydrotestosterone administration to newborn female Sprague Dawley rats reduces *Dnmt3a* levels in developing amygdala (Kolodkin and Auger 2011).

There is additional evidence linking hormone actions to specific DNA methylation patterns in hormone-responsive tissues. Estrogen and progesterone influence DNA methylation patterns and mRNA abundance of endometrial genes involved in transcription, apoptosis, proliferation, extracellular matrix and blood vessel

morphogenesis (Houshdaran et al. 2014). Neonatal testosterone exposure masculinizes DNA methylation at sexually dimorphic CpG sites in female mouse brain (Ghahramani et al. 2014).

Evidence links hormone induced DNA methylation patterns to specific anatomical and physiological endpoints. Estrogen and androgens are responsible for mouse gender dependent differences in DNA methylation and expression of cardiomyocyte gene, *calsequestrin 2* in cardiac ventricle, leading to sexually dimorphic cardiac structure and function (Sebag et al. 2011). Further, DNA methylation, ventricle structure and function are reversed upon gonadectomy (Sebag et al. 2011). Non-neuronal serotonin drives a switch in *Shh* promoter DNA methylation in mouse embryonic fibroblasts and mouse mammary gland tissue, leading to increased *Shh* expression and increased circulating calcium concentrations during lactation (Laporta et al. 2014). Together these results reveal that hormones can influence expression of DNA methylation regulators, DNA methylation patterns, expression of target genes, and physiological responses elicited by target genes. Further investigation into these interactions will likely shed light on both developmental and disease processes driven by steroid hormones. These results will also inform future studies to determine whether hormones influence DNA methylation in developing prostate. DNA methylation could be a means by which androgens or andromedins (genes responsible for mediating the actions of androgens) alter gene expression to drive prostate development.

A future research opportunity: Examine known roles of DNA methylation in other organs and use them to test new hypotheses in prostate development

My work has revealed several roles of DNA methylation in prostate development, including regulation of: E-cadherin expression, epithelial outgrowth and differentiation, *Ar* expression and prostate responsiveness to androgens. My work has just begun to open the door in terms of identifying roles of DNA methylation during prostate development. Therefore, looking at which developmental mechanisms, genes and pathways are affected by DNA methylation in other developing organs may inform future studies in the prostate. Global inactivating mutations in mouse *Dnmt1*, *Dnmt3b*, or *Dnmt3a+Dnmt3b* cause mid-gestational mortality (0.5-11.5 dpc) (Lei et al. 1996; Okano et al. 1999), and mutations in mouse *Dnmt3a* associate with severe growth retardation and mortality around 4 weeks of postnatal age (Okano et al. 1999). Early embryonic mortality and growth retardation of *Dnmt* mutant mice limits their utility in testing DNMT requirements in early organ development. However, the growing number of genetically modified mice that express cell type specific *cre* drivers facilitates an organ- and cell-type specific approach towards understanding the role of DNA methylation in development. Using currently available mouse strains, several studies have revealed context-dependent roles for *Dnmt1* and DNA methylation.

Selective *Dnmt1* deletion in developing excitatory neurons and astroglia of cortex and hippocampus causes cortical degeneration and persistent learning and memory deficits (Hutnick et al. 2009). Interestingly, a fraction of hypomethylated neurons survive postnatally but exhibit increased dendritic branching and impaired excitability (Hutnick et al. 2009). Developmental mechanisms responsible for these phenotypes include changes to genes responsible for cell layer-specification, cell death and ion transport (Hutnick et al. 2009). *Dnmt1* deletion in retinal pigment epithelium or neural retina

disrupts retinal pigment epithelia morphology and polarization, photoreceptor development and neural retinal differentiation (Nasonkin et al. 2013). These events are accompanied by disrupted actin cytoskeleton morphology, decreased retinal adhesion and DNA methylation changes in Hedgehog, Notch and Wnt pathway genes (Nasonkin et al. 2013). DNA methylation also mediates key events in mouse hair follicle development and regeneration. Epidermal *Dnmt1* deletion causes uneven epidermal and hair fiber thickness, altered follicle size and an aging-related decline in hair fiber quantity (Li et al. 2012). Focal cell proliferation defects partially account for uneven epidermal thickness (Li et al. 2012). Molecular mechanisms responsible for changes in hair follicle size are unknown largely because few molecules are known to influence hair size. Proposed targets include *Bmp* and *Wnt* pathway members which can influence hair diameter and thickness (Sharov et al. 2009; Enshell-Seijffers et al. 2010). Together these studies indicate that DNA methylation is not only important for cell survival, but can also influence tissue morphogenesis and function.

Developing prostate relies upon several developmental mechanisms regulated by DNA methylation in retina, skin and hair follicle. Cytoskeleton rearrangement, cell adhesion and epithelial polarity are all essential for prostate epithelial development and differentiation (Chambers et al. 2011). Signaling pathways including Hedgehog, Notch, Bmp and Wnt have all been linked to prostate epithelial budding, branching and differentiation (Vezina et al. 2008; Mehta et al. 2013; Omori et al. 2014). Whether DNA methylation regulates these pathways in prostate, as it does in retina and hair follicles, is an area of future study.

A future research opportunity: Testing whether mechanisms of establishing and erasing DNA methylation marks in embryogenesis are conserved in prostate development

Understanding how the DNA methylome is rapidly and extensively remodeled during embryonic development may inform future studies on whether aspects of this process occur during prostate development, especially in terms of understanding DNA demethylation. A rapid wave of paternal genome demethylation occurs shortly after fertilization in pre-implantation embryos. DNA methylation patterns are then re-established largely through *de novo* methylation to permit embryonic specification in the blastocyst (Monk et al. 1987; Santos et al. 2002). Rapid genomic reprogramming also occurs in primordial germ cells (PGCs) prior to and once reaching the embryonic gonad around 10-13 dpc in mouse (Surani 2007; Sasaki and Matsui 2008; Hemberger et al. 2009).

The identification of active mechanisms for removing DNA methylation marks filled a longstanding gap in understanding epigenetic programming. It was first thought that passive loss of DNA methylation or presence of a demethylase enzyme capable of removing methyl groups was the only mechanism to mediate demethylation. While some evidence points to methyl CpG binding domain 2 (*Mbd2*) as having independent demethylase activity (Bhattacharya et al. 1999) this is still controversial. In contrast, DNA demethylation has been shown to occur in a stepwise fashion, involving first modification of methylated cytosines and then replacement by DNA repair pathways. DNA hydroxymethylation is a cytosine modification catalyzed by ten eleven

translocation (TET) gene family members, which leads to base excision repair and demethylation (Tahiliani et al. 2009; Ito et al. 2010; Tan and Shi 2012). Deoxycytidine deamination occurs via the activation induced cytidine deaminase (*Aicda*, or *Aid*) and apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1–3 (*Apobec1*, 2 and 3) family members (Morgan et al. 2004; Bhutani et al. 2010). Deoxycytidine deamination generates DNA mismatches which trigger DNA base excision repair, activation of mismatch repair pathways (Modrich 2006; Popp et al. 2010; Franchini et al. 2012), or activation of DNA glycosylases which remove damaged bases or T:G and U:G mismatches (Cortellino et al. 2011). Active mechanisms of DNA demethylation provide for potentially rapid and context-specific addition and removal of DNA methylation marks during specific morphogenetic events.

Like the mRNAs of DNMT enzymes responsible for establishing DNA methylation, the mRNAs for *Tets*, *Aid*, *Apobec*s, DNA glycosylases, and other DNA repair enzymes, capable of removing DNA methylation, are expressed in male mouse UGS during prostate development (Keil et al. 2013). Many of the mRNAs encoding these enzymes are abundant in UGS mesenchyme prior to prostate bud formation before diminishing therein and localizing instead to prostate bud epithelium (Keil et al. 2013). A temporal change in localization and expression of the chromatin modifier, enhancer of zeste 2 (*Ezh2*) also occurs in developing prostate (Duan et al. 2013), suggesting that machinery for modifying histone structure, like DNA methylation machinery, is dynamically regulated during prostate development. These results raise the possibility that transient changes in DNA methylation and demethylation may

contribute to morphogenetic changes associated with AR activation and onset of prostate development.

A future research opportunity: Mechanisms involving DNA methylation underly fetal basis of adult disease

The fact that the epigenomic landscape is established during embryonic development also makes it a period of susceptibility to perturbations by environmental and endocrine disrupting chemicals. Chemically-induced epigenomic reprogramming during embryonic development can lead to changes in gene expression and altered histology and physiology later in life. This is one aspect of a fetal basis of adult disease (reviewed in (Barker 1994)). Two estrogenic chemicals receiving considerable research focus are diethylstilbesterol (DES) and bisphenol A (BPA). Some mouse and rat strains exposed *in utero* to DES and BPA exhibit altered developmental morphogenesis, reprogrammed DNA methylation patterns and altered adult histology/physiology of prostate and other organs.

The developing prostate is extremely sensitive to endocrine disrupting chemicals. CD-1 mice exposed *in utero* to estradiol, DES or BPA exhibit changes in the number of prostate buds formed and fetal UGS morphology (Timms et al. 2005). Mice exposed during fetal and neonatal development to high doses of DES (200ug/kg) generally develop fewer prostatic buds, while mice exposed to low doses of BPA (10ug/kg) and DES (0.1ug/kg) form more prostatic buds and exhibit increased UGS AR expression (vom Saal et al. 1997; Timms et al. 2005; Richter et al. 2007). How DES and BPA elicit their actions on prostate morphogenesis is not entirely known. Our results reveal DNA

methylation of AR as a regulator of prostatic bud development, this raises the hypothesis that these chemicals may disrupt AR DNA methylation to perturb prostate development.

This hypothesis is supported by further evidence that BPA-induced changes in DNA methylation alter gene expression to disrupt homeostatic regulation of prostatic growth later in life. In Sprague-Dawley rats, *in utero* BPA exposure increases incidence of carcinogen-induced hyperplastic and precancerous lesions in adult prostate (Ho et al. 2006). Male rats exposed *in utero* to BPA exhibit increases in prostatic *Dnmt3a* and *3b* expression at day 10 and 90 which diminishes by day 200 (Tang et al. 2012). This *in utero* BPA exposure induces hypo- or hypermethylation in a gene specific context in prostate. Some of these marks persist throughout life, while others are not observed until after sexual maturity or stimulation with pro-oncogenic factors in adulthood (Tang et al. 2012). Similarly, effects of perinatal testosterone exposure on DNA methylation is modest in sexually dimorphic forebrain regions during development but the number of genes with altered DNA methylation increases 20 fold in adulthood (Ghahramani et al. 2014). These studies provide evidence that early life exposure to endocrine disruptors, and potentially endogenous hormones, do not necessarily elicit immediately detectable changes in DNA methylation or gene expression. In some cases, these responses are not detectable until later in life-puberty or in response to a 'second hit' stimulus such as changes in hormone levels. These results have important implications in assessing potential mechanisms of hormone action and health risks associated with endocrine disrupting chemical exposures in that timing of assessment is potentially as important as doses tested and endpoints measured.

Collectively, in prostate and other organs, there are links between endocrine disruptor exposure and altered development, changes in DNA methylation and abnormal organ function and histology associated with disease later in life. These observations together with our results raise one potential mechanism, that *in utero* exposure to endocrine disrupting chemicals could influence prostate morphogenesis and homeostasis throughout life by altering DNA methylation during the embryonic period when DNA methylation guides prostate ductal development.

Future studies focused on a reawakening of developmental signals involving DNA methylation may lead to improved understanding of prostate cancer and BPH

Prostate Cancer

It has been proposed that a reawakening of developmental signals drives inappropriate growth in prostate cancer and BPH (Bierhoff et al. 1997). Since DNA methylation is critical for controlling expression of key prostate development genes (*Cdh1* and *Ar*), it is possible that aberrant DNA methylation patterns of these genes and others reawakens prostate growth mechanisms to drive onset and progression of BPH and prostate cancer. This notion is supported by multiple studies. Recently, a chromatin remodeling gene (high mobility group AT-hook 2, *Hmga2*) was identified as being selectively activated during prostate development (Zong et al. 2012). When *Hmga2* was overexpressed in adult prostate stromal cells, combined with prostate epithelium and grown as xenografts, the grafts formed high-grade prostate intraepithelial neoplasia (PIN) (Zong et al. 2012). PIN precursor lesions precede cancer formation in

predisposed mice and in humans (Lee et al. 2010). This study provides evidence that inappropriate expression of epigenetic modifiers in prostate stroma may be sufficient to alter epithelial proliferative activity. In another study, our group determined that during the course of embryonic mouse prostate development, *Dnmt* abundance declines in prostate stroma and restricts to a prostate epithelial cell subpopulation (Keil et al. 2013). DNMT protein abundance and activity are inappropriately elevated in human prostate cancer tissue and cell lines versus non-malignant controls (Patra et al. 2002; Gravina et al. 2013). DNMT protein expression also increases in prostate intraepithelial neoplasia and well-differentiated tumors in transgenic adenocarcinoma of mouse prostate (TRAMP) mice that are genetically predisposed to prostate cancer (Morey Kinney et al. 2008).

Androgen Receptor

DNA methylation of *Ar* occurs during prostate development to control onset and rate of androgen dependent prostate bud formation, confining initiation of prostate development to a specific embryonic development window (Keil 2014). There is evidence that this developmental role of *Ar* DNA methylation could also be hijacked to drive prostate cancer. DNA methylation silences *AR* gene expression in primary human prostate cancer tissues (Kinoshita et al. 2000; Yamanaka et al. 2003) as well as advanced (androgen-insensitive) prostate cancer cell lines (Jarrard et al. 1998). Chemical inhibition of *Ar* DNA methylation can restore *AR* expression and androgen responsiveness (Izbicka et al. 1999). Administration of DNA methylation inhibitor 5'-aza-2'deoxyctidine to TRAMP mice impairs progression to androgen independent prostate

cancer and when combined with castration increases survival (Zorn et al. 2007). These findings raise new hypotheses about the role of DNA methylation in progression of prostate cancer to an aggressive form that loses its growth dependence on androgens and can be lethal.

E-cadherin

DNA methylation of cell adhesion molecule, *Cdh1*, allows for prostate epithelial outgrowth during prostate development (Keil et al. 2014). Epigenetic regulation of *Cdh1* also influences epithelial cell migration and behavior in prostate cancer. *CDH1* is inappropriately silenced by DNA methylation in prostate cancer (Graff et al. 1995). Silencing of *CDH1* is associated with tumor invasion and metastasis and is predictive of poor patient outcome (Li et al. 2001). Treatment with DNA methylation inhibitor restores *Cdh1* expression in prostate cancer cell lines and decreases invasion potential (Kwon et al. 2010). Together these results suggest that DNA methylation is not only an important aspect of development but inappropriate DNA methylation activity is associated with prostate disease and progression.

Benign Prostate Hyperplasia (BPH)

It was first hypothesized by the pathologist John McNeal that the proliferative growth pattern in BPH nodules resembled developing prostate ducts undergoing branching morphogenesis (McNeal 1978). He hypothesized that inappropriate reawakening of prostate development signaling pathways could contribute to BPH pathogenesis (Cunha and Ricke 2011). Our findings that DNA methylation controls

developing prostatic growth raises the hypothesis that DNA methylation may also control aspects of inappropriate proliferative growth seen in BPH. Evidence now exists to support the hypothesis that epigenetic changes co-occur with histological prostate hyperplasia. Global 5-methylcytosine levels are reduced (Bedford and van Helden 1987) and localized DNA hypermethylation is observed in tumor suppressor genes (Henrique et al. 2005) in benign hyperplastic prostate compared to histologically normal prostate tissue.

Epigenetic abnormalities in the immune system may contribute to BPH associated with LUTS (symptomatic BPH). Inflammation is one of the major factors commonly observed in prostates from patients with BPH/LUTS. Men with chronic prostatic inflammation are 6.8 times more likely to have histological BPH than individuals without (Zlotta et al. 2014). Men with higher prostate inflammation also report more bothersome LUTS (Nickel et al. 2008). The cause of inflammation is not well understood and since not all incidences are accompanied by histological or culturable presence of bacteria (Nickel et al. 1999), multiple inflammatory mediators are likely involved. Recently a DNA demethylation pathway has been shown to be active in BPH. The innate anti-viral immune response is activated in prostate tissue from patients with BPH and LUTS (symptomatic) versus those with histologic BPH but few LUTS (asymptomatic) (Madigan et al. 2012). The cytosine deaminase, APOBEC3G, is upregulated in prostates from patients undergoing surgery for symptomatic BPH compared to patients with asymptomatic BPH or histologically normal prostate tissue (Madigan et al. 2012). Concurrently, demethylation and increased expression of LINE-1 retrotransposable elements occurs in BPH tissues from patients with symptomatic BPH

compared to histologically normal tissues (Madigan et al. 2012). LINE-1 demethylation activates the immune response in autoimmune disorders (Mavragani and Crow 2010). Together these results raise the hypothesis that *ApoBec* mediated demethylation of LINE-1 may occur in prostate to drive inflammation contributing to BPH and symptomatic LUTS.

Changes in the hormonal milieu have also been hypothesized as a driving factor in onset and progression of BPH and LUTS. As men age, testosterone levels decline while estrogen levels remain the same or even rise (Harman et al. 2001). Severe bladder outlet obstruction and increased prostate wet weight is observed in rodent models mimicking this hormonal change (Nicholson et al. 2012). Interplay between the epigenome and hormone action is also evident in BPH. Testosterone is converted to the more potent dihydrotestosterone (DHT) by steroid 5 alpha reductase, alpha polypeptide 2 (SRD5A2) within prostate, driving proliferative growth in development, BPH and prostate cancer. 5 alpha reductase inhibitors like finasteride are commonly used in men to alleviate symptoms of BPH with varying success. Currently it is not understood why some men respond to these therapies better than others. *SRD5A2* has CpG regions and DNA methylation has been shown to regulate its expression in prostate (Niu et al. 2011). Interestingly, men exhibit varying levels of *SRD5A2* expression in prostate (Niu et al. 2011). The possibility that men with highly methylated *SRD5A2* may be more resistant to 5 alpha reductase inhibitor therapy has been raised (Bechis et al. 2014). What regulates *SRD5A2* DNA methylation and why it is only seen in some men experiencing BPH is unknown but a promising field of study for designing personalized therapies.

Future studies are needed to divulge interaction between diet and DNA methylation on upper and lower urinary tract function

One of the intriguing aspects of DNA methylation is that it is a malleable event. Environmental factors, genetics, maternal nutrition and diet can all impact the epigenome throughout life. One of the major dietary influences is folic acid. Folic acid acts as a methyl donor in catalyzing DNA methylation events. For this reason it is recommended that women of childbearing age or are pregnant take 0.4-4mg/day to prevent neural tube closure defects (Barua et al. 2014). However, the other effects of folic acid on DNA methylation and fetal development are not clearly understood. In 1998 the United States became the first country to require folic acid fortification in cereal grains, since that time serum folic acid levels have risen ~2.5 fold in the US population (Pfeiffer et al. 2012). Folic acid is also included in one-a-day vitamins and occurs naturally in some foods. Over 40% of American men over age 60 take dietary supplements containing folic acid, which are likely responsible for the fact that over 34% of American men over age 60 exhibit biomarkers consistent with folic acid excess (Bailey et al. 2010). What this means in the context of aging and disease progression is not well understood. Thus there are future research opportunities to determine developmental and adulthood implications of folic acid excess and what this does to DNA methylation, prostate growth and urinary function.

My work has just begun to determine effects of a folic acid enriched diet on prostate development. While it appears prostate bud number is unaltered by maternal folic acid enriched diet (chapter 8), whether DNA methylation changes occur and

whether alterations in DNA methylation affect prostate growth, histology, disease or urinary function later in life is a future area of study.

To parallel developmental studies my work has also just begun to determine effects of folic acid excess in adulthood and the potential impact on prostate growth and urinary function. My data indicate that folic acid enriched diet in utero throughout adulthood provide beneficial outcomes on voiding function in mice challenged with T+E2 (chapter 8). Whether these actions occur through changes in DNA methylation and what genes and signaling pathways are affected is a future area of study. This line of research has potential clinical implications. It is possible that folic acid may be used as a therapeutic intervention in some cases of LUTS.

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