Chromatin Remodeling Proteins ARID4A and ARID4B Regulate Tight Junction Protein Claudin-3 Expression

by Ming Jiang

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Thesis directed by

Ray-Chang Wu
Assistant Professor of Biochemistry and Molecular Biology

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Abstract of Thesis

"Chromatin Remodeling Proteins ARID4A and ARID4B Regulate Tight Junction Protein Claudin-3 Expression"

Claudins are a family of proteins that are the most important components of the tight junctions. Claudin3 (Cldn3), a member of Claudin family, is a component of the newly formed tight junction. Functionally, tight junctions between the somatic Sertoli cells form the basis of the blood-testes barrier that is important for nourishing the developing sperm cell throughout the stage of spermatogenesis and testes development. Cldn3 has also been reported to be expressed in the tight junctions within the nephric duct and ureteric bud, and promotes tubule formation in kidney development. Moreover, Cldn3 was reported to be overexpressed in ovarian cancer cell. It is believed that Cldn3 overexpression in ovarian cancer enhances tumor cell motility, invasiveness and survival. In this thesis, we describe a novel regulatory mechanism governing Cldn3 expression. By chromatin remodeling protein Arid4a and Arid4b.

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List of Abbreviation

Cldn3	Claudin-3
Arid4a	AT rich interactive domain 4A
Arid4b	AT rich interactive domain 4B
PWS-IC	Prader-Willi syndrome cis-acting imprinting center
AS	Angelman syndrome
HEK293T	Human embryonic kidney 293T cell line
RT-PCR	Reverse Transcription Polymerase Chain Reaction
QRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RB	Retinoblastoma protein
TFIID	Transcription factor II D
AR	Androgen receptor
UB	Ureteric bud
MM	Metanephric mesenchyme
RCE	RB control elements

Introduction

ARID4A and ARID4B are members of the Retinoblastoma remodeling complex.

The human ARID4A and ARID4B gene, also known as retinoblastoma-binding protein 1(RBBP1, RBP1) and RBBP1-like protein 1(RBBP1L1), respectively, are components of the retinoblastoma protein (RB) remodeling complex (Jullien et al., 2008). These two proteins share 74% amino acid identity sequence within their AT rich interactive (ARID) domains and 40 to 50% identity across the full length of each protein. Structurally, while both proteins contain a Tudor domain and a chromodomain, ARID4A and ARID4B contain an LXCXE (pRB-binding) motif that is responsible for interaction with RB. A major target of RB is the E2F family transcription factors, which control the expression of many genes that are required for DNA synthesis and cell cycle progression (DeGregori et al., 1995). However, RB also contains a domain known as RB pocket that can bind to other proteins. The pocket domain of RB family members were shown to repress E2F-dependent transcription by recruiting HDAC1, HDAC2, HDAC3 (Lai et al., 2001). Also, RBP1/ARID4A can bind to the RB pocket directly by its conserved LXCXE motifs and recruit Class1 HDACs to suppress transcription. In addition, RBBP1/ARID4B can bind to RBP1 and be recruited as a part of large protein complex. Figure 1 shows the RB remodeling model.

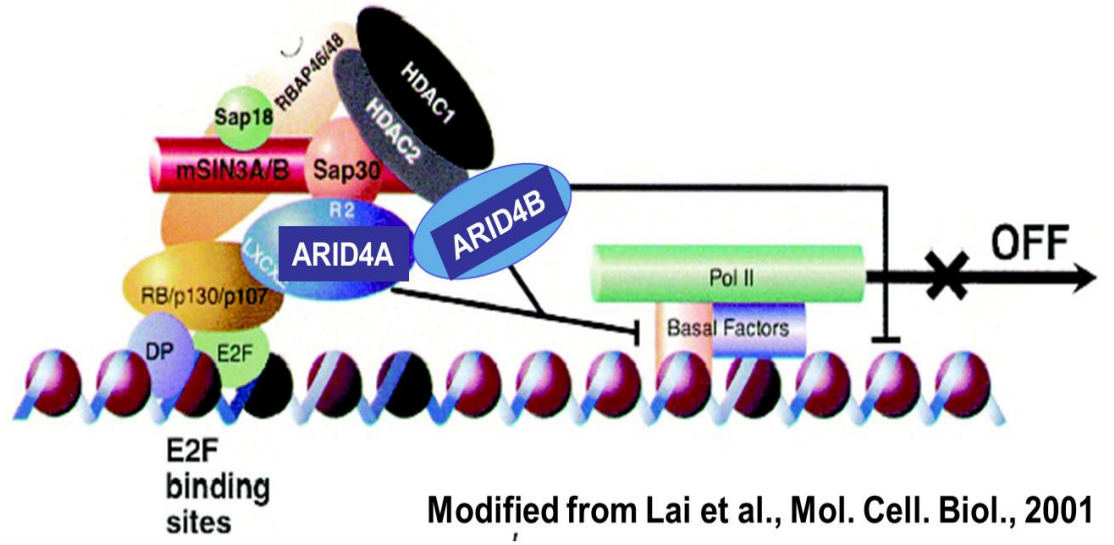

Figure1. Model of transcription repression by pRB-E2F complex. During quiescence and the early G1 phase of the cell cycle, recruitment of the mSIN3-HDAC complex through the pocket-dependent association of ARID4A and ARID4B repressed gene transcription..

Arid4A and Arid4B knockout mouse model

Arid4a null (Arid4a$^{-/-}$) mice and Arid4b null (Arid4b$^{-/-}$) mice have been generated and the phenotypes have been reported (Wu et al., 2008). Mice with homozygous deficiency for Rbbp1/Arid4a and heterozygous deficiency for Rbbp1l1/Arid4b (Arid4a$^{+/-}$ Arid4b$^{+/-}$) showed a reduced tri-methylation of histone H4K20 and H3K9 and reduced DNA methylation at the Prader-Willi syndrome cis-acting imprinting center (PWS-IC). Importantly, mutations of Rbbp1/Arid4a, Rbbp1l1/Arid4b, suppressed an Angelman syndrome (AS) imprinting defect caused by a mutation at

the AS-IC. Also, Arid4a and Arid4b are ahown to be leukemia suppressor genes through control of epigenetic modifications (Wu et al., 2008).

In addition, we found that Arid4A$^{-/-}$ Arid4B$^{+/-}$ mice manifested an age-related reduced fertility phenotype. Specifically, 10% of mutant mice are born infertile and by the end of their reproductive period, more than 80% of male mutant mice are infertile. Moreover, the male mice displayed morphological defects in their urogenital system, For example, some of the mutant mice have reduced size of testes compared to wild type mice. We also observe absence seminal vesicle or abnormal seminal vesicle in some of mutant mice.

These phenotypes suggest that Arid4a andArid4b may play an important role in the development and function of male reproductive system.

Androgen receptor (AR) in male reproduction

The androgen receptor (AR) is a member of nuclear receptor superfamily and a ligand-activated transcription factor. AR can be activated by binding of the androgenic hormones testosterone or dihydrotestosterone in the cytoplasm and then translocate into the nucleus (Roy et al., 1999). The androgen receptor regulates expression of genes that are critical for the development and maintenance of male sexual phenotype (Heinlein and Chang, 2002). In the testis, Sertoli cells are critical for spermatogenesis, and the function of l Sertoli cells depends on AR (Zhou et al., 2011).

Cldn3 is the potential target gene of Arid4a and Arid4b

To better understand how Arid4a and Arid4b regulate male fertility, a microarray analysis was performed using in the testes fron Arid4a$^{-/-}$Arid4b$^{+/-}$ mice to identify potential genes important for testis development and function (data not shown). Since the male infertility phenotype from our rid4a and Arid4b knockout mouse is very similar to that found in AR knockout and RB knockout mouse, we compared our array results to that performed on Sertoli cells derived from the AR and RB knockout mice (Denolet et al., 2006) (Nalam et al., 2009). We found Cldn3 is the common one that decreased in all three microarrays.

CLDN3 is important for testes and kidney development

Cldn3 is a member of the claudin protein family and a component of the tight junction strands. Tight junctions form continuous seals around cells and serve as a physical barrier to prevent solutes and water from passing freely through the paracellular space (Kniesel and Wolburg, 2000). In testes, Sertoli cell is located in seminiferous tubule and its main function is to nourish the developing sperm cell through the stage of spermatogenesis (Gary et al., 2007). Tight junction in Sertoli cell form blood-testis barrier, which is the interstitial compartment to partition the testis from the adluminal compartment of the seminiferous tubules, and is important for sperm maturity (Meng et al., 2005). Since Cldn3 is a component of the tight junction, it suggests that Cldn3 protein is important for the function of Sertoli cells.

Mammals's kidney originates from two primordial tissues that are nephric duct and nephrogenic mesenchyme. After these two tissues have elongated to the level of the hindlimbs, the ureteric bud (UB) emerges from the nephric duct and forms a mutual signaling network with metanephric mesenchyme (MM). Then UB will elongate and form tubules and eventually bifurcate repeatedly to form the adult collecting duct system.

In addition to its role in testis and spermatogenesis, CLDN3 was shown to be important for kidney development (Haddad et al., 2011). In the adult mouse kidney, Cldn3 is expressed on the apical surfaces of epithelial cell within the ascending limb of Henle, the distal nephron, and the collecting ducts. Cldn3 in these compartments acts as a barrier-forming protein that seals the paracellular pathway. This study showed that Cldn3 is expressed in tight junctions within the nephric duct and UB and over-expression of Cldn3 can lead to increasing tubule formation in kidney. .

The aim of this study is to confirm Arid4a and Arid4b regulate Cldn3 expression in testes and kidney and to understand how expression of Cldn3 is regulated by Arid4a and Arid4b.

Materials and Methods

Cell culture

TM4 cell line (a mouse testicular embryonic Sertoli cell line) and HEK393T cell line (human embryonic kidney 293T cell line) were maintained in DMEM supplemented with 5% Fetal Bovine Serum. Cells were reseeded every 2 days.

Mouse model and care

The *Arid4a* null (*Arid4a*$^{-/-}$) mice, mice heterozygous for the *Arid4b* deletion (*Arid4b*$^{+/-}$), and mice null for *Arid4a* and heterozygous for the *Arid4b* deletion (*Arid4a-/-Arid4b+/-*) have been described previously (Wu et al., 2006). These mutant mice are maintained on a hybrid C57BL6/J and 129/SvEv genetic background.

The mice (Wild type mice, Arid4A $^{-/-}$ mice, Arid 4B$^{-/-}$ mice and Arid4a$^{-/-}$Arid4b$^{+/-}$ mice) were all bred and maintained according to a protocol (protocol number: A208) approved by the George Washington University Institutional Animal Care and Use Committee. The animal research facility is approved by the American Association for Accreditation of laboratory Animal Care and is operated in accordance with current regulations and standards of US departments of Agriculture and of Health and Human Services.

Pups are weaned 20days after birth. . NTES buffer was used to lyse the tails overnight at 50°C. The lysate was used to do PCR to determine the genotype of new born pups.

Mice were anesthetized with isoflurane and then killed by cervical dislocation. The mice were dissected, and organs such as kidney and testes were removed and stored in liquid nitrogen.

Western blot analysis

Mouse testes were collected from WT and Arid4a-/-Arid4b+/- mouse when they are two months old. At least three mice from each genotype are used in each experiments. Testes samples were homogenized and lysed in NP-40/SDS buffer {1% Nonidet P-40, 0.01% SDS, 0.1 M Tris-HCl, pH 7.2, and complete Protease Inhibitor Cocktail Tablet (Roche Applied Sciences, Indianapolis IN). Sixty micrograms of mouse testes protein were used for electrophoresis on 10% Tris-Cl ready gels (Bio-Rad, Hercules CA). The proteins were transferred to nitrocellulose membrane (Bio-Rad). The membranes were then incubated with the appropriate primary antibodies. The membranes were then incubated with either goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) or donkey anti-goat HRP (ac-2004 or ac-2020, respectively, Santa Cruz Biotechnology). The signals were detected by enhanced chemiluminesence method (ECL, GE healthcare). The levels of Cldn3 were normalized against the levels of actin in each sample.

qRT-PCR

Testes and kidney of WT and Arid4a$^{-/-}$Arid4b$^{+/-}$ mice were collected at two months of age. Total RNA was purified from mouse whole testes dissected from pups at two months of age using an RNeasy plus kit (Qiagen, Hilden, Germany). Five μg of DNase I-treated total RNA was used for reverse transcription to synthesize the first strand cDNA by the Superscript III First-strand synthesis system (Invitrogen, Carlsbad, CA). Quantitative RT-PCR analysis was performed using LightCycler Fast-Start DNA Master SYBR Green I (Roche) on the ABI StepOne Plus machine using TaqMan Gene expression assays (Applies Biosystems). PCR primer sequences are list as follows, Human: ARID4A-F GTTGCAACCATAGACAGGAGGAG, ARID4A-R CACCTGCATTCTACAGCAAGTAC; ARID4B-F GGAGGAGGAGAGGAATATAATACC, ARID4B-R CTTTTTCCTGATCAGAATGTGTAGG; CLDN3-F CGCGAGAAGAAGTACACGGCC, CLDN3-R CCCTTAGACGTAGTCCTTGCGG;

Mouse: Gapdh-F CTCCACTCACGGCAAATTCA, Gapdh-R ATGGGCTTCCCGTTGATGA. Gapdh transcripts were amplified as controls for gene expression. For quantification experiments, we used at least 3 of mice from every genotype. The levels of gene expression were normalized against the levels of an endogenous control in each sample. In each set of experiments, the normalized level of gene expression from the wild-type mouse was always set as 1.

Luciferase Assay

TM4 and 293T cells are plated into 24-well plate. Trasfection was performed according to manufacturers' instruction. For RNAi knockdown experiment,

Lipofectamine RNAiMAX (Invitrogen) was used. Luciferase reporter plasmids containing different length of cldn3 promoter region, including cldn3-Luc-pro2 1.2Kb, cldn3-Luc-pro2 1.0Kb, cldn3-Luc-pro2 0.8Kb and cldn3-Luc-pro2 0.2Kb are used . For protein expression, pCMV-Tag-hRBBP1, pCDNA3.1-mRbbp111, androgen receptor or Retinoblastoma protein were transfected into cells using Lipofectamine (Invitrogen). On the day of plasmid DNA transfection, cells were grown up to 70% confluence in 24 well tissue culture plate. Cells are lysed 48h after transfection. For combined siRNA and plasmid DNA transfection, cells were transfected first with siRNA followed by transfection by plasmid DNA. Seventy-two hours later, cells are collected. Cells were treated with Passive lysis buffer (Promega) and incubated at room temperature with constant shaking. The cell lysate was collected and 30 ul of the supernatant was mixed with 50 ul of reconstituted luciferase assay substrate (Promega) and measured immediately using Luminometer (Lumat LB 9507, Berthold Technologies).

Results

Decreased expression of CLDN3 in the Arid4$^{-/-}$Arid4B$^{+/-}$ mouse testes

To determine whether Arid4A, Arid4B can regulate Cldn3 expression in Arid4a$^{-/-}$ Arid4b$^{+/-}$ mouse testes, qRT-PCR and Western blot were conducted to measure the mRNA levels and protein levels of in the Arid4$^{-/-}$Arid4B$^{+/-}$ mouse testes. Testes from the wild type and Arid4$^{-/-}$Arid4B$^{+/-}$ mouse testes were collected and used to compare the Cldn3 expression.

In Figure 2A, qRT-PCR results showed that the levels of Cldn3 mRNA were modestly and significantly reduced in Arid4a$^{-/-}$ and Arida4$^{-/-}$Arid4b$^{+/-}$ mouse testes, respectively, compared to that from the wild type mouse. In agreement with reduced Cldn3 mRNA levels, Western blot results in Figure4 B showed that the Cldn3 protein levels from the testes of Arid4a$^{-/-}$Arid4b$^{+/-}$ mouse were markedly decreased compared to that from the wild type mouse. Together, these results showed that deletion of Arid4a and Arid4b leads to reduced expression of Cldn3 mRNA and protein, suggesting that Arid4a and Arid4b may regulate Cldn3 expression in mouse testes.

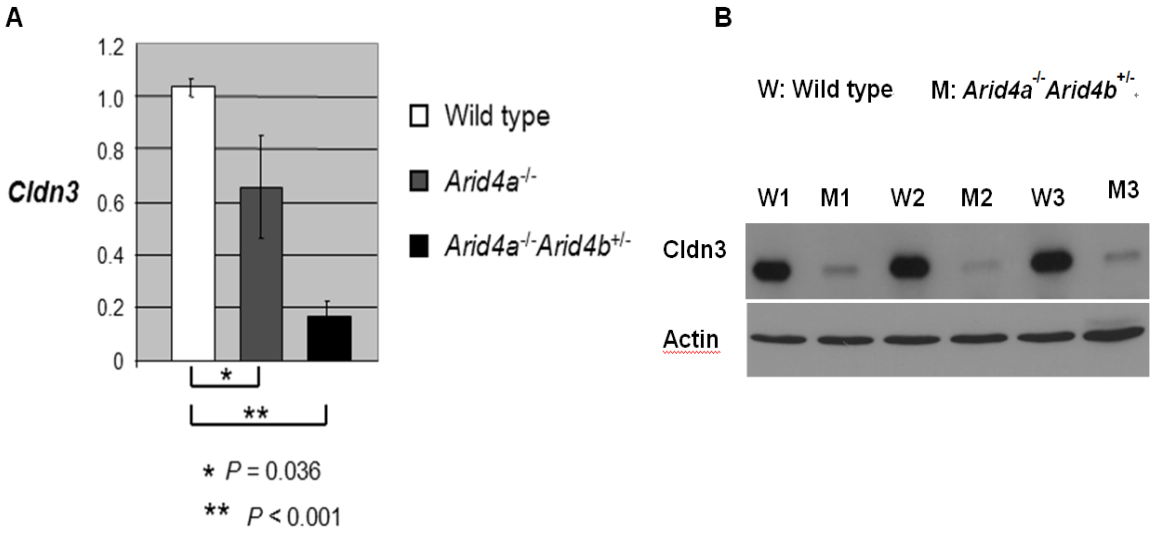

Figure2. (A) Comparison of Cldn3 mRNA levels in the *Arid4a*$^{-/-}$ and *Arid4a*$^{-/-}$ *Arid4b*$^{+/-}$ mouse testes by qRT-PCR analysis. The results showed the reduced level of *Cldn3* mRNA expression from the *Arid4a*$^{-/-}$, and the Arid4a$^{-/-}$Arid4b$^{+/-}$ mice compared to the wild type mice. Three mice from each genotype were used. The statistical analysis was performed by Excel. A p value of less than 0.05 is considered significant. The levels Cldn3 mRNA from the wild-type mice were set as 1. (B) Western blotting analysis was performed to compare Cldn3 protein levels from the testes of Arid4a$^{-/-}$Arid4b$^{+/-}$ and wild type mice. Three sets mouse were analyzed: W1, M1; W2, M2; W3, M3. (W refers to wild type; M refers to Arid4a$^{-/-}$Arid4b$^{+/-}$). The levels of actin were used as a loading control.

Arid4a and Arid4b co-activate with AR on the CLDN3 promoter in mouse Sertoli cell

Similar to deletion of Arid4a and Arid4b, deletion of androgen receptor (AR) was previously shown to reduce Cldn3 expression in testes. It thus raises an interesting

possibility that Arid4a and Arid4b may function together with AR to regulate Cldn3 expression. To test this possibility, we first determined whether Arid4a and Arid4b interact with AR by a co-immunoprecipitation (Co-IP) assay. Indeed, our results showed that Arid4a and Arid4b can interact with AR an AR ligand independent manner (data not shown here). Next, we determined whether Arid4a, Arid4b or AR can activate the cldn3 promoter in mouse Sertoli cell using a luciferase reporter assay. Our results showed that transfection of AR alone activated the Cldn3 promoter in a ligand-dependent manner (Figure 3A). Transfection of either Arid4a or Arid4b alone with AR modestly whereas co-transfection of Arid4a and Arid4b significantly enhanced the activation of Cldn3 promoter by AR(Figure 3A). On the other hand, although knockdown of Arid4a or Arid4b alone only slightly reduced the activation of Cldn3 promoter by AR (likely a result of functional redundancy between Arid4a and Arid4b), knockdown both Arid4a and Arid4b significantly inhibited the Cldn3 promoter activity (Figure 3B).

Importantly, knockdown of Arid4a and Arid4b also effects the expression of endogenous *Cldn3*. Results in Figure 3C showed that knockdown Arid4a or Arid4b alone resulted in a modest decrease of endogenous Cldn3 expression, and combined knockdown of Arid4a and Arid4b further decreased Cldn3 expression. Knockdown of Arid4a and Arid4b in this experiment was confirmed by qRT-PCR (Figures 3D and 3E). These results showed that AR activates the *CLDN3* promoter, and Arid4a and Arid4b may co-activate with AR and further increase the *CLDN3* promoter activity in mouse Sertoli cell.

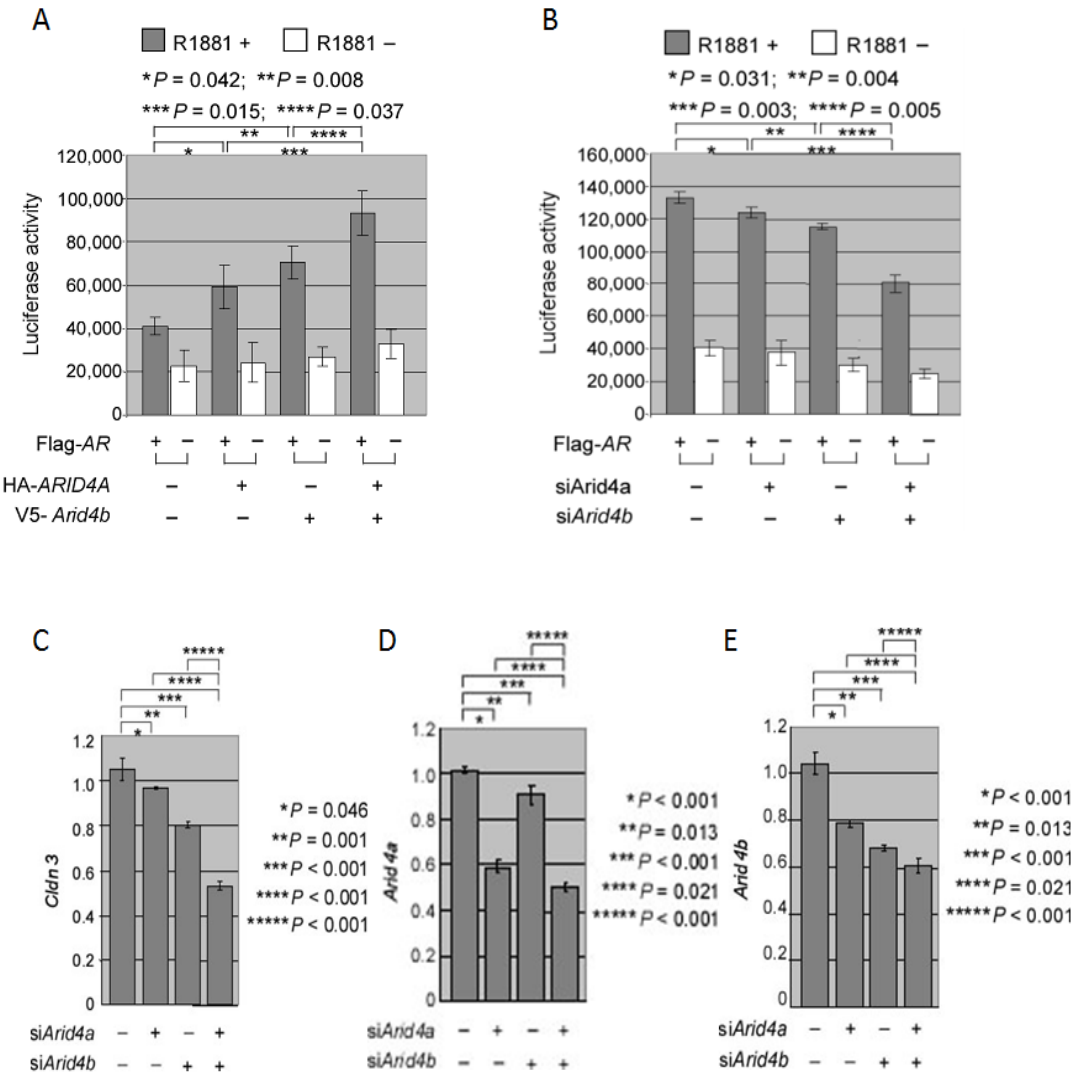

Figure3. Arid4a and Arid4b co-activate with AR on *Cldn3* promoter. (A) Mouse Sertoli (TM4) cells were transfected with *Cldn3* promoter-regulated luciferase reporter combined with expression vectors for Flag-*AR*, HA-*ARID4A*, or V5-*Arid4b* as indicated. The transfected cells were treated with vehicle or R1881 for 24 h. The luciferase activity is determined as described in Materials and Methods. (B) TM4 cells that were transfected with siRNAs against Arid4a, Arid4b or both for 24 h were further co-transfected with *Cldn3* promoter-regulated luciferase reporter and expression vectors for AR for additional 24 h. The luciferase activity was determined as described above. (C-E) qRT-PCR analysis confirmed decreased

expression endogenous *Cldn3* (C) after knockdown of *Arid4a* (D) and *Arid4b* (E). The statistical analysis was performed by Excel. A p value of less than 0.05 is considered significant.

Arid4a and Arid4b co-activate with RB on the CLDN3 promoter in mouse Sertoli cell.

Arid4a and Arid4b, also known as Retinoblastoma-binding protein 1 and Retinoblastoma-binding protein-like 1, respectively, have been shown to interact with RB protein (Jullien et al., 2008). Therefore, it is also possible that Arid4a and Ardi4b may function together with RB to regulate Cldn3 expression. To confirm whether this is indeed the case, a luciferase reporter assay was performed. We transfected the TM4 cells with Cldn3 luciferase reporter gene driven by the Cldn3 promoter together with expression vectors for RB, Arid4a or Arid4b. Our results showed that transfection of RB alone induced Cldn3 promoter. Transfection of Arid4a or Arid4b alone modestly enhanced the induction of RB on CLDN3 promoter. Co-transfection of Arid4a and Arid4b with RB significantly increased the activation of Cldn3 promoter by RB. These results indicated that RB can induce the CLDN3 promoter activity, and Arid4a and Arid4b can co-activate with RB on CLDN3 promoter activity.

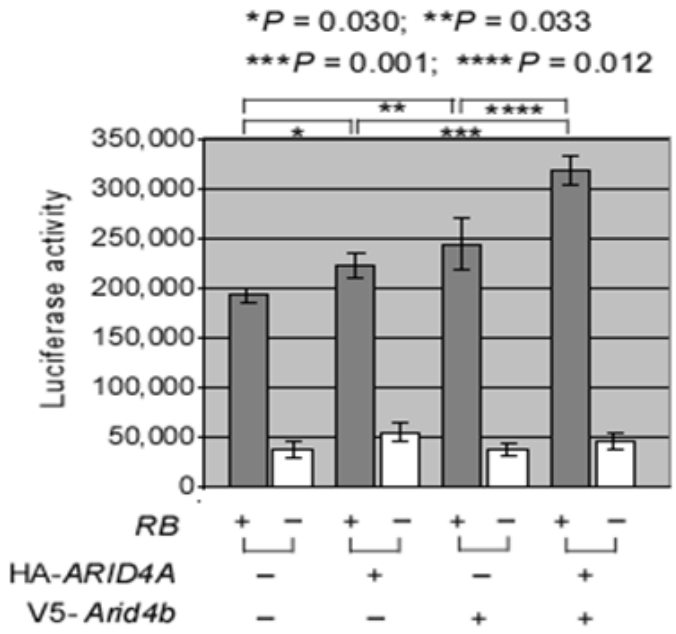

Figure4. Arid4a and Arid4b co-activate with AR on Cldn3 promoter activity. TM4 cells were transfected with *Cldn3* promoter-regulated luciferase reporter combined with expression vectors for RB, HA-*ARID4A*, or V5-*Arid4b* as indicated. The luciferase activity was determined as described. The statistical analysis was performed by Excel. A p value of less than 0.05 is considered significant.

Identify the regions on the CLDN3 promoter responsible for activation by AR and RB

To identify the region(s) on the Cldn3 promoter critical for induction by AR and RB, we generated a series of reporter plasmids containing various lengths of the 5' Cldn3 promoter region. We first used these constructs to identify which region of the Cldn3 promoter is critical for the induction by AR. These constructs were each individually transfected with AR into TM4 cells and then the luciferase activity was

determined. As shown in Figure 5A, we found that the promoter region from -972 to +129 bp can be activated by AR. Deletion of regions from -741 to –972 bp and from -518 to -972 bp did not affect the activation by AR. On the other hand, deletion of -65 to -517 bp significantly reduced but did not abolish the activation by AR. These results suggest that sequences between -65 and -517 bp are important for activation by AR.

Next, we identified which region on the promoter is important for activation by RB using a similar approach by transfecting these constructs individually with RB into TM4 cells and determined the luciferase activity. Our results showed that RB activated the promoter region from -972 to +129. Deletion of -741 to -972 bp and -65 to -517 bp but not deletion of -518 to -740 bp reduced the activation by RB. The results suggest sequences from -972 to 741 bp and from -517bp to -65bp are important for the activation by RB.

It should be pointed out that PROMO3.0 predicts seven putative AR binding sites (http://alggen.lsi.upc.es/cgibin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) and several so-called RB responsive elements (Banchio et al., 2007). were located from -972 to +129 bp on the Cldn3 promoter area (Figures 5C and 5D). Study to pinpoint exactly which AR binding site or RCE is required for the activation by AR and RB, respectively, is required.

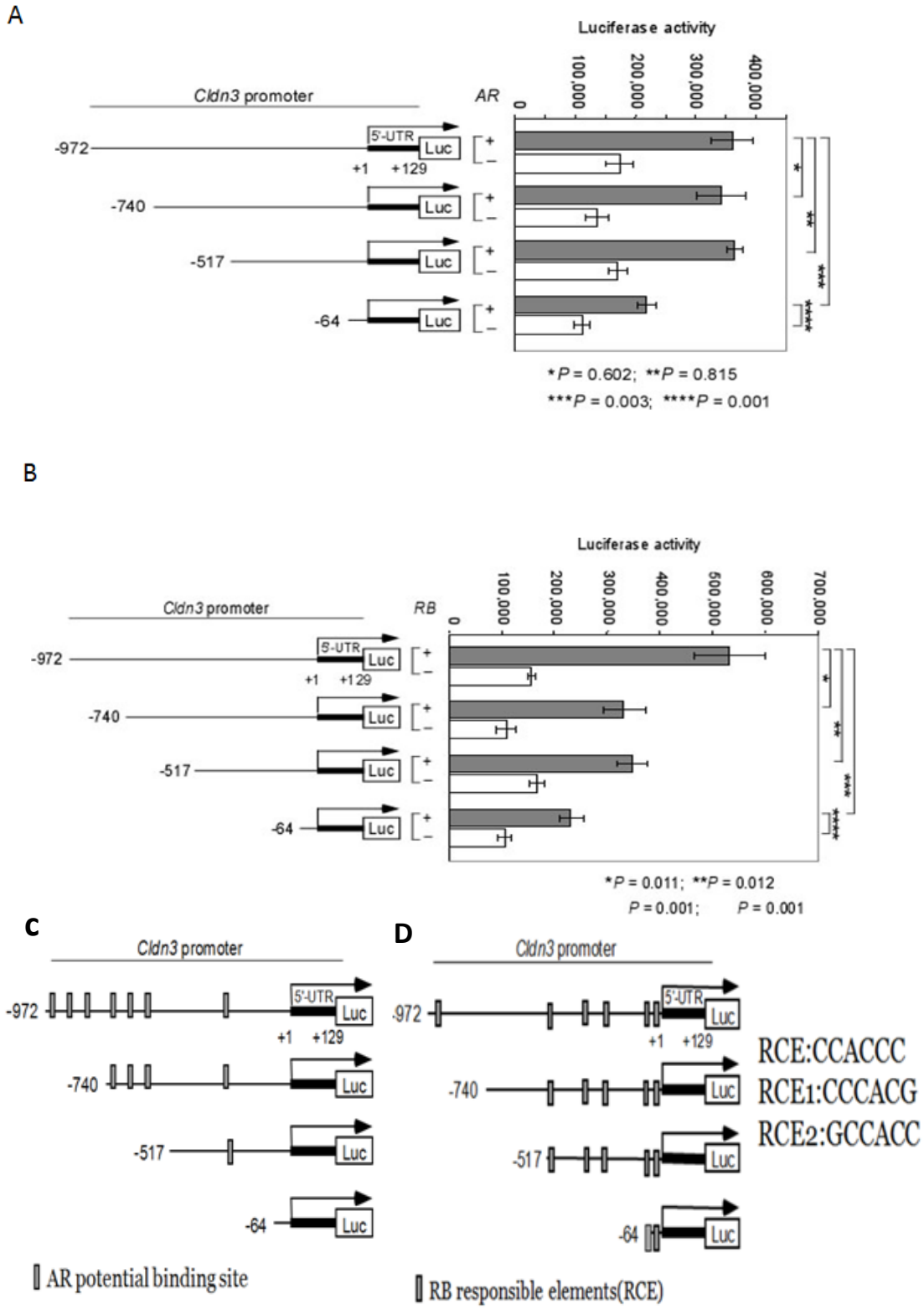

Figure5. Identify the regions of the Cldn3 promoter responsible for activation by AR and RB. (A) The luciferase activity from TM4 cells co-transfected with AR and

various 5' truncated *Cldn3* promoter-regulated luciferase reporters was determined and compared to identify the region important for the activation by AR. (B) The luciferase activity from TM4 cells co-transfected with RB and various 5' truncated *Cldn3* promoter-regulated luciferase reporters was determined and compared to identify the region important for the activation by RB. (C) Putative AR binding site(s) on the Cldn3 promoter predicted by PROMO3.0. (D) Putative RB responsible element(s) on the Cldn3 promoter.

CLDN3 level decrease in Arid4a$^{-/-}$Arid4b$^{+/-}$ mouse kidney.

Cldn3 is expressed in tight junctions within the nephric duct and Ureteric bud during critical stages of kidney development in the mouse (Haddad et al., 2011). Since we have shown that expression of Cldn3 is regulated by Arid4a and Arid4b (Figure 3) and deletion of Arid4a and Arid4b reduced Cldn3 expression in the testes (Figure 2), it is possible that Cldn3 may involve in kidney development. Therefore, we examined whether Arid4a$^{-/-}$ Arid4b$^{+/-}$ mouse exhibited kidney defect.

Interestingly, we found that some but not all the Arid4a$^{-/-}$Arid4b$^{+/-}$ mouse contain only one kidney (Figure 6A). Although we have shown that Arid4a$^{-/-}$ Arid4b$^{+/-}$ mouse expressed decreased levels of cldn3 in the testes, qRT-PCR assay was performed to verify whether the cldn3 levels also decrease in Arid4a$^{-/-}$Arid4b$^{+/-}$ mouse kidney. The qRT-PCR results showed that expression of Cldn3 was reduced in Arid4a$^{-/-}$ Arid4b$^{+/-}$ mouse kidney. These results suggest that Cldn3 may be involved in kidney development.

Figure6. Decreased Cldn3 expression in Arid4a$^{-/-}$ Arid4b$^{+/-}$ mouse kidney. (A) A comparison of the urogenital system between the wild type and Arid4a$^{-/-}$ Arid4b$^{+/-}$ mouse.(B) qRT-PCR revealed reduced Cldn3 expression in the *Arid4a*$^{-/-}$*Arid4b*$^{+/-}$ kidney. Shown are results from at least three mice from each genotype. The levels from the wild-type mice were set as 1.

Arid4A and Arid4b promote CLDN3 expression in HEK293T cell

Next, we investigated whether Arid4a and Arid4b regulate Cldn3 expression in a normal human embryonic kidney HEK293T cells. As shown in Figure 7A, over-expression of Arid4a or Arid4b alone increased the mRNA levels of endogenous Cldn3 in HEK293T cells as measured by qRT-PCR. Over-expression of both Arid4a and Arid4b further increased the Cldn3 expression. Over-expression of Arid4a and Arid4b was also confirmed by qRT-PCR in the same experiment (Figure 7B-C).

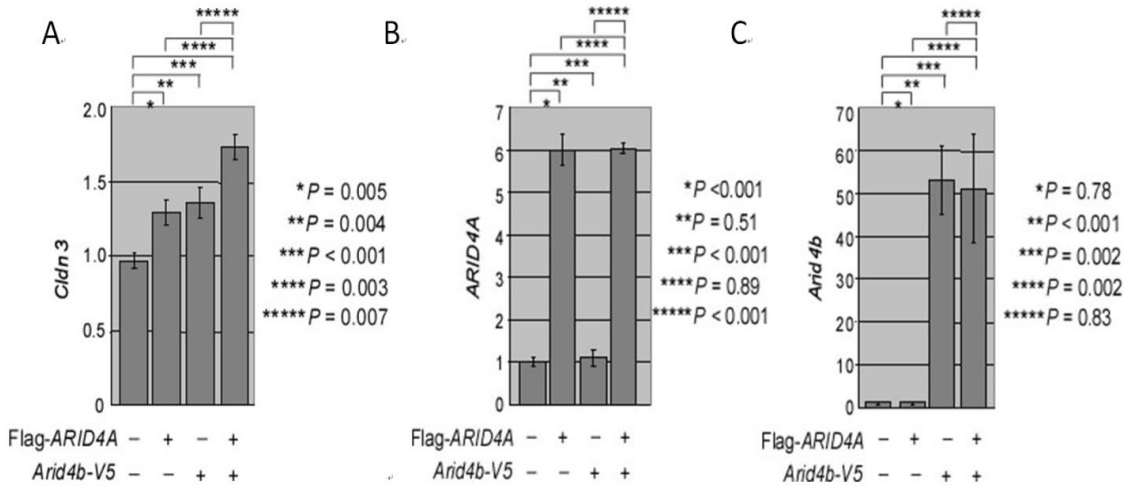

Figure 7. Arid4a and Arid4b promote *Cldn3* expression in HEK 293T cell. (A) After transfection of Flag-*ARID4A* and *Arid4b*-V5 expression vectors into HEK293T cells, qRT-PCR analysis confirmed increased expression of endogenous *Cldn3*. (B) Also shown is the confirmation of ARID4A (B) and Arid4b (C) over-expression from the experiments described in (A). Shown are means ± SD from three experiments performed in triplicate. The mean of gene expression from the control was set as 1.

Discussion

In this thesis, I described regulation of a tight junction protein, Cldn3, by two related chromatin remodeling proteins, Arid4a and Arid4b, in testes and kidney. Cldn3 is a very important protein that forms the tight-junction in testes and helps the formation of nephric duct and ureteric bud during critical stages of kidney development. The major findings of this work are the following. First or all, we found that Arid4a and Arid4b regulate male reproductive function, and this function may require expression of a tight junction gene Cldn3 as deletion of Arid4a and Arid4b reduced Cldn3 expression in mouse testes. Accordingly, we showed that Arid4a and Arid4b co-activated with AR and RB and up-regulated Cldn3 expression in mouse embryonic Sertoli cells. Secondly, our results also suggest that Arid4a and Arid4b may be involved in kidney development in mouse and activate Cldn3 expression in human embryonic kidney HEK293 cells. Compared to wild type mouse, $Arid4a^{-/-}Arid4b^{+/-}$ male mouse showed a gradual decrease in fertility as they aged with only 20% of $Arid4a^{-/-}Arid4b^{+/-}$ male mouse remains fertile. Morphologically, the infertile male mice have smaller testes compared to the wild type mice. To better understand how deletion of Arid4a and Arid4b regulated male infertility, we performed microarray analysis to identify downstream molecular targets of Arid4a and Arid4b. Our array results identified Cldn3 as one of the potential targets of Arid4a and Arid4b from the $Arid4a^{-/-}Arid4b^{+/-}$ male mouse testes. Cldn3 is a tight junction protein that form the blood-testes barrier and plays an important role in testes development and spermatogenesis (McCabe et al., 2012). Reduced Cldn3 expression resulted in increased permeability across the blood-testes barrier and

affect cytoskeletal development of testes (Meng et al., 2011) (Willems et al., 2010). In addition, Cldn3 disruption could lead to epididymal dysfunction, which is responsible for post-testicular sperm maturation (Dube et al., 2010). More importantly, the phenotypes in the reproductive system from the Cldn3 defective mouse are very similar that found in our Arid4a$^{-/-}$Arid4b$^{+/-}$ male mouse (Willems et al., 2010)

This work also provides a better understanding on how expression of Cldn3 is regulated at the molecular levels. Previously, it has been shown that expression of Cldn3 is regulated by androgen receptor (AR) (Willems et al., 2010). AR is essential for male reproductive system development (Merlet et al., 2007). AR also regulates the permeability of blood-testes barrier by regulating the expression of Cldn3 (Meng et al., 2005). Deletion of AR resulted in delayed and incomplete formation of the Sertoli cells barrier and changes the expression of several genes involved in cell adhesion and cytoskeletal development (Willems et al., 2010). In mouse AR Sertoli cell knockout Microarray analysis using Sertoli cells derived from AR knockout mouse identified Cldn3 as one the genes whose expression is down-regulated (Denolet et al., 2006). Since knockout of AR and knockout of Arid4a and Arid4b shared similar testis defects in mouse, we investigated whether Arid4a and Arid4B may cooperate with AR to regulate Cldn3 expression. As a first step, we determined whether Arid4a and Arid4b can interact with AR since neither Arid4a nor Arid4b binds to DNA directly. In fact, our Co-IP results showed that Arid4a and Arid4b can be co-immunoprecipitated with AR. To demonstrate this interaction is functionally important, we performed report gene assay. Our results further showed that AR can

activate the Cldn3 promoter and over-expression of Arid4a, Arid4b alone or combined further enhanced the ability of AR to activate the Cldn3 promoter. On the other hand, suppression of Arid4a or Arid4b expression by siRNAs inhibited activation of the Cldn3 promoter by AR. To identify which region of the Cldn3 promoter is important for the activation by AR, we generated a series of Cldn3 promoter deletion mutants. These mutants enabled us to narrow down the region important for AR activation to -65 to -574 bp. Our results showed that deletion of region between -65 to -574 bp which contains one of the predicted AR binding sites reduced the activation by AR. However, it should also be pointed out that AR still activated the promoter which did not contain the predicted AR binding sites despite at a lesser extent (compare -64 to +129 bp to -517 to +129 bp). Although these results suggest that AR binding site located between -65 and -574 bp may be important for activation by AR, it is also possible that AR can be recruited to Cldn3 promoter by tethering to other transcription factors that bind to the region between -64 to +129 bp and activates CLDN3 promoter. More study is needed to investigate these possibilities.

In addition to the interaction with AR shown in current study, Arid4a and Arid4B are known to associate with RB and form a multi-protein complex (Lai et al., 2001). RB is a tumor suppressor whose function is lost in several major cancers (Murphree and Benedict, 1984). One function of RB is to prevent the cell from replicating damaged DNA by preventing its progression along the cell cycle from G1 into S phase (Das et al., 2005). Our data also showed that RB can significantly induce the Cldn3 promoter activity and the ability of RB to activate the Cldn3 promoter is

further activated by Arid4a and Arid4b. Our results are also consistent with results from microarray analysis using Sertoli cells derived from RB knockout mouse where Cldn3 expression was shown to be reduced (Nalam et al., 2009). Sequence analysis identified several possible RB control element (RCE) on the Cldn3 promoter. Although our preliminary results indicated that the RCEs may be important for the activation of Cldn3 promoter by RB, further investigation is needed to confirm their importance.

Cldn3 is required for formation of tight junction which is the contact points between epithelial cells in the ureteric bud. Ureteric bud form tubules and branch, that is critical for kidney development. A recent report showed that Cldn3 was expressed in the nephric duct and ureteric bud (Haddad et al., 2011) and over-expression of Cldn3 in mouse inner medullary collecting duct cell resulted in significant increase in tubule formation. Since Arid4a$^{-/-}$Arid4b$^{+/-}$ male mouse exhibited defect in kidney development and showed reduced Cldn3 expression in mouse kidney, together these results suggest that Cldn3 may be involved in kidney development. In summary, in this thesis we presented evidence to support that ablation of chromatin remodeling proteins Arid4a and Arid4b resulted in important defects, including male infertility and abnormal kidney development. We identified Cldn3 which encodes a tight junction protein as a potential target gene of Arid4a and Arid4b. We hypothesize that Arid4a and Arid4b regulate Cldn3 expression through recruitment by AR and RB and expression of Cldn3 plays an important role maintaining male fertility and kidney development.

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