THE ROLE OF HISTONE H3K79 METHYLTRANSFERASE DOT1L IN RENAL DEVELOPMENT, INJURY AND REPAIR

AN ABSTRACT

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FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

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ABSTRACT

Histone modifications are critical effectors of gene expression thereby regulating cell lineage, identity and cell fate. Dot1l, as the sole histone methyltransferase specific for Histone 3 lysine 79, plays an essential role in embryonic development. This study examines the role of H3K79 methylation in renal development, injury and repair. We demonstrate that Dot1l and H3K79 methylation are developmentally up-regulated and are highly enriched in chromatin of differentiated epithelial cells of the ureteric bud (UB) lineage, which gives rise to the renal collecting system. Surprisingly, conditional deletion of Dot1l in the UB lineage ($UB^{Dot1l-/-}$) did not produce an abnormal phenotype, with preservation of UB branching morphogenesis and renal medullary development. However, embryonic $UB^{Dot1l-/-}$ kidneys exhibit expansion of the progenitor p63+ UB-tip cell domain and ectopic expression of p63 in UB stalks. Later in development, collecting ducts lacking Dot1l/H3K79me3 exhibit a significantly higher number of dividing ($P\text{-}H3^{+}$) cells, a lower intercalated cell/collecting duct ratio, and decreased expression of the intercalated cell master regulator, Foxi1. Our results indicate that chromatin-based mechanisms mediated via Dot1l/H3K79 methylation control the terminal differentiation fate of progenitor UB-tip cells. We propose a model whereby the absence of Dot1l disrupts the balance between proliferation and differentiation of UB-tip cells (p63 vs Foxi1), which alters the fate of collecting duct precursor cell types. Our data also suggest that in addition

to its role in terminal epithelial differentiation, Dot1l may play a role in regeneration following renal ischemia-reperfusion (I/R) injury or unilateral ureteral obstruction (UUO). IR and UUO provoke a marked reduction in tubular epithelial cell Dot1l and H3K79me3 at 24-72h, while regeneration at 7d correlates with re-expression of Dot1-H3K79methylation. Moreover, $UB^{Dot1l-/-}$ mice show a more severe injury and fibrosis when subjected to UUO, suggesting a role of Dot1l-H3K79methylation in renal injury and repair.

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TO ALL THE PEOPLE WHO CARE ABOUT ME

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CHAPTER 1: INTRODUCTION

1. Overview of epigenetics regulation

1.1 Epigenetics

Gene regulation has been traditionally attributed to transcription factor binding to DNA sequences and recruitment of the RNA polymerases to gene promoters. However, since each cell has identical genetic material but different cell fate during development, there must be some other regulatory mechanisms affecting the cell fate in tissue-specific gene regulation. Chromatin-based mechanisms acting via through histone modifications and DNA methylations (Dambacher et al. 2010) cooperate with transcription factors in the regulation of gene expression.

Epigenetics was initially described as “the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being” by Conrad Waddington (Waddington 1942). Nowadays, epigenetics has evolved to be illustrated as inheritable changes which occur in gene expression or cellular phenotype without alterations in the DNA sequence. This inheritability can be passed through the germ line or in somatic cells (Dressler 2008). The epigenetic code can be divided into two mechanisms including DNA methylation and histone modifications (Figure 1.1) (Qiu 2006).

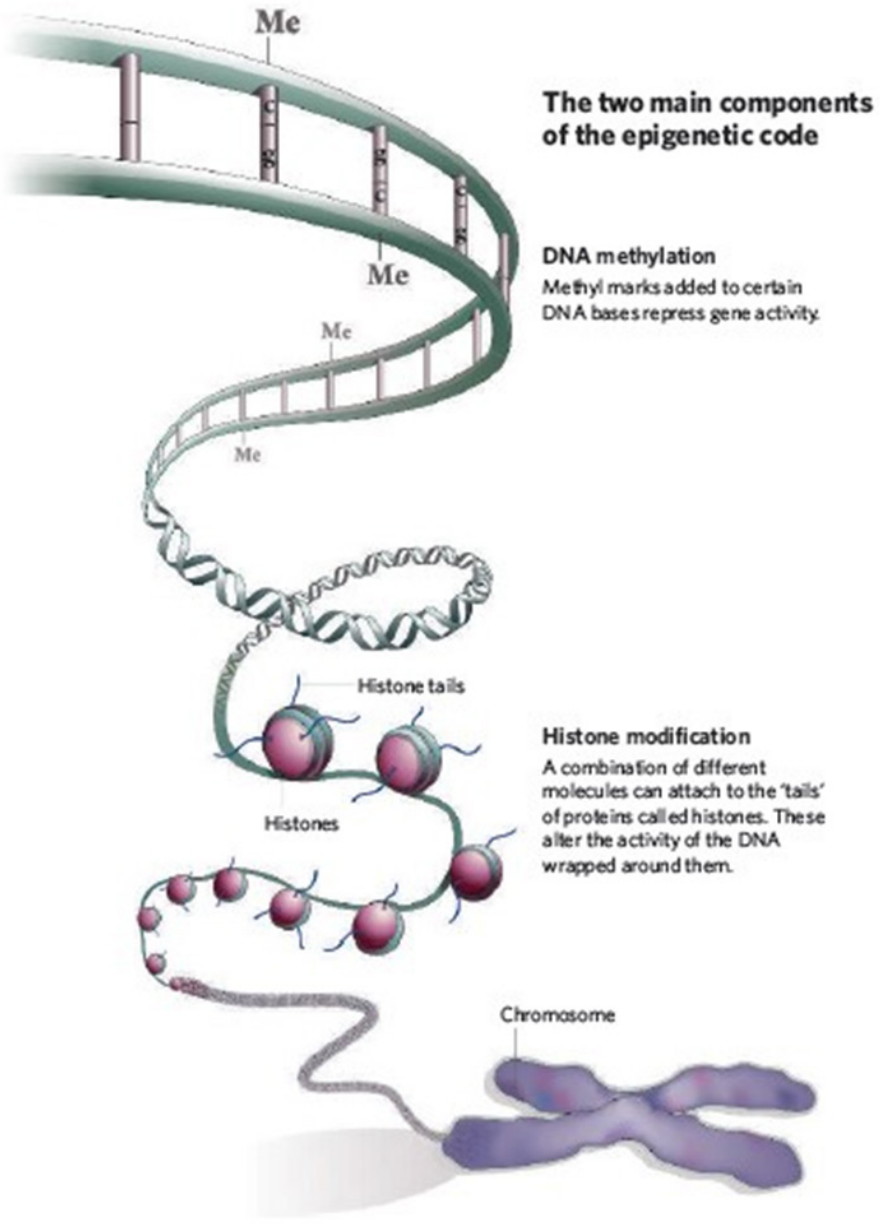

Figure 1.1. Epigenetic code includes DNA methylation and Histone modification.

DNA methylation represses gene activity by adding methylation marks to CpG bases. Histone modification, on the other hand, alters the affinity of histone-DNA interaction as well as hitone-histone interaction. The histone code can either silence gene expression through recruiting effector proteins at heterochromain or activate gene expression through recruiting effector proteins at euchromatin (Qiu 2006).

Adapted from figures by Qiu 2006.

1.1.1 Histones

The basic unit of chromatin, the nucleosome, consists of an octamer of core histones (two H2A/H2B dimers and H3/H4 heterotetramer) wrapped around by 147bp DNA helix(Kornberg et al. 1999). A spacer region bound by histone H1 or H5 serves to separate the adjacent nucleosomes (Dressler 2008). In addition to the central globular domain, histones are also comprised of unstructured N-terminal and C-terminal tails which protrud from the nucleosome. The protruding tails of histones are the major sites subjected to extensive posttranslational modifications including methylation, ubiquitination, phosphorylation, acetylation, ribosylation and sumoylation (Figure 1.2)(Strahl et al. 2000).

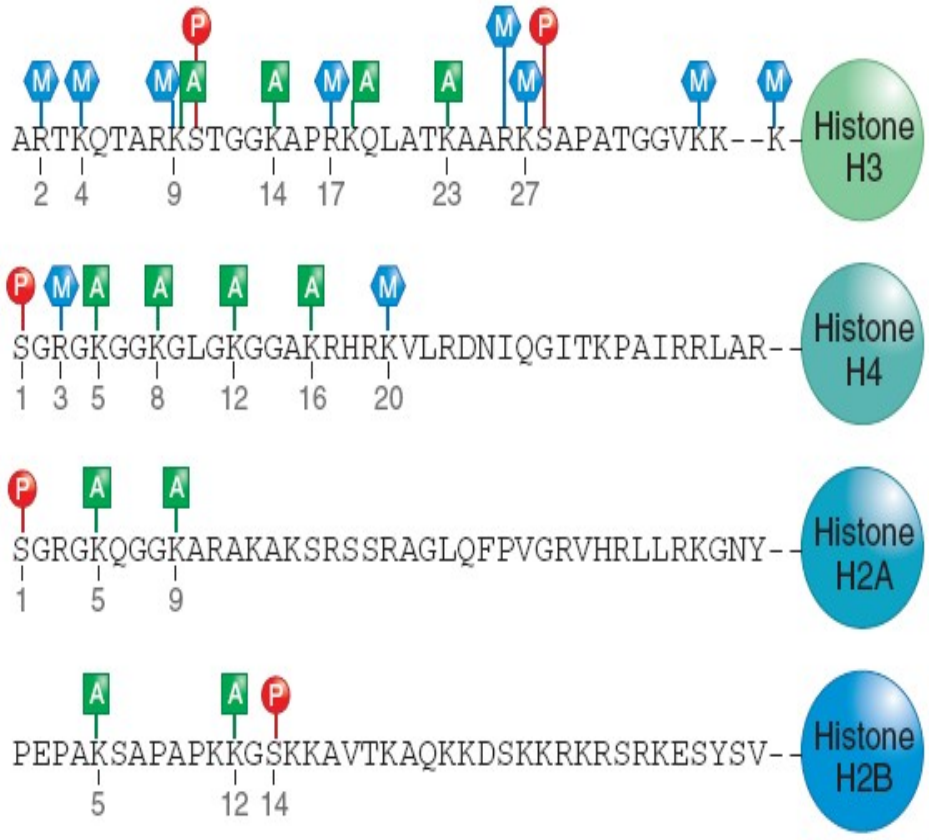

Figure 1.2. Summary of histone modifications.

The residues subjected to histone modifications are delineated. M represents methylation on arginines (R) or lysines （K） while acetylation represented by M usually takes place on lysine residues and serine residues (S). Phosphorylation (P) can happen on serine residues of histone 2, histone 3 and histone 4.

Adapted from Dressler 2008.

1.1.2 Histone acetylation

Acetylation and methylation of histone tails are generally considered the most important modifications which remodel the chromatin and are responsible for cell

lineage commitment, transcriptional regulation and development (Dressler 2008). Histone H3 lysine 9, 14, 18, 23 are the major acetylation sites (Thorne et al. 1990). Acetylations on the tails of Histone 2A, 2B, 3, and 4 induce transcription activation through binding with bromodomain-containing transcription factors. The histone acetyltransferases (HATs) and histone deacetylase (HDACs) are responsible for maintaining a steady-state reversible balance of the modifications with HATs adding acetyl groups to lysine residues and HDACs, on the other hand, removing those acetyl groups (Strahl et al. 2000, Selvi et al. 2010). HDACs are classified into four main groups as following:

Class 1: HDAC1, HDAC2, HADC3 and HDAC8 with a homology to yeast RPD3

Class 2a: HDAC4, HDAC5, HDAC7, HDAC9 with two catalytic sites

Class 2b: HDAC6 with two catalytic domains and HDAC10

Class 3: sirtuins with a homology to yeast Sir2

Class 4: HDAC11 with a homology deacetylase domain to class I and II HDAC

Histone hyperacetylation generally correlates with active gene transcription, whereas histone deacetylation correlates with repression. However, this is only a general rule and there are many examples where HDACs are associated with active trajnscription. HDACs are ubiquitously expressed in all tissues with a significant variation in different tissue types. The critical role of HDACs and their inhibitors are also recognized in organogenesis, especially clarified in the development of mouse kidney and intestines where HDACs regulate the molecular signaling program, including gene expression and cell proliferation/survival in development (Chen et al.

2011).

1.1.3 Histone methylation

Histone methylation generally occurs on lysine/arginine of histones H3 and H4 and is catalyzed by arginine methyltransferases (PRMTs) and lysine methyltransferases (KMTs). Most lysine methylation sites are located on histone tails such as H3K4, H3K9, H3K27, H3K36 and H4K20 while H3K79methylation distinctly occurs in the nucleosome core region of Histone H3 (Dambacher et al. 2010). Figure 1.3 illustrates the various sites of methylation and their corresponding methyltransferase/demethylase (Martin et al. 2005). Unlike histone acetylation, histone methylation is generally related with either transcription activation or repression or even both depending on the sites of methylation. Each residues can be mono, di, and tri-methylated which will correspondingly lead to different functional outcomes.

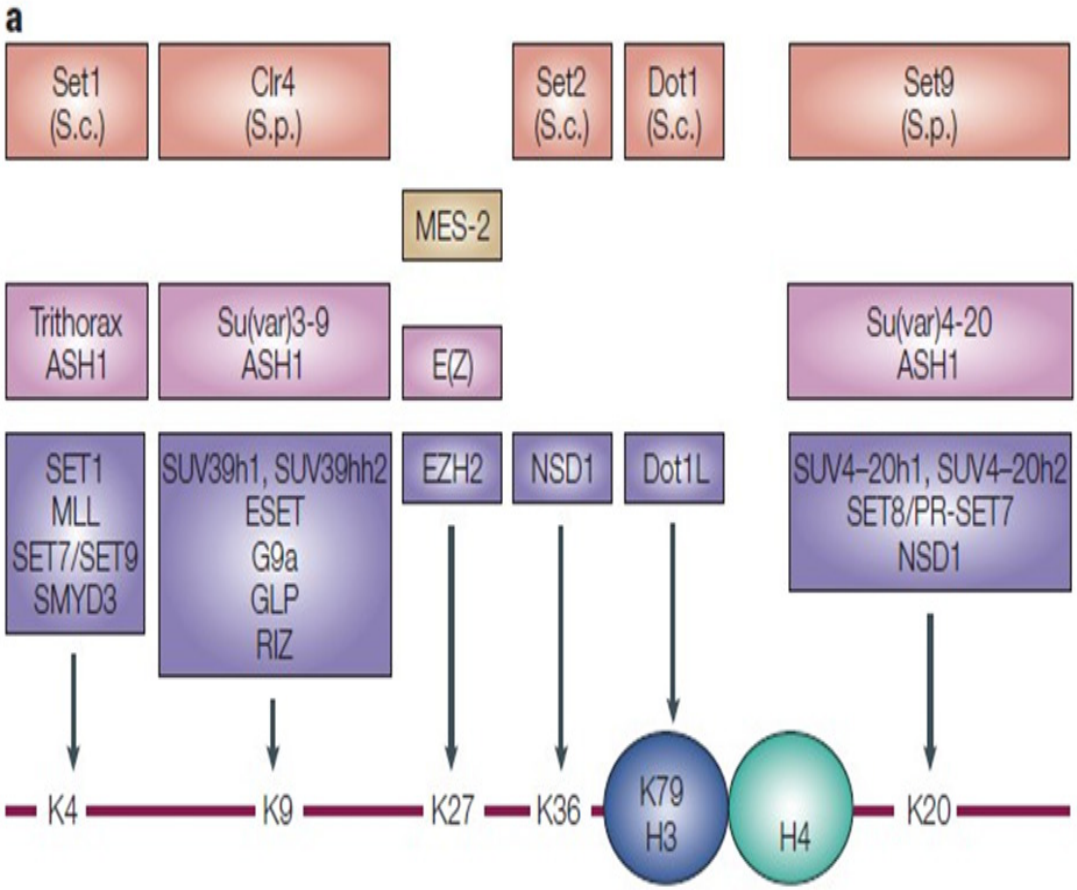

Figure 1.3. Schematic figure of methylations and their catalyzing enzymes on H3 and H4.

Methyltransferases were marked according to the specific lysine residue for methylations. Colors were coded according to their origin as red for yeast, pink for fly and purple for mammalian. Specific methyltransferase and demethylase work together for maintaining the balance state of methylation on each lysine. Dot1l acts as the sole methyltransferase for H3K79 and no demethylase has been found yet.

Adapted from figures by Martin, C 2005.

Functional studies have divided the histone methylations into two groups with one group (H3K4, H3R17, H3K36, H3K79 and H4R3) correlating with transcriptional activation, and the other group (H3R2, H3K9, H3K27, H4K20)

implicated in gene repression (Dambacher et al. 2010). Such kind of transcriptional regulation mainly occurs by recruiting effector proteins. The transcription activation marks, such as H3K4methylation, can recruit activating proteins and histone acetyltransferases (Ingvarsdottir et al. 2007). Transcription repression mark H3K9 methylation, on the other hand, recruits HP1 (chromodomain protein heterochromatin protein 1) to initiate the heterochromatin formation (Eskeland et al. 2007).

Most lysine methyltransferases catalyze the transfer of methyl group from S-Adenosyl Methionine (SAM) to lysine residues and harbor a specific 130-amino-acid SET (suppressors of variegation enhancers of zeste and Tristae) domain with the exception of Dot1l (disruptor of telomere silencing), the sole methyltransferase for H3K79, which contain Non-SET domain (Selvi et al. 2010). Histone methyltransferases have been shown to be involved in extensive cell biological processes. For example, EZH2, the histone 3 lysine 27 methyltransferase, is reported to regulate allogeneic T cell responses mediating graft-versus-host disease (He et al. 2013) and malignant peripheral nerve sheath tumorigenesis (Zhang et al. 2013). Our lab has recently shown that EZH2-mediated H3K27me3 influences the fate of metanephric mesenchymal cells in renal development (McLaughlin et al. 2013). The H3K79 methyltransferase Dot1l plays critical roles in DNA damage, cell cycle, embryonic development, leukemogenesis and stem cell reprogramming (Jones et al. 2008, Barry et al. 2009, FitzGerald et al. 2011, Nguyen et al. 2011, Onder et al. 2012).

For a long time, histone methylation has been considered an irreversible and stable post-translational modification until the discovery of histone demethylases. The

first identified demethylase is LSD1 (Lysine specific demethylase 1) which was discovered in 2004 (Shi et al. 2004). LSD1 acts on the mono- and di- methylation sites of both H3K4 and H3K9 methylation in a context dependent manner (Selvi et al. 2010). Several other demethylases have been identified since then with most of them having jumonji domains. The discovery of histone demethylases has changed the old definition of histone methylation into a reversible and dynamic process regulated by histone methyltransferases and demethylases.

Table 1 | **The two histone demethylase families**

Name	Synonyms	Protein structure*	Histone substrates‡	Non-histone substrates‡
LSD demethylases				
LSD1	AOF2, BHC110, KDM1A	SWIRM, Amine oxidase, Spacer region	H3K4me1, H3K4me2, H3K9me1, H3K9me2	p53, E2F1, DNMT1
LSD2	AOF1, KDM1B	CW	H3K4me1, H3K4me2	
JMJC demethylases				
JMJD7		JMJC		
HIF1AN				
HSPBAP1				
JMJD5	KDM8		H3K36me2	
JMJD4				
JMJD6	PSR, PTDSR		H3R2, H4R3	
JMJD8				
FBXL10	JHDM1B, KDM2B	CXXC, PHD, FBOX, LRR	H3K36me1, H3K36me2, H3K4me3	
FBXL11	JHDM1A, KDM2A		H3K36me1, H3K36me2	p65, NF-κB
KIAA1718	JHDM1D		H3K9me1, H3K9me2, H3K27me1, H3K27me2	
PHF8	JHDM1F		H3K9me1, H3K9me2, H4K20me1	
PHF2	JHDM1E		H3K9me2	ARID5B
HR				
KDM3B				
JMJD1A	JHDM2A, TSGA, KDM3A		H3K9me1, H3K9me2	
JMJD1C				
JMJD3	KDM6B		H3K27me2, H3K27me3	
UTX	KDM6A	TPR	H3K27me2, H3k27me3	
UTY				
JMJD2A	JHDM3A, KDM4A	JMJN, TUDOR	H3K9me2, H3K9me3, H3K36me2, H3K36me3, H1.4K26me2, H1.4K26me3	
JMJD2C	JHDM3C, GASC1, KDM4C		H3K9me2, H3K9me3, H3K36me2, H3K36me3, H1.4K26me2, H1.4K26me3	
JMJD2B	JHDM3B, KDM4B		H3K9me2, H3K9me3, H3K36me2, H3K36me3, H1.4K26me2, H1.4K26me3	
JMJD2D	JHDM3D, KDM4D		H3K9me2, H3K9me3, H3K36me2, H3K36me3, H1.4K26me2, H1.4K26me3	
JARID1B	PLU1, KDM5B	ARID, C5HC2	H3K4me2, H3K4me3	
JARID1C	SMCX, KDM5C		H3K4me2, H3K4me3	
JARID1D	SMCY, KDM5D		H3K4me2, H3K4me3	
JARID1A	RBP2, KDM5A		H3K4me2, H3K4me3	
JARID2				
MINA				
NO66			H3K4me2, H3K4me3, H3K36me2, H3K36me3	

ARID, AT-rich interacting domain; amine oxidase, amine oxidase domain; C5HC2, C5HC2 zinc-finger domain; CW, CW-type zinc-finger domain; CXXC, CXXC zinc-finger domain; DNMT1, DNA methyltransferase 1; FBOX, F-box domain; FBXL, F-box and Leu-rich repeat protein; HIF1AN, hypoxia-inducible factor 1A inhibitor; HR, hairless domain; HSPBAP1, heat shock protein-associated protein 1; JARID, Jumonji domain–ARID-containing protein; JMJC, Jumonji C domain; LRR, Leu-rich repeat domain; LSD, Lys-specific demethylase; MINA, MYC induced nuclear antigen; NF-κB, nuclear factor-κB; NO66, nucleolar protein 66; PHD, plant homeodomain; SWIRM, Swi3p, Rsc8p and Moira domain; TPR, tetratricopeptide domain; TUDOR, Tudor domain; UTX, ubiquitously transcribed X chromosome tetratricopeptide repeat protein; UTY, ubiquitously transcribed Y chromosome tetratricopeptide repeat protein. *Grouped according to levels of homology. ‡Substrate specificity for residues of histone tails and non-histone targets are indicated for all proteins with demonstrated demethylase activity.

Table 1.1: There are two families of histone demethylases: LSD demethylases and JMJC demethylases divided by different reaction mechanisms (Kooistra et al. 2012). Adapted from table by Kooistra and Helin 2003

There are two major demethylases family as described in Table 1.1. LSD1 and LSD2 are the major demethylases responsible for mono and dimethylation on

H3K4and H3K9 while JMJC family demethylate other lysine methylations, including mono-, di-, and trimethylation (Kooistra et al. 2012). LSD family demethylases mainly utilize the FAD (flavin adenine dinucleotide)-dependent amine oxidation reaction. On the other hand, Fe(II) and α-ketoglutarate dependent dioxygenase reaction is the responsible mechanism for JMJC family demethylases regulated demethylation (Kooistra et al. 2012). Enzymes which demethylate H4K20methylation and H3K79methylation have not been described yet.

1.1.4 Epigenetics and development

Epigenetic mechanisms have long been known to participate in cancer and development (Baldewijns et al. 2008, Dressler 2008, Maher 2013). Differentiation and development require a stable inherited genomic organization to preserve the cellular memory through the epigenetic machinery (Dressler 2008). In embryonic development, epigenetics is not only just inherited via the germline but also in somatic cells. This type of epigenetic regulation plays a critical role in the self-renewal process of stem cells and the lineage commitment in embryonic development (Shen et al. 2013). There are two groups of proteins exerting essential role in this epigenetic regulatory mechanism, including Trithorax group (TrxG) and polycomb group (PcG) proteins. TrxG proteins are concerned with gene activation, such as the activation of Hox genes, through H3K4 trimethylation. On the other hand, PcG proteins are mostly linked to gene silencing by trimethylating histone 3 lysine27 (H3K27). Thus TrxG and PcG oppose each other in the regulation of gene

transcription in development and cancer (Grimaud et al. 2006). Furthermore, in early embryonic development, TrxG proteins play a critical role in X-chromosome inactivation (Pullirsch et al. 2010). Not surprisingly, aberrant expression of these two groups of proteins is commonly observed in cancer.

1.2 Dot1l-H3K79methylation

Unlike most histone H3 modifications which occur on residues on the N-terminal tail of histone, H3K79 is located in the globular domain on the nucleosome surface. Dot1l, the sole methyltransferase of H3K79 methylation, does not have a SET domain (as other histone methyltransferase) but contains four conserved sequence motifs (I, post I, II, and III) of class I SAM-dependent methyltransferases. The crystal structure of yeast and human Dot1 has also revealed an alpha-helical N-terminal domain, an active SAM binding site, a lysine binding site and a central open alpha/beta structure (Min et al. 2003) (Figure 1.4).

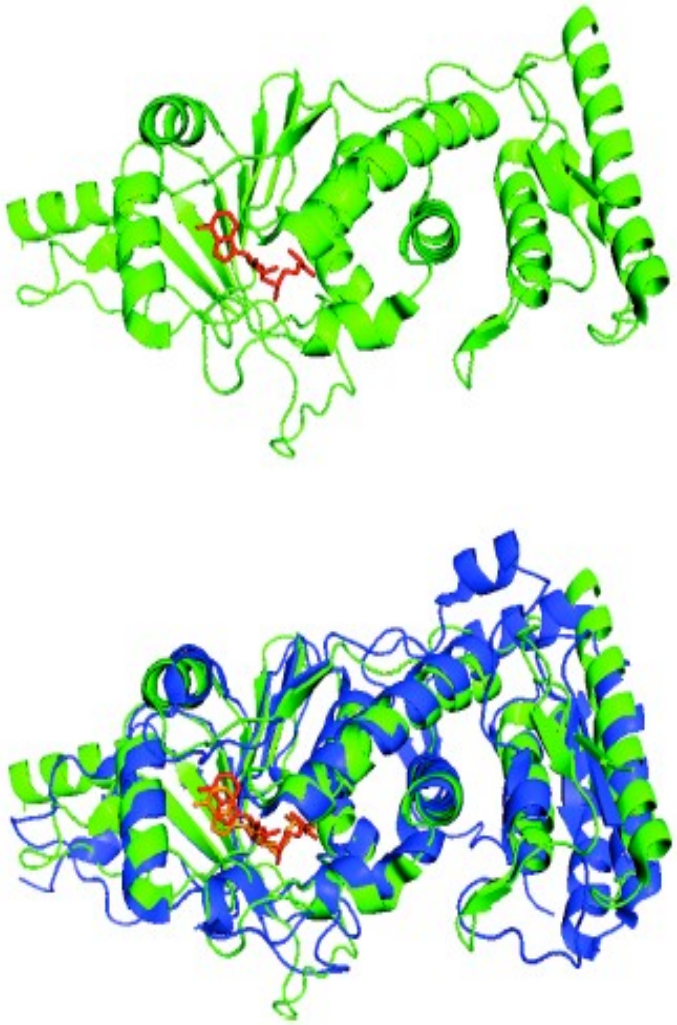

Figure 1.4. Schematic structures of human Dot1l catalytic domain (upper) and yeast Dot1l (lower) (Min et al. 2003, Sawada et al. 2004)

Red color represents the S-adenosylmethionine(SAM) domain in the upper figure. The catalytic domain of human Dot1l in green are structurally aligned with yeast Dot1l in blue in the lower figure. SAM bound to yeast Dot1l is shown in orange. It can be readily seen from the figure that human Dot1l and yeast Dot1l are highly conserved.

Dot1l was initially identified to affect telomeric silencing in Saccharomyces cerevisiae (hence the name represents disruptor of telomeric silencing), and is highly conserved from yeast to human (Zhang et al. 2004). Since its discovery more than 10 years ago, the yeast protein Dot1l and its human homolog have been shown to participate in multiple processes such as cancer, cell cycle, leukemogenesis, transcription and development. Similar to Dot1l, the methylation of histone H3 at lysine 79 is also highly conserved in eukaryotic species. H3K79methylation results in loss of telomeric silencing in yeast by reducing the level of SIR protein bound to telomere (Shilatifard 2006). Dot1l-H3K79methylation is also required for the recruitment of Rad6/Bre1 complex and the subsequent process of monoubiquination of histone H2B Lysine 123 by uibiquitin-conjugating E2 enzyme Rad6 and the ubiquitin E3 ligase Bre1 complex (Wood et al. 2005). Other studies have suggested a role for Dot1l-H3K79methylation in meiotic check point control and DNA double-strand break repair.

1.2.1 The role of Dot1l in telomeric silencing

Previous studies have shown that H3K79methylation is enriched at euchromatin. Dot1l was initially identified as a disruptor of telomeric silencing found in Saccharomyces cerevisiae. As described above, Dot1l actually recruits and binds SIR (silent information regulator) to lead to telomeric silending. In yeast, H3K79methylation is present through the yeast genome but highly deficient at the

regions where genes are controlled by Sir (silent information regulator) proteins (Sir2, Sir3, and Sir4), such as heterochromatic telomeric loci and ribosomal DNA (Steger et al. 2008). Dot1l overexpression, inactivation, loss of Dot1l or mutation of H3K79methylation, can all lead to a decrease on Sir expression and thus attenuate the repressive effects of Sir proteins on genes. Interestingly, Sir proteins, especially Sir3, can reciprocally inhibit H3K79methylation through competing the enzymatic activity required binding sites with Dot1 (Fingerman et al. 2007). This interaction indicates that the balance between Sir proteins and H3K79methylation dictate telomere heterochromatin formation. Although extensive research supports the role of Dot1l-H3K79methylation in opposing heterochromatin formation, some groups have raised a new question regarding this issue. Rossmann's group has proposed that Dot1l is not actually required in general telomere silencing shown by their URA3 telomere reporter system (Rossmann et al. 2011).

1.2.2 Dot1l-H3K79 methylation correlates with transcription activation

Genome-wide profiling studies on Drosophila euchromatic genes have shown that hyperacetylation on H3&H4, as well as hypermethylation at H3K4 and H3K79 are found on active genes, while an opposite pattern, hypomethylation and deacetylation at the same residues is detected in inactive genes (Schubeler et al. 2004). This analysis supports the hypothesis that H3K79methylation correlates with transcription activation. Dot1l-H3K79methylation also correlates with transcription activation in various mammalian cell types. Recent studies have indicated that the

conversion from H3K79monomethylation to di- or tri-methylation is accompanied by enhanced gene transcription levels. Also, Dot1l occupies the transcribed region of active genes as described in Drosophila with a robust H3K79 dimethylation and H3K79 trimethylation. However, H3K79 methylation regulates transcription repression in aldosterone regulated genes, such as ENaC (epithelial Na+ Channel) (Zhang et al. 2009).

1.2.3 Dot1l-H3K79methylation in DNA damage response and checkpoint control

Studies in yeast and mammalian cells have demonstrated a role of Dot1l-H3K79methylation in DNA damage checkpoint control and repair (Martin et al. 2005). The tandem tudor domain of 53BP1, a tumor suppressor protein which is critical in DNA damage and repair, is recruited to DNA double strand breaks (DSBs) upon binding to methylated H3K79. The DNA double strand breaks (DSBs), induced by ionizing radiation, are believed to induce the change of H3K79methylation chromatin structure and then recognized by 53BP1 since there is not much change in H3K79methylation levels upon the DNA damage. Mutations in the tudor domain of 53BP1 will disrupt the interaction with H3K79methylation and thus fail to recruit 53BP1 to the DSBs (Huyen et al. 2004). However, there is controversy on the role of H3K79 methylation with 53BP1 as one group has reported that it is H4K20me2 which binds directly to 53BP1. It is possible that both H3K79methylation and H4K20me2 are involved in the recruitment of 53BP1 (FitzGerald et al. 2011).

The role of H3K79methylation in DNA damage and repair is evolutionarily

conserved, as studies have shown that the recruitment and activation of budding yeast 53BP1 homologue Rad9, requires Dot1l-H3K79methylation (Giannattasio et al. 2005). When Dot1l-H3K79methylation is lost, the recruitment and activation of Rad9, as well as the phosphorylation of Rad53 (checkpoint regulator in cell cycle) are all compromised. In mouse embryonic fibroblasts, deletion of Dot1l induces UV hypersensitivity, which impedes the recovery process after UV radiation (Giannattasio et al. 2005). Surprisingly, in the nucleotide excision repair pathway (NER)，no evidence has been shown that Dot1l is implicated in UV-induced DNA damage removal process (Oksenych et al. 2013).

There are two major pathways involved in repair of DNA double strand breaks (DSBs), including homologous recombination repair (HRR) and the nonhomologous end-joining (NHEJ). HRR is the primary DSB repair mechanism in S. cerevisiae including the involvement of multiple proteins, including cohesin. Dot1l facilitates the recruitment of cohesin to help the repair process besides the recruitment of Rad9 (Lazzaro et al. 2008). The role of Dot1l in NHEJ is not well characterized yet.

Previous studies have shown that Dot1l-H3K79methylation participates in repairing other types of DNA damage such as that induced by UV radiation and alkylating agents (Conde et al. 2008). UV radiation induced DNA damage requires the repair mechanism by NER (nucleotide excision repair), PRR (post-replication repair) and RR (recombination repair). Dot1l is critically important in the repair process as the loss of Dot1l-H3K79methylation influences all three processes.

Recent studies on H3K79methylation in stem cells have also demonstrated

that the deficiency of Dot1l leads to an arrest in G2/M-phase cell cycle along with increased aneuploidy, as well as a disrupted regulation on cell cycle controlling genes (Barry et al. 2009).

In conclusion, Dot1l-H3K79methylation plays an evolutionarily conserved role in the DNA damage repair process and later checkpoint control.

1.2.4 Dot1l-H3K79methylation is required for mammalian embryonic development

Dot1l fly ortholog Grappa has been implicated in the Wnt/Wingless signaling pathway in Drosophila. H3K79methylation level fluctuates with gene activity during development. Interestingly, both Polycomb group (PcG) and Trithorax group (TrxG) proteins are influenced by the mutation in Grappa, suggesting a unique role of this chromatin modification in regulating the gene activity in Drosophila development. Moreover, knockdown of Grappa in Drosophila leads to a reduction in Wingless target gene expression, which is essential in wing morphogenesis (Mohan et al. 2010). Previous studies have also shown that the thyroid hormone receptor in Xenopus metamorphosis can activate histone H3K79 methyltransferase Dot1l and thus influence the metamorphosis. Dot1l binds to the promoter region of thyroid hormone (T3) response element, which plays a critical role in vertebrate development. In addition, the transformation of intestine and tails in Xenopus correlates with Dot1l-H3K79methylation (Matsuura et al. 2012).

Recent studies have documented the essential role of Dot1l in embryogenesis

of mammals. In the mouse zygotes, H3K79methylation is almost undetectable and does not start appearing until the blastocyst stage (Ooga et al. 2008).

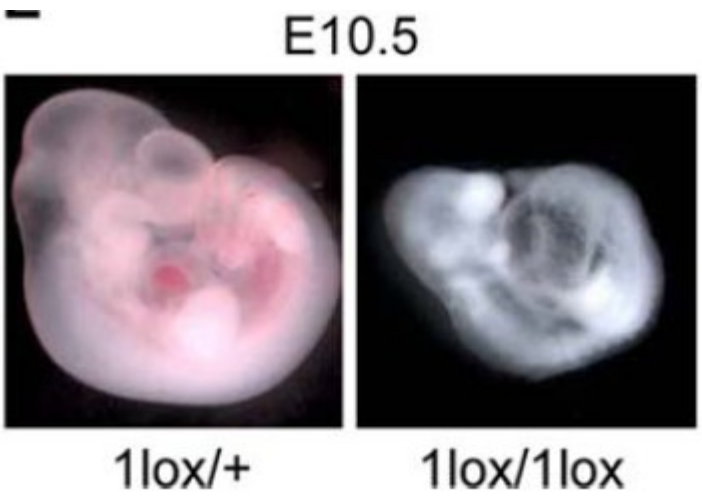

Figure 1.5. Representative pictures of a 10.5-dpc Dot1L1lox/1lox embryo (right) and a heterozygous littermate

Adapted from figure by Jones et al., 2008.

Dot1l is also required for embryonic development in mice as germ-line inactivation of Dot1l in mice leads to an embryonic lethality around E10.5. At E9.5, Dot1l mutant mice are much smaller than the wild type mice with enlarged hearts, thinner and disorganized yolk sac vasculature, and short tails (Jones et al. 2008). The conditional knockout of Dot1l in cardiomyocytes leads to a severe phenotype, similar to patients with dilated cardiomyopathy by regulating dystropin transcription, which is essential in the viability of cardiomyocytes (Nguyen et al. 2011). In addition, Dot1l removal from AQP2 expressing principal cells results in 13%-16% more intercalated cells, as well as 20% fewer principal cells. Interestingly, there is a complete loss of

H3K79me2 in most intercalated cells though the deletion of Dot1l is only in AQP2 expressing progenitor cells. Thus, Dot1l deletion might induce a transition from AQP2-expressing progenitor cells to intercalated cells (Wu et al. 2013).

1.2.5 Dot1a regulates ENaC expression in renal collecting duct

ENaC (epithelial sodium channel) is expressed mainly on the apical membrane of principal cells in kidney and regulates urinary sodium excretion in collecting duct. ENaC consists of three subunits, α subunit is mainly responsible for translocation while the β and γ are the regulatory subunits (Kone 2013). The histone H3K79 methyltransferase Dot1 splice variant a is actively involved in the regulation on ENaC α through interaction with transcription factor Af9, Sgk1 (serum and glucocorticoid-induced kinase-1) or Sirt1, the nicotinamide adenine dinucleotide (NAD) dependent protein deacetylase (Zhang et al. 2006, Zhang et al. 2007, Zhang et al. 2009)(Figure 1.6). ENaC α promoter is maintained in a hypermethylated state by the complex of Dot1a/Af9 or Dot1a/Sirt1. This hypermethylation state actually represses ENaC α gene transcription.

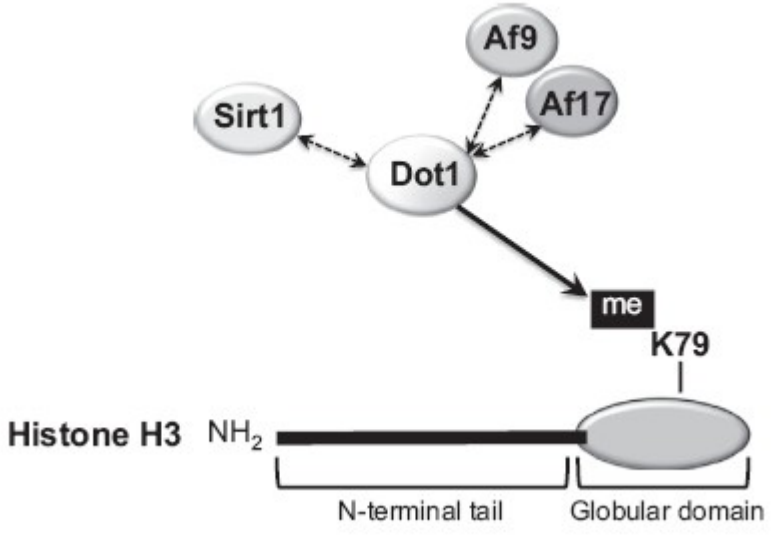

Figure 1.6. Dot1a forms complexes with other partners as Af9, Af17 and Sirt1

Adapted from figure by Kone. B 2013.

Aldosterone and Sgk1 can relieve the Dot1a complex mediated repression on ENaC α by disrupting the formation of Dot1a complexes, such as Dot1a/Af9 and Dot1a/Sirt1. Aldosterone up-regulates serine/threonine protein kinase Sgk1, which phosphorylates Af9 Ser435 and impedes the interaction between Af9 and Dot1l. Af9-Dot1l complex represses ENac α expression. Thus, aldosterone relieves the repression of ENac α by affecting Af9-Dot1l complex (Figure 1.7). Previous studies have demonstrated another potential mechanism of aldosterone regulated ENac α expression by inhibiting Sirt1 mRNA level (Zhang et al. 2006, Zhang et al. 2007, Zhang et al. 2009, Kone 2013) (Figure 1.7). Most recent studies on Af17 have shown that overexpression of Af17 increases ENaC expression by competing with Af9 on Dot1a binding sites and stimulating Sgk1 expression (Reisenauer et al. 2009, Kone 2013).

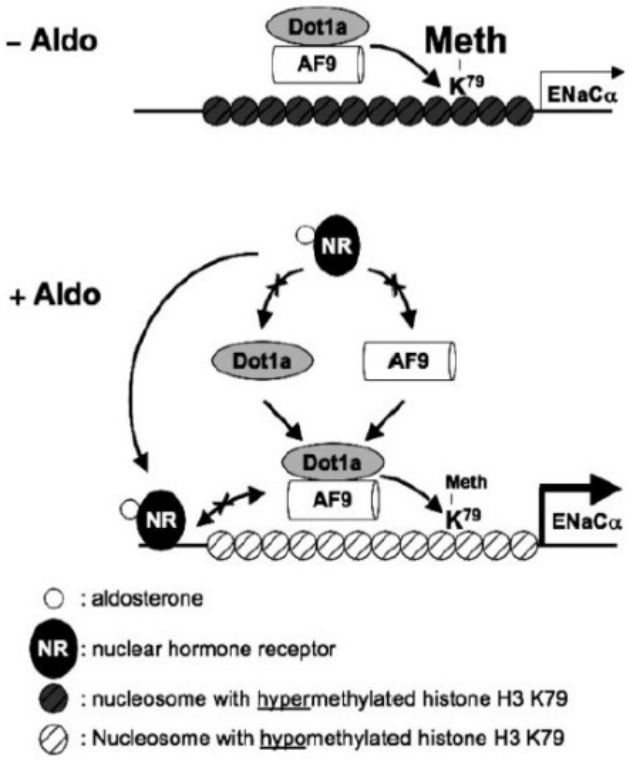

Figure 1.7. Hypothetical model for aldosterone regulated ENaC repression modulated by Dot1la-Af9 complex through H3K79 hypermethylation.

In the absence of aldosterone, Dot1a forms a complex with Af9 which hypermethylate H3K79 and repress ENaCα expression. When aldosterone is present, it can relieve the repression of ENaCα through two different mechanisms. Nuclear hormone receptor can bind with aldosterone and directly transactivate ENaCα through targeting the glucocorticoid response element in the ENaCα promoter. In the newly found pathway, aldosterone can decrease the expression of Dot1a and Af9 thus relieve the repression of ENaCα. Aldosterone can also upregulate serine/threonine protein kinase Sgk1 which can phosphorylate Af9 Ser435 and impede the interaction between Af9 and Dot1l.

Adapted from figure by Zhang. W 2013.

2. Mammalian renal development

2.1 Kidney morphogenesis

The mammalian kidney arises from strips of mesodermal cells named intermediate mesoderm (IM), lying between the lateral plate mesoderm and paraxial somitic mesoderm. Signals released by paraxial somitic mesoderm counteract BMP signals from the lateral plate mesoderm to induce the development of IM around E8.5. After gastrulation, the intermediate mesoderm develops along an anterior to posterior axis into anterior kidney structures such as pronephros (transient embryonic structure) and mesonephros (functional embryonic kidney) and finally posterior metanephros (adult kidney). Pronephros, mesonephros and metanephros comprise three developmental stages in the mammal kidney (Dressler 2009) (Figure 2.1). All three types of kidneys are comprised of the basic functional units of kidney, nephrons, but different in number and structural complexity. For example, mesonephros contains twelve nephrons while metanephros usually have up to 11,000 nephrons in mice (Liang et al. 2002, James et al. 2006).

Since the pronephros and mesonephros are transient structures in mammals, we will focus mainly on the metanephros.

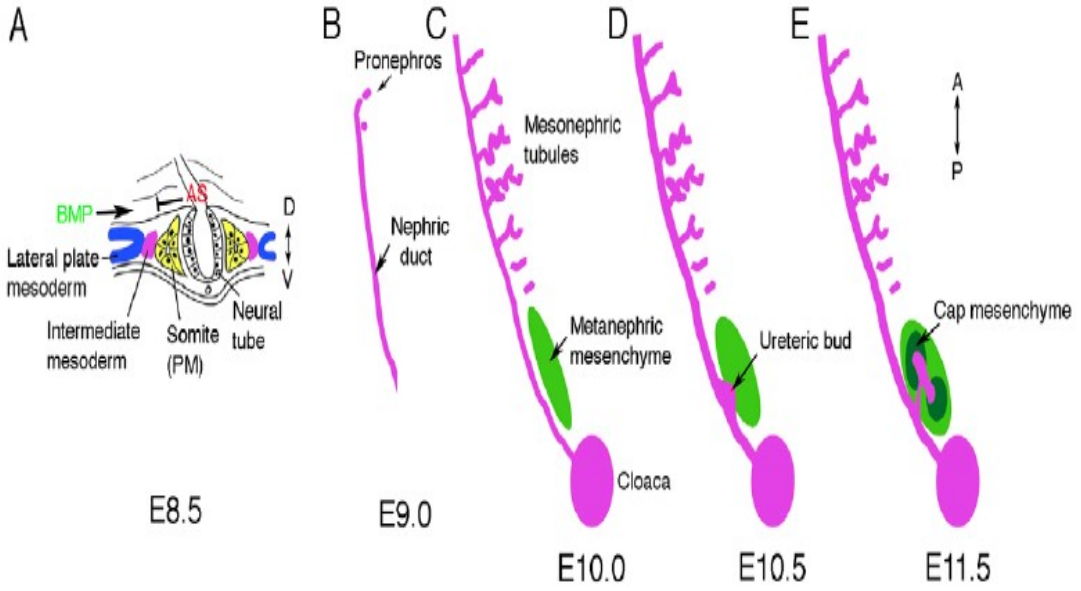

Fig. 2.1. The intermediate mesoderm: its origin and derivatives.

(A) A schematic figure for section through mouse embryo at E8.5 showing the intermediate mesoderm (purple) locates between the lateral plate mesoderm (yellow) and paraxial somatic mesoderm (blue).The paraxial somatic mesoderm can release antagonistic signals (AS) to counteract the BMP signals generated by lateral plate mesoderm to induce the pronephric structures. (B-E) Figure B-E is showing the process when intermediate mesoderm develops into metanephric kidney. (B) Nephric duct (Wolffian duct) appears from the caudal end at embryonic day 9 (E9) and induces the proliferation and differentiation of epithelial tubules derived from mesenchymal cells. (C) At E10, cells adjacent to the cloaca region of nephric duct form metanephric mesenchyme in green color. (D) Ureteric bud （UB） outgrows from nephric duct and starts invasion into metanephric mesenchyme at E10.5. (E) By E11.5, UB induces mesenchyme to aggregate surround UB tips and form cap mesenchymal cells which serve as nephron progenitors.

Adapted from Dressler, 2009.

2.1.1 Metanephros

Metanephros, the permanent kidney in mammals, forms from the caudal section of the mesonephric duct. The basic structures in mammalian metanephros consist of cortical region, inner medullary region and outer medulla region. Collecting ducts and long loops of Henle comprise the inner medullary region while outer medullary region includes short loops of Henle and collecting ducts. Other structures of nephrons are located in the cortical region (Fenton et al. 2007).

Metanephric kidney development starts around E10.5 when the outgrowth of ureteric bud (UB) invades the surrounding metanephric mesenchyme (MM). Previous studies have shown that the release of GDNF (Glial cell line-derived neurotrophic factor) induces the outgrowth of the UB. The interaction between UB and MM induces the latter to condense around the tip of UB and form cap mesenchyme. Cap mesenchyme undergoes MET-mesenchymal to epithelial cell transition- to form renal vesicles which will fuse into collecting ducts later. UB continues to generate new branches which in turn induce more UB tip-associated aggregates.

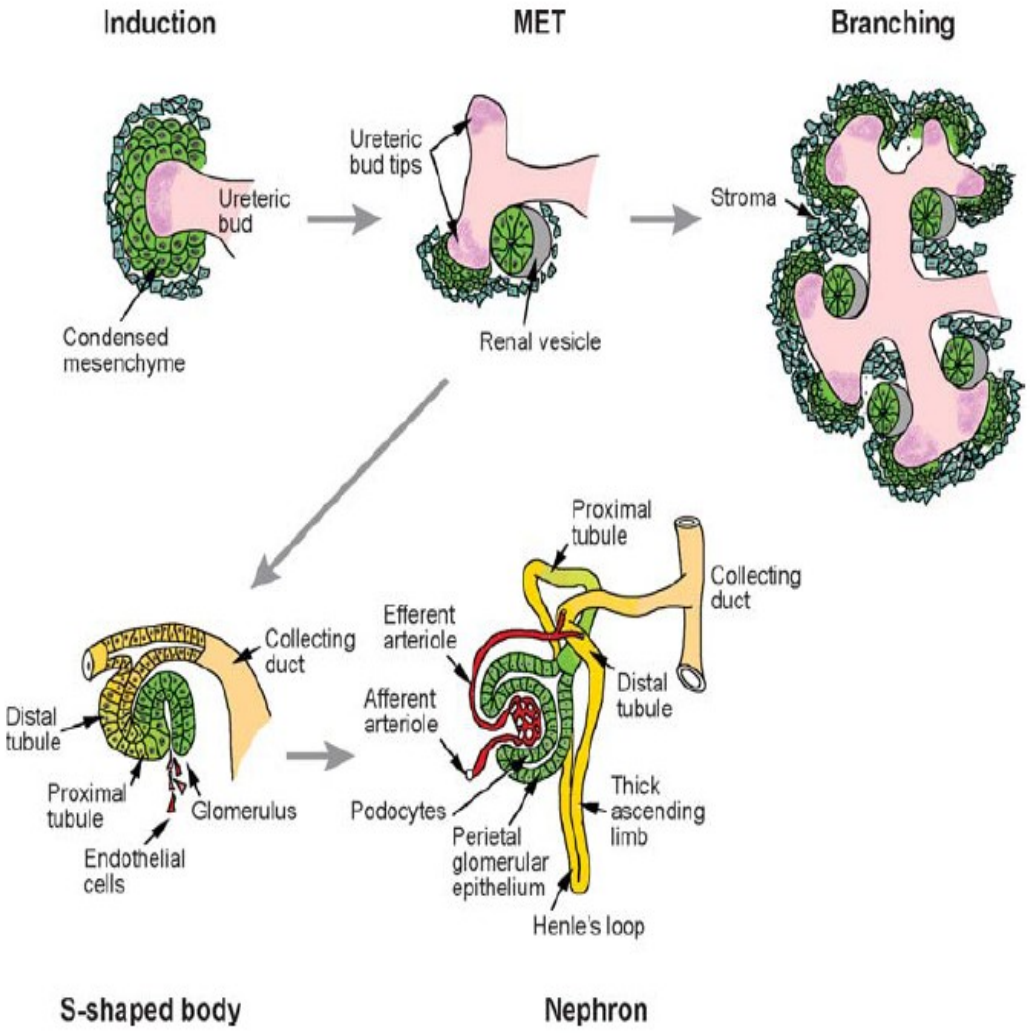

Figure 2.2 Sequential steps of nephrogenesis.

Ureteric bud invades into metanephric mesenchyme and induces metanephric mesenchyme cells to condense around ub tips around E11.5 and form cap mesenchymal cells. Cap mesenchymal cells will polarize and undergo mesenchymal to epithelial cell transition (MET) into renal vesicle. Uncondensed metanephric mesenchymal cells become stromal cells. Renal vesicle fuses into ureteric stalk to generate S-Shaped body with a proximal and distal cleft. Endothelial cells invaded proximal cleft and form glomerular tuft. Along with glomerular development, nephron forms and proximal tubules elongate to form loop of henle.

Adapted from figure by Dressler, 2006.

2.2 Molecular basis of renal development

2.2.1 UB branching morphogenesis

UB branching morphogenesis can be generally described as a multi-step process: a) UB outgrowth from nephric duct; b) UB undergoes a rapid iterative branching; c) UB branching occurs simultaneously as MM undergoes differentiation, and d) termination of UB branching as well as mesenchymal differentiation (Nigam et al. 2009). Around embryonic day E10.5 in mice (fifth week of gestation in human), the UB starts outgrowing from the caudal end of Wolffian duct and then invades the surrounding MM. The continuous iterative branching of UB leads to formation of renal collecting duct system including collecting ducts, ureter, pelvis and calyces (Costantini et al. 2010, Song et al. 2012). The condensed mesenchymal cells surrounding UB tips then undergo EMT to form nephron structures such as renal corpuscle, proximal/distal tubules, loop of Henle and the epithelial glomerulus. During this process, Wingless 9b (Wnt9b) is released by the UB to induce Wnt4 expression which then regulates the MET process (Song et al. 2011).

The reciprocal induction between the UB and surrounding MM is regulated by GDNF together with its receptor tyrosine kinase Ret and coreceptor GPI-linked protein Gfr α 1 (Nigam et al. 2009). GDNF is released by the MM and activates Ret to stimulate UB outgrowth and the invasion of MM. Ret marks the population of UB distal “tip cells” other than tubular “trunk cells” (Shakya et al. 2005). The trunk cells are actually the population that is going to differentiate into collecting duct system

while tip cells are the UB progenitors (Schmidt-Ott et al. 2005).

Extensive in-vitro and in-vivo studies have been performed to unveil the intrinsic mechanism of UB morphogenesis. Mouse knockout models have demonstrated the essential role of GDNF and the two receptor proteins in renal development. For example, failure of UB outgrowth is characteristic of GDNF or Ret mutant mice. However, recent reports have shown that in some cases, the UB is still capable of initiating outgrowth even in the absence of GNDF. This suggests that an alternate signaling pathway for GDNF/Ret should exist, though the compensatory effect might not be sufficient to take place of the role of GDNF/Ret. Indeed FGFs (fibroblast growth factors), especially FGF10, have been reported to replace the function of Gdnf/Ret under certain circumstances in vivo (Costantini et al. 2010).

A complicated signaling network has been described for the patterning of UB branching and elongation downstream of GDNF/Ret- Gfr α 1 signaling (Figure 2.3). It has been shown that mitogen activated protein kinases Erk1/2 and phosphatidylinositol 3-kinase (PI3K) are generally acting as the downstream effectors of GDNF/Ret signaling pathway. The activation of PI3K-Akt signaling pathways and Ras/Erk MPA kinases by Ret is essential for kidney development. GDNF/Ret signaling via PI3K up-regulates the expression of Etv4 and Etv5. The double knockout mice of these two transcription factors cause a severe UB branching defect and hypodysplasia syndrome (Costantini et al. 2010). Recent studies have also suggested the critical role of FGFRs in this process. Inactivation of FGFR2 in UB lineage leads to fewer nephron numbers and UB branching numbers (Qiao et al. 2001).

BMPs can inhibit GDNF signaling while gremlin, the inhibitor of BMPs, can stimulate GNDF signaling pathway. Spry1, tyrosine kinase inhibitor, acts as the repressive regulator for Ret signaling and forms a negative feedback loop. More interestingly, Spry1 removal in Ret or Gdnf knockout mice can rescue the phenotype and regain normal kidney development. The mutations in Pax2, Eya1, Six1 and Hox11 result in renal agenesis, suggesting that they are required for the GDNF signaled UB outgrowth (Costantini et al. 2010).

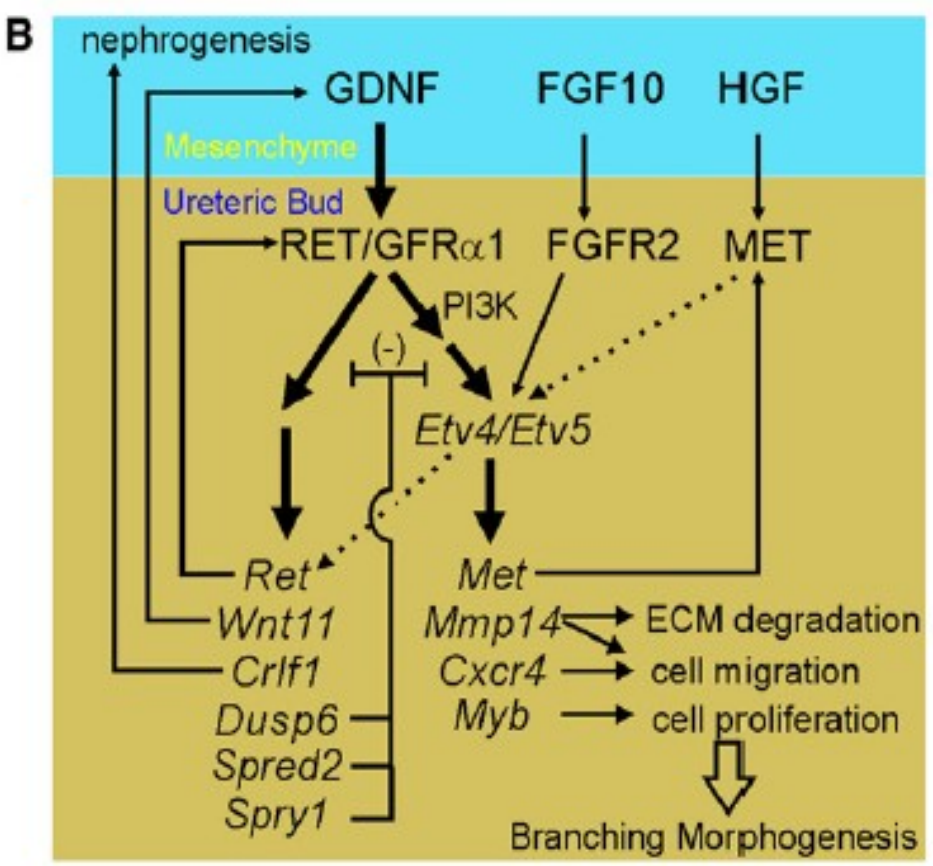

Figure 2.3 Genetic network mechanisms for ureteric bud branching morphogenesis.

Adapted from figure by Costantini et al., 2010.

2.2.2 The formation of collecting duct

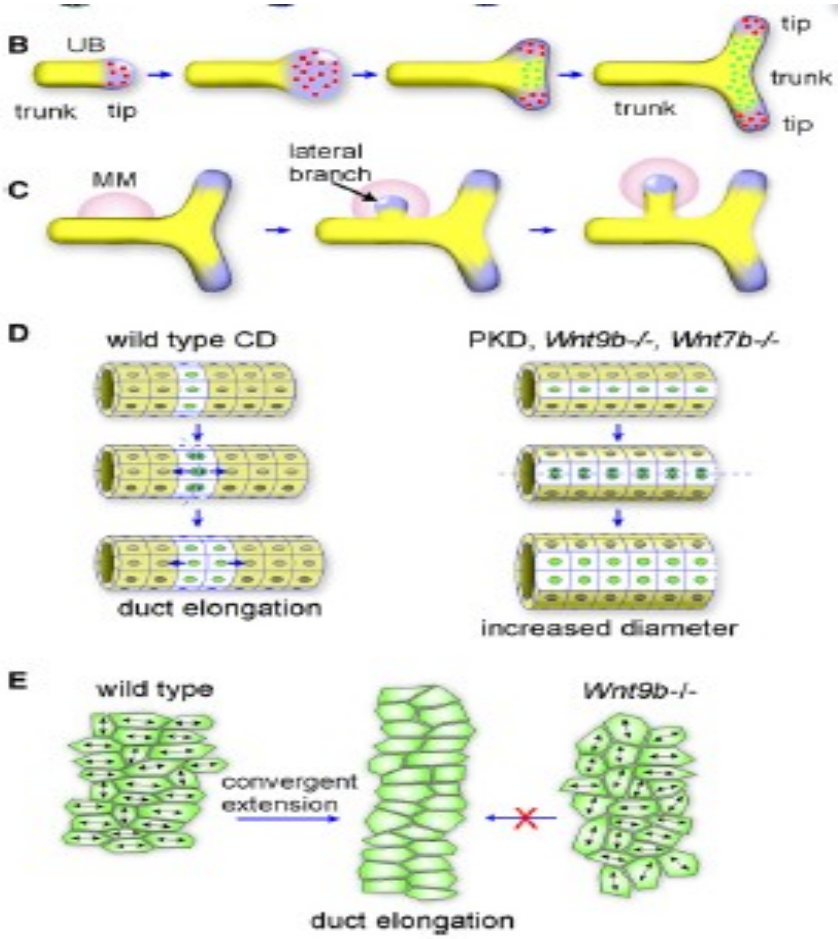

Figure 2.4 Ureteric bud/collecting duct morphogenesis.

(B) UB tips cells have shown bipotential features. The UB tips are marked in light purple color while UB trunks are shown in yellow color. Initially UB tips cells will proliferate to generate new UB tips cells shown in red but there is also another population of tip cells (green) left in the developing UB trunks. (C) Tip cells (light purple) have been shown to be regenerated from the yellow trunk cells during the lateral branching process. (D) In PKD, Wnt9b and Wnt7b mutants the mitotic orientation of collecting ducts have been disorganized and thus increased diameter instead of length of collecting ducts compared with wild type. (E) In Wnt9b mutants, the orientation of cells in collecting ducts has been changes and lost the ability to

elongate collecting ducts. This is suggesting the convergent extension movement, which induces the collecting duct elongation in wild type, is disrupted in Wnt9b mutants.

Adapted from figure by Costantini et al., 2010.

The initial phase of UB branching is a rapid iterative branching process with a Ret-dependent proliferation of UB tip cells (Schmidt-Ott et al. 2005). The subsequent branching process induces the elongation of collecting duct accompanied with UB trunk cell proliferation (Costantini et al. 2010). Part of UB tip cells remain in the tip domain for self-renewal process while the remainder proliferate to enlarge the terminal ampulla and form the trunks (Figure 2.4). There is evidence that the “tip to trunk” process can be reversible though the intrinsic mechanisms remain unclear. Previous studies have stated that UB tip cells have a distinct and highly specific gene expression pattern which is different from UB trunk cells. Ret, Wnt11 and CLF-1 are the common markers defining the UB tip cells. Wnt9b is firstly expressing in the UB trunk cells and later in medullary collecting ducts.

As discussed above, renal collecting ducts in medulla and papilla are formed by the later continuous branching of UB after E15.5 in mouse. The MM-derived nephrons are connected to the collecting duct by the connecting tubules. The major function of collecting duct is to collect urine, regulate the osmolarity, pH and extracellular volume (Wu et al. 2013). Oriented cell division (OCD) is generally considered an important mechanism in collecting duct elongation. Another important cellular mechanism is convergent extension. Convergent extension here in the

collecting duct mainly refers to the process when the intercalation of cells stimulates the collecting duct to converge along one axis and elongate (Costantini 2012). Wnt9b in canonical Wnt pathways, as well as noncanonical Wnt signal Fat4, can regulate both the OCD and convergent extension in collecting ducts and thus influence the collecting duct elongation and formation. Wnt7b can also regulate OCD and thus the medulla and papilla development through signaling to the adjacent interstitial cell (Costantini et al. 2010, Costantini 2012). Wnt7b, HGF, EGF and laminin are also required for cell survival during the formation of collecting ducts (Costantini 2012).

Mature collecting ducts consist of three distinct cell types: principal cells, alpha and beta intercalated cells. Intercalated cells are mainly responsible for acid-base homeostasis. The difference between alpha and beta intercalated cells is that alpha intercalated cells secrete acid but reabsorbs bicarbonate while beta intercalated cells act in a totally opposite way. Principal cells regulate sodium and potassium balance through influencing the sodium and potassium channels located on the apical membrane of principal cells. This sodium/potassium balance is regulated by aldosterone and ENaC. Previous studies have suggested that aldosterone regulates sodium and potassium transport through increasing the Na^+/K^+-ATPase pumps. Vasopressin (ADH, antidiuretic hormone) regulates the aquaporin-2 channel thus controls water absorption in principal cells (Schlatter et al. 1987). These three types of cells work together to maintain the normal function of collecting ducts in the kidney.

3. Renal injury and repair

3.1 Acute Renal injury

Acute kidney injury (AKI), or acute renal failure, is a clinical syndrome with a rapid loss of renal excretory function induced over a short time period (hours to days), which is different from the more gradual developing chronic kidney disease (CKD). Various reports have shown that the prevalence of AKI is up to 7.1% in hospitalized patients, especially common in critically ill patients. A high mortality from 30% to 70% highlights the significance of the clinical problem of AKI (Bellomo et al. 2012, Patschan et al. 2012). AKI can result from various reasons, including low blood volume such as in renal ischemia/reperfusion model, obstruction of urinary tract and the exposure to harmful substances to kidney (Lelarge et al. 1992, Molina et al. 2005).

3.1.1 Renal ischemia/reperfusion (I/R) model

Renal ischemia/reperfusion injury acts as a major cause of acute renal injury and kidney allograft dysfunction. Thus it has been demonstrated to be critically important in kidney transplantation (Nelson 2007, Bonventre et al. 2011). Partial or complete blockade of renal blood flow followed by reperfusion can lead to AKI (Kusch et al. 2013). Under normal physiologic conditions, the renal cortex is the major site receiving about 90% of renal blood flow (RBF) due to the abundance of glomeruli. The other 10% of RBF goes into the renal medulla. The decrease of RBF is actually more prominent in outer medulla than renal cortex under ischemic conditions (Munshi et al. 2011). The interrupted blood supply will lead to a reduction in GFR

and disturbance of oxygen supply in kidney structures. In mouse, renal I/R injury is induced experimentally by clamping the renal pedicle for certain length of time and then released (van den Akker et al. 2013). The mechanisms of renal I/R injury are not completely understood, but it has been shown that hypoxia, inflammatory responses as well as free radical damage all play an important role (Williams et al. 1997).

3.1.2 Morphologic changes in renal ischemia and reperfusion injury

Previous studies have shown that the major morphologic changes induced by renal I/R injury include loss of proximal tubule brush border, proximal tubular dilation, cysts in distal tubules as well as cellular regeneration (Devarajan 2006). Also, necrosis can be detected in outer medullary regions although glomerular defects are not normally found in the ischemic acute renal injury. Furthermore, peritubular capillaries show significant vascular obstruction with endothelial cell injury (Bonventre et al. 2004, Devarajan 2006).

3.1.3 Signaling mechanisms in renal ischemia and reperfusion injury

The renal ischemia-induced ATP depletion can lead to severe disruption in the selective transport mechanisms for macromolecules, as wells as ions and water transport in proximal tubules. Proximal tubular cell migration, differentiation, and proliferation are the major repair processes following I/R injury. Several cellular mechanisms participate in this reperfusion injury, including oxidative stress, disturbance of calcium balance, pH changes and the trafficking of inflammatory cells

into postischemic renal structures (Williams et al. 1997, Chatterjee 2007).

3.2 Unilateral ureter obstruction (UUO) model

The hallmark of renal injury caused by obstructive nephropathy is interstitial fibrosis (Bascands et al. 2005). However, the underlying cellular and molecular mechanisms of renal fibrosis are not completely understood. Since 1970s, the unilateral ureter obstruction (UUO) model has been widely used as a useful in vivo animal research model for the investigation of progressive renal fibrosis (Chevalier et al. 2009). UUO, performed by the surgical ligation of the ureter, can efficiently mimic the key processes of obstructive renal injury including interstitial fibrosis, fibroblast proliferation, cellular infiltration, tubular apoptosis, epithelial to mesenchymal transition (EMT), inflammation and tubular atrophy (Bascands et al. 2005, Chevalier et al. 2009). These processes are illustrated in Fig. 3.1. Table 3.1 shows the various genetic animal models which investigated UUO-induced fibrosis.

3.2.1 Cellular processes and underlying mechanisms in unilateral ureter obstruction (UUO) model

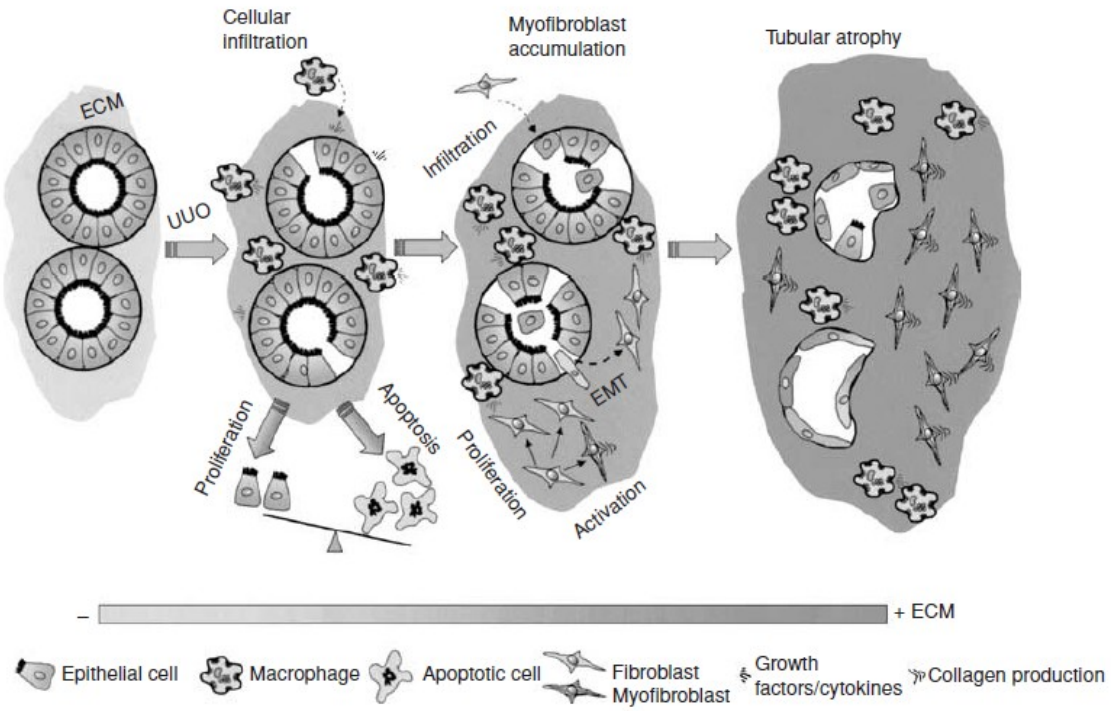

Figure 3.1 Overview of the different stages of the development of obstructive nephropathy.

Adapted from figure by Bascands and Schanstra, 2005.

Table 2 Selected potential molecular therapeutic targets in AKI and CKD identified by functional studies in the UUO model

Molecule	Targeting technique
Renin-angiotensin system	
AT1	receptor antagonist
	KO
AT2	Receptor antagonist
Kinin B1 receptor	Receptor antagonist
MAS (Ang-(1-7) receptor)	KO
ACE	Pharmacological inhibition
Inflammation	
NFκB	NF-κB decoy double-stranded ODN
	Pharmacological inhibition
TNFα receptors 1 and 2	KO
TNFα	Antibody neutralization
TWEAK	KO
CCR2 and CCR1	Pharmacological inhibition
	KO
OPN	KO
MCSF-1	Receptor antagonist
CCL21/CCR7	CCL21 antibody neutralization
	CCR7 KO
ET-1	Receptor antagonist
TGF-β1	
TGF-β1	Antibody neutralization
BMP-7	Administration
Smad3	KO
	KO
Smad7	Transgenic mice overexpression
Alk3 (THR-123)	Receptor agonist
Kinases	
HIPK2	KO
JNK	Pharmacological inhibition
CDK	Pharmacological inhibition
Serine proteases	
tPA	KO
PAI-1	Transgenic mice overexpression
	KO
HE4	Antibody neutralization
Others	
VDR (paricalcitol)	Receptor agonist
p53	Pharmacological inhibition
Nerve-dependent signaling	Receptor antagonist
NMDAR	Receptor agonist

KO knock out

Table 3.1 Selected potential molecular therapeutic targets in AKI and CKD identified by functional studies in the UUO model.

Adapted from the table by Ucero et al, 2013.

4. The role of the p53 family member, p63

4.1 Overview of p63 structure

P63 and p73 are members of p53 gene family. P53 is a well-known tumor suppressor gene crucial not only for prevention of cancer but also critical in cellular damage response and development (King et al. 2013). Human p63 gene resides on chromosome 3q 27 and the p63 protein shares similar functional domains with p53, such as N-terminal transactivation domain, DNA binding domain and carboxy-oligomerization domain. The use of alternative promoters at 5' end of p63 gene allows p63 to exist as two major isoforms due to the alternative splicing: TA isoform which contains an N-terminal transactivation domain and ΔN isoform which harbors a truncated N-terminal domain (Amelio et al. 2012, King et al. 2013). C-terminal sequence splicing also gives rise to other specific subclasses isoforms for TAp63 and ΔNp63 (Figure 4.1) (Murray-Zmijewski et al. 2006). As indicated by structure, TAp63, which contains the transactivation domain, will act as transcription activator for genes. On the other hand, ΔNp63 acts as a dominant-negative inhibitor for TAp63 function. However, ΔNp63 also harbors potential transactivation capability as some studies have discovered an alternate transactivation domain (TA2) existing in exon 11 and 12 of C-terminus gene (Dohn et al. 2001). Although TAp63 can transactivate and regulate most p53 target genes as they share highly similar sequences and functional domains, the results of knocking out p63 and p73 in mice have surprisingly shown a defect in development but no increase in cancer incidence. This result suggests a distinct role of p63 from p53.

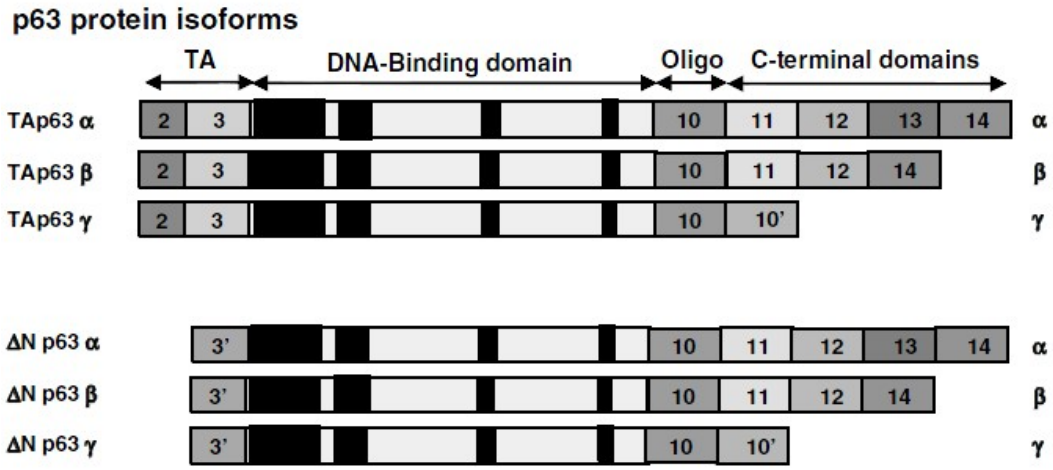

Figure 4.1 Different p63 protein isoforms

Adapted from figure by Murray-Zmijewski et al., 2006.

Previous studies have shown that p63 is essential in the regulation of embryonic development (especially in limb formation and epidermal development), DNA damage repair as well as pathological processes (Yang et al. 1999). It has also been suggested that the balance between TAp63 and ΔNp63 may be involved in the regulation of cell fates, such as the differentiation and proliferation state of stem cells and progenitors (Yao et al. 2012).

4.2 The role of p63 in embryonic development

P63 null mice exhibit severe limb development defects as well as failure of skin development. The expression patterns of specific isoforms for TAp63 and ΔNp63 have remained unclear due to the lack of specific antibodies. However, the individual function of TAp63 and ΔNp63 has been investigated by several groups.

P63 null mice die soon after birth due to developmental defects in stratified

epithelial barrier in skin (Yang et al. 1999). P63 knock-out mice also show distinct features such as lack of epithelial to mesenchymal transition as well as the absence of all squamous epithelial structures (mammary and salivary glands) (Yao et al. 2012). Reconstitution of TAp63 or ΔNp63 both show a rescue for the epithelialization however ΔNp63 reintroduction seems to do a better job (Candi et al. 2006). Previous studies have indicated that ΔNp63 is more abundant in postdevelopmental tissues while TAp63 expression is higher in oocytes compared with ΔNp63 (Mikkola 2007, Livera et al. 2008). Furthermore, TAp63 has been shown to be expressed in early embryogenesis and is essential for the start of epithelial stratification. TAp63 also inhibits the terminal differentiation process (Koster et al. 2004). ΔNp63, in an opposite way, is required for the later maturation of embryonic epidermis (Koster et al. 2004). The shift in expression from TAp63 to ΔNp63 is believed to be critical to counteract the terminal differentiation inhibition effect by TAp63. After terminal differentiation has finished and the mature epidermis has formed, high expression of ΔNp63 persists to maintain the proliferative capacity of keratinocytes through inhibition of p21 (Koster et al. 2004). In conclusion, p63 is critically important in the maintenance of progenitor cells and terminal differentiation of keratinocytes in skin epidermal development.

The role of p63 in limb development is quite similar to skin with TAp63 knockdown leads to an inhibited proliferation but promoted differentiation (Wang et al. 2005). Accordingly, ΔNp63 deficiency results in inhibited proliferation but does not affect differentiation (Wang et al. 2005). Taken together, these results suggest the

potential roles of p63 in the proliferation and differentiation process of limb development.

4.3 P63 in cell cycle and cancer

P53 has a recognized role in control of cell cycle and death pathways. Recent studies have also suggested the potential role of p53 family member gene p63 in the related mechanisms. The antagonistic effect of ΔNp63 on TAp63 is involved in cell cycle arrest since TAp63 can up-regulate p21 expression whereas ΔNp63 inhibits p21 transcription. More interestingly, TAp63 and ΔNp63 can both suppress the G2/M regulator Cyclin B2 and cdc2, suggesting a complicated interaction pattern between these two isoforms of p63 (De Cola et al. 2012). The in-vivo model utilizing p63 knockout mice have shown that p63 is required for the p53 dependent apoptosis (Flores 2007). It has also been suggested that TAp63 can activate the mitochondrial apoptosis pathway through upregulation of Bcl-2 family proteins as well as RAD9 (Gressner et al. 2005).

Considering the role of p63 in apoptosis and cell cycle, it can be hypothesized that p63 may also serve as a tumor suppressor similar to p53. Various mouse models have been established to investigate the role of p63 in cancer. P63 has been found to lose heterozygosity in squamous cell carcinomas (Flores 2007). Several groups have shown that p63 mutant mice can develop multiple tumors (Gaiddon et al. 2001, Keyes et al. 2006, Flores 2007). To be more specific, TAp63 has been shown to act as tumor suppressor while its dominant negative regulator ΔNp63 harbors oncogenic

activities. Moreover, deficiency of TAp63 and/or overexpression of ΔNp63 can result in invasive tumor development. Mutant p53 can also inhibit TAp63 and lead to a similar development of invasive tumor. The delicate balance between TAp63 and ΔNp63, as occurs in embryonic development, is also believed to affect tumorigenesis (Flores 2007).

5. The aim of the study

In view of the essential roles of Dot1l-H3K79methylation in embryonic development, ENaC regulation, as well as transcriptional control of genes, the major aim of this study is to delineate the ontogeny and biological functions of Dot1l-H3K79methylation in renal development, injury and repair.

The major aims are as follows:

Aim 1: To delineate the developmental expression pattern of Dot1l-H3K79methylation during nephrogenesis.

Aim 2: To determine the biological function of Dot1l in kidney development.

Aim 3: To determine the role of Dot1l-H3K79methylation in renal epithelial cell injury and repair in I/R injury and UUO.

CHAPTER 2: MATERIALS AND METHODS

1. Protein extraction, histone extraction and western blotting

Whole cell proteins were extracted from cells and tissues using RIPA buffer (Invitrogen) through centrifugation at 8000g for 10min at 4℃. Protein concentration was assayed by Pierce BCA Protein Assay Kit (Thermo Scientific). Histones were extracted by acid-extraction method. Briefly, cells or tissue are homogenized and then lysed in Triton Extraction Buffer (TEB/PBS containing 0.5% Triton X-100 (v/v), 2 mM phenylmethylsulfonylfluoride (PMSF) and 0.02% (w/v) NaN3) at a cell density of 10^7 cells per ml and 0.1g/2.5ml TEB for tissues with gentle stirring on ice for 10min. Following centrifugation at 2000g for 10 minutes at 4℃, the supernatant is collected and washed in half volume TEB and centrifuged again. Pellets were resuspended in 0.2N HCl for overnight at 4℃ followed with centrifugation at 2000g for 10min. Supernatants containing histones were collected and stored at -80C.

Western blotting was performed using the following primary antibodies:

Antibody	Species	Company	Catalog Number	Dilution
H3K79me3	Rabbit	Abcam	ab2621	1:1000
H3K4me3	Mouse	Abcam	ab1012	1:1000
H3K9me3	Rabbit	Abcam	ab8898-100	1:1000
H3K27me2	Rabbit	Abcam	ab24684	1:1000
H3K27me3	Rabbit	Abcam	ab6002	1:1000
Pan H3	Rabbit	Millipore	06-755	1:1000

2. RNA isolation and quantitative Real-time PCR

RNA was isolated from kidneys and mouse kidney cell lines (MK3, MK4, IMCD3 and UB) as well as human proximal tubule cell line HK-2 utilizing RNeasy Minikit (QIAGEN). One-step Brilliant quantitative RT-PCR master mix kit (Stragagene) was used to perform Taqman real-time PCR. Relative mRNA levels were normalized to GAPDH for mouse and beta-actin for human species. All QPCR probes were ordered from Applied Biosystems:

Active beta-actin (human species): Hs01060665-g1

Dot1l (human species): Hs00287200_m1

JMJD3 (mouse species): Mm01332672_g1

EZH1 (mouse species): Mm00468440_m1

EZH2 (mouse species): Mm00468464_m1

Dot1l (mouse species): Mm01173481_m1

Suv39 (mouse species): Mm01347696_g1

UTX (mouse species): Mm00801998_m1

GAPDH (mouse species): Mm99999915-g1

The qPCR conditions were as following:
Segment 1: 50℃ 30min, 1cycle
Segment 2: 95℃ 10min, 1 cycle
Step 3: 95℃ 15s , 56℃ 1min, 72℃ 30s, 40cycles

3. Generation of ureteric bud lineage-specific Dot1l deficient mice

The original chimeric Dot1l3lox/+ mice were supplied by Dr. Taiping Chen (Novartis, MA) through frozen embryo rederivation (Charles River). Dot1l-floxed mice were maintained on a C57BL/6-129Sv mixed background. Generation of Dot1l 2lox/+ and Dot1l 1lox/+ mice was achieved by crossing Dot1l 3lox/+ mice with zp3-cre transgenic mice expressing Cre recombinase in the germline. Dot1l 2lox/+ mice and Dot1l 1lox/+ mice were then crossed with Hoxb7-cre mice to generate Dot1l 2lox/2lox Hoxb7-cre/+ or Dot1l 2lox/1lox Hoxb7-cre/+ mice in which Dot1l was inactivated with methyltransferase function specifically in ureteric bud lineage cells. Genotyping primers were as follows:

CT-132: 5’-ggaactcaagctatagacaggctg-3’

CT-226: 5’-atcagccatatcacatctgtagag-3’

CT-484: 5’-caactgcaaacatcactacggag-3’

CT-485: 5’-atcctctctcctgaggaggcagc-3’

1lox: CT-132+CT-485. Expected size: 1lox ~230 bp

2lox: CT-484+CT-485. Expected sizes: WT ~240bp, 2lox~380bp

3lox: CT-226+CT-485. Expected size: 3lox ~570 bp

PCR conditions:

95℃ 1 min	
95℃ 20 sec	
55℃ 30 sec	
72℃ 30 sec	Go to step 2 for 5 cycles
95℃ 20 sec	
62℃ 30 sec	
72℃ 30 sec,	Go to step 6 for 25 cycles
72℃ 5 min	4℃ forever.

4. Immunofluorescence

Kidneys were harvested and fixed in 10% formalin overnight, then processed for paraffin sections at 5uM thickness. Sections were deparaffinized in xylene and rehydrated with series of ethanols. Antigen retrieval was performed by immersing the slides in 10nM sodium citrate (PH 6.0) in electric steamer for 45min. Primary antibodies and secondary antibodies were prepared in antibody buffer with 2% normal donkey serum (Jackson Lab).

Primary antibodies used were as follows:

Primary antibody	Species	Company	Catalog Number	Dilution
H3K79me2	Rabbit	Active Motif	#39143	1:200
H3K79me3	Rabbit	Abcam	ab2621	1:200
Pho-H3	Rabbit	Cell signaling	#9701S	1:200
Cytokeratin	Mouse	Sigma	C2562	1:200
AQP2	Goat	Santa Cruz	sc-9882	1:200
Carbonic anhydrase Ⅱ	Rabbit	Santa Cruz	sc-25596	1:200
Jagged-1	Rabbit	Santa Cruz	sc-8303	1:200
Laminin	Rabbit	Sigma	L9393	1:200
Six2	Rabbit	Proteintech Group	11562-1-AP	1:200
WT1	Rabbit	Abcam	ab15249	1:200
Dot1l	Rabbit	Abcam	ab64077	1:1000
Calbindin	Rabbit	Abcam	ab25085	1:200
Pax2	Rabbit	Invitrogen	71-6000	1:200
4a4p63	Mouse	Abcam	ab3239	1:200
LEF1	Rabbit	Cell signaling	#2230S	1:200
Foxi1	Goat	Abcam	ab20454	1:200
E-cadherin	Mouse	BD biosciences	610181	1:200
Beta-catenin	Rabbit	Cell signaling	#9582S	1:200
Lhx1	Mouse	Developmental Hybridoma Bank	4F2-S	1:50
B1-V ATPase	Rabbit	Dr. Dennis Brown's lab at Harvard		1:200

Secondary antibodies used were as following table:

Secondary antibody	Company	Catalog Number
Alexa Fluor 555 Donkey anti rabbit IgG	Invitrogen	A31572
Alexa Fluor 488 Donkey anti rabbit IgG	Invitrogen	A21206
Alexa Fluor 647 Donkey anti rabbit IgG	Invitrogen	A31573
Alexa Fluor 555 Donkey anti mouse IgG	Invitrogen	A31570
Alexa Fluor 488 Donkey anti mouse IgG	Invitrogen	A21202
Alexa Fluor 647 Donkey anti mouse IgG	Invitrogen	A31571
Alexa Fluor 555 Donkey anti goat IgG	Invitrogen	A21432
Alexa Fluor 488 Donkey anti goat IgG	Invitrogen	A11055
Alexa Fluor 647 Donkey anti goat IgG	Invitrogen	A21447
TNB blocking reagent	PerkinElmer	FP1012
TSA-Plus Cyanine 3&5, TMR and fluorescein system	PerkinElmer	NEL760001KT
Peroxidase-AffiniPure F(ab')2 Frag Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch	711-036-152
Peroxidase-AffiniPure F(ab')2 Frag Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	715-036-150
Peroxidase-AffiniPure F(ab')2 Frag Donkey Anti-Goat IgG (H+L)	Jackson ImmunoResearch	705-036-147

5. Whole Mount Immunofluorescence

Microssected E12.5 kidney explants were cultured on membranes of six-well transwell plates (Corning) in advanced DMEM-F12 medium with 10% FBS (invitrogen) for 4 hours and then fixed in ice-cold 100% methanol for 10min followed by twice washing of PBS, 0.1%Tween 20 (PBST) at 4℃. Primary antibodies were diluted in PBST and added to kidneys for overnight incubation in 4℃ fridge. The samples were subjected to multiple washes in PBST and incubated with secondary antibody overnight in 4℃. After 8h PBST washing, kidneys were mounted on a slide with fluorescent mounting media (Dako). Images were captured using Olympus BX51 fluorescence microscope. Primary antibodies used were

anti-cytokeratin (Mouse monoclonal, 1:200, catalog number C2562, Sigma), anti-Pax2 (Rabbit polyclonal, 1:200, catalog number 71-6000, Zymed Laboratories Inc) and secondary antibodies included Alexa Fluor 555 Donkey anti rabbit IgG (1:400, catalog number A31572, Invitrogen), Alexa Fluor 488 Donkey anti mouse IgG (1:400, catalog number A31571, Invitrogen) and DAPI (1:400, catalog number D1306, Invitrogen).

6. Cell apoptosis assay

Cell apoptosis was assessed by TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) staining. TUNEL was performed on paraffin sections utilizing the Deadend Fluorometric TUNEL System (Promega) and counterstained with DAPI.

7. Renal ischemia/reperfusion experiment in vivo

Paraffin sections were made from kidneys of adult male mice subjected to renal ischemia/reperfusion (I/R) injury by clamping the left renal artery for 45 min then relasing it. Kidneys were harvested from animals at 0h, 24h, 72h and 7days after I/R injury. The paraffin sections were kindly supplied by Dr. DeCasteker from Vanderbilt University.

8. Induced hypoxia in HK-2 cells

To mimic the effect of I/R in vitro, HK-2 cells (human proximal tubule cell line) were treated with mineral oil (Sigma) to induce hypoxia-like conditions. HK-2 cells were plated in 100mm X 20mm petri dish (Corning) with Keratinocyte Serum Free Medium (K-SFM) supplemented with 5ng/ml EGF and 0.05mg/ml BPE. (Invitrogen)

After twice through wash with PBS, cells were covered with 4ml medium and 6ml mineral oil carefully added onto the top of medium and incubated in 37°C incubator for 90min. The cells were then washed six times with PBS to remove the extra mineral oil. This was followed by adding 6ml fresh medium to the cells and culture for additional 6h, 12h or 24h followed by RNA extraction.

9. Section In Situ Hybridization

In situ Hybridization was performed on paraffin sections with digoxigenin-labeled antisense probes as described at http://www.hhmi.ucla.edu/derobertis. Kidneys were harvested at different developmental stages（E14.5， P1， Adult）and fixed in 10% formalin for overnight, then processed for paraffin sections at 10uM thickness on Gold Plus slides. Paraffin sections were then treated with xylene and ethanols for rehydration followed by refixation in 4% Paraformaldehyde in PBS at room temperature for 15 minutes. Proteinase K (1ug/ml) was added to section slides to make RNA more accessible to hybridization. Slides were initially incubated in 0.2M HCl (1:60 dilution in PBS) at room temperature for 15 minutes and then in hybridization buffer in humid chamber at 65℃ overnight followed by anti-digoxigenin antibody incubation. BM purple was used as substrate for alkaline phosphatase to stain color. Color reactions could develop from overnight to over several days. After developing, wash in PBSw and mount the slides with Immuno Floure Mounting Medium (Dako). Digoxigenin-dUTP–labeled RNA probes used for staining were: Wnt7b, Wnt9b, Wnt11, Ret, Foxi1, Wnt4 at 0.5ug/ml.

10. Unilateral Ureter Obstruction

Adult male and female wild-type and Dot1l$^{f/f}$/Hoxb7-Cre mice were anesthetized using avertin (200 mg/kg body weight, intraperitoneally, Sigma, St. Louis, MI). The left ureters were isolated from the surrounding tissues via an abdominal or back incision and then double-ligated and cut between two ligated points. The incision was closed under aseptic conditions and then maintained for 3days, 7days and 14days separately. The unilateral ureteral obstruction-treated (UUO) mice were allowed to recover. The right intact kidney served as control.

11. Statistical Analysis

The results were presented as mean ± SEM. P-values were calculated using two-tailed t-test and differences were considered as significant when p value was less than 0.05.

CHAPTER 3: RESULTS

1. Developmental expression of H3K79methylation and its sole methyltransferase Dot1l in kidney development

To determine whether Dot1l-mediated H3K79methylation is a developmentally regulated process in the kidney, we performed western blotting on acid-extracted histones from various stages of kidney development. H3K79me3 levels are relatively low during fetal life and become highly abundant after birth, while other histone methylations, such as H3K4me3, H3K9me3, H3K27me2 and H3K27me3, maintain a stable expression level (Figs 5 and 6A). The expression of Dot1l, the sole enzyme responsible for H3K79methylation, was examined by quantitative real-time RT-PCR. We found an up-regulated expression of Dot1l mRNA level postnatally, consistent with the developmental changes observed in H3K79 methylation (Fig 6. B). We further performed experiments to check the expression of Dot1l in multiple mouse kidney cell lines. Dot1l mRNA expression was more abundant in differentiated epithelial cell lines IMCD3 than embryonic metanephric mesenchyme cell lines MK3 and MK4 (Fig. 6, C). Collectively, these results indicate that Dot1l and H3K79 methylation are developmentally up-regulated and are highly enriched in chromatin of differentiated renal epithelial cells, suggesting a potential role for this epigenetic modification in renal cell differentiation.

Figure 5. Expression of various histone modifications in mice kidney of embryonic day 13.5, day 17.5, postnatal day 1, postnatal day 20 and adult stage. Western blot analysis of H3K79 tri-methylation (H3K79me3), H3K4 tri-methylation (H3K4me3), H3K9 tri-methylation (H3K4me3), H3K27 di-methylation (H3K27me2) and H3K27 tri-methylation (H3K27me3) at different developmental ages of mouse kidneys (E13.5, E15.5, E17.5, Postnatal day 1(P1), Adult). Equal loading was monitored by Ponceau S staining and pan H3 expression.

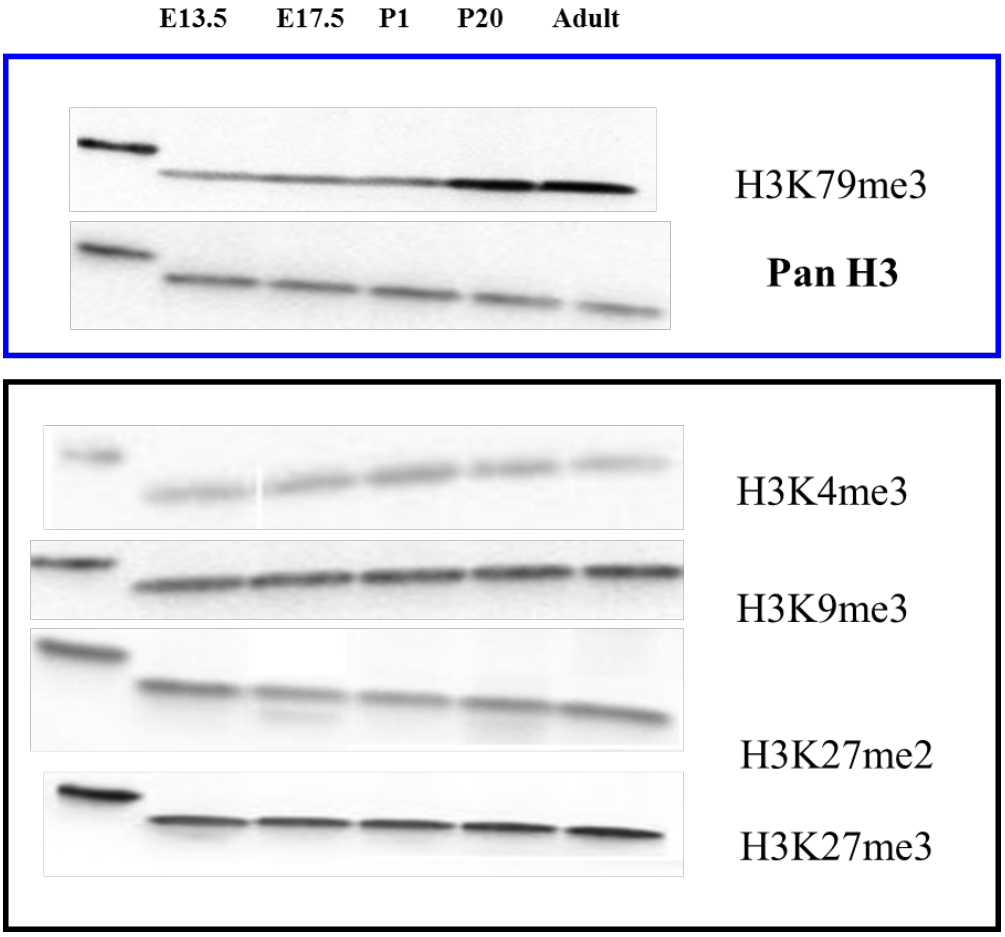

Figure 5. Expression of various histone modifications in mice kidney of embryonic day 13.5, day 17.5, postnatal day 1, postnatal day 20 and adult stage

Figure 6. Developmental expression of Dot1l-H3K79methylation in mouse kidney and mouse kidney cell lines. (A) Densitometric analysis of western blots was expressed as fold change compared to E13.5 expression level. (B) Quantitative real-time RT-PCR of H3K79 methyltransferase, Dot1l, showed up-regulation with postnatal renal maturation during renal development. Dot1l mRNA levels were normalized to Gapdh and expressed as fold change relative to E13.5 level. (C) RT-QPCR of Dot1l expression in various kidney cell lines (MK3, MK4, IMCD3, UB) with fold change expressed relative to MK3 expression levels. Dot1l was more abundant in renal epithelial cell lines (IMCD3, UB) than mesenchymal cells (MK3, MK4). *$P<0.05$

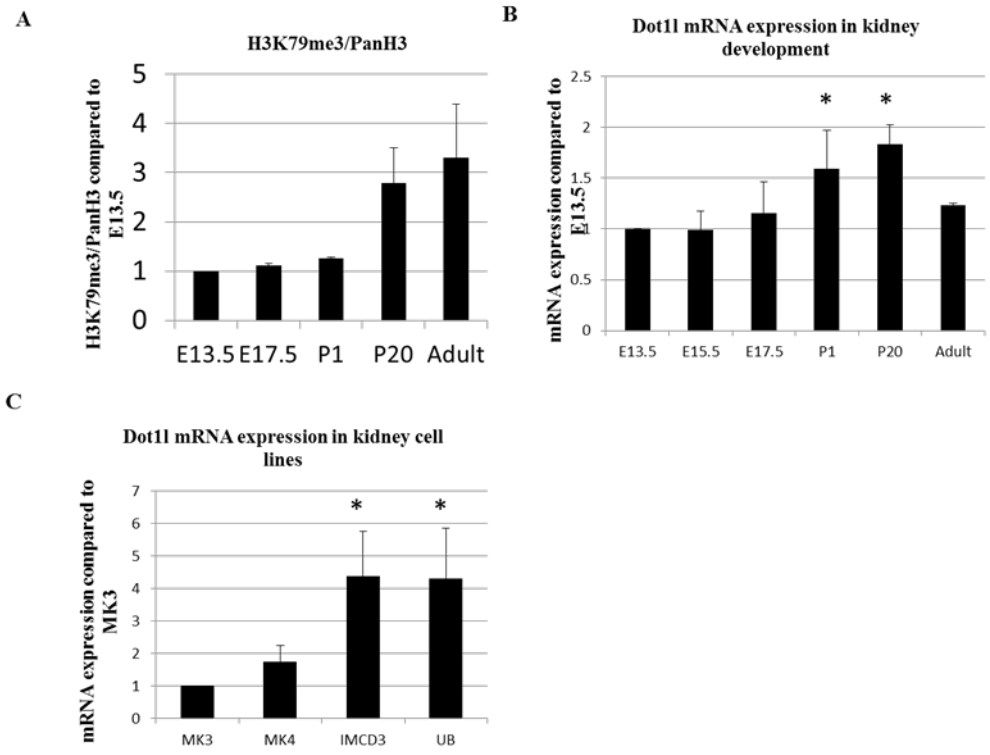

Figure 6. Developmental expression of Dot1l-H3K79methylation in mouse kidneys and mouse kidney cell lines

2. Spatial expression of H3K79 methylation in developing kidney

To further study the potential function of H3K79methylation and Dot1l in mouse kidney, we profiled the localization of this mark and its methyltransferase by immunostaining at different ages of kidneys. At the newborn stage, the outer cortical region of the kidney is still undergoing new nephron formation, while deeper regions are more differentiated and mature. H3K79me3 was found to be more abundant in more differentiated tubules than the nascent nephron region in postnatal day 1 (P1) kidney (Fig. 7, A). Differentiated tubules, marked by cytokeratin, were lined by H3K79me3 expression (Fig 7, B). In adult kidney, H3K79me3 and Dot1l were predominantly expressed in collecting duct principal cells marked by AQP2 as well as intercalated cells marked by Carbonic Anydrase Ⅱ (Fig 7, C). The co-localization of H3K79me3 with the terminal differentiation markers such as AQP2 and CAII suggests the role of Dot1l-H3K79me3 in terminal differentiation process.

Figure 7. Spatial expression of H3K79 methylation in developmental kidney.

(A) Upper figure: low power view (x4). Dotted line indicates the renal capsule. Low (x10) (middle figure) and high (x40) power views (lower figure) of the renal cortex showing a progressive increase in H3K79me2/3 in differentiating E-cadherin+ tubules. The nephrogenic zone and nascent nephrons (arrowheads) are almost devoid of H3K79me2/3. (B) P1 kidney sections were co-stained with H3K79me3 (green) and collecting duct marker cytokeratin (red). (C), Adult kidney sections were co-labeled with H3K79me3 (green) with principal cell marker AQP-2 (blue) and intercalated cell marker carbonic anhydrase (red). Arrow, PC, principal cells; asterisk: IC, intercalated cells.

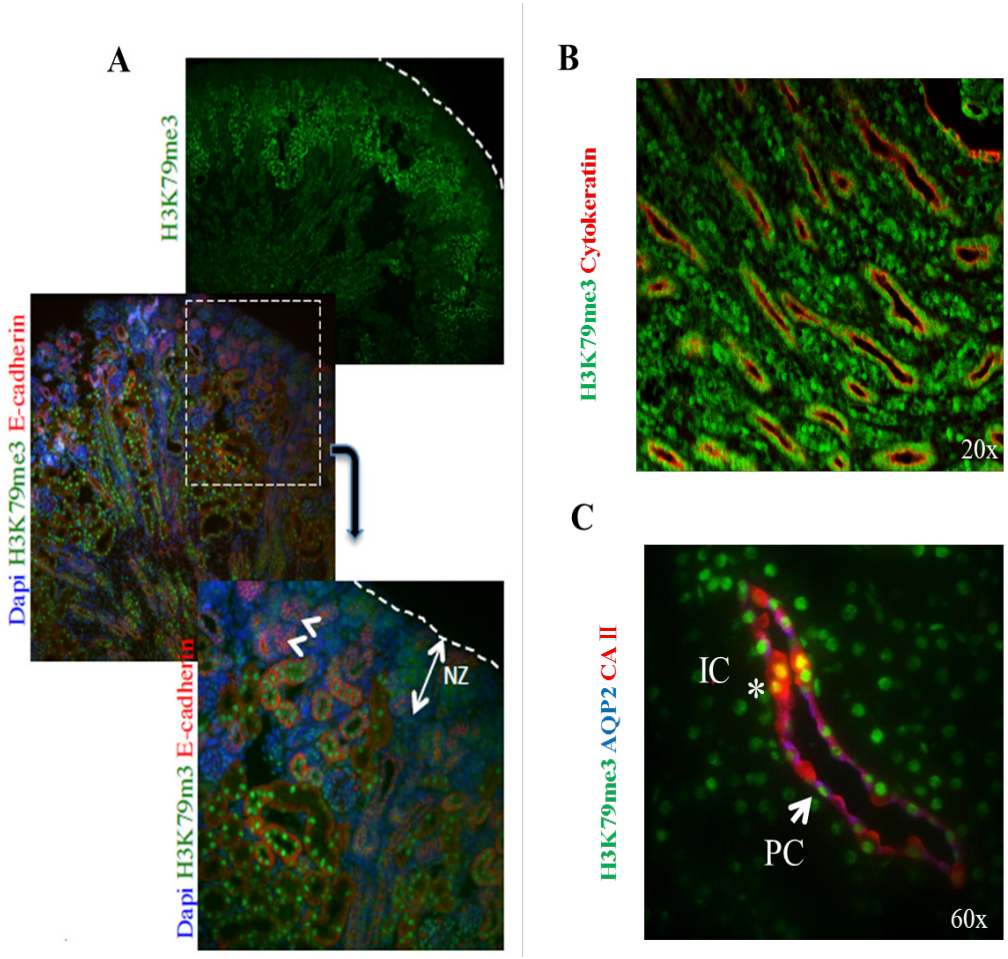

Figure 7. Spatial expression of H3K79 methylation in developmental kidney

3. Generation of ureteric-bud lineage cell specific Dot1l conditionally inactivated mice

Germline deletion of Dot1l causes embryonic lethality around E10.5 with stunted growth phenotype and cardiac failure (Jones et al. 2008). To characterize the physiological role of Dot1l in renal development, we generated conditional Dot1l KO mice in which Dot1l is specifically inactivated in UB lineage. Dot1l 3lox/+ mice were supplied by Dr. Chen from Novartis and then crossed with zp3 germline cre expression mice to yield Dot1l 2lox/+ or Dot1l 1lox/+ mice (Fig 8, A). We then generated UB lineage specific KO mice model through crossing with Hoxb7-cre mice. Dot1l 2lox/2lox Hoxb7-cre or Dot1l 2lox/1lox Hoxb7-cre mice served as conditionally Dot1l knockout mice with genotype results showed in Fig 8. B and are referred to as $UB^{Dot1-/-}$. Since Dot1l is the only responsible methyltransferase for H3K79 methylation, we examined H3K79me2 expression in E14.5 and P1 kidneys to check the cre recombination efficiency (Fig. 8, C-H). At E14.5 and P1, H3K79me2 expression was almost completely deleted from UB lineage cells compared with wild type mice (Fig. 8, C-H). $UB^{Dot1-/-}$ mice were born at normal Mendelian ratios. Thus, the successful Dot1l inactivation in UB lineage did not cause any embryonic lethality.

Figure 8. Generation and characterization of conditionally mutant mice.

(A), Loxp sites removal was achieved by crossing with zp3-cre germ line cre mice. Strategy of genetic crosses between Dot1l 2lox/+ mice and Dot1l 1lox/+ mice with Hoxb7-cre mice generated conditional inactivation of Dot1l in renal ureteric bud lineage cells. (B), Genotyping PCR was based on DF1&DR1 primers for Dot1l loxp alleles (320bp) and wild type alleles (240 bp) and EGFP primers for Hoxb7-cre transgene. (160bp) (C),Successful deletion of H3K79 methylation from UB lineage in mutant mice (right panel) versus wild type mice (left panel) revealed by co-staining of H3K79me2 (red) with ureteric bud lineage marker cytokeratin (first row, green) in E14.5 kidney sections and collecting duct marker AQP2 (second row, green) in P1 kidney.

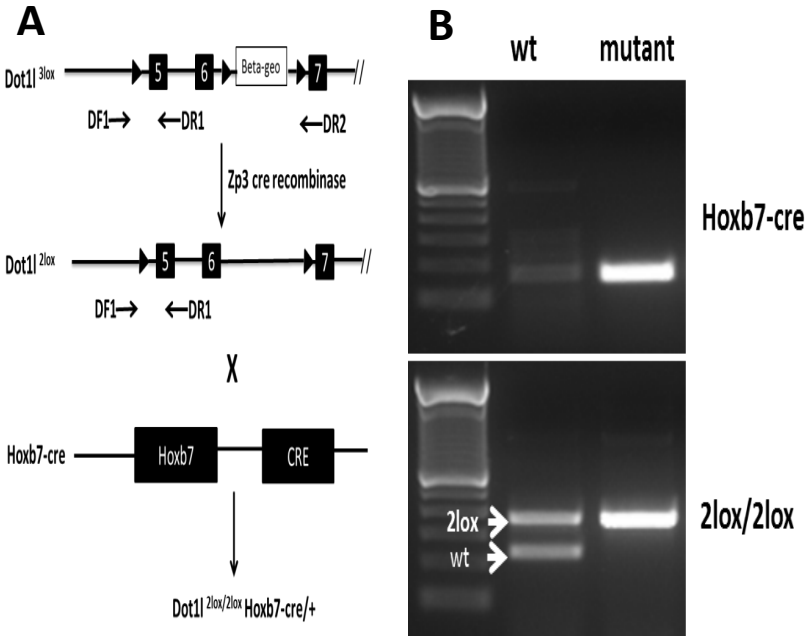

Figure 8. A-B Generation and characterization of conditionally mutant mice.

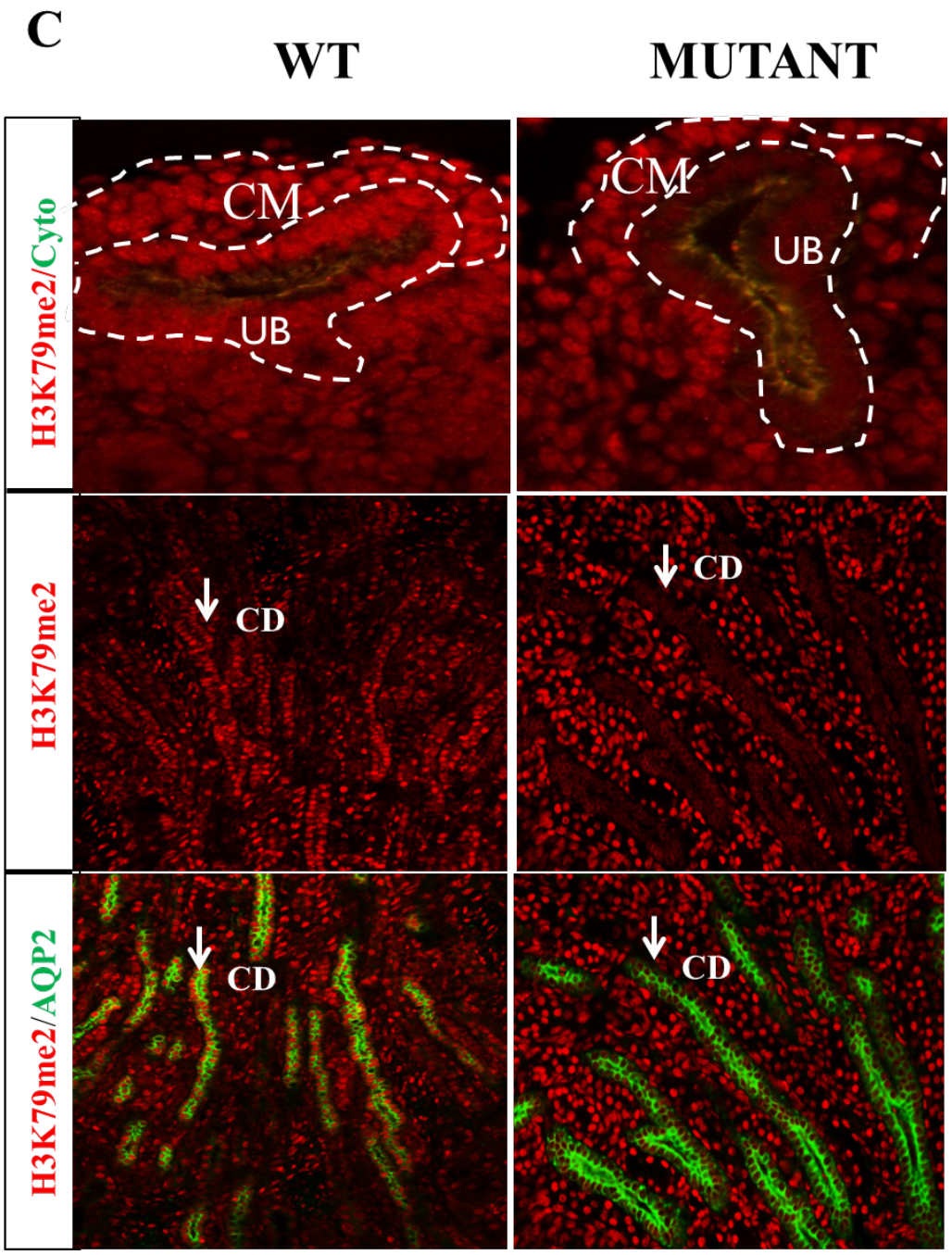

Figure 8. C Successful removal of H3K79methylation in ureteric bud lineage cells

4. Gross morphology of $UB^{Dot1-/-}$ mice

The gross morphology of mutant kidneys harvested at the newborn stage showed no abnormalities as compared to wild type kidneys (Fig. 9, A). Histological analysis of mutant and wild type kidneys at different renal development stages (E14.5, P1 and adult) displayed normal nascent nephrons, well-developed cortex and renal medulla (Fig. 9, B). The size of mutant kidneys during development was similar to wild type kidneys. Kidney weight were also comparable between several groups of wild type kidneys and mutant kidneys at P1 and P5 stage (n=3). In summary, the gross phenotype of the kidney was not affected by Dot1l inactivation.

Figure 9. Renal phenotype of conditionally mutant mice versus wild type mice.

(A), Representative images of newborn mutant mice (right panel) displayed a normal gross phenotype compared with wild type mice (left panel). (B), Statistical analysis for kidney weight at P1 and P5 stage between wild type groups (blue) and mutant groups (red). n=3. (C), H&E staining of paraffin sections showed normal histologic features of mutant mice kidney at different ages (E14.5, P1, Adult) (Left panel: wild type mice, Right panel: mutant mice). C represented cortex and M represented medulla part in the kidney.

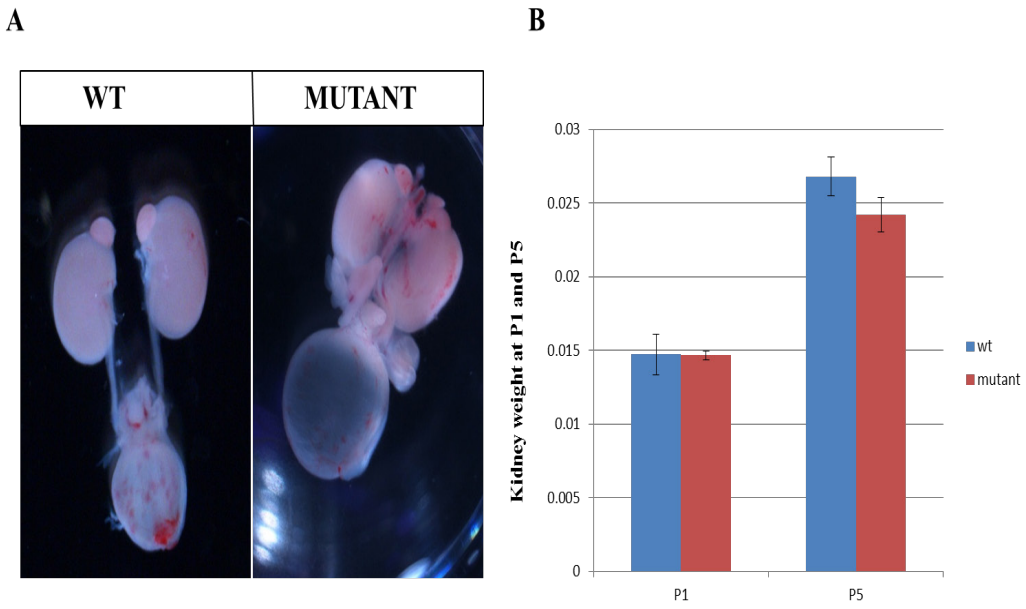

Figure 9. A. Renal phenotype of conditionally mutant mice versus wild type mice at newborn age

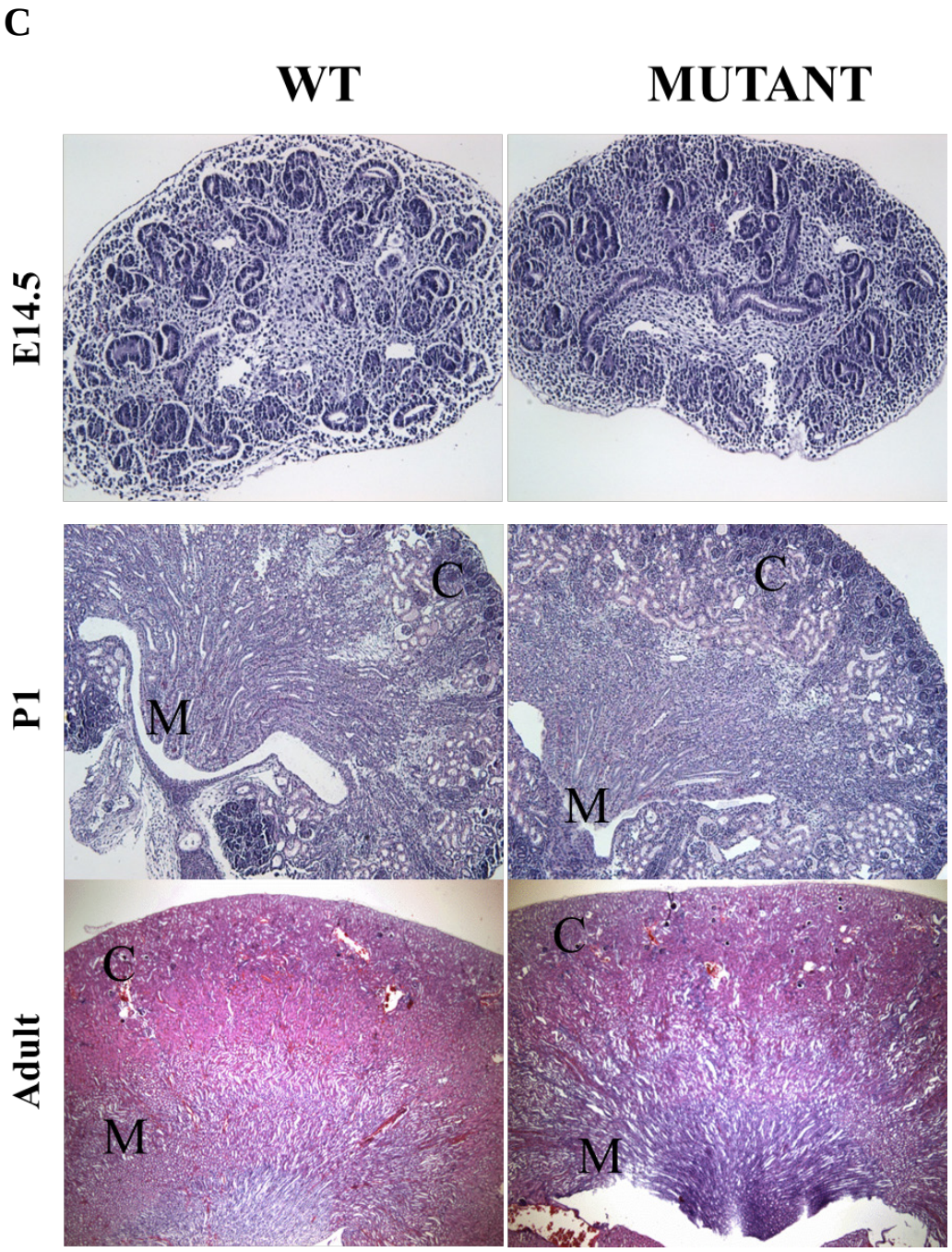

Figure 9. C Renal histological phenotype of conditionally mutant mice versus wild type mice at different developmental ages (E14.5, P1, Adult)

5. Effect of Dot1l deficiency on markers of renal maturation

We next asked whether Dot1l deletion alters the expression of renal developmental genes. Histologic sections from E14.5 WT and $UB^{Dot1l-/-}$ metanephric kidneys were subjected to immunofluorescence staining of multiple essential genes in early renal development, including Six2 (Fig.10 A-B), Lhx1 (Fig.10 C-D), Pax2 (Fig.10 E-F), LEF1 (Fig.10 G-H), Jagged-1 (Fig.10, I-J), Beta-catenin (Fig.10, K-L) and Laminin (Fig.10, M-N). Pax2 activates Gdnf expression and is required for the early establishment of nephric duct and UB formation (Little et al. 2012). Pax2 is expressed in the nephric duct, early uninduced metanephric mesenchymal cells adjacent to UB as well as the epithelial derivatives of mesenchymal cells (Brophy et al. 2001). Six2 marks cap mesenchymal cells and serves to renew and maintain nephron progenitor cells (Mugford et al. 2009). A subset of cap mesenchymal cells is induced by the UB to undergo the mesenchymal to epithelial transition. The canonical Wnt/beta-catenin signaling is involved in this MET process as well as in UB outgrowth and branching morphogenesis. During this process, Six2 is downregulated in association with activation of Lhx1, Wnt4 and Pax8 to form the pretubular aggregates. Lhx1 (also known as Lim1) is required to mediate the maturation of nephron progenitors to comma-shaped bodies and S-shaped bodies (Kobayashi et al. 2005). LEF1 (lymphoid enhancer factor), is a known target of the Wnt/beta-catenin signaling pathway and is expressed in condensed mesenchymal cells and differentiating renal vesicles. Laminin lines the basement membrane in developing UB and is required for UB branching morphogenesis (Brophy et al. 2001). Laminin is also found in comma- and S-shaped bodies, glomerulus and mature renal tubules (Virtanen et al. 1995). Jagged-1 is a member of

Notch signaling pathway which is essential in nephron progenitor differentiation to proximal tubules.

Consistent with the normal gross phenotype of $UB^{Dot1l-/-}$ mice, no significant difference was observed in expression of the above-mentioned developmental genes between the mutant and wild type kidneys. Accordingly, early steps of kidney development are not affected by Dot1l conditional inactivation in UB lineage.

Figure 10. Immunostaining of developmental genes in mutant kidneys versus wild type kidneys at E14.5 stage

Paraffin sections from E14.5 kidney of Dot1l mutant mice and wild type mice were labeled with different antibodies. The upper panels represented the wild type kidneys and lower panels represented the mutant kidneys. (A-B) Six2 was stained in red while DAPI in blue was utilized to label the nucleus staining. (C-D) E14.5 kidney sections were stained with Lhx1, which labeled the renal vesicles in green. (E-F) Renal developmental regulator Pax2 was stained in green to mark the nephron progenitors. (G-H) Representative figure for the co-staining of LEF1 (red) and cytokeratin (green) in Dot1l mutant kidney (H) versus wild type kidney (G). (I-J) Epithelial structures were outlined by the co-staining of E-cadherin (green) and Jagged-1 in red. (K-L) Immunofluorescence labeling on E14.5 kidney sections with beta-catenin antibody in green showed no difference between wild type kidney (K) and mutant kidney (L). (M-N) Laminin was stained in red to delineate the comma- and S-shaped bodies as well as renal tubules.

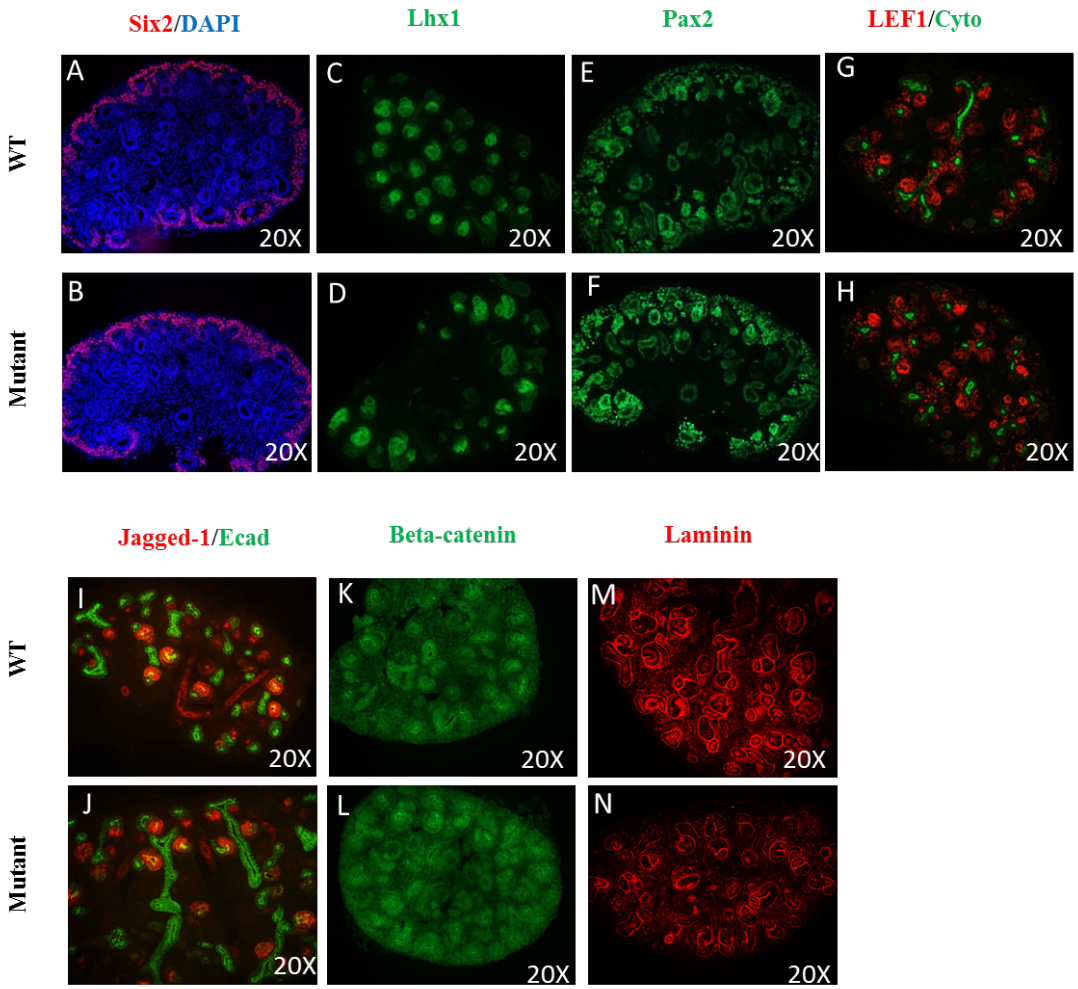

Figure 10. Immunostaining of developmental genes in mutant kidneys versus wild type kidneys at E14.5 stage

6. $UB^{Dot1l-/-}$ kidneys exhibits abnormalities in Wnt signaling

Although the gross morphology Dot1l mutant kidneys appears normal compared with wild type kidneys, it was still possible that Dot1l inactivation in the UB lineage impairs UB-specific developmental processes such as branching and terminal differentiation. In situ hybridization was therefore performed to examine the expression of UB-expressed developmental genes. Wnt7b is required for medulla formation and the coordinated growth of Loop of Henle (Yu, J. 2009). Wnt7b expression displayed no difference between wild type and mutant kidneys at E14.5 (Fig 11, A). However, the results in Fig.10 B revealed that Wnt7b expression level was down-regulated in $UB^{Dot1l-/-}$ kidneys at P1 stage. This result suggests a potential participation of Dot1l in medulla development. Wnt9b expression, which mediates MET, is also not altered in E14.5 kidneys but increased in P1 kidneys (Fig.11, A-B). In situ hybridization for Ret and Wnt11 revealed that both transcripts are higher in $UB^{Dot1l-/-}$ kidneys; interestingly, the Ret/Wnt11+ UB tip domain is enlarged in mutant kidneys (Fig.11, A-B). The transcription factor Foxi1, which mediates differentiation of intercalated cells from UB progenitors, is downregulated in E14.5 and P1 mutant kidneys vs. wild type kidneys (Fig.11, A-B). This result was quite surprising as it had been reported by Zhang's group that Dot1l deletion from Aqp2+ cells results in an increment in intercalated cell numbers (Wu et al. 2013). These observations implicated a potential new role of Dot1l-H3K79methylation in renal intercalated cell differentiation.

Figure 11. In situ hybridization with probes of renal functional genes in E14.5 and P1 kidneys

(A-B), Wnt7b and Wnt9b expression in E14.5 (A) showed no significant changes between wild type and mutant mice. In newborn kidney P1 sections (B), Wnt7b showed a decrease expression while Wnt9b expression was strongly enhanced in medulla collecting ducts of mutant kidney. Conditionally inactivation of Dot1l in ureteric bud lineage cells in E14.5 and P1 kidney displayed an expanded Ret/Wnt11+ ureteric bud tip region. In situ hybridization of Foxi1, which was the master regulator of intercalated cell fate, decreased in mutant kidney compared with wild type kidney at both E14.5 and P1 stage (Left panel: wild type mice, Right panel: mutant mice; A: E14.5 kidney sections; B: P1 kidney sections).

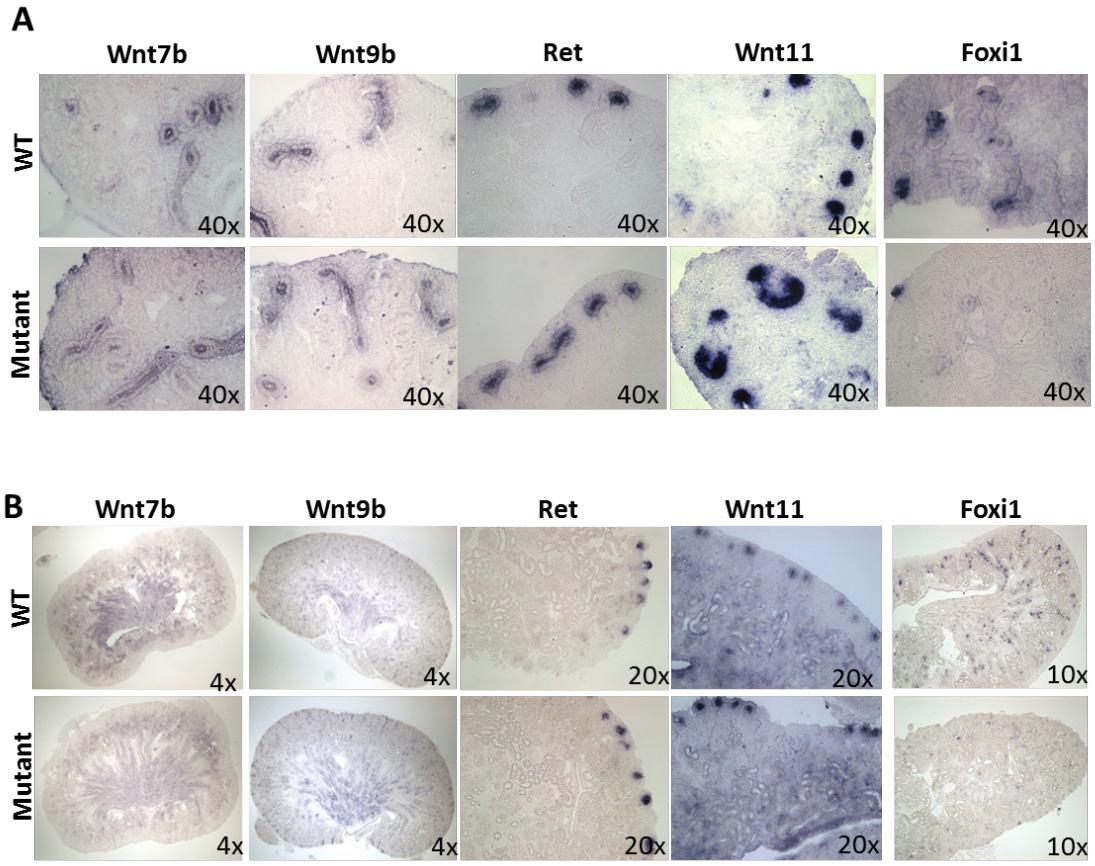

Figure 11. In situ hybridization with probes of renal functional genes (Wnt7b, Wnt9b, Ret, Wnt11, Foxi1) in E14.5 and P1 kidneys

7. UB branching and overall kidney size were not affected by conditional inactivation of Dot1l

Since Wnt genes and Ret were regulated by Dot1l conditional inactivation, we wished to examine whether UB branching was affected. Metanephric organ culture was utilized to monitor the impact of Dot1l inactivation on UB branching morphogenesis and overall kidney size. E12.5 embryonic kidneys were harvested from both mutant and wild type mice and cultured ex vivo. UB branching morphogenesis was carefully followed for 72h. Explants were then fixed in 100% methanol and stained with cytokeratin (UB marker) and Pax2 (epithelial nephron progenitor marker) antibodies. Numbers of UB tips and branches were counted in mutant and wild type kidneys. The results revealed that mutant kidneys exhibited normal UB branching morphogenesis: overall kidney size from 0h-72h and UB tip numbers at 72h were similar in wild type and mutant kidneys (Fig.12).

Figure 12. UB branching and overall kidney sizes were not affected by Dot1l deletion revealed by whole mount immunostaining of E12.5 kidneys cultured for 72h

E12.5 kidneys explants were cultured on transwell plates. Pictures were shot every 24h to monitor the growth of mutant and wild type kidneys until 72h. Organ cultured kidneys were then harvested at 72h for whole mount immunostaining with antibodies of pax2 (red) and cytokeratin (green). UB branching numbers were counted based on the immunostaining results and no statistical significance observed (N=10 kidneys).

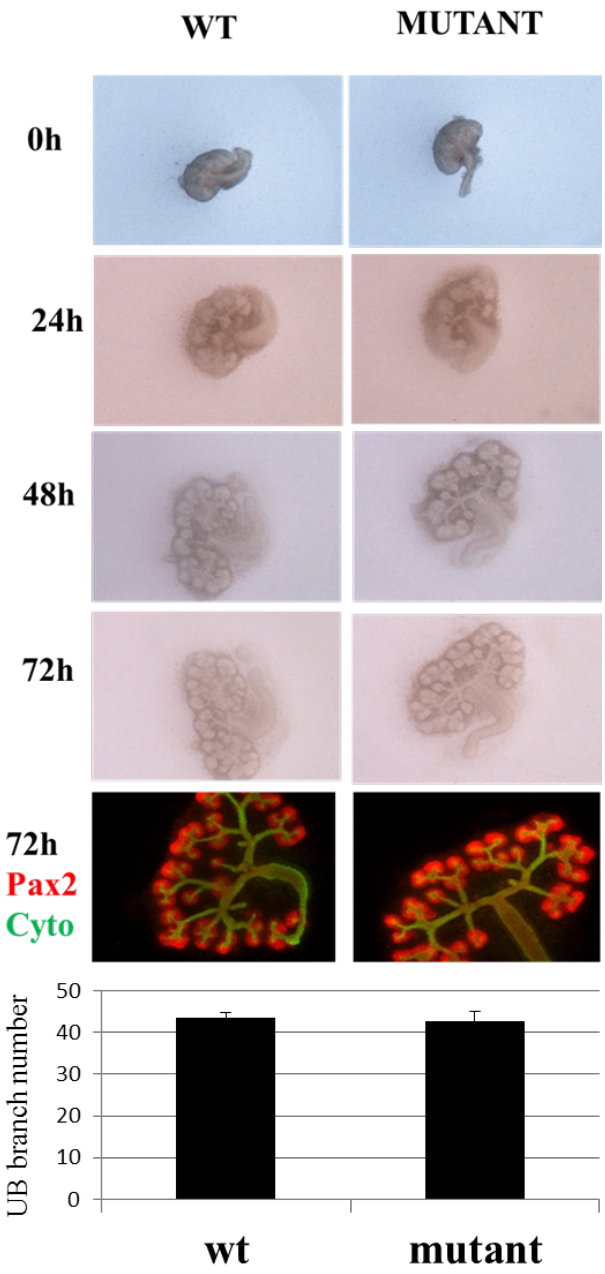

Figure 12. UB branching and overall kidney sizes were not affected by Dot1l deletion revealed by whole mount immunostaining of E12.5 kidneys cultured for 72h

8. Dot1l inactivation induces premature onset of p63-expressing UB tip cells and increases the number of p63+ and Pho-H3+ cells in collecting ducts at P1

We showed earlier that the intercalated cell master regulator Foxi1 is downregulated upon Dot1l inactivation. Current models suggest that UB epithelial progenitors give rise to plastic principal Aqp2+ cells, which have the potential to give rise to intercalated cells (Wu et al. 2013). Since $UB^{Dot1l-/-}$ mutant kidneys exhibit enlarged Ret/Wnt11+ UB-tip cell (UB-TC) domain, we asked whether UB tip cell differentiation was affected by the inhibition of H3K79methylation in UB lineage. Unpublished studies from our laboratory have shown that a subset of Ret^{+} UB-TC express the p53 family member p63. P63 is a master regulator of epithelial cell renewal and differentiation in skin and prostate (Candi, Rufini et al. 2006). In wild type embryonic kidneys, p63 expression is expressed in the ureteric and pelvic epithelium and in UB-TC and diminishes dramatically after postnatal day 5. Notably, loss of H3K79methylation in $UB^{Dot1l-/-}$ mice induced premature expansion of p63 expression domain as early as E12.5 and E13.5 in UB tip cells (Fig.13 A-B). At E14.5, p63 expression decorated UB-TC where H3K79methylation was lost (Fig.13 C). More interestingly, in mutant kidneys, p63 exhibited a much higher expression in UB tips at P1 stage along with a significantly higher number of p63+ cells in elongating collecting ducts (Fig.14, A, lower panels). This population of p63+ cells did not exist in the collecting ducts of wild type kidneys (Fig.14, A, upper panels). Co-staining of p63 and p-H3 (a marker of proliferating cells) in the mutant kidneys revealed a higher p-H3+ cell number in the mutant collecting ducts (Fig. 14B). Quantitative analysis of total p-H3+ cell number in the whole kidney also demonstrated a significant increase in p-H3 inDot1l mutant than wild type kidneys (Fig. 14C). Together, our findings

show that inactivation of Dot1l in the UB lineage causes premature expression of p63 and robust expression of p63 in the UB-TC and elongating collecting ducts. Moreover, the collecting ducts of mutant kidneys contained a higher number of proliferating cells than wild type kidneys.

The collecting ducts are comprised of two cell types which express Dot1l and its mark H3K79me2/3. Intercalated cells are responsible for acid-base homeostasis and are marked by carbonic anhydrase or H^+-ATPase. The principal cells are marked by AQP2 and are mainly involved in sodium-potassium and water balance. Previous studies had indicated that Dot1l depletion in $AQP2^+$ principal cells increases the proportion of intercalated relative to principal cells and eliminates H3K79 methylation in both cell types, suggesting that principal cells give rise to intercalated cells (Wu et al. 2013). Thus it was necessary to investigate the distinct features of the newly induced $p63^+$ cell subsets in collecting ducts of $UB^{Dot1l-/-}$ kidneys.

Figure 13. Dot1l inactivation induces premature onset of p63 expression

(A) Conditional inactivation of Dot1l in ureteric bud lineage induced premature onset of p63 expression in UB tip as early as E12.5, shown by immunostaining with 4A4 p63 antibody (green) and calbindin (red). Calbindin was utilized as an epithelial structure marker here. (B-C) Similar premature induced p63 expression had been observed in E13.5 mutant kidney (B) and E14.5 mutant kidney (C) compared with wild type kidneys (Upper panel: wild type kidney; Lower panel: mutant kidney).

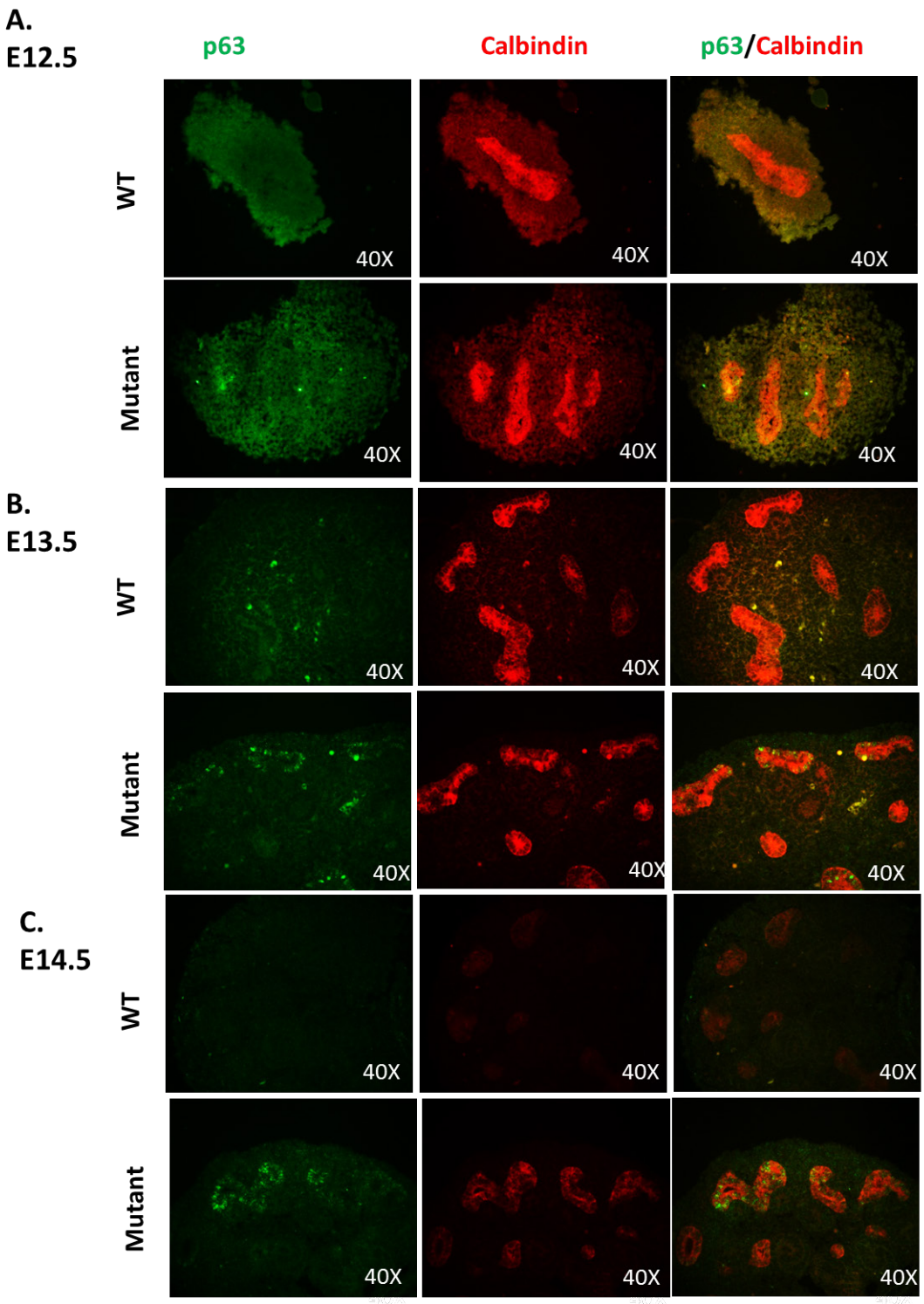

Figure 13. Dot1l inactivation induces premature onset of p63 expression

Figure 14. Loss of H3K79 methylation increased the number of p63+ and P-H3+ cells in collecting ducts at P1

(A)Immunostaining with anti-4A4 p63 (green) and AQP2 (red) in P1 kidney sections. Significant increase of p63 expression was found in UB tip cells; also, p63+ cells were present in elongating collecting ducts of mutant mice marked by AQP2. (B) P-H3 (red), cell proliferation marker, was increased in Dot1l mutant kidneys versus wild-type kidneys, especially in the p63 (green) populating collecting ducts marked by AQP2 in blue. (C) Quantitative analysis of whole kidney p-H3 cell number in wild type (left column) and mutant kidneys (right column). N=3 animals per group. * means P value is less than 0.05.

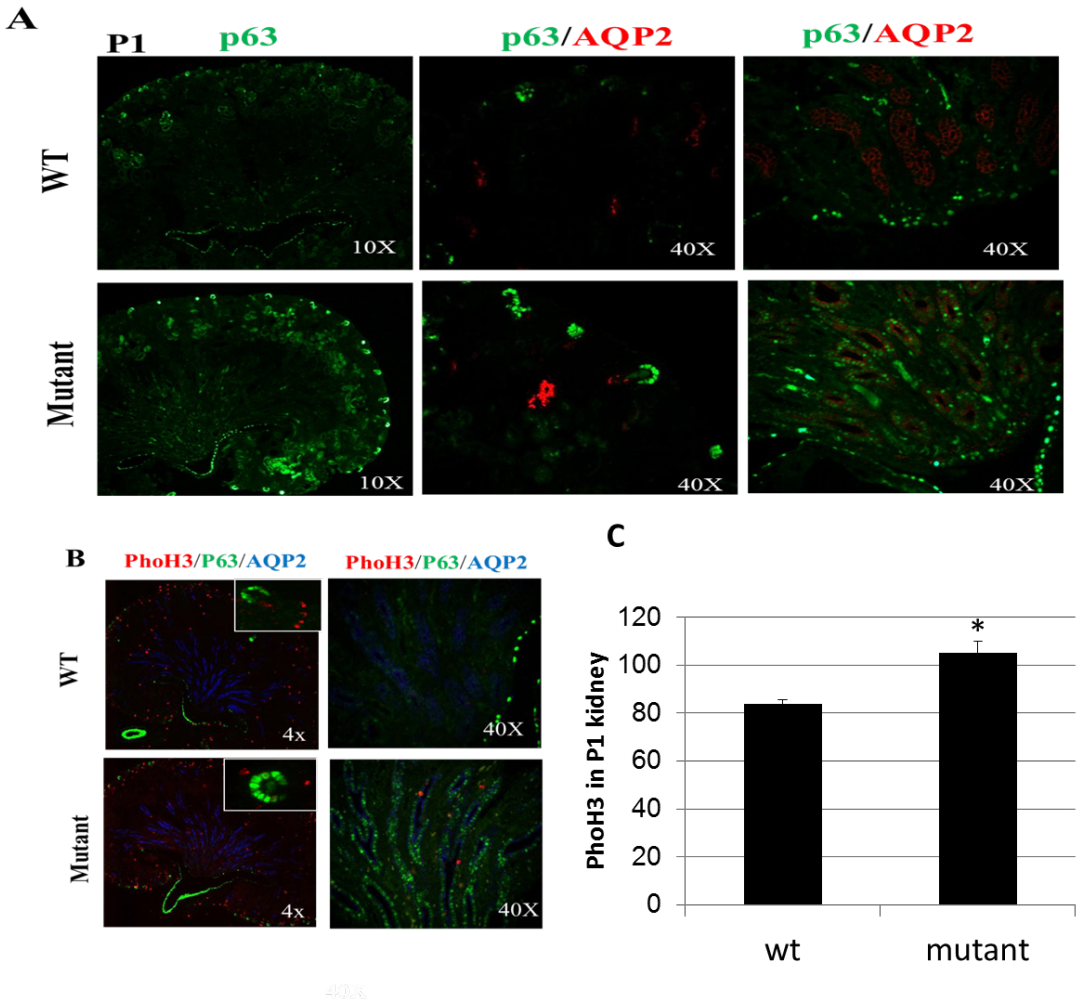

Figure 14. Loss of H3K79 methylation increased the number of p63^{+} and Pho-H3^{+} cells in collecting ducts at P1

9. H3K79methylation deficient collecting ducts showed a significant increase of p63 expression exclusively in H^+-ATPase negative cells along with decreased intercalated cell number

To delineate the nature of $p63^+$ cells in $UB^{Dot1l-/-}$ collecting ducts, immunofluorescence staining was performed on P1 kidney sections for p63, intercalated cell marker H^+-ATPase and principal cell marker AQP2 (Fig. 15). The results revealed the existence of a $p63^+$ cell population in the collecting ducts of mutant kidney (right panel) which was not observed in the wild type kidney (left panel) (Fig. 15 A-B). Under most circumstances, p63 was expressed in $AQP2^+$ cells (Fig. 15 E-F). However, we also observed a small population of cells which expressed p63 but were negative for AQP2 and H^+-ATPase (Fig.15 G-H). Interestingly, p63 was exclusively expressed in H^+-ATPase negative cells along the collecting ducts (Fig.15 G-H). Thus the p63+ subset of cells could be divided into two types: $p63^+$ $AQP2^+$ H^+-$ATPase^-$ cells and $p63^+$ $AQP2^-$ H^+-$ATPase^-$ cells, though the latter type comprised a modest percent of the whole cell population.

As shown earlier, Foxi1 expression is decreased at E14.5 and P1 mutant kidneys. As Foxi1is the master regulator of intercalated cell formation, it could be predicted that intercalated cells might be fewer in number in mutant vs. wild-type kidneys. Consistent with the Foxi1 in situ hybridization results, the relative number of carbonic anhydrase II^+ intercalated cells was decreased in the mutant kidneys (Fig.16 A, B, blue bar). It was intriguing that the relative percentage of $AQP2^+$ principal cells did not change when compared to the total cell number (Fig. 16, B, red bar). However, we observed that the percentage of non-principal/non-intercalated cells, considered as progenitor cells, was increased in the mutant kidneys (Fig. 16, B, green bar).

Collectively, p63, induced by the loss of H3K79methylation, was only expressed in non-intercalated cells. This was associated with a decrease in intercalated cell number in collecting ducts of mutant kidneys and an increase in progenitor cells. Taken together with the fact that Foxi1 was decreased upon the loss of H3K79methylation, these data suggest that Dot1l-H3K79methylation might affect the balance between intercalated cells and principal cells in the collecting duct through inducing proliferation of $p63^{+}$ progenitor cells and inhibiting the expression of Foxi1.

Figure 15. H3K79methylation deficient collecting ducts showed a significant increment of p63 expression exclusively in H^+-ATPase negative cells

In Dot1l-mutants, p63 (green) is not expressed in H-ATPase (red) marked intercalated cells but is expressed in some AQP2 (purple) marked principal cells. (A-B) Green color stained p63 expression was only observed in the Dot1l mutant kidneys (B) but hardly seen in the wild type kidneys (A). (C-D) H^+-ATPase was utilized to mark the intercalated cells in red. Much fewer H+-$ATPase^+$ cells had been observed in the mutant kidneys. (E-F) Immunostaining revealed the co-localization of p63 (green) and AQP2 (red) pointed out by arrowheads. (G-H) Immunofluorescence staining had shown that p63 was not expressed in H^+-$ATPase^+$ cells (red), which were pointed out by the empty arrowheads. The H^+-$ATPase^-$ $AQP2^-$ cells with p63 expression were pointed out by the arrows.

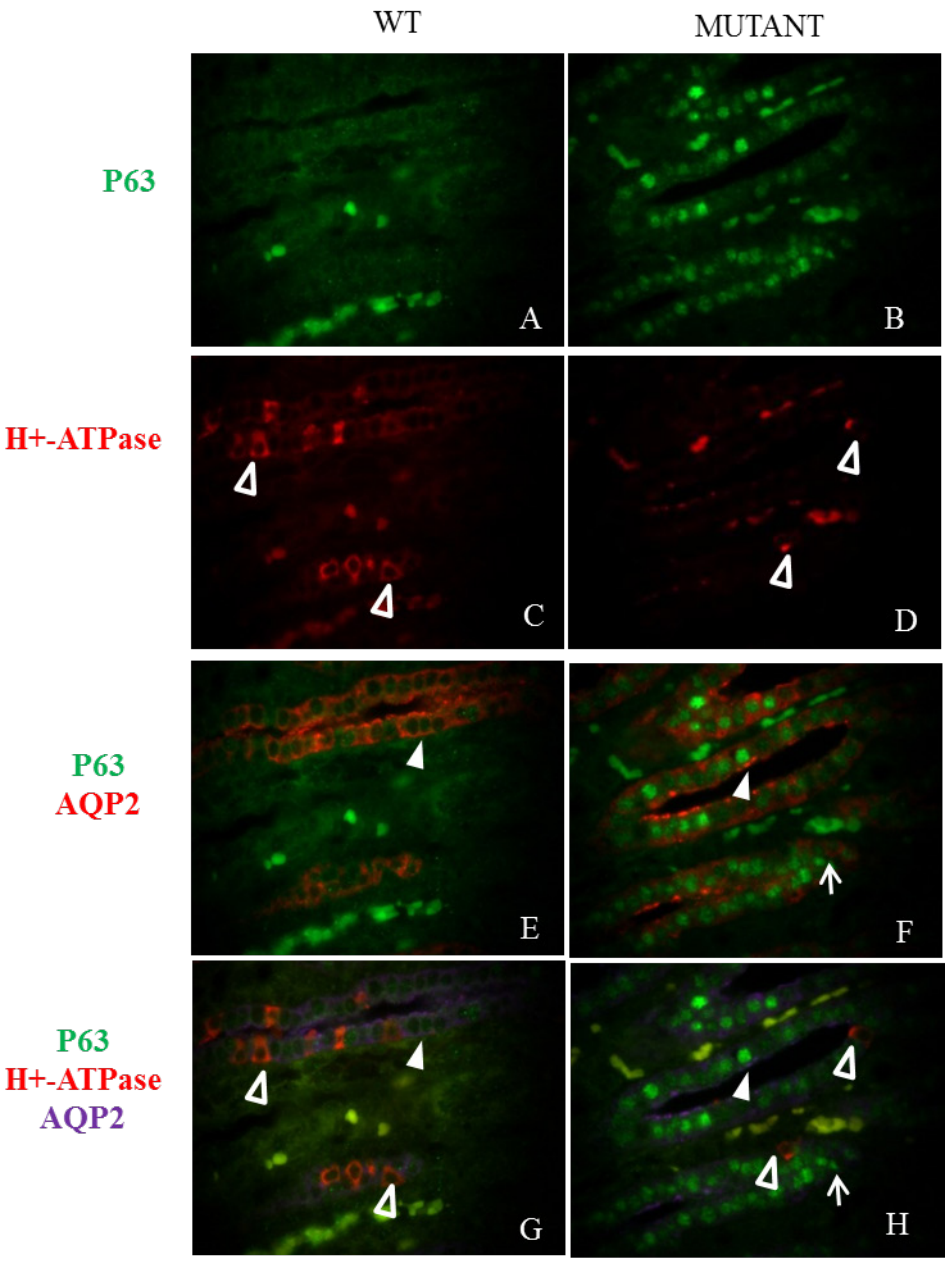

Figure 15. H3K79methylation deficient collecting ducts show a significant increment of p63 expression exclusively in H+-ATPase negative cells

Figure 16. Conditional inactivation of Dot1l in UB lineage cells leaded to a decrease in intercalated cell numbers along the collecting ducts

The two types of cells in collecting duct were marked by immunostaining of carbonic anhydrase II (red) and AQP2 (green). (A) CA2 expression was decreased in P1 mutant mice (right panel) compared with wild type mice (left panel). (B) Dot1l mutant mice at P1 stage had a lower ratio of intercalated cells/collecting duct cells (blue bar) but higher non-intercalated/principal cells ratio (green bar) compared with WT (Right panel: mutant kidney; left panel: wild type kidney). P-values were calculated as described in Chapter 2. * means $P \leq 0.05$. N=4.

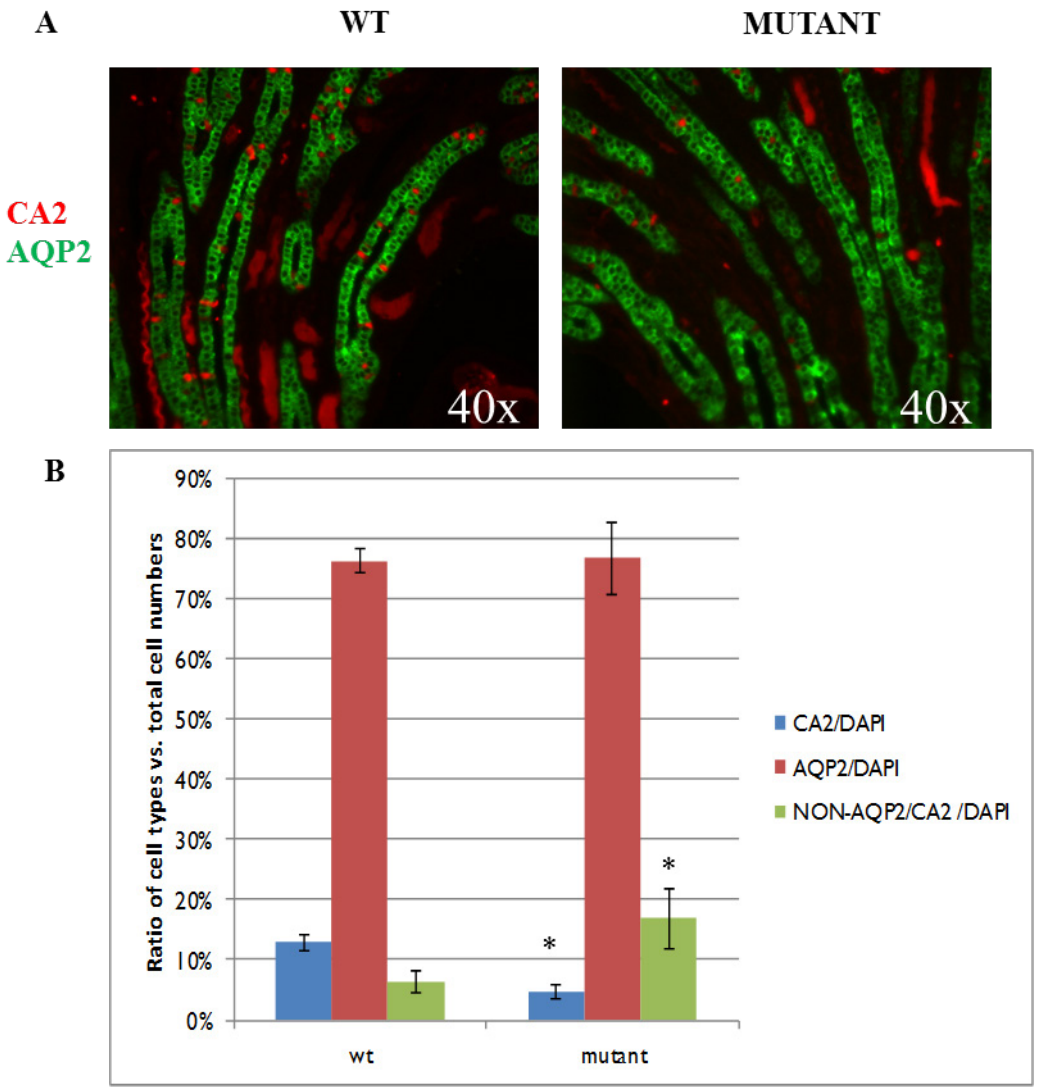

Figure 16. Conditional inactivation of Dot1l in UB lineage cells leaded to a decrease in intercalated cell numbers along the collecting ducts

10. H3K79me3 is decreased after ischemic injury and regained upon tissue recovery

Our previous data had shown that H3K79me2/3 is preferentially expressed in more differentiated renal tubules, suggesting a role for Dot1l in renal differentiation. Thus we wanted to determine the function of Dot1l-H3K79methylation in renal injury, tissue repair and regeneration. To this end, a renal I/R mouse model was utilized for in vivo studies while the mineral oil induced hypoxia in IMCD3 cells served as the in vitro study model. The in vivo I/R mouse model was performed by Dr. DeCasteker from Vanderbilt University. The overall strategy is depicted in Fig. 17A. The left side of renal artery of the mouse was clamped for 30min to generate ischemic injury and then released for reperfusion. The left and right kidneys were harvested 24h, 72h and 7days later. Immunofluorescence staining revealed that H3K79methylation was decreased in the ischemic kidney at 24h, and reached the lowest expression level at 72h. However, when the recovery or repair process began around 7d, H3K79methylation regained its expression (Fig.17 B). The decrease of H3K79me3 in the injured proximal tubules was also accompanied with an increase in cell proliferation indicated by PCNA (Fig.17 C). Similar findings were found in the in-vitro model. When the human proximal tubule cell line (HK-2 cells) was exposed to hypoxia treatment, Dot1l mRNA expression was reduced rapidly at 6h (Fig. 17 D). These findings together indicated the potential role of Dot1l-H3K79methylation in ischemia/reperfusion induced renal injury and repair process.

Figure 17. The expression change of Dot1l-H3K79methylation in renal ischemia/reperfusion model

(A) Schematic figure for experiment design of renal ischemia/reperfusion model establishment. (B) H3K79me3 (red) were co-stained with proximal tubule marker LTA (green) to reveal the expression change of H3K79me3 upon injury, especially in most injured proximal tubules at cortex region. H3K79me3 was initially reduced at 24h and most decreased at 72h. When it came to 7d, H3K79me3 was increased along with the renal tissue repair. Immunofluorescence staining in the medullary part with Dot1l (red) and collecting duct marker AQP2 (blue) showed a similar expression pattern as H3K79me3. (C) Cell proliferation marked by PCNA (blue) was increased in injured proximal tubules together with decreased H3K79me3 (red). Upper panel was referred to the control kidneys subjected to no injury and lower panel was showing the injured kidneys subjected to I/R for different time length as indicated. (D) HK-2 cells were treated with mineral oil for 90min to induce hypoxia. Cells were then harvested for mRNA extraction. QPCR results showed decreased Dot1l mRNA expression 6h after hypoxia treatment compared with expression in control cells. * means p value is less than 0.05. N=3.

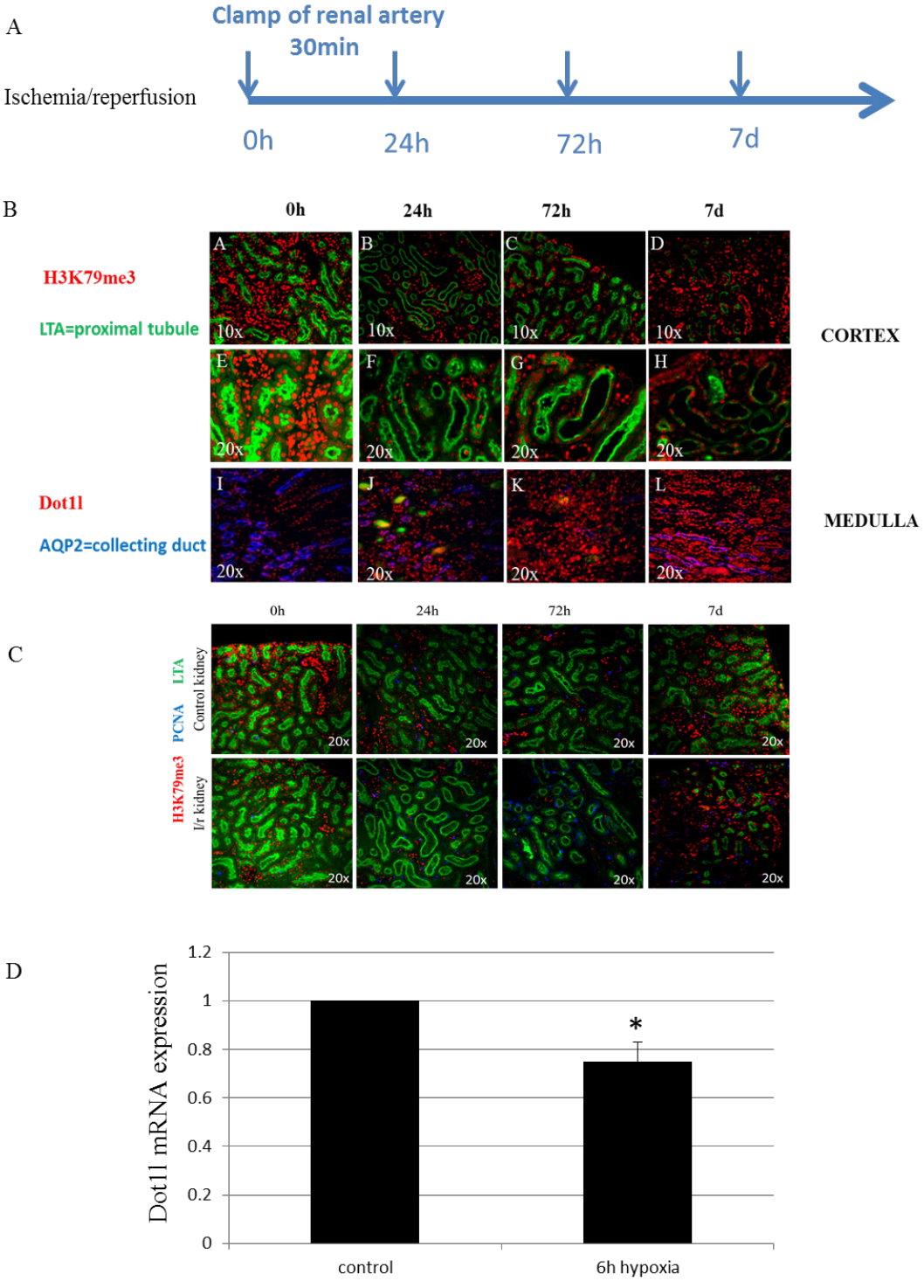

Figure 17. The expression change of Dot1l-H3K79methylation in renal ischemia/reperfusion model

11. Obstructed Dot1l mutant kidneys demonstrated more severe injury and fibrosis.

Since the adult Dot1l conditionally mutant kidney is structurally normal, we examined the effect of Dot1l deficiency superimposed on renal cell injury on tubular integrity; in this case, we chose the UUO model in which medullary collecting ducts will be the direct target site for injury. Male and female adult wild-type and $UB^{Dot1l-/-}$ mice were subjected to left unilateral ureter obstruction (UUO) for 7days. The left obstructed and right intact kidneys were harvested after 7 days of UUO and processed for histological and immunohistochemical analysis. Masson Trichrome staining was utilized to examine the extent of renal fibrosis (Fig. 18 A). The Dot1l mutant UUO kidneys demonstrated more severe injury and fibrosis (indicated by the blue area of MT staining) than wild-type UUO kidneys. Section immunofluorescence staining was performed for LTA (proximal tubule marker) in green, AQP2 (collecting duct marker) in blue and H3K79me2 in red. The results revealed more dilated and injured collecting ducts in Dot1l mutant mice (C, E) when compared with the wild type mice (B, D). Therefore, the loss of Dot1l-mediated H3K79methylation aggravates the severity of UUO injury.

Figure 18. Dot1l mutant mice demonstrate more severe injury and fibrosis when subjected to UUO injury

(A) Masson Trichrome staining in Dot1l mutant mice (right panel) showed more fibrosis and injury compared to wild type mice (left panel) when subjected to 7day UUO surgery. (B-E) Section immunofluorescence staining was performed to demonstrate more injury happened on the cortex and medullary part of Dot1l mutant kidney sections (C, E) compared with wild type kidney sections (B, D). H3K79me2 (red) was co-stained with renal tubule markers LTA (green) and AQP2 (blue). Arrows pointed out the dilated and injured collecting ducts.

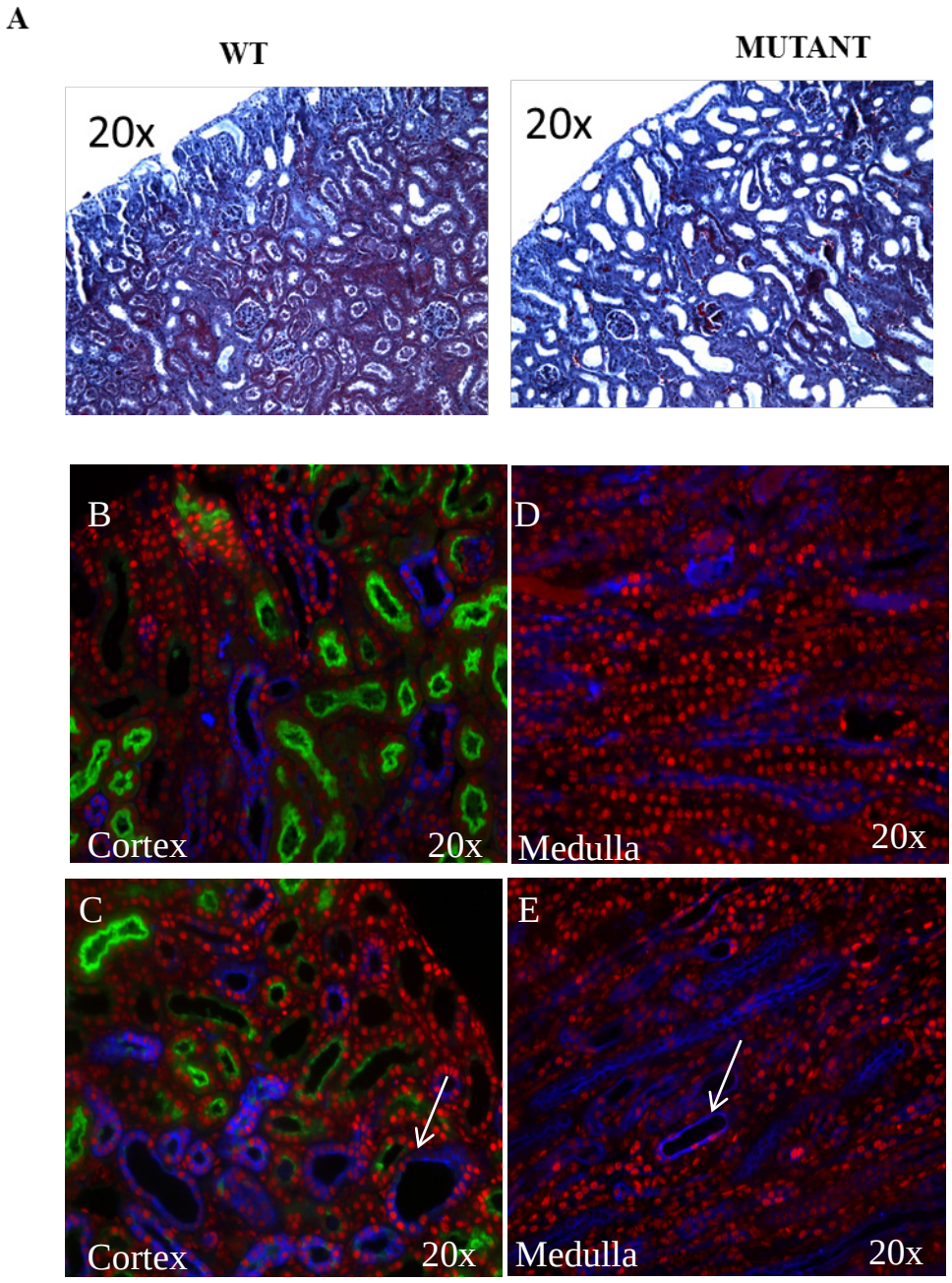

Figure 18. Dot1l mutant mice demonstrate more severe injury and fibrosis when subjected to UUO injury

CHAPTER 4: DISCUSSION

Epigenetic mechanisms play a key role in multiple biological processes, such as cancer and embryonic development. The current study investigates the role of histone methylation, specifically H3K79methylation and its sole methyltransferase Dot1l, in renal development, injury and tissue repair. The major findings of this study are as follows:

1. Global chromatin H3K79me3 levels increase markedly during kidney development together with its only known methyltransferase, Dot1l. Dot1l/H3K79methylation correlates with terminal differentiation of the renal epithelium.

2. H3K79 methylation is dispensable for early ureteric bud branching morphogenesis and collecting duct system development.

3. Chromatin-based mechanisms mediated via Dot1l/H3K79 methylation modulate the terminal differentiation fate of UB-TC presumably via induction of p63 and repression of Foxi1.

4. Dot1l-H3K79methylation is actively involved in renal injury and repair as illustrated by the mouse ischemia/reperfusion and UUO models.

1. Developmental expression of histone methylation in the kidney

Our studies have demonstrated a unique postnatal expression pattern for H3K79methylation and its methyltransferase Dot1l during renal development. Dot1l is the only methyltransferase for H3K79methylation since Dot1l inactivation leads to a complete loss of all three states of H3K79methylation (Barry et al. 2009). The demethylase for H3K79methylation has yet to be identified; however the demethylation of H3K79me2 has been found in early embryonic development (Ooga et al. 2008). This might explain the low expression level of H3K79methylation during early kidney development. We have also shown a relatively stable expression level for most other histone methylation marks during kidney development. In case of H3K27me3, the lack of developmental change may be related to the reciprocal maturational expression pattern of the two methyltransferases for H3K27: EZH1 and EZH2. Hence EZH2 is responsible for the early regulation of H3K27methylation in kidney, while EZH1 gradually takes over this role after birth.

2. Dot1l conditional inactivation in UB lineage

Wnt and their target genes are amongst the essential developmental renal regulators. The in situ hybridization results for Wnt target genes in the Dot1l mutant mice are quite interesting. Wnt7b, Wnt9b, Wnt4 did not show detectable changes at

early stages (E14.5) in Dot1l mutant mice. Postnatally, Wnt7b expression was downregulated while Wnt9b showed an increase compared with wild type kidneys. Previous studies have reported that Dot1l deficiency is accompanied by repression of Wingless target genes in Drosophila (Mohan et al. 2010). It has also been shown that Dot1l is required for the recruitment and regulation for Wnt target genes in a beta-catenin dependent manner in colorectal cancer (Mahmoudi et al. 2010). However, later studies in intestinal epithelium have demonstrated that the loss of H3K79methylation has no appreciable effect on intestinal homeostasis, Wnt target gene expression or the overall health of mice (Ho et al. 2013). Our results demonstrate that conditional inactivation of Dot1l in the UB lineage did not affect UB branching morphogenesis and nephrogenesis. Thus, Dot1l-H3K79methylation appears to be dispensable for embryonic kidney development. This may be the result of compensatory mechanisms mediated by other epigenetic regulations. For example, the histone code could be modulated to compensate for the loss of transcription activation caused by the loss of H3K79 methylation (e.g., H3K36 methylation may be activated to compensate for H3K79 methylation).

However, Dot1l-H3K79methylation might have an instructive role in disease states as suggested by the studies performed in pathological states (Ho et al. 2013). This conclusion is supported by our findings of Dot1l-H3K79methylation in renal ischemia/reperfusion and UUO models. It’s interesting to find increased cell proliferation is accompanied with the less H3K79me3 expression in renal I/F model. The decrease of H3K79me3 in the injured proximal tubules leads to the increased cell

proliferation as we find deficiency of H3K79me3 in renal collecting ducts can also induce cell proliferation. The decrease of H3K79me3 might be required for the initial injury response process and thus generate more cells to help participate the later repair. Additional work is required to determine the potential gene targets and signaling pathways in the injury/repair models. For example, Dot1l might modulate EMT through interacting with Tcf4/beta-catenin in the renal fibrosis caused by UUO.

3. A role of Dot1l-H3K79methylation in UB-TC fate and the balance between intercalated and principal cells in collecting ducts

13%-16% more intercalated cells have been found in collecting ducts of mutant mice harboring a deletion of Dot1 in AQP2-expressing principal cells. The newly generated intercalated cells are also deprived of H3K79methylation, which supports the idea that that intercalated cells are at least partly derived from the principal cells (Wu et al. 2013). Our findings challenge this conclusion, however. We found that intercalated cell/collecting duct ratio is actually lower in $UB^{Dot1l-/-}$ mice. We also find that Dot1l inactivation induce de novo expression of the epithelial stem cell marker, p63, exclusively in non-intercalated cells, specifically in either $AQP2^{+}$ cells or $AQP2^{-}$ H^{+}-$ATPase^{-}$ double negative progenitor cells. Indeed, quantitative analysis confirmed a significant increase in the $p63^{+}$ cell population upon the loss of H3K79methylation. Taken together with the fact that Foxi1 expression is also

decreased by Dot1l inactivation, we propose a model in which Dot1l inactivation provokes an expansion of p63 stem/progenitor cells which populate UB tips and transit into stalk/trunk cells. $P63^{+}$ trunk progenitors differentiate into principal cells or intercalated cells (Fig. 19). According to this model, inhibition of Foxi1 by Dot1l inactivation prevents the progenitor cells from differentiating into intercalated cells. It is interesting to note that Dot1l is expressed at very low levels at the 4-16 cell embryo stage and is only detectable by the blastocyst stage where it appears to mediate differentiation of ESC (Barry et al. 2009). This fits with our model where loss of H3K79methylation negatively affects further differentiation of $p63^{+}$ stem/progenitor cells. A possible explanation to the conflict with the model proposed by Zhang et al could be that, only the early deletion of Dot1l in UB lineage cells can induce a premature onset of p63 expression which induces more progenitor cells in early renal development. Since their model is knocking out Dot1l from cells expressing AQP2 which comes later around E17.5, collecting duct cells might have determined their destiny at that time. It could be highly possible that the population of progenitor cells will diminish postnatally with the maturation of medullary collecting ducts. However they might appear again when induced by injury or stress, as indicated by our findings of that the mice subjected to UUO injury show an induced expression of p63.

Collectively, the results of our study strongly support the hypothesis that although not essential in renal UB morphogenesis, Dot1l-H3K79methylation plays a novel role in terminal differentiation of the renal collecting duct by modulating the differentiation fate of UB tip and trunk progenitors into principal and intercalated

cells. Our study also identified p63 and Foxi1 as two novel transcription factors downstream of the Dot1l-H3K79me pathway.

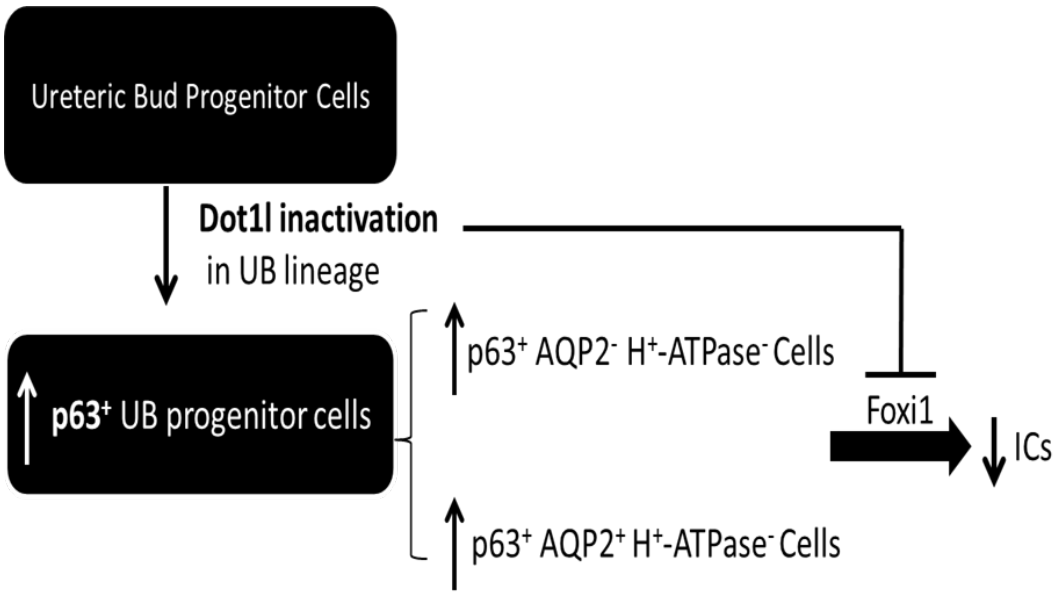

Figure 19. A proposed model for the role of Dot1l-H3K79methylation in regulation the transition balance of intercalated cells and principal cells

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BIOGRAPHY

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