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Aldosterone-sensitive repression of *ENaC* transcription by a histone H3 lysine-79 methyltransferase

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Abstract

Aldosterone is a major regulator of epithelial Na⁺ absorption. One of its principal targets is the epithelial Na⁺ channel α -subunit (*ENaC*), principally expressed in the kidney collecting duct, lung, and colon. Models of aldosterone-mediated *trans*-activation of the *ENaC* gene have focused primarily on interactions of liganded nuclear receptors with the *ENaC* gene promoter. Herein, we demonstrate that the murine histone H3 lysine-79 methyltransferase, murine disruptor of telomeric silencing alternative splice variant “a” (mDot1a), is a novel component in the aldosterone signaling network controlling transcription of the *ENaC* gene. Aldosterone downregulated mDot1a mRNA levels in murine inner medullary collecting ducts cells, which was associated with histone H3 K79 hypomethylation in bulk histones and at specific sites in the *ENaC* 5'-flanking region, and *trans*-activation of *ENaC*. Knockdown of mDot1a by RNA interference increased activity of a stably integrated *ENaC* promoter-luciferase construct and expression of endogenous *ENaC* mRNA. Conversely, overexpression of EGFP-tagged mDot1a resulted in hypermethylation of histone H3 K79 at the endogenous *ENaC* promoter, repression of endogenous *ENaC* mRNA expression, and decreased activity of the *ENaC* promoter-luciferase construct. mDot1a-mediated histone H3 K79 hypermethylation and repression of *ENaC* promoter activity was abolished by mDot1a mutations that eliminate its methyltransferase activity. Collectively, our data identify mDot1a as a novel aldosterone-regulated histone modification enzyme, and, through binding the *ENaC* promoter and hypermethylating histone H3 K79 associated with the *ENaC* promoter, a negative regulator of *ENaC* transcription.

Keywords

gene regulation; collecting duct; Na⁺ transport; chromatin; epigenetic; Dot1

The maintenance and restoration of Na⁺ balance are critical to the normal physiology of all eukaryotic cells. The epithelial Na⁺ channel (ENaC), a heteromultimeric ion channel composed of α -, β -, and γ -subunits, mediates the rate-limiting step for Na⁺ absorption across epithelial cells that line the distal part of the renal tubule, the distal colon, the ducts of several exocrine glands, and the lung airways (8). In the renal tubule, Na⁺ reabsorption by ENaC is one of the essential mechanisms involved in the regulation of Na⁺ balance, extracellular fluid volume, and blood pressure (10,19,26,30,34). Activating mutations in

channel subunits, which alter the rate of Na^+ transport, can cause hypertension with hypokalemia and alkalosis (31,34). ENaC is also an important molecular target of aldosterone. Increased circulating mineralocorticoids, as demonstrated in primary hyperaldosteronism, also promote hypertension with hypokalemic alkalosis from increased Na^+ reabsorption via ENaC (26).

The biological actions of aldosterone on target gene transcription have generally been believed to be mediated principally or exclusively through the mineralocorticoid receptor (MR), a member of the nuclear hormone receptor family. Aldosterone has both immediate (<3 h) effects on ENaC, attributed to increased trafficking or activity of ENaC in the apical membrane, and delayed actions (>3 h) that involve the synthesis of new ENaC subunits (1,2,15,18,33). In the cortical collecting duct, aldosterone administration or hyperaldosteronism induced by a low- Na^+ diet increases *ENaCa* gene transcription, without increasing β - or γ -subunit expression (3,6,15,23,28), and without a separate effect on *ENaCa* mRNA turnover (17). Therefore, in these cells, synthesis of the *ENaCa* gene is believed to be the rate-limiting step in Na^+ channel formation. A highly conserved imperfect glucocorticoid-response element (GRE) has been identified in the 5'-flanking regions of the human, mouse, and rat *ENaCa* genes and found to be necessary for *trans*-activation mediated by either the glucocorticoid receptor or MR, upon stimulation of glucocorticoid or aldosterone (12,17,35). The proximal 1.56 kb of 5' upstream sequence of murine *ENaCa* (*mENaCa*) contains the conserved GRE and multiple GRE half sites and is required for cell-specific expression and corticosteroid-mediated regulation (12).

The available data suggest that both GRE-dependent and -independent mechanisms control *ENaCa* transcription, and tissue-specific transcriptional controls on the gene are operative (3,6,14,28). Aside from interaction with liganded GREs and the finding that high mobility group protein I-C interacts with protein inhibitor of activated signal transducer and activator of transcription-3 in a cooperative way to inhibit GR: dexamethasone-stimulated transcription of the *ENaCa* gene (35), only limited information exists regarding the specific mechanisms governing transcriptional regulation of this gene, and no information is available about epigenetic mechanisms exerting such controls. Before interacting with the cognate hormone response element, the ligand-receptor complex must have accessibility to the DNA, which is compacted into the chromatin. According to the histone code hypothesis, dynamic changes in chromatin structure induced by sequential or combinatorial histone modifications, such as acetylation and methylation, play an essential role in many biological processes (29). Although previous studies (9,11,25,27) have discovered numerous aldosterone-regulated genes in various settings, none of the identified genes has a known function in histone modifications or chromatin remodeling. Therefore, it is largely unknown how aldosterone induces chromatin alterations to regulate transcription.

Histone methylation is catalyzed by histone methyltransferases (HMTs), which consists of three families: the suppressor of variegation, enhancer of zeste, and trithorax domain family of lysine HMTs, the protein arginine methyltransferase family of arginine HMTs, and the Dot1 (disruptor of telomeric silencing) family. Unlike the other two types of HMTs, which target the NH_2 terminal tails of histones, Dot1 specifically methylates histone H3 K79, which is located in the globular domain. Histone H3 K79 methylation is enriched at the recombination-active rather than at the inactive loci in mammalian cells, and has been considered to be a conserved hallmark of active chromatin regions (20). We previously characterized mDot1 and its mRNA expression in the mouse kidney and mIMCD3 cells (derived from the mouse inner medullary collecting duct) and identified five alternative splicing variants (mDot1a-e) (36). Because mIMCD3 cells share many of the phenotypic properties of the inner medullary collecting duct in vivo (24), including responsiveness to aldosterone (Ref. 9, and results herein) and expression of mENaCa and express mDot1a as

the principal mDot1 splicing variant (Ref. 36 and data herein), we used mIMCD3 cells as our model. In this study, we report that mDot1a mediates a novel signaling network participating in the control of aldosterone-sensitive *transactivation* of *mENaCa* that directly links aldosterone-elicited chromatin modifications to *mENaCa* transcriptional activation and reveals new functional and regulatory aspects of mDot1a and thus histone H3 K79 methylation in general.

MATERIALS AND METHODS

Reagents

Antibodies against dimethyl H3 K79, acetylated H3 K9 or the NH₂ terminal tail of H3 (Upstate), α -tubulin (Santa Cruz), or EGFP (Clontech) were used at 1:1,000 for Western blot analysis. Lipofectamine 2000 reagent (Invitrogen), chromatin immunoprecipitation (ChIP) kits (Upstate), and aldosterone (Sigma) were purchased and used according to the manufacturer's instruction.

Plasmids

EGFP-Dot1a constructs (36) have been described. To clone mDot1a and the methyltransferase-dead mutant mDot1a^{R_{CR}} into pcDNA3.1 (+), we used the annealed oligonucleotides (WZ756 5' TTAAGATGGCTCATATGCTCGAGCGAATTCTGCGGTACCG-GATCCGGGCC 3' and WZ757 5' CGGATCCGGTACCGCA-GAATTCGCTCGAGCATATGAGCCATC 3') to replace the *Afl*III-*Apa*I fragment of pcDNA3.1 (+). The resulting plasmid was named pcDNA3.1(+)-V2. Next, an *Eco*RI-*Kpn*I fragment containing mDot1a isolated from pEGFPC3-mDot1a was cloned into pcDNA3(+)-V2 at *Eco*RI-*Kpn*I, generating pcDNA-mDot1a. pcDNA-mDot1a^{R_{CR}} was similarly constructed. A 1.3-kb fragment containing a Zeocin-expressing cassette under control of the EM-7 promoter was generated by PCR, using pcDNA4/TO/Myc-HisC (Invitrogen) as the template, and ligated into the luciferase reporter vector pGL3Basic (Promega) at the *Bam*HI-*Afe*I sites to generate pGL3Zeocin, which allows selection with Zeocin (Invitrogen) for stable transfections. The *mENaCa* 5'-flanking region [1.3 kb; -850 to +536, relative to the major transcription start site (12)] was amplified by PCR with the genomic DNA isolated from a C57BL/6 adult mouse tail as a template and inserted into pGL3Zeocin at the *Mlu*I-*Xho*I sites. The resulting plasmid was designated pGL3Zeocin-1.3 *mENaCa*. Three mDot1a target sequences (RNAi1 AACAACTACGAGCCCT-TCTCC, encoding aa 125–132; RNAi2 AATACACACTGGAAC-GAGGTG, encoding aa 214–221; and RNAi3 AACAGTCGGAA-CAGCTGGAGA, encoding aa 577–584) were annealed and cloned into pSilencer4.1-cytomegalovirus (CMV)-Neo (Ambion) at *Bam*HI-*Hind*III according to the manufacturer's instructions. The resulting plasmids were named pSCN-RNAi1, pSCN-RNAi2, and pSCN-RNAi3, respectively. The authenticity of all constructs was verified by sequencing the junctions and inserts before transfection.

Generation and characterization of anti-mDot1 antiserum

The anti-mDot1 antiserum was generated exactly as we previously described (37), except for the different immunizing peptide CETMDRE-FRKWMKWY (aa 196–209 of mDot1a) used to raise the antibody in rabbits (SigmaGenosys, The Woodland, TX). The amino acid sequence, excluding the NH₂ terminal cysteine residue for linkage to keyhole limpet hemocyanin, is also identically present in mDot1b (36) and hDot1L (7).

Cell culture, aldosterone treatment, transient and stable transfections, and RNA interference

mIMCD3 cells were maintained with DMEM/F12 plus 10% FBS. For aldosterone treatment, cells were seeded and cultured with DMEM/F12 plus 10% FBS for 24 h, then switched to DMEM/F12 plus 10% charcoal-stripped FBS for 16 h before the addition of 1 μ M aldosterone or 0.1% ethanol as vehicle, and harvested at the time points indicated in the text and figure legends. In experiments to demonstrate the dose-dependent response, 100, 10, and 1 nM aldosterone, respectively, were used and cells were harvested after 2 or 7 h. In experiments involving transient transfection, cells that were similarly seeded and cultured as above were transfected using the Lipofectamine 2000 reagent as described previously (36) with the plasmids indicated in the text and figure legends. Five hours after transfection, the medium was replaced with DMEM/F12 supplemented with 10% charcoal-stripped FBS. Sixteen hours later, the cells were harvested directly or treated with aldosterone (1 μ M) or 0.1% ethanol for the indicated time. To establish stable mIMCD3 cell lines, mIMCD3 cells were transfected as above with *AfeI*-linearized pGL3Zeocin-1.3mENaCa. Forty-eight hours after the transfection, cells were cultured and selected in DMEM/F12 plus 10% FBS and Zeocin (800 μ g/ml) for 3 wk, with medium changes every 2–3 days. Five independent colonies (designated C9–C13) were isolated and expanded into cell lines. The remaining survival cells were pooled as a mixed line (C14). All six cell lines were then tested for aldosterone induction of *mENaCa* promoter-driven luciferase activity. Cell lines C10, C12, and C14 exhibited 3- to 6-fold induction 24 h after aldosterone administration (data not shown). C14, which might be more representative, was chosen for further study. For the generation of mIMCD3 cells stably expressing untagged or EGFP-tagged mDot1a, the cells were transfected similarly as above with plasmids derived from pcDNA3.1-V2 or pEGFP-C3 harboring wild-type and mutant mDot1a sequences, and selected with Geneticin (G418, 800 μ g/ml) for 4 wk. Six to ten surviving individual or pooled colonies from each transfection were characterized for expression of the full-length mDot1a by Western blot analysis with the antibodies against mDot1a or EGFP. To knock down mDot1a mRNA levels by RNA interference, pSCN-RNAi1, pSCN-RNAi2, and pSCN-RNAi3 were transfected individually into mIMCD3 cells to establish stably transfected cell lines after selection with Geneticin (800 μ g/ml). Ten to fifteen colonies from each transfection were investigated with real-time RT-quantitative PCR (qPCR) for the efficiency of silencing mDot1a expression, and those with the lowest mDot1a mRNA level were chosen for further analysis. pSilencer4.1-CMV-neo negative control (Ambion) was similarly transfected and used as a negative control.

Western blot analysis, histone preparations, and luciferase assays

Western blot analysis was performed according to our published protocols (13,38), with the exception of a modified cell lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, pH 8.0, 1% Triton X-100, 1% SDS, and a cocktail of protease inhibitors) used to prepare whole cell lysates. Acid extracts abundant in free histones were prepared as described (36). Luciferase activity was measured using the Dual-Luciferase kit according to the manufacturer's instruction (Promega) and reported as a ratio of firefly luciferase to *Renilla* luciferase.

Indirect immunofluorescence microscopy

Indirect immunofluorescence microscopy was performed as described in our previous publications (13,38). In brief, 6-wk-old adult mice were anesthetized with ethyl ether and perfused via the abdominal aorta with 4% paraformaldehyde in PBS. Kidneys were isolated and fixed in 10% neutral buffered formalin, embedded in paraffin blocks, and cut into 5- μ m-thick sections. Paraffin sections were pretreated with a microwave oven for antigen recovery and then blocked with 1% normal sheep serum in 1 $\times$ antibody dilution buffer (1% BSA in 0.3% Tween-PBS) for 1 h. The sections were incubated with 1:100 dilution of the

polyclonal antibody against dimethyl H3 K79 antiserum, raised in rabbits, and 1:100 dilution of anti-aquaporin-2 LC54, generated in chickens (from Dr. James B. Wade, University of Maryland) overnight at 4°C in a humidified chamber. After being washed three times in 0.3% Tween-PBS for 10 min, slides were incubated with Alexa Fluor 488 goat anti-rabbit IgG and 594 goat anti-chicken IgG (both at 1:1,000 and from Molecular Probes, Eugene, OR) for 1 h at room temperature in a dark humid chamber and then washed in 0.3% Tween-PBS four times for 10 min/wash and rinsed in deionized water. Slides were stained with 4',6-diamidino-2-phenylindole, mounted with Gel/Mount (Biomedica, Foster City, CA) and visualized. Samples without primary antibody were used as control. Images were acquired with a Nikon TE2000-U microscope outfitted with epifluorescence and a Cascade digital camera (Roper Scientific, Tucson, AZ), and images were acquired and processed using Metamorph software version 6.0 (Universal Imaging, Downingtown, PA).

ChIP, real-time qPCR, or RT-qPCR and statistical analysis

ChIP assays were performed as described previously (16), except for use of our modified cell lysis buffer described above and the sonication setting (3×10 s, duty cycle: 50% and output control: 6) using a Sonifier Cell Disruptor 350 (Branson). Gradient annealing temperatures were employed to optimize the PCR conditions for each primer pairs, yielding a single sharp band with correct size and identity revealed by agarose gel and sequencing analyses. Real-time qPCRs were then performed on a DNA Engine Opticon System 2 (MJ Research) with 0.5× SYBR Green Supermix (Bio-Rad). Each sample was amplified in triplicate. Serially diluted input DNA derived from the vehicle-treated sample was used as a positive control and assigned an artificial copy number to establish a standard curve for each pair of primers at a fixed threshold cycle detection level. The copy number of each test sample was then automatically calculated according to its threshold cycle value, measured from cycle-dependent product amplification curves. The relative dimethylated histone H3 K79 at each region was calculated by measuring the apparent immunoprecipitation efficiency (the ratio of the copy number of the ChIP sample to that of the corresponding input) (21). In real-time RT-qPCR, all RNA samples were pretreated with DNase I to eliminate potential genomic contamination and verified by RT-PCR in which the reverse transcriptase was omitted (data not shown). cDNA was then synthesized with iScript cDNA Synthesis kit (Bio-Rad) and similarly analyzed with primers specific for mDot1a, mENaCa, or actin in separate wells. The copy number of mDot1a or mENaCa transcripts was normalized to that of actin from the same sample. The sequences of all primers and the detailed PCR conditions are available from the authors upon request. Unpaired Student's *t*-test was performed and significance was set at $P < 0.05$. All experiments were replicated a minimum of three times, with each independent observation representing a single *n* in the figure legends. The triplicates in RT-qPCR or qPCR experiments were counted as a single *n* because they were derived from a single sample, and their average was used to represent the corresponding sample to calculate the final average and standard error, which were further normalized to the control as indicated in the figure legend.

RESULTS

Generation and characterization of an antiserum directed against mDot1a

A polyclonal IgG specific for mDot1a was generated in rabbits with the use of an immunizing peptide corresponding to aa 196–209. The specificity of the antiserum for its target was then tested by Western blot analysis of lysates prepared from mouse kidney, as well as whole cell lysates from mIMCD3 cells that had been transfected with pEGFP-mDot1a 2–478 or empty vector pEGFPC3 (Fig. 1). Because the EGFP-tagged NH₂ terminal portion of mDot1a encoded by pEGFP-mDot1a 2–478 contains the peptide sequence (aa 196–209) used to generate the antibody, it is recognized by the antibodies against EGFP and

Dot1a. As shown in Fig. 1, *lane 1*, the mDot1a antibody specifically labeled a band approximating the expected size of mDot1a (~170 kDa) from the mouse kidney lysate. Shorter exposure time revealed that the band consists of a doublet (data not shown). In addition, a doublet in the size range (~80 kDa) expected for the protein encoded by pEGFP-mDot1a 2–478 was evident in the cells expressing the EGFP-mDot1a fusion (Fig. 1, *lane 3*). This doublet was absent from the vector-transfected cells (Fig. 1, *lane 2*), when an excess of the immunizing peptide was included in the binding mixture (Fig. 1, *lanes 4 and 5*), or when preimmune serum was substituted for the antiserum (Fig. 1, *lanes 6 and 7*). Furthermore, the EGFP antibody recognized the proteins (Fig. 1, *lanes 8 and 9*), further confirming their identities. The doublet may represent different isoforms of the EGFP-mDot1a fusion differing in posttranslational modifications that will require further definition. Therefore, we conclude that the mDot1a antiserum specifically recognizes proteins containing its epitope. It should be noted that the signal of the EGFP-mDot1a fusion detected by the mDot1a antibody was much weaker than that detected with the EGFP antibody, suggesting that the mDot1a antibody might have a lower affinity toward the fusion protein.

In agreement with our previous Northern blot analysis suggesting that mIMCD3 cells express predominantly, if not exclusively, mDot1a (36), we found that a cDNA fragment encoding aa 548 to 632 of mDot1a was readily amplified by RT-PCR using total RNA from mIMCD3 cells (Fig. 2B). Nevertheless, the endogenous mDot1a protein in the mIMCD3 cells was undetectable with the mDot1a antiserum (Fig. 1, *lanes 2 and 3*). This is possibly due to the relative weak affinity of the antibody toward the mDot1a protein that is expressed and maintained at low levels in the mIMCD3 cells. Similar observations were reported about another antibody against the human homologue, hDot1L (22). Nonetheless, because we have demonstrated mDot1a mRNA expression and functional histone H3 K79 methyltransferase activity in mIMCD3 cells (36), functional mDot1a protein is clearly expressed in these cells.

Aldosterone dynamically regulates the histone H3 K79 methylation and mDot1a mRNA expression in mIMCD3 cells

To determine whether aldosterone regulates histone H3 K79 methylation, mIMCD3 cells were cultured in charcoal-stripped serum and treated with aldosterone (1 μ M) for 0 to 72 h, and then acid extracts rich in free histones were isolated and examined by Western blot analysis using antibodies specific for histone H3 dimethylated K79 (designated “ α mK79”) or H3 acetylated K9 (designated “ α AcK9”) as control. As shown in Fig. 2A, the amount of histone H3 K79 methylation in bulk histones was lower than baseline in cells treated with aldosterone for 7 h, whereas cells exposed to aldosterone for longer time periods exhibited progressively higher levels. In contrast, acetylated histone H3 K9 did not change significantly over the time course studied (Fig. 2A). Reprobing of these blots with an anti- α -tubulin antibody revealed that approximately equal loading among the lanes (Fig. 2A). Coomassie staining of an identical gel run in parallel showed that there was no obvious degradation of histones in any of the samples (Fig. 2A). Similar results were obtained using human embryonic kidney-293T cells (data not shown). Thus aldosterone promotes time-dependent changes in the steady-state level of histone H3 K79 methylation in these cell lines.

To test the hypothesis that aldosterone regulates the steady-state level of histone H3 K79 methylation by affecting mDot1a mRNA abundance, real-time RT-qPCR was performed with total RNA isolated from mIMCD3 cells that had been treated with aldosterone or vehicle (ethanol) as above for 2, 4, or 7 h. The forward primer was positioned around the junction of exon 17 and 18 and the reverse one was located in exon 19 of mDot1a, and these successfully amplified a 252-bp cDNA fragment encoding aa 548 to 632 of mDot1a (Fig. 2B). Aldosterone-treated cells exhibited mDot1a mRNA levels that were ~70% and ~35% less than controls at the 2- and 4-h time points, respectively, in keeping with the effects on

histone H3 K79 methylation (Fig. 2B), whereas no significant change was observed 7 h after the treatment. The mRNA abundance of the housekeeping control α -actin across the samples was constant (Fig. 2B). No significant changes in methylated H3 K79 or mDot1a mRNA level were detected at earlier time points (0.5 and 1 h, data not shown). Furthermore, lower concentrations of aldosterone (100 nM, 10 nM) also elicited similar, but less pronounced effects at 2 or 7 h on the abundance of either histone H3 mK79 in bulk histones or mDot1a mRNA. No significant changes were observed when 1 nM aldosterone was used in parallel experiments (data not shown). Therefore, 1 μ M aldosterone was used for further studies because it elicited the maximum effects. Taken together, these data suggest that aldosterone at an early time points (2 h) downregulates the steady-state level of mDot1a mRNA and that this precedes, and is associated with, downregulation of histone H3 K79 methylation in mIMCD3 cells.

Aldosterone causes histone H3 K79 hypomethylation at mENaCa 5'-flanking region coupled with increased endogenous mENaCa expression in mIMCD3 cells

We next sought to determine whether the ability of aldosterone to downregulate mDot1a mRNA and histone H3 K79 methylation also participates in aldosterone-mediated activation of *mENaCa* transcription. We hypothesized that histone H3 K79 methylation specifically at the 5'-flanking region of *mENaCa* might serve to regulate *mENaCa* transcription. To test this hypothesis, we used a combination of ChIP and real-time qPCR techniques to monitor the changes of histone H3 K79 methylation associated with the -988 to +494 bp [relative to the major transcription start site of *mENaCa* (12)] of the *mENaCa* promoter. This region contains a highly conserved GRE at -811 bp and multiple putative GRE half sites at -983, -416, -325, -241, and -234 bp (Fig. 3A) and responds to aldosterone by activating promoter-luciferase constructs as reported by others (12). Four sequential subregions, designated as R0 to R3, spanning -988 to +494 of the *mENaCa* promoter were selected for study (Fig. 3A), and primers were designed to amplify these regions. As expected, aldosterone treatment (2 h) resulted in a 10-fold increase in the *mENaCa* mRNA level (Fig. 3B), whereas the abundance of the housekeeping gene actin was unchanged (Fig. 3B). In keeping with our hypothesis, ChIP assays demonstrated that aldosterone-treated cells exhibited much lower levels of chromatin-associated histone H3 K79 methylation in R0 (~30 fold), R1 (~8 fold), and R3 (~3 fold), but not in R2, as measured by real-time qPCR and verified by agarose gel analysis of the amplified fragments (Fig. 3C). Under the same conditions, ChIP assays with anti-bodies recognizing histone H3 acetylated K9 or the NH₂ terminal tail of histone H3 did not show significant changes in any of the regions examined after aldosterone treatment (Fig. 3C), indicating that the aldosterone-induced H3 K79 methylation changes are not generalized to other histone H3 modifications and are not due to a general reduction in total histone H3. In addition, the background signals from the ChIP with normal rabbit IgG were generally at least 100 times lower than those from the ChIP with the antibodies and undetectable by the agarose gel analysis (Fig. 3C). In the aggregate, the data support the concept that endogenous mDot1a-mediated histone H3 K79 methylation is associated with all four subregions of the *mENaCa* 5'-flanking region examined, that this association is differentially regulated by aldosterone, and that it contributes to regulation of *mENaCa* transcription.

Overexpression of mDot1a induces histone H3 K79 hyper-methylation associated with mENaCa 5'-flanking region and decreases endogenous mENaCa expression

To test directly the hypothesis that mDot1a represses endogenous *mENaCa* expression through histone H3 K79 hypermethylation at specific sites in the *mENaCa* 5'-flanking region, mIMCD3 cells were transiently transfected with pEGFP or its derivatives encoding EGFP-tagged wild-type or methyltransferase-dead mutant mDot1a (pEGFP-mDot1a and pEGFP-mDot1a^{RCR}, respectively). Similar sets of transfected cells were examined by real-time RT-

qPCR (to monitor *mENaCa* and actin mRNA levels), Western blot analysis (to determine the expression level of transfected EGFP-tagged mDot1a fusions), or ChIP coupled with real-time qPCR (to detect changes in histone H3 K79 methylation associated with the 5'-flanking region of *mENaCa*). In agreement with our hypothesis, overexpression of wild-type mDot1a resulted in 40% lower *mENaCa* mRNA levels compared with vector-transfected cells, whereas *mENaCa* mRNA levels in cells transfected with the methyl-transferase-dead mutant pEGFP-mDot1a^{RCR} were almost twice that of the vector-transfected cells (Fig. 4). This doubling of *mENaCa* mRNA suggests that pEGFP-mDot1a^{RCR} acts as a dominant-negative mutant. The changes in *mENaCa* mRNA levels were not the result of a general effect on transcription, because the actin mRNA levels were constant in these cells (Fig. 4). In addition, the Western blot analyses with anti-EGFP and anti- α -tubulin antibodies revealed comparable expression levels of the pEGFP-mDot1 and pEGFP-mDot1a^{RCR} fusions and equal loading across the samples (Fig. 4). Thus mDot1a represses *mENaCa* mRNA expression in a methyltransferase-dependent manner.

To determine whether histone H3 K79 methylation at the subregions of the *mENaCa* promoter was dependent on the methyltransferase activity of mDot1, mIMCD3 cells were transiently transfected with pEGFP, pEGFP-mDot1a, or pEGFP-mDot1a^{RCR}, and ChIP assays were performed, using antibodies to histone H3 methylated at K79, histone H3 acetylated at K9, or the NH₂ terminus of histone H3, and PCR primers to amplify the R0-R3 subregions. As seen in Fig. 5, overexpression of wild-type mDot1a resulted in a significant increase of the histone H3 methylated at K79 associated with the R0, R1, and R3 subregions (2-, 4- and 3-fold, respectively; Fig. 5), but not with the R2 subregion. As stated earlier, R0, R1, and R3 subregions exhibited decreased H3 K79 methylation on aldosterone-mediated downregulation of endogenous mDot1a expression. Therefore, it appears that H3 K79 methylation at the R0, R1, and R3 subregions of the *mENaCa* promoter is somehow more sensitive to the level of mDot1a expression than at R2. In all cases, overexpression of the mutant mDot1a failed to alter significantly the H3 K79 methylation pattern in the four fragments examined (Fig. 5). ChIP assays with antibodies recognizing histone H3 acetylated K9 or the NH₂ terminal tail of histone H3 revealed no obvious differences under the various transfection conditions over the entire *mENaCa* 5'-flanking region examined (Fig. 5), indicating that the changes in histone H3 K79 methylation were specific and not the result of a generalized effect on histones. Furthermore, ChIP with IgG yielded background signals that were undetectable by agarose gel analysis (Fig. 5). We also vigorously attempted to establish stable cell lines expressing untagged or EGFP-tagged wild-type or mutant mDot1a to perform a similar set of experiments as above. However, constant overexpression of the wild-type or mutant mDot1a was apparently lethal, because the full length of mDot1a was never detected in any of the surviving cells after selection (data not shown). In the aggregate, these data strongly suggest that mDot1a directly or indirectly associates with and hypermethylates histone H3 K79 at specific portions of the *ENaCa* 5'-flanking region, inducing a local alteration of the chromatin structure that facilitates *ENaCa* mRNA expression.

mDot1a represses *mENaCa* promoter-luciferase activity in a methyltransferase-dependent manner

To test the hypothesis that mDot1a limits endogenous *mENaCa* mRNA expression by repressing *mENaCa* promoter activity, mIMCD3 cell lines carrying a stably transfected *mENaCa* promoter-luciferase reporter were established, as detailed in MATERIALS AND METHODS. To eliminate any potential effects imposed by the EGFP tag on the reporter expression, untagged wild-type or methyl-transferase-dead mDot1a constructs were transiently transfected into the established mIMCD3 cells harboring the reporter. Cells overexpressing wild-type mDot1a exhibited *mENaCa* promoter-luciferase activity about two

times lower than control, whereas the methyltransferase-dead mutant mDot1a was without effect (Fig. 6). Western blot analysis revealed comparable expression of both wild-type and mutant mDot1a, whereas the endogenous mDot1a was again undetectable from the empty vector-transfected cells (Fig. 6). Furthermore, Western blots probed with anti- α -tubulin showed equal loading of the samples (Fig. 6).

Knockdown of mDot1a mRNA levels by RNA interference augments endogenous mENaCa expression and activity of mENaCa-promoter luciferase reporter

To confirm further that mDot1a represses mENaCa expression, we employed RNA interference technology to silence mDot1a and examined the effects on expression of endogenous mENaCa and the transiently transfected mENaCa-promoter luciferase reporter. Three RNAi constructs specifically targeting mDot1a and a negative control construct were constructed and transfected into mIMCD3 cells to establish stable cell lines, as detailed in MATERIALS AND METHODS. No adverse effects on cell growth or morphology were grossly apparent in any of the RNAi-transfected cell lines. Real-time RT-qPCR revealed that RNAi1 and RNAi2 knocked down mDot1a mRNA levels to ~71% and 59%, respectively, compared with the control cell line transfected with the negative control construct harboring an unrelated RNAi target sequence (Fig. 7). No cell lines with significant reduction of mDot1a expression were established from pSCN-RNAi3 transfected cells. In contrast, mENaCa expression in the cells transfected with RNAi1 and RNAi2 was increased to 157% and 337%, respectively (Fig. 7) of control, whereas the level of actin mRNA remained relatively constant in these cells (Fig. 7). Transient transfection of the mENaCa-promoter luciferase reporter into these cells revealed a more dramatic effect. RNAi1- and RNAi2-transfected cells exhibited luciferase activity that was 3- or 7-fold greater than the control cells (Fig. 7). The collective data strongly argue that mDot1a limits mENaCa expression, at least partially, via directly or indirectly binding the mENaCa promoter and mediating H3 K79 methylation associated with the promoter.

Histone H3 K79 methylation occurs in mouse collecting duct

To determine whether mDot1a is expressed in aldosterone target cells of the adult mouse kidney, we performed immunohistochemistry with purified α mDot1a on mouse kidney sections. However, the antibody yielded high background signals that were difficult to interpret. Instead, we used α mK79 to detect the modified histone H3, as an indicator of Dot1 methyltransferase activity, and an anti-aquaporin-2 antibody as a marker of collecting duct principal cells, a primary target of aldosterone action in the kidney. As shown in Fig. 8, histone H3 K79 methylation was detected in all collecting duct epithelial cells, regardless of the presence or absence of aquaporin-2 expression. No immunofluorescence was observed when the primary antibodies were omitted. Unfortunately, generation of an isoform-specific RNA probe to perform in situ hybridization or an antibody to determine the spatial expression pattern of each mDot1 variant was extremely impractical because 1) there are five alternative splicing variants (mDot1a-e) generated by the *mDot1* gene (36), and the complete coding region has only been determined for mDot1a and mDot1b (36); and 2) Western blot analysis of the whole kidney lysate with our Dot1 antibody revealed a doublet (Fig. 1, lane 1) and Northern blot analysis revealed two major mRNA variants in kidney, most likely to be mDot1a and mDot1d (36). In brief, although we could not directly demonstrate that mDot1a in fact catalyzed the histone H3 K79 methylation detected or the specific cellular localization of mDot1a in the kidney, our immunolocalization data concerning histone H3 K79 methylation are supportive of the hypothesis that Dot1a-mediated repression of *mENaCa* occurs in all renal collecting duct epithelial cells and such repression is only relieved in aldosterone target cells, such as principal cells.

DISCUSSION

The molecular mechanisms controlling *mENaCa* gene transcription and the effects of aldosterone on target gene transcription are complex and incompletely defined. In this report, we identify mDot1a as the first histone modification enzyme significantly involved in the aldosterone-sensitive control of *mENaCa* transcription and additionally establish *mENaCa* as a novel target of mDot1a. These conclusions are based on several novel findings. First, aldosterone downregulates mDot1a gene expression in mIMCD3 cells, followed by histone H3 K79 hypomethylation in bulk histones and at specific regions of the *mENaCa* 5'-flanking region in vivo, and consequent upregulation of *mENaCa* mRNA levels in mIMCD3 cells. Second, mDot1a associates directly or indirectly with specific regions of the *mENaCa* 5'-flanking region. Third, mDot1a-mediated histone H3 K79 hypermethylation and repression of *mENaCa* transcription require mDot1a methyl-transferase activity. Fourth, reducing mDot1a expression by RNA interference yielded substantially higher expression of both the endogenous *mENaCa* and the transiently transfected luciferase reporter under control of the *mENaCa* promoter. Finally, mDot1a-catalyzed histone H3 K79 methylation occurs ubiquitously in mouse kidney tubular epithelial cells. On the basis of these findings and the discussion below, we propose a model for mDot1a-mediated *ENaCa* repression in the presence or absence of aldosterone (Fig. 9).

Aldosterone is known to increase *mENaCa* gene expression by promoting its transcription rather than by a separate effect on *mENaCa* mRNA turnover (17). Our data are consistent with and extend these findings. First, manipulations of histone H3 K79 methylation at the *mENaCa* promoter, resulting from overexpressed mDot1a or from aldosterone-induced reductions in mDot1a mRNA expression, correlated with changes in *mENaCa* mRNA expression and the activity of the *mENaCa* promoter-luciferase reporter. These data directly link aldosterone-mediated transcriptional control of *mENaCa* to changes in local chromatin structure of its promoter and strongly argue that Dot1a regulates *mENaCa* mRNA expression at least partially through controlling *mENaCa* transcription. It should be noted that the aldosterone-induced decrease in either mDot1a mRNA level or association of histone H3 mK79 at specific sites of the *ENaCa* 5' flanking region precedes a detectable decrease of methylated histone H3 K79 in bulk histone (2 vs. 7 h). These results can be reconciled when the following factors are considered. First, these observations were made with the use of different experimental approaches (RT-qPCR, ChIP-coupled qPCR, or Western blot analysis). It is unlikely that these various techniques share precisely the same sensitivity. We cannot rule out the possibility that aldosterone induced subtle changes in the overall histone H3 K79 methylation at 2 h and the changes escaped detection by Western blot analysis. Second, it has not been established whether a significant change in a specific histone modification associated with a specific site requires or correlates with a significant corresponding change of such modification in bulk histones. In this case, it is possible that a decrease or increase of chromatin-associated histone H3 K79 methylation at the *ENaCa* promoter occurs in the absence of a concomitant alteration in bulk histones, as observed in our hands. Finally, aldosterone exerts its effects on mDot1a mRNA and *ENaCa* promoter-associated histone H3 mK79 via multiple mechanisms. Downregulation of mDot1a expression is only one of them. Other possibilities include, but are not limited to, a negative regulation of a mDot1a-interacting partner(s). We are currently testing these hypotheses.

The repression effect on the endogenous *mENaCa* or the *mENaCa* promoter-luciferase reporter imposed by mDot1a was not quantitatively dramatic. Presumably this relates to the fact that mDot1a is only one component of a complex that regulates *mENaCa* transcription in an aldosterone-sensitive manner. In addition, the time and scale of the induction by aldosterone vary significantly between the endogenous *mENaCa* mRNA and the *mENaCa* promoter-luciferase reporter. The difference in these two experiments might be explained

because one was designed to measure the induction at mRNA level, whereas the other was to detect the changes at the protein level (luciferase). The differential sensitivities of real-time RT-PCR and luciferase assays may also contribute to the discrepancy. Finally, the silencing efficiency of RNA interference with the three stably transfected different targets was relatively low. It is possible that the cells with higher silencing efficiency resulted in even lower levels of mDot1a expression constantly experienced growth defects as reported (22) and were thus unfavorably selected.

Many genes up- or downregulated by aldosterone have been identified in different systems including the renal collecting duct (11,25,27) and mIMCD3 cells (9). However, mDot1a represents the first aldosterone-regulated gene known involved in histone modifications or chromatin remodeling. Our findings indicate that aldosterone dynamically regulates mDot1a gene expression in mIMCD3 cells, which results in a delayed overall reduction of H3 K79 methylation. Analysis of the 2 kb 5'-flanking region of mDot1a with the Transcription Element Search System (<http://www.cbil.upenn.edu/tess/>) reveals multiple GRE-like elements throughout the region (-283, -359, -649, -716, -748, -900, -1,274, -1,553, -1,613, -1,755, -1,805, and -1,846; relative to the putative transcription start site 238 bp upstream of the translation initiation codon of mDot1a). Whether aldosterone regulates mDot1a mRNA levels by controlling its transcription through these GRE-like sites or by affecting its stability/degradation remains to be defined. In addition, further experiments are required to determine whether the Dot1 mRNA variants are also similarly regulated in vivo in kidney and distal colon, two major physiological targets of aldosterone action.

Whereas histone H3 K79 methylation is not evenly detected throughout the genome in yeast or mammalian cells (20,32), Dot1 is generally considered to be a nontargeted methyltransferase (20,21,32). However, our data raise the additional possibility that targeted interactions of mDot1a with the *mENaCa* 5'-flanking region not only occur, but also are regulated. First, aldosterone negatively regulated to varying degrees the association of methylated histone H3 K79 at specific areas of the *mENaCa* 5'-flanking region, depending on the subregions (R0-R3) examined (Fig. 3). Second, mDot1a overexpression resulted in increased chromatin-associated histone H3 K79 methylation at R0, R1, and R3 (Fig. 5). Finally, these results argue against non-sequence-specific DNA binding activity of Dot1 proteins in this promoter context. We favor the hypothesis that mDot1a is ushered to these specific regions, presumably by an mDot1a-interacting protein that exerts DNA binding activity to the implicated regions of the *mENaCa* promoter (Fig. 9). Studies are underway to test this hypothesis because no mDot1a-interacting proteins with DNA binding activity have thus far been reported.

Although aldosterone clearly plays a regulatory role, it appears that mDot1a-mediated effects on *mENaCa* transcription are at least partially independent of aldosterone-MR interactions with the *mENaCa* promoter because manipulation of mDot1a expression or its methyltransferase activity in the absence of aldosterone was sufficient to elicit changes in histone H3 K79 methylation and *mENaCa* transcription. It is unknown whether aldosterone regulates an upstream component in the pathway. Other factors might also cause the changes. For example, Boyd et al. (4) compared the levels of *mENaCa* mRNA in mouse CCD cells stably expressing either full-length serum- and glucocorticoid-induced kinase 1 (SGK1) or a kinase-dead, dominant-negative (K127M)-SGK1 mutant. They reported that SGK1 regulates *mENaCa* gene expression, whether cells were maintained in steroid-free media or in the presence of corticosteroids. The loss of SGK1 function caused a ~45% decrease in *mENaCa* expression, which varied directly with the level of functional SGK1, and the authors hypothesized that the effect of SGK1 on *mENaCa* transcription is mediated by the activation of unidentified transcription factors (4).

In the broader context of other genes affected by histone H3 K79 methylation status, our data indicate that this modification may have bimodal effects on transcription. On the basis of studies in yeast of Sir-dependent silencing loci (20) and in mammalian cells of recombination-active vs. -inactive genomic loci, histone H3 K79 hypermethylation has been proposed to be a marker of active chromatin regions (20). In contrast, our data indicate that histone H3 K79 hypermethylation can also correlate with transcriptional repression. There are several explanations for this apparent bimodal effect on transcription. First, even in yeast, the role of histone H3 K79 methylation in association of the Sir proteins and thus in gene silencing is still controversial. Deletion or overexpression of Dot1 displayed a similar phenotype: defects in Sir protein binding and silencing at telomeric and HM loci. To account for these paradoxical results, Sir proteins are proposed to associate preferentially with histone H3 harboring methylated (21) or nonmethylated K79 (20,32). Second, a mixture of non-, mono-, di- or trimethylated H3 K79 coexists (32) and a mechanistic insight of differential methylation states is unknown. Nevertheless, the cross-reactivity of the anti-dimethyl H3 K79 antibody towards mono- and trimethylated H3 K79 (from Upstate Biotechnology) has not been rigorously examined. Nonetheless, the specificity of the antibody to methylation of K79 in histones has been established (20,21,36). We do not know for certain whether hypomethylation induced by aldosterone or hypermethylation caused by overexpression of mDot1a represents a change in the relative ratio of each K79-methylated form to the nonmodified one. Because the same antibody was employed in the previous studies (20,21), it is possible that a similar increase or decrease in histone H3 K79 methylation observed by ChIP with the antibody in different systems may represent a different shift of the spectrum of differential methylation status and thus point to different functions. Finally, histone H3 K79 methylation, like H3 S10 phosphorylation (5), may be associated with both gene activation and repression, depending on the patterns of other histone modifications leading to either the association or the dissociation of distinct effector proteins.

The model for aldosterone-sensitive and MR-independent signaling mechanisms that result in control of *mENaCa* transcription is incomplete, and there have been few reports about the mechanisms for basal repression of *mENaCa* transcription or how aldosterone modifies chromatin to alter gene transcription. In addition, there has been extremely limited information about the functions and interactions of mDot1a in mammalian cells. The novel findings described in this report revealed a new signaling network linking aldosterone action to chromatin modifications and *mENaCa* transcriptional control via mDot1a. In a broader context, our studies also provide important insights applicable to the transcriptional control of other complex genes, the molecular actions of mineralocorticoids, and the functions and regulation of mDot1a.

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Fig. 1.

Characterization of a murine disruptor of telomeric silencing alternative splice variant “a” (mDot1a)-specific antibody. Whole cell lysates from mouse kidney (*lane 1*) or from mouse inner medullary collecting ducts (mIMCD3) cells transfected with either plasmid epidermal growth factor protein C3 (pEGFPC3) vector control (*lanes 2, 4, 6, and 8*) or pEGFPC3-mDot1a 2–478, encoding aa 2–478 of mDot1a fused to EGFP (*lanes 3, 5, 7, and 9*), were analyzed by Western blots with an antiserum against mDot1 (α Dot1) or anti-EGFP antibody (α EGFP). As controls for specificity, blots were also prepared using α Dot1 plus a molar excess of the immunizing peptide (Pep) or preimmune serum (Pre). Shorter exposures revealed that the band of ~170 kDa in the mouse kidney samples (*lane 1*) consists of a doublet (data not shown). The identity of the ~25 kDa protein that comigrated with EGFP (*lane 8*) and was weakly labeled in all cases is unknown and nonspecific (NS).

Fig. 2.

Aldosterone dynamically regulates the levels of Dot1a mRNA and H3 K79 methylation in mIMCD3 cells. *A*: acid extracts rich in histones isolated from mIMCD3 cells that had been cultured in charcoal-stripped serum and treated with 1 μ M aldosterone for the indicated times were separated on identical gels, and immunoblotted with antibodies specific for methylated H3 at K79 (α mK79), acetylated H3 at K9 (α AcK9) or stained with Coomassie blue dye. The blots were stripped and reprobed with an antibody against α -tubulin (α Tubulin). *B*: total RNA prepared from mIMCD3 cells treated with vehicle (ethanol) or 1 μ M aldosterone for 2, 4, or 7 h was analyzed by agarose gel electrophoresis and by real-time RT-quantitative PCR (qPCR; presented in the histogram) with primers specific for mDot1a or actin. The mDot1a mRNA levels were normalized against invariant actin mRNA, as

measured by real-time PCR. The mDot1a mRNA level of the vehicle-treated cells for 2 h was arbitrarily set as 1 and used to calculate the relative levels of all other samples. $*P < 0.05$ vs. -Aldo; $n = 3$.

Fig. 3.

Aldosterone induces hypomethylation of histone H3 K79 at the epithelial Na⁺ channel α -subunit (*mENaC α*) 5'-flanking region and *ENaC α* expression in mIMCD3 cells. mIMCD3 cells were cultured with charcoal-stripped serum and treated with either 1 μ M aldosterone or vehicle for 2 h. Similar sets of cells were harvested for RT-qPCR, Western blot analysis, or chromatin immunoprecipitation (ChIP). **A**: diagram of the 5'-flanking region of *mENaC α* fragments designated R0-R3 are shown along with their relative positions to the major transcription start site (+1) of *mENaC α* . • and ■ represent the putative GRE site (-811) and GRE half sites (-983, -416, -325, -241, and -234), respectively. **B**: the *mENaC α* mRNA levels were examined by agarose gel electrophoresis and RT-qPCR (see Fig. 2B). * P < 0.05 vs. -Aldo; n = 3. **C**: DNAs coimmunoprecipitated by the indicated antibodies were analyzed

by agarose gel electrophoresis and by real-time qPCR (bottom histogram) with primers designed to specifically amplify R0 to R3, followed by agarose gel analysis. Antibodies against the acetylated histone H3 K9 (α AcK9), the NH₂ terminal tail of histone H3 (α H3), and normal rabbit IgG were used as controls. The relative histone H3 K79 methylation was defined as ChIP efficiency, i.e., the immunoprecipitated amount of material to that of the input sample, as determined by real-time PCR and verified by agarose gel analysis. * $P < 0.05$ vs. -Aldo; $n = 3$.

Fig. 4.

Overexpression of wild-type mDot1a, but not its methyltransferase-dead mutant, decreases mENaC α mRNA levels in mIMCD3 cells. mIMCD3 cells were transiently transfected with an empty vector pEGFP-C3 (Vec) or its derivatives expressing EGFP-tagged wild-type (WT) or methyltransferase-dead mutant (Mut). RNA samples were prepared and analyzed for ENaC α and actin expression by agarose gel electrophoresis and RT-qPCR (bottom histogram). Antibodies against EGFP (α EGFP) or tubulin (α Tubulin) were used to monitor the expression of EGFP-mDot1a fusions and to verify equal loading on Western blots. * $P < 0.05$ vs. corresponding vector control; $n = 3$.

Fig. 5.

Overexpression of wild-type mDot1a, but not its methyltransferase-dead mutant, increases histone H3 K79 methylation at specific portions of the *mENaCa* 5'-flanking region in mIMCD3 cells. ChIP assays coupled with real-time qPCR were carried out with the indicated antibodies to determine the association of EGFP-mDot1a fusions with the R0-R3 subregions of the *mENaCa* 5'-flanking region. * $P < 0.05$ vs. corresponding vector control; $n = 3$.

Fig. 6.

Overexpressed mDot1a represses the activity of an *mENaCa* promoter-luciferase reporter gene in a methyltransferase-dependent manner. An mIMCD3-derived cell line harboring a stably transfected firefly luciferase reporter driven by the *mENaCa* promoter (−850 to +536, just before the translation start site ATG) was cultured in charcoal-stripped serum and transiently transfected with an empty vector pcDNA 3.1-V2 (Vec) or its derivatives expressing untagged wild-type mDot1a (WT) or methyltransferase-dead mDot1a mutant (Mut). *Renilla* luciferase reporter pRL-SV40 was included as an internal control. Firefly luciferase activity of each sample was normalized to its *Renilla* luciferase activity. The firefly luciferase activity of the vector-transfected cells was designated as 1 and utilized to determine the relative level and the significance of the other samples. * $P < 0.05$ vs. Vec; $n = 3$. Overexpressed mDot1a proteins were monitored by Western blot analyses with the αDot1 characterized in Fig. 1, and equal loading was verified by an identical blot probed with anti-tubulin.

Fig. 7.

Knockdown of mDot1a mRNA expression by RNA interference increases expression of endogenous mENaC α and a mENaC α promoter-luciferase reporter. **A**: three independent cell lines stably transfected with pSilencer4.1-CMV-neo negative control (Vec) or mDot1a silencing constructs pSCN-RNAi1 (RNAi#1) or pSCN-RNAi2 (RNAi#2) were examined by real-time RT-qPCR for expression of mDot1a, mENaC α , or actin. The level of mDot1a and mENaC α was first normalized to that of the actin in the same sample and then compared with that of the control cells. **B**: the same cells as in **A** were transiently transfected with the same mENaC α promoter-luciferase reporter, followed by luciferase assay as in Fig 6.

Fig. 8.

Histone H3 methylation at K79 occurs in nuclei of kidney collecting duct cells. Tissue sections of the mouse kidney were fixed and indirect immunofluorescence performed as detailed in MATERIALS AND METHODS using rabbit polyclonal histone H3 dimethylK79 antiserum and anti-aquaporin-2 raised in chickens as primary antibodies, with Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 594 goat anti-chicken IgG as secondary antibodies. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Samples without primary antibodies were used as a negative control (Neg. Control). Image analysis was performed as detailed in the MATERIALS AND METHODS. A representative image of the renal outer medulla is shown (magnification $\times 600$); $n = 3$.

Fig. 9.

Hypothetical model for aldosterone-sensitive repression of *ENaCa* expression by an mDot1a-containing complex modulating histone H3 K79 hypermethylation associated with the *ENaCa* promoter. Under basal conditions, mDot1a and its putative interacting partner “X” form a nuclear complex that directly or indirectly binds to the 5'-flanking region of *ENaCa*, leading to hypermethylation of histone H3 K79 at this location and repression of *ENaCa* (A). In this model, aldosterone (○) stimulates *ENaCa* transcription by regulating two antagonistic complexes. Aldosterone binds and activates the nuclear hormone receptors (NR) that are either glucocorticoid receptor and/or mineralocorticoid receptor (MR) homo- or heterodimers to bind the GRE for transactivation of *ENaCa*. In parallel, aldosterone downregulates the amount of the Dot1a complex, possibly by multiple mechanisms, including reducing the abundance of mDot1a and its partner X (shown) via NR-dependent (shown) or NR-independent mechanisms (not shown) to limit the mDot1a complex availability, leading to histone H3 K79 hypomethylation at specific *ENaCa* promoter subregions and release of repression of *ENaCa*. In either case, free mDot1a binds DNA nonspecifically and catalyzes histone H3 K79 methylation throughout the genome at the basal level (not shown). The putative counter-interaction between the NR-aldosterone complex and the mDot1a-“X” complex is shown with a dotted line and may tune the ultimate level of *ENaCa* transcription. The relative position of the site(s) associated with the mDot1a complex to the *ENaCa* transcription start site and GRE remains to be determined. Similar mechanisms may be applicable to other aldosterone-regulated genes.