

# KLF15 negatively regulates estrogen-induced epithelial cell proliferation by inhibition of DNA replication licensing

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In the epithelial compartment of the uterus, estradiol-17 $\beta$  (E<sub>2</sub>) induces cell proliferation while progesterone (P<sub>4</sub>) inhibits this response and causes differentiation of the cells. In this study, we identified the mechanism whereby E<sub>2</sub> and P<sub>4</sub> reciprocally regulate the expression of minichromosome maintenance (MCM)-2, a protein that is an essential component of the hexameric MCM-2 to 7 complex required for DNA synthesis initiation. We show in the uterine epithelium that Kruppel-like transcription (KLF) factors, KLF 4 and 15, are inversely expressed; most importantly, they bind to the *Mcm2* promoter under the regulation of E<sub>2</sub> and P<sub>4</sub>E<sub>2</sub>, respectively. After P<sub>4</sub>E<sub>2</sub> exposure and in contrast to E<sub>2</sub> treated mice, the *Mcm2* promoter displays increased histone 3 (H3) methylation and the recruitment of histone deacetylase 1 and 3 with the concomitant deacetylation of H3. This increased methylation and decreased acetylation is associated with an inhibition of RNA polymerase II binding, indicating an inactive *Mcm2* promoter following P<sub>4</sub>E<sub>2</sub> treatment. Using transient transfection assays in the Ishikawa endometrial cell line, we demonstrate that *Mcm2* promoter activity is hormonally stimulated by E<sub>2</sub> and that KLF15 inhibits this E<sub>2</sub> enhanced transcription. KLF15 expression also blocks Ishikawa cell proliferation through inhibition of MCM2 protein level. Importantly, in vivo expression of KLF15 in an estrogenized uterus mimics P<sub>4</sub>'s action by inhibiting E<sub>2</sub>-induced uterine epithelial MCM-2 expression and DNA synthesis. KLF15 is therefore a downstream physiological mediator of progesterone's cell cycle inhibitory action in the uterine epithelium.

endometrium | implantation | menstrual cycle | endometriosis

Estradiol-17 $\beta$  (E<sub>2</sub>) and progesterone (P<sub>4</sub>) directs uterine preparation for pregnancy. These hormones synthesized cyclically in humans cause a sequential series of proliferative and differentiation events in the uterine stroma and epithelium that result in the epithelium being receptive to the blastocyst for attachment and subsequent implantation (1). Despite this close control of uterine cell proliferation, aberrant proliferative conditions of the human endometrium are common. For example, endometrial polyps and endometriosis are caused by inappropriate proliferation of the uterus, while unopposed estrogen stimulation is associated with menstrual irregularities and endometrial hyperplasia/adenocarcinoma (2). Endometrial cancer is the most common female genital tract malignancy and is responsible for 6% of cancer deaths of women in the United States and more worldwide (3). However, the molecular mechanisms underlying these pathologies are still obscure, as are the molecular mechanisms involved in normal hormonal regulation of cell proliferation in the endometrium, which is essential for successful pregnancy (4).

The mouse uterus provides a unique in vivo model to study the regulation of epithelial cell proliferation as the physiological actions of E<sub>2</sub> and P<sub>4</sub>E<sub>2</sub> can be recapitulated in ovariectomized animals by treatment with exogenous hormones (4). Physiologically in mice, E<sub>2</sub>, synthesized at proestrus, induces a wave of epithelial DNA synthesis followed by cell division. This E<sub>2</sub>-induced wave of epithelial DNA synthesis is completely inhibited

by the P<sub>4</sub> that is synthesized following copulation. Implantation is precipitated by a surge of estrogen on day four of pregnancy (4). Similar mechanisms of hormone action are found in humans where the E<sub>2</sub> induced epithelial cell proliferation is also inhibited by P<sub>4</sub> (5). There is doubt whether E<sub>2</sub> is required for implantation in humans, as implantation can occur without ovaries providing P<sub>4</sub> is supplied. However, given that the mouse uterus can synthesize E<sub>2</sub> at implantation, the endometrium may be the source of this hormone in humans (6).

The effects of E<sub>2</sub> and P<sub>4</sub> on cell proliferation in the uterus are mediated by their cognate transcription factors estrogen receptor (ER $\alpha$ ) and progesterone receptor (PR), respectively. These receptors act in cell autonomous and nonautonomous fashion together with cell type specific transcriptional cofactors to exercise their biological functions. Recent cell-specific gene targeting shows that E<sub>2</sub> induced epithelial cell proliferation is regulated through stromal localized ER (7) that causes the synthesis of paracrine factors, including IGF-1, that act on the epithelial cells (8). PR is required in the uterine epithelium and stroma and causes inhibition of E<sub>2</sub>-induced epithelial cell proliferation (9, 10). Despite these cell-specific requirements for the receptor, each hormone induces a cascade of downstream effectors including transcription factors (TF) that propagate the hormonal signal (1).

In uterine epithelial cells, P<sub>4</sub> exerts its inhibitory effects on the E<sub>2</sub>-induced cell cycle in G1 within 3 h of E<sub>2</sub> treatment (11). In order to dissect the TFs and the pathways involved in the P<sub>4</sub>-inhibition of uterine epithelial DNA synthesis, we examined the transcriptome in this epithelium 3 h posttreatment of P<sub>4</sub>E<sub>2</sub> or E<sub>2</sub> alone. We found the coordinate down-regulation by P<sub>4</sub>E<sub>2</sub> treatment of greater than 20 genes associated with DNA replication and chromatin assembly. These genes include *Mcm* family members, *Pcna*, *Mki67*, *Fen1*, *Lig1*, *Cdk2*, *Tk1*, and *Mad2l1* (12). This coordinated response suggests action of TFs in the epithelial cells that mediate the cascade of events through the regulation of gene batteries involved in DNA replication. Several TFs were identified that were expressed differentially in response to E<sub>2</sub> and P<sub>4</sub>E<sub>2</sub> (12) including transcripts for the Kruppel-like transcription factors, *Klf4* and *Klf15*, whose expression was increased by E<sub>2</sub> and P<sub>4</sub>E<sub>2</sub> treatment, respectively.

KLFs belong to the specificity family of proteins. They are zinc finger containing TFs named after the segmentation gene product Kruppel originally identified in *Drosophila melanogaster*, in which it plays an essential role in embryogenesis (13). In mammals they constitute a large family of factors containing at least

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seventeen members that bind to a CACCC or CGCCC DNA motif (14, 15). KLFs have pleiotropic functions that both enhance and inhibit transcription (16), and through competition for binding to their target sequences, they can mutually antagonize each other (15, 17). They have a wide range of functions in regulating cell cycle, apoptosis, and differentiation (15). KLFs affect expression of proliferative genes such as E, D and A type cyclins. They also regulate several antiproliferative genes, for example p53 and E cadherin (17). KLF9 expressed in the uterus has a role in reproduction; its loss results in a modest reduction in fertility in mice caused by an implantation failure secondary to the attenuation and delay of cell proliferation in the luminal and glandular epithelium in response to E<sub>2</sub> (18–20). KLF9 acts as a PR coregulator and, depending on cell context, acts as a negative or positive regulator of ER signaling (18, 21). In this study, because of its regulation by P<sub>4</sub>E<sub>2</sub> in the uterine epithelium at the important 3 h time point, we focused on KLF 15 and, as a contrast, analyzed KLF4 as an E<sub>2</sub> regulated TF.

A major concept in cell cycle regulation is the assembly of the prereplication complex (RC) upon origins of replication at the end of mitosis and in early G<sub>1</sub> by the association of CDC6 and CDT1 proteins to the origin recognition complex (ORC). The association of CDC6 and CDT1 to the ORC is a prerequisite for the loading of a hexameric complex containing six different minichromosome maintenance proteins (MCM 2–7) to chromatin, licensing the origin for a new replication round. At this stage, MCMs that are members of the AAA+ class of ATPases (22) acquire their helicase activity to unwind DNA. The molecular components and the basic interaction of pre-RC components are strikingly conserved from yeast to metazoa. However, the dynamics of the ancillary protein assembly vary in different species. After DNA synthesis is initiated, the MCM2–7 complex dissociates from the DNA and CDT1 is degraded, thus ensuring only one round of DNA replication per cell cycle. MCMs play a critical role in the initiation of DNA synthesis, and the removal of any one MCM blocks DNA synthesis in a wide range of organism from yeast to humans (23–27). Given their essential role in proliferation, they are used as a biomarker and prognostic indicator for different cancers (26).

In the uterine epithelium, E<sub>2</sub> stimulates the expression of the MCMs and of the loading factor CDT1 (12, 28). Consequentially, chromatin association of the MCM2–7 complex is stimulated by E<sub>2</sub> at the G<sub>1</sub>/S phase transition. P<sub>4</sub>E<sub>2</sub> inhibits the transcript abundance of MCM 2 to 6, causes nuclear egress of the residual MCM proteins, and inhibits CDT1 synthesis. Thus the binding of the MCM2–7 complex to chromatin is inhibited and prereplication licensing is blocked (12). A similar action can be ascribed to P<sub>4</sub>E<sub>2</sub> in human endometrial epithelium as a dramatic down-regulation of MCM2 transcripts; and loss of protein occurs in the secretory and therefore P<sub>4</sub> dominated phase of the menstrual cycle (5). Despite the importance of this regulation in mice and humans, the molecular basis for the P<sub>4</sub>E<sub>2</sub> regulation of DNA replication licensing is not understood.

In this study we tested the hypothesis that KLF15 mediates the actions of P<sub>4</sub>E<sub>2</sub> in the uterine epithelium through inhibition of the transcription of MCM2, and thereby, of DNA replication licensing. We show that KLF15 binds to the MCM2 promoter in a P<sub>4</sub>E<sub>2</sub> dependent fashion and that it negatively regulates RNA Pol II association. KLF15 expression suppresses E<sub>2</sub> mediated MCM2 transcription. In vivo, KLF-15 expression in the E<sub>2</sub> exposed uterus mimics P<sub>4</sub> action by inhibiting MCM2 expression and epithelial cell DNA synthesis. These data establish KLF-15 as a downstream mediator of the antiproliferative action of progesterone on E<sub>2</sub>-induced epithelial cell proliferation.

## Results

**KLF4 and KLF15 Are Regulated by E<sub>2</sub> and P<sub>4</sub>E<sub>2</sub>.** To identify the molecular basis of uterine epithelial DNA synthesis, it is necessary to

identify the steroid hormone regulated cell type specific TFs that are responsible for this process. Because KLFs have diverse functions in cell cycle regulation, and KLF4 and KLF15 transcripts are differentially regulated in the uterine epithelium 3 h after E<sub>2</sub> and P<sub>4</sub>E<sub>2</sub> treatment, we chose to study these two TFs. First, to ensure whether the differential expression in transcript abundance leads to changes in relative protein expression, we performed Western analysis of epithelial lysates isolated at different times after physiologically relevant hormonal treatment of ovariectomized mice. E<sub>2</sub> treatment resulted in an up-regulation of KLF4 protein within 3 h that persisted through 14 h posttreatment compared to vehicle treated controls (Fig. 1A). P<sub>4</sub> pretreatment inhibited this E<sub>2</sub> up-regulation of KLF4 and resulted in a significant decline over the first 3 h of treatment, followed by an increase that was still significantly lower than the E<sub>2</sub> treated group through the 14 h observation period (Fig. 1A). KLF 15 protein concentration was reduced by E<sub>2</sub> treatment at the 3 h mark compared to the oil treated control. Although there is some recovery in concentration, this suppression persisted until 14 h posttreatment. In contrast, P<sub>4</sub>E<sub>2</sub> treatment significantly elevated KLF15 protein concentration over the control and E<sub>2</sub> treated groups through the 14 h time course (Fig. 1A). KLF3, whose mRNA was not shown to be differentially regulated by P<sub>4</sub>E<sub>2</sub> but is expressed in the uterine epithelium, responded to E<sub>2</sub> treatment modestly in protein concentration regardless of P<sub>4</sub> pretreatment (Fig. 1A).

Cell type specificity of expression at the important 3 h time point posthormonal treatment was investigated by immunohistochemistry (IHC) in transverse uterine sections and by Western blotting of lysates of separated epithelial and stromal cells using anti-KLF4 and KLF15 antibodies. KLF4 as assessed by IHC is highly expressed in the uterine epithelium of E<sub>2</sub> treated mice with minimal expression in the stroma (Fig. 1B, *i*). In the P<sub>4</sub>E<sub>2</sub> mice, staining was dramatically reduced (Fig. 1B, *iii*), almost to the control level of staining seen with the IgG control (Fig. 1B, *v*). In contrast, KLF15 immunostaining shows a dramatic increase in expression in the uterine epithelium of P<sub>4</sub>E<sub>2</sub> treated mice with only background levels detected in the E<sub>2</sub> treated epithelium (Fig. 1B, *ii* and *vi*). There is some immunostaining in the stroma of the P<sub>4</sub>E<sub>2</sub> group, but only slightly more than the background obtained with a control antibody (Fig. 1B, *iv* and *vi*). These data are confirmed by Western blotting of separated epithelial and stromal uterine cell types prepared as described in the *Materials and Methods*. KLF4 is expressed at high levels only in the epithelium isolated from E<sub>2</sub> treated mice (Fig. 1C). In contrast, KLF15 is expressed predominantly in the P<sub>4</sub>E<sub>2</sub> treated uterine epithelium with a low level in the stroma and only minimal expression in the two tissues isolated from the estrogenized uterus (Fig. 1C). It should also be noted that the stromal preparations are contaminated by glandular epithelium (29) and are not as pure as the >95% purity of the epithelial fraction (30). Thus, the expression of KLF15 in stromal cells is likely even lower than the low level detected on the Western. From these data we can conclude that there is a preferential expression of KLF4 and KLF15 in the uterine epithelium following E<sub>2</sub> and P<sub>4</sub>E<sub>2</sub> treatment, respectively. Thus the relative balance of the concentration of these TFs in the uterine epithelium depends on hormonal treatment.

**KLF4 and KLF15 Bind to the *Mcm2* Promoter and Predict RNA Pol II Association.** The up-regulation of KLF15 by P<sub>4</sub>E<sub>2</sub>, coincident with the down-regulation of *Mcm2* transcript abundance, led to the hypothesis that KLF15 could negatively regulate *Mcm2* transcription. To test this hypothesis we first determined whether this transcription factor binds to the putative *Mcm2* promoter. As a control, we also determined the binding of the E<sub>2</sub> induced KLF4 to the same promoter region. In order to do this analysis, a region containing 5,000 bp upstream and 2,000 bp downstream, encompassing the first two introns from the MCM2 transcriptional start



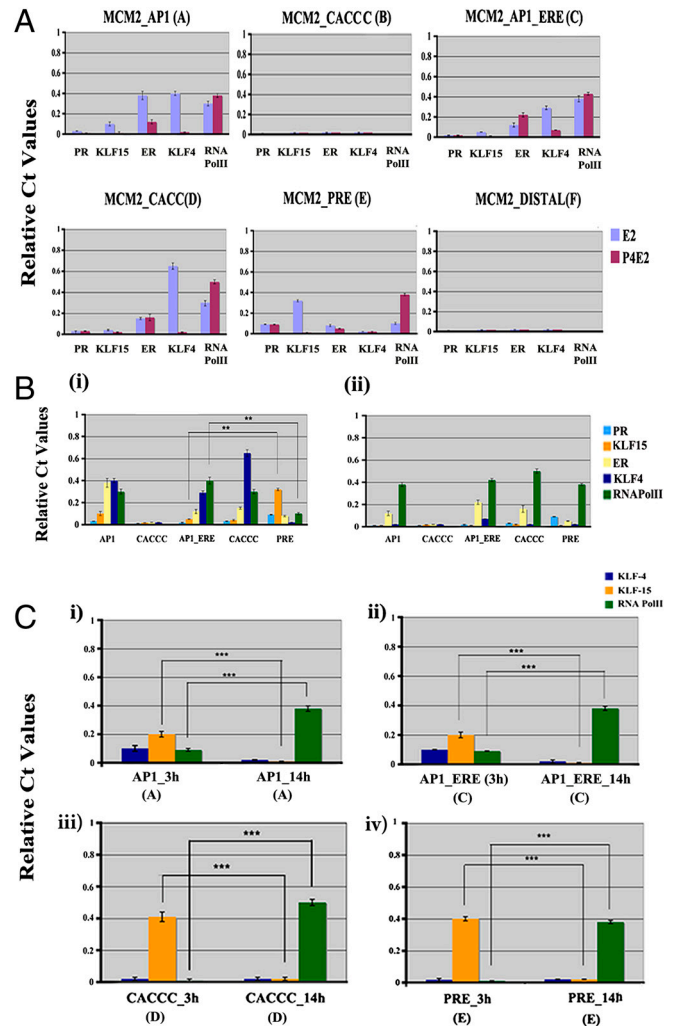


polymerase II followed by PCR (to test whether the band size corresponds with the expected one; Fig. 2*B*, *i*) as well as Quantitative real-time-PCR (Q-PCR; Fig. 2*B*, *ii*) 3 h after E<sub>2</sub> or P<sub>4</sub>E<sub>2</sub> treatment. There was minimal binding detected at this cycle number with an irrelevant IgG or with any of the antibodies used to the distal region (F) or to the downstream CACCC (B) sequence (Fig. 2*B*). This lack of significant binding with the nonspecific IgG was found in all experiments and was always run as a specificity control. In contrast, hormone specific binding of individual transcription factors was readily detected at this low cycle number. KLF15 bound to the AP1 (A), AP1\_ERE (C), CACCC (D), and PRE (E) in chromatin extracted from P<sub>4</sub>E<sub>2</sub> treated mice (Fig. 2*B*). This binding of KLF15 was significantly diminished in chromatin from the E<sub>2</sub> treated group (Fig. 2*B*). In contrast, KLF4 was bound to the AP1 (A), AP1\_ERE (C), and CACCC (D) sites in chromatin from the E<sub>2</sub> treated uteri, but was significantly diminished in chromatin from the P<sub>4</sub>E<sub>2</sub> group (Fig. 2*B*). This ChIP analysis also revealed that PR bound to the PRE (E) and ERα to the AP1 (A) and AP1\_ERE (C) sites again in a hormone regulated fashion (Fig. 2*B*). In addition to these TFs, the binding of RNA Pol II was also determined. RNA Pol II binding was highly up-regulated by E<sub>2</sub> treatment at the AP1 (A), AP1\_ERE (C), and PRE (E) sites. RNA Pol II association is at least sixfold higher in the E<sub>2</sub> than P<sub>4</sub>E<sub>2</sub> treated uterus at site A (AP1); on sites C (AP1\_ERE) and E (PRE), its association is dramatically higher as it is essentially undetectable in P<sub>4</sub>E<sub>2</sub> treated samples (Fig. 2*B*). Thus RNA Pol II is significantly recruited to most of these binding sites in an estrogen dependent manner and this was significantly inhibited, in many cases to levels at the limit of detection, by P<sub>4</sub>E<sub>2</sub> pretreatment (Fig. 2*B*, *i* and *ii*).

ChIP demands a significant input of material together with rapid cross-linking to obtain reliable data from *in vivo* sources and with multiple antibodies. Therefore, given the predominant expression of KLF4 and 15 in the uterine epithelium of E<sub>2</sub> and P<sub>4</sub>E<sub>2</sub> treated mice respectively, we used chromatin from whole uteri to perform the experiments. However, to ensure the data is representative we also performed ChIP on isolated epithelium 3 hrs post-E<sub>2</sub> and P<sub>4</sub>E<sub>2</sub> treatment. These data show the same pattern of binding of KLF15, KLF4, and RNA Pol II that was determined in whole uterine homogenates (Fig. S1). This result indicates that the data from whole uteri are representative because of the preferential expression of both KLF4 and KLF15 in the uterine epithelium.

Because binding of these TFs is hormone responsive, in Fig. 2*C* and *D* we display the Q-PCR data from the ChIP analysis of the *Mcm2* promoter in uteri according to their E<sub>2</sub> (Fig. 2*C*) and P<sub>4</sub>E<sub>2</sub> (Fig. 2*D*) treatment, respectively. These data show that following E<sub>2</sub> treatment of KLF4, ERα and RNA Pol II are bound to the putative MCM2 promoter with minimal binding of KLF15 (Fig. 2*C*) at each position. In contrast, P<sub>4</sub>E<sub>2</sub> treatment ablates binding of KLF4, ERα, and RNA Pol II while PR and particularly KLF 15 binding is highly enriched (Fig. 2*D*). Together, these data show that P<sub>4</sub>E<sub>2</sub> treated mice have higher amounts of KLF-15 binding on each of three positions and that this binding corresponds with lower RNA Pol II association.

**The Temporal Regulation of KLF15 Binding Controls RNA Pol II Association.** Physiologically, by 14 h the effects of P<sub>4</sub> on cell proliferation are attenuating in preparation for the next wave of E<sub>2</sub> stimulation, and there is a rebound in the expression of many of the genes that are inhibited by P<sub>4</sub> early in the process (28, 31, 32). Thus, to test the hypothesis that KLF-15 inhibits *Mcm2* transcription by blocking RNA polymerase association with the chromatin, we determined the changing pattern of recruitment of KLF-4, KLF-15, and RNA Pol II at 14 h after treatment using ChIP and Q-PCR of uterine chromatin. Notably, at 14 h, RNA Pol II is recruited to the AP1 (A), AP1\_ERE (C), CACCC (D), and PRE (E) sites (Fig. 3*A*) in the P<sub>4</sub>E<sub>2</sub> treated uterine samples to



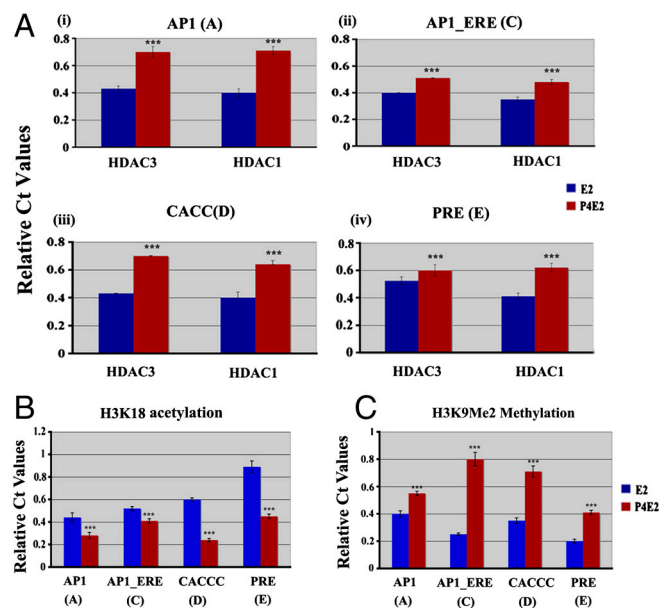
**Fig. 3.** Temporal binding patterns of KLF4 and KLF15 compared to RNA Pol II on the *Mcm2* promoter. (A) Analysis of recruitment of ERα, PR, KLF-4, KLF-15, and RNA Pol II by ChIP on the uterine *Mcm2* promoter 14 h after hormone treatment. The results presented are compared to input and normalized against IgG control. (B) Correlation between KLF15 recruitment and RNA Pol II recruitment 14 h after E<sub>2</sub> (*i*) and P<sub>4</sub>E<sub>2</sub> (*ii*) treated samples. Shown are representative bar diagrams that established a reciprocal relationship between KLF15 and RNA Pol II association. (Differences between binding  $***p < 0.001$ ,  $n = 5$ ). (C) Identification of a direct link between KLF15 and RNA Pol II association by comparing 3 h vs. 14 h in the P<sub>4</sub>E<sub>2</sub> treated samples at four discrete positions on the *Mcm2* promoter. In each case a reduction in KLF15 binding at 14 h is correlated with an increase in RNA Pol II binding. Differences in binding at each position  $***p < 0.001$ ,  $n = 5$ . Bar identification shown in figure is as described in Fig. 2.

an even greater extent than in the samples treated with E<sub>2</sub> alone. This result is shown in more detail in Fig. 3*B*, where the two hormone treatments are separated graphically. Specifically, binding of KLF-15 vs. RNA Pol II at the site AP1\_ERE (C) vs. PRE (E) shows a significantly higher binding of KLF-15 at the PRE (E) that corresponds with reduced RNA Pol II association (Fig. 3*B*, *i*). Furthermore, KLF15 binding that dominated at 3 h after P<sub>4</sub>E<sub>2</sub> treatment is now lost, and RNA Pol II is associated at each of the positions (Fig. 3*A* and *B*, *ii*). We hypothesized that there is an inverse relationship between KLF-15 binding with RNA Pol II recruitment; therefore, we compared their binding at 3 h versus at the 14 h time point after P<sub>4</sub>E<sub>2</sub> treatment at four individual motifs (Fig. 3*C*, *i-iv*). In each position, the significantly higher association of KLF-15 at 3 h after P<sub>4</sub>E<sub>2</sub> treatment ( $p < 0.001$ ) is correlated with a corresponding lower RNA Pol II association

( $p < 0.001$ ). Our data strongly suggest that KLF15 negatively regulates RNA Pol II binding.

**E<sub>2</sub> and P<sub>4</sub>E<sub>2</sub> Alter Histone Acetylation and Methylation of the MCM2 Promoter.** The activity of promoters is partially defined by the state of histone acetylation and methylation. Hyper-acetylated histones are linked to transcriptionally active domains, whereas hypo-acetylated histones are generally associated with transcriptionally silent domains (33–35). Therefore we examined these epigenetic signatures in response to hormone exposure utilizing ChIP on the uterine *Mcm2* chromatin 3 h after hormone treatment. Assays were performed with antibodies against the histone modifying enzymes, HDAC1 or HDAC3, to determine their association with these positions (A, C, D, and E). In P<sub>4</sub>E<sub>2</sub> treated mice, uterine binding of HDAC3 was higher at all four sites compared to E<sub>2</sub> treated samples. Similarly, HDAC1 association was significantly higher in P<sub>4</sub>E<sub>2</sub> treated samples at the AP1 (A), AP1\_ERE (C), CACCC (D), and PRE (E) sites compared to chromatin from E<sub>2</sub> treated mice. Consistent with this binding of the HDACs to the *Mcm2* promoter region, ChIP using antibodies specific for the acetylated lysine 18 of Histone H3 (H3K18) indicated that the acetylation state of the *Mcm2* promoter was lower at all four sites in the P<sub>4</sub>E<sub>2</sub> treated versus the E<sub>2</sub> treated group (Fig. 4B).

Inactive promoters also harbor higher levels of methylation of lysine 9 on histone 3 (H3K9Me2) (36, 37). Therefore, we performed ChIP on the *Mcm2* promoter with an antibody against H3K9Me2 in P<sub>4</sub>E<sub>2</sub> and E<sub>2</sub> treated samples 3 h after treatment. Our results detected this modification at the AP1 (A), AP1\_ERE (C), CACCC (D), and PRE (E) positions in the *Mcm2* promoter. Consistent with an inactive promoter in the P<sub>4</sub>E<sub>2</sub> treated samples, there was a greater amount of H3K9Me2 at all four positions (Fig. 4C).



**Fig. 4.** Acetylation and methylation signature on *Mcm2* chromatin 3 h after hormone treatment. Uterine tissues were collected from ovariectomized mice after 3 h of E<sub>2</sub> or P<sub>4</sub>E<sub>2</sub> treatment and subjected to ChIP using antibodies against (A) HDAC1 or HDAC3, as described in the *Materials and Methods*. The results presented are compared to input and normalized against IgG control. Each site is independently shown. Differential binding as indicated \*\*\* $P < 0.001$ ,  $n = 5$ . (B) H3K18Ac levels at different sites were determined by ChIP-QPCR 3 h after E<sub>2</sub> or P<sub>4</sub>E<sub>2</sub> treated uterine chromatin. (C) Levels of H3K9Me2 on different sites were determined by ChIP-QPCR 3 h after E<sub>2</sub> or P<sub>4</sub>E<sub>2</sub> treated uterine chromatin. Statistical analysis (B and C) represents the difference between E<sub>2</sub> treated samples with P<sub>4</sub>E<sub>2</sub> treated one at four different sites, \*\*\* $P < 0.001$ ,  $n = 5$ .

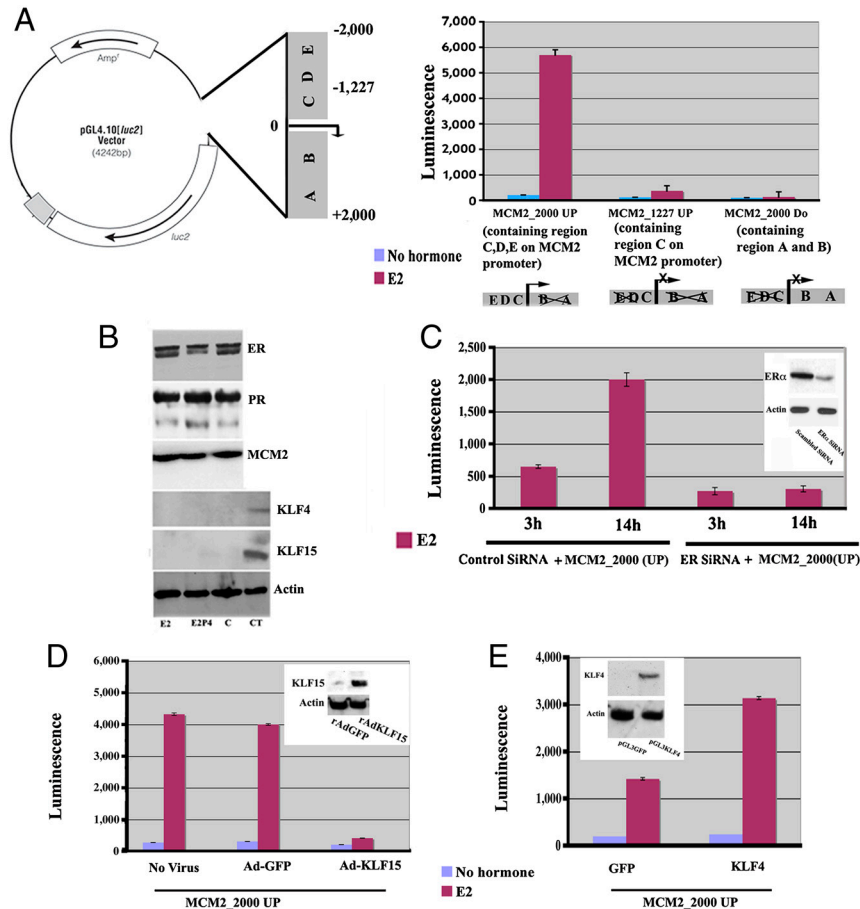
Together, these data confirmed that the uterine *Mcm2* promoter is in a more active configuration in E<sub>2</sub> than in P<sub>4</sub>E<sub>2</sub> treated mice.

**The *Mcm2* Promoter Region Is Regulated by E<sub>2</sub> and Inhibited by KLF15.**

Our results from the ChIP analysis define the temporal and hormone dependent recruitment of TFs to the putative *Mcm2* promoter region. These in vivo studies, particularly those pertaining to RNA Pol II recruitment and histone acetylation and methylation, also suggest that female sex steroid hormones regulate *Mcm2* promoter activity. To test this hypothesis directly, we performed luciferase (LUC) assays using constructs containing the putative promoter regions separately fused to a promoterless LUC expression vector (pGL4-Luc basic) (Fig. 5A). These constructs were transfected into the endometrial adenocarcinoma Ishikawa cell line, which is one of the few uterine cell lines that expresses functional ER $\alpha$  and PR (38). This cancer cell line is modestly responsive to E<sub>2</sub> but has lost its inhibitory response to P<sub>4</sub> despite expression of receptors for both hormones. Before performing the luciferase assays, we determined the expression of ER $\alpha$ , PR, KLF4, KLF15, and MCM2 in our clone of Ishikawa cells by Western blotting with appropriate antibodies. This analysis demonstrated that ER $\alpha$ , PR, and MCM2 proteins are abundantly expressed in Ishikawa cells although no hormonal regulation of these proteins was obtained in this cell line (Fig. 5B). In contrast, we could not detect KLF4 or KLF15 in Ishikawa cell lysates even though these proteins were readily detected in the control (CT) uterine lysates (Fig. 5B). Having established that Ishikawa cells express ER $\alpha$ , we next analyzed promoter activity of the various genomic DNA fragments by transient transfection methods. Serum deprived Ishikawa cells were separately transfected with one of the three constructs containing MCM2\_2000-Luc (2000 bp upstream of the TSS UP), MCM2\_1227-Luc (1227 bp upstream from the TSS UP), and MCM2\_2000-Luc (2000 bp downstream from the TSS Do) region together with a Renilla luciferase plasmid followed by hormonal treatment; the normalized firefly activity was determined. Fourteen hours after hormone treatment, only the MCM2\_2000 (up) sequence exhibited significant promoter activity, and this activity was dependent on E<sub>2</sub> with very little luciferase activity being detected in the untreated control (Fig. 5A). Neither the downstream nor the immediate 1227 base pair up-stream regions exhibited significant transcriptional activity (Fig. 5A).

As E<sub>2</sub> causes a stimulation of *Mcm2* promoter activity, we determined whether this was ER $\alpha$  dependent by knocking down this receptor expression using a SiRNA. This SiRNA reduced ER $\alpha$  protein by approximately 80%, and significantly reduced *Mcm2* transcriptional activity (Fig. 5C). This result indicates that the ER $\alpha$  drives the transcriptional response of the *Mcm2* promoter and that the MCM2 upstream region acts as an E<sub>2</sub> responsive element with the regulatory region between 2000 and 1227 bp upstream from the TSS.

Our ChIP data obtained above suggests negative regulation of *Mcm2* transcription by KLF 15. To test this hypothesis directly, serum deprived Ishikawa cells were infected with adenovirus containing KLF-15 or control GFP followed by transfection with the MCM2\_2000 UP (LUC) construct. The resultant cells that express KLF15 (Fig. 5D) were treated with E<sub>2</sub> and the level of luciferase activity was directly determined in cell extracts. The expression of GFP from the adenovirus had no effect on luciferase activity (Fig. 5D). However, expression of KLF15 almost completely inhibited *Mcm2* transcription in the E<sub>2</sub> treated cells (Fig. 5D). As a control for specificity of the KLF, KLF4 was also overexpressed (Fig. 5E) in a parallel experiment. In contrast to KLF15, KLF4 increased MCM2-2000 luciferase expression (Fig. 5E). Thus the effect of KLF15 on *Mcm2* transcription is not simply due to a KLF indiscriminately inhibiting its transcription.



**Fig. 5.** Transcriptional activity of the *Mcm2* promoter in Ishikawa cells. (A) Shown is the representative diagram of PGL4 (*luc2*) vector containing the putative *Mcm2* promoter region containing the specific sites for binding of transcription factors identified in Fig. 2. Ishikawa cells were transiently cotransfected with a control renilla plasmid and the luciferase derivatives containing three separate genomic fragments 2000 bp upstream region of TSS (MCM2\_2000 UP), downstream region containing 2000 bp downstream in relation to TSS (MCM2\_2000 DO), and region containing 1227 upstream in relation to TSS (MCM2\_1227 UP) for 36 h followed by vehicle alone (blue bar) or E<sub>2</sub> (red bar) treatment and assessed for luciferase activity 14 h after hormone treatment. Results in all panels are presented as normalized by firefly luciferase activity compared to Renilla control vector. (B) Serum deprived Ishikawa cells were treated with E<sub>2</sub> or P<sub>4</sub>E<sub>2</sub> and whole cell lysates were collected and analyzed by western blotting for expression of ER $\alpha$ , PR, MCM2, KLF4 and KLF15. The vehicle treated cells (C) along with uterine epithelial tissue lysates (CT) are used as control for KLF4 and 15, respectively. (C) Effects of silencing of ER $\alpha$  expression on *Mcm2* transactivation. Ishikawa cells were cotransfected with ER $\alpha$  SiRNA along with the MCM2\_2000(UP) construct followed by E<sub>2</sub> treatment for 3 h or 14 h when the cells were harvested as described in the *Materials and Methods*. Insert is the Western blot analysis of ER $\alpha$  expression compared to actin control after transfection of specific SiRNA or scrambled SiRNA in Ishikawa at the end of the experiment. (D) Role of KLF15 in MCM2\_2000 transactivation. Ishikawa cells were transfected with MCM2\_2000 UP followed by infection with AdKLF15 or Ad-GFP. Cells were incubated for 36 h, then treated with E<sub>2</sub> or vehicle for 14 h. Luciferase assays were performed on the isolated cells extracts. Insert is the Western blot analysis of KLF15 expression after infection with AdGFP (control) or AdKLF15 (experiment) compared to actin control in Ishikawa followed by E<sub>2</sub> treatment for 14 h. (E) Role of KLF4 in MCM2\_2000 transactivation. Ishikawa cells were cotransfected with MCM2\_2000 UP along with KLF4 or vector containing GFP. Cells were incubated for 36 h then treated with E<sub>2</sub> or vehicle for 14 h. Luciferase assays were performed on the cells extracts. Insert is the Western blot analysis of KLF4 expression compared to actin control after transfection of specific pGL3KLF4 (experiment) or pGL3GFP (control) in Ishikawa followed by E<sub>2</sub> treatment for 14 h.

Together, these data show that the *Mcm2* promoter is transcriptionally regulated by E<sub>2</sub> and that this transcription can be significantly inhibited by KLF15.

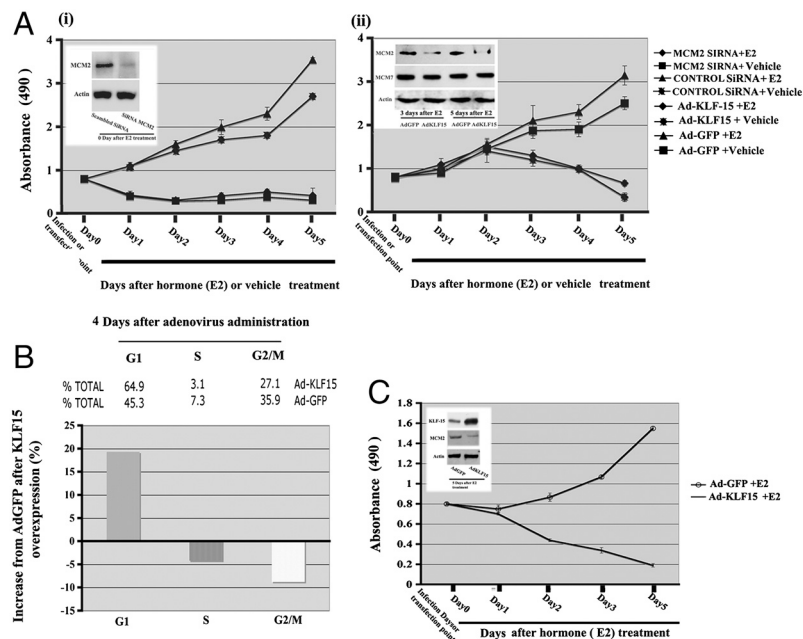
**Ishikawa Cell Proliferation Is Negatively Regulated by KLF15 and Requires MCM2.** The rapid down-regulation of MCM proteins by P<sub>4</sub>E<sub>2</sub> (28) led to the hypothesis that inhibition of the MCM mediated pathway plays a major role in the P<sub>4</sub> negative regulation of uterine epithelial cell proliferation. This hypothesis was based on observations in lower organisms that reduction in any of the MCM's level resulted in an inhibition of DNA synthesis (39). In this study, we showed that KLF15 is a negative regulator of *Mcm2* transcription. Therefore we hypothesized that KLF15 is the mediator of P<sub>4</sub> inhibition of DNA synthesis in uterine epithelial cells by suppression of *Mcm2* transcription.

To test this hypothesis, we first inhibited *Mcm2* expression using a SiRNA and determined the effects on cell proliferation

in Ishikawa cells. In these experiments, E<sub>2</sub> modestly stimulates Ishikawa cell proliferation, as determined using a MTT assay (Fig. 6A, *i*) as previously reported (38). This proliferative response was unaffected by transfection with a scrambled SiRNA. However, transfection with a specific SiRNA against *Mcm2* mRNA, which reduced MCM2 protein levels by approximately 80%, resulted in a complete suppression of cell proliferation in both the control and E<sub>2</sub> treated groups. This effect was seen within 24 h of the nonhormone treated state.

Next we tested the effects of KLF15 overexpression in Ishikawa cells. These cells were infected with a KLF15 expressing adenovirus (rAdKLF15) or control GFP virus (rAdGFP) 24 h before hormone addition (Fig. 6A, *ii*). In the control virus treatment group, there was no effect on cell proliferation. However, infection of the cells with KLF15 expressing adenovirus reduced cell proliferation in both vehicle and E<sub>2</sub> treated groups. But in contrast to the response to the MCM2 SiRNA, this reduction in





**Fig. 6.** Effects of MCM2 knock down and KLF15 expression on Ishikawa cell proliferation. (A) Ishikawa cells were cultured in hormone free media for 24 h after MCM2 or scrambled SiRNA transfection (i) or infection with Ad-KLF15 or Ad-GFP (ii) followed by E<sub>2</sub> or vehicle (ethanol) treatment. Cell proliferation was assayed in triplicate wells daily for 5 d after hormone treatment as described in the *Materials and Methods*, using the MTT assay (absorbance at 490 nm). Inserts (i, ii) indicate the Western blot analysis of equivalent amounts of protein isolated from these cells and detected with antibodies against MCM2, MCM7 (only ii) or actin as control after silencing of MCM2 SiRNA (i) or after KLF-15 overexpression (ii) for the indicated times. (B) Role of KLF15 overexpression on cell cycle parameters. Four days after postinfection, Ishikawa cells were collected and analyzed by flow cytometry to determine distribution of cells in each phase of the cell cycle. (C) Role of KLF15 overexpression on T47D cell proliferation. Human breast cancer T47D cells in culture were infected with rAdKLF-15 or rAd-GFP. Twenty-four hours after infection, cells were treated with E<sub>2</sub> or vehicle and cell proliferation was assessed by the MTT method from 0 d to 5 d after treatment. Insert shows the Western blot analysis using antibodies against KLF15 and MCM2 compared to the control actin 5 d after treatment, indicating the reciprocal expression of KLF15 and MCM2. Values in (A) and (C) are presented as mean ± SE of five independent experiments (A and C).

proliferation occurred after approximately 2 d of expression. To determine whether this KLF15 overexpression affected MCM2 protein levels, we measured its expression. Within 4 d of infection (3 d after E<sub>2</sub> treatment), KLF15 overexpression reduced MCM2 expression in these cells, an effect that was enhanced by 5 d of treatment (Fig. 6*A*, ii, insert). To determine specificity of the effect of KLF15, we also examined MCM7 expression because, in vivo, its expression is unresponsive to hormone treatment. KLF15 overexpression did not inhibit expression of MCM7 in Ishikawa cells, indicating specificity of the response (Fig. 6*A*, ii). We determined the effects of KLF15 on cell cycle progression by flow cytometry. This flow cytometric analysis indicated that, following KLF15 expression, cells accumulated in G1, showing that KLF15 inhibited cell cycle progression in the G1 phase of the cell cycle (Fig. 6*B*).

The inhibition of cell proliferation in Ishikawa cells by KLF15 was profound. Therefore, we sought to determine whether this was the case in other hormone responsive cell lines. We used the breast cancer cell line T47D to perform these experiments. Infection with an adenovirus expressing KLF15 inhibited T47D cell proliferation with a time-course similar to that observed in Ishikawa cells, whereas the control GFP virus had no effect (Fig. 6*C*).

Therefore, we can conclude that KLF15 is a negative regulator of cell proliferation in hormone responsive cell lines, at least in part through its inhibition of *Mcm2* transcription.

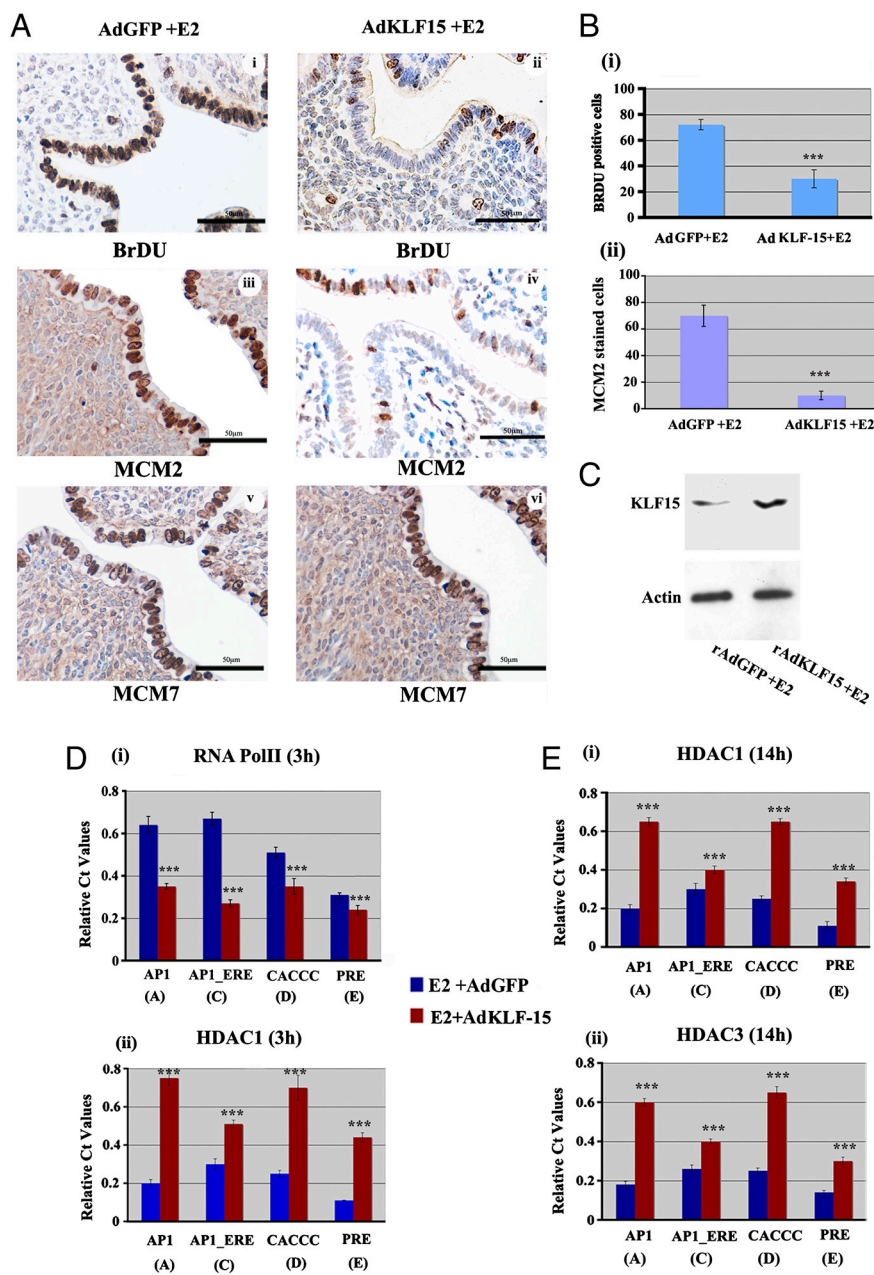
**KLF15 Inhibits E<sub>2</sub> Induced Epithelial Cell Proliferation in Vivo and Deacetylates the *Mcm2* Chromatin.** The hypothesis tested in this paper is that KLF 15 inhibits transcription of genes such as *Mcm2*, and thereby inhibits E<sub>2</sub> induced epithelial DNA synthesis, and therefore cell proliferation. This is clearly the case in tissue culture, so we tested whether KLF15 could block DNA synthesis in the mouse uterine epithelium in vivo. In order to test this hypothesis, we infected ovariectomized mice with the adenovirus expressing KLF15 or GFP as a control through intravenous and uterine intraluminal routes and subsequently treated the mice with E<sub>2</sub>. Mice were killed 15 h after E<sub>2</sub> injection at the peak of DNA synthesis (40) and 2 h after an ip injection of BrdU. Following histological preparation of the uterus, the level of DNA synthesis was measured by BrdU incorporation per cell (Fig. 7*A*). Compared with the GFP control virus, the overexpression of KLF-15 (Fig. 7*C*) significantly inhibited E<sub>2</sub> induced uterine DNA synthesis by approximately 65% in response to E<sub>2</sub> (Fig. 7*A*, i and ii;

and B, i). To determine whether this inhibition of DNA synthesis by KLF15 was coincident with an inhibition of *Mcm2* expression, we immunostained uterine transverse sections for MCM2. KLF15 expression compared to the control GFP expressing virus resulted in an approximately 80% reduction in the number of MCM2 expressing cells in presence of E<sub>2</sub>, similar in extent to that observed for BrdU incorporation (Fig. 7*A*, iii and iv; B).

Our previous results indicated that neither the expression of *Mcm7* transcript nor the protein is significantly changed in uterine luminal epithelium in E<sub>2</sub> vs. P<sub>4</sub>E<sub>2</sub> treated samples (28). Thus we determined the effect of KLF15 on MCM7 expression. In contrast to the effect on MCM2 expression, there was no significant difference in MCM7 levels between the GFP and KLF15 treated groups in presence of E<sub>2</sub> (Fig. 7*A*, v and vi). These data, together with the normal structure of the cells in the epithelium and stroma, showed that the effects of KLF15 overexpression are specific and not the result of toxicity.

Because we demonstrated an inverse relationship between KLF15 association with RNA Pol II binding on *Mcm2* uterine chromatin during E<sub>2</sub> and P<sub>4</sub>E<sub>2</sub> treatments, we determined the status of RNA Pol II association after KLF15 overexpression in the uterus 3 h after E<sub>2</sub> treatment. ChIP analysis was performed using RNA Pol II specific antibodies with uterine tissues collected from rAdKLF-15 or rAdGFP treated mice. Our results showed that KLF15 administration inhibited RNA Pol II association at the AP1 (A), AP1\_ERE (C), CACCC (D), and PRE (E) sites on the *Mcm2* chromatin even in the presence of E<sub>2</sub> (Fig. 7*D*, i). Because E<sub>2</sub> inhibits HDAC association and increases histone acetylation, next we determined the effect of KLF15 expression on HDAC association, on this *Mcm2* promoter. KLF15 facilitated HDAC1 recruitment on chromatin in the estrogenized uterus at 3 h (Fig. 7*D*, ii) and 14 h (Fig. 7*E*, i) after E<sub>2</sub> administration when compared to rAd-GFP treated mice. Increased HDAC-3 binding was also found 14 h after hormone treatment in rAd-KLF-15 mice (Fig. 7*E*, ii). Overall, KLF15 facilitates the recruitment of the HDACs onto the *Mcm2* promoter even in the presence of E<sub>2</sub> administration, consistent with its negative regulation of *Mcm2* transcription.

In summary, we conclude that KLF15 is a negative regulator of E<sub>2</sub> induced cell proliferation and, physiologically, is a downstream mediator of P<sub>4</sub> action.



**Fig. 7.** KLF15 expression in the mouse uterus suppresses MCM2 expression and inhibits  $E_2$ -induced epithelial DNA synthesis. (A) Ovariectomized CD1 mice were exposed to rAdGFP (control) (*i, iii, v*) or rAdKLF15 (*ii, iv, vi*) as described in the *Materials and Methods*, followed by injection of  $E_2$  for 14 h. Two hours before killing, mice were given BrDU ip. (*i* and *ii*). Transverse sections of formalin fixed uteri were immunostained for (*i, ii*) BrDU incorporation, (*iii* and *iv*) MCM2 and (*v* and *vi*) MCM7 followed by hematoxylin staining as described. Brown staining indicates positive reactivity. The data described here are representative of the analysis of at least five different mice for each group. Scale bar: 50  $\mu$ m. (B) Differential BrDU incorporation (*i*) or MCM2 staining (*ii*). Percentage of positive epithelial cells staining for nuclear BrDU (*i*) or MCM2 (*ii*) in Ad-KLF15 or Ad-GFP infected mice 14 h after  $E_2$  administration. \*\*\* $P < 0.001$ ,  $n = 5$ . (C) KLF15 protein expression after rAdKLF15 or rAdGFP treated uterus. Equivalent amount of protein lysate isolated from uterine epithelial cells of mice treated with rAdKLF15 (experiment) or rAdGFP (control) followed by  $E_2$  for 14 h were separated by SDS PAGE and subjected to Western blotting with specific antibodies as shown. Actin was used as a protein loading control. (D) Analysis of RNA Pol II and HDAC1 association on *Mcm2* promoter after KLF15 overexpression. Uterine tissues were collected after administration of rAdKLF15 (experiment: red bars) or rAd-GFP (control: blue bars) as described in *Materials and Methods* followed by  $E_2$  treatment for 3 h. ChIP analysis on isolated chromatin was performed with antibody against RNA Pol II (*i*) and HDAC1 (*ii*) on the indicated regions of *Mcm2* gene promoter. Data was analyzed by Student *t* test. Difference in binding in each position \*\*\* $P < 0.001$ ,  $n = 5$ . (E) Analysis of acetylation signature on *Mcm2* promoter after AdKLF-15 overexpression. ChIP analysis was performed and data expressed as defined in Fig. 2 using chromatin isolated from uterine tissues after rAd-KLF-15 (red bars) or rAd-GFP (blue bars) infection followed by  $E_2$  treatment for 14 h with antibody against HDAC1 (*i*) or HDAC3 (*ii*). (Difference between binding \*\*\* $P < 0.001$ ,  $n = 5$ ).

## Discussion

$E_2$  induces and  $P_4$  inhibits uterine epithelial cell proliferation. In this study, as summarized in Fig. 8, our results show that two members of the Kruppel-like family of transcription factors, KLF4 and KLF15, mediates these hormones effects within the uterine epithelium. This is achieved by modulating the expression of *Mcm2*, whose product is required for DNA replication.  $E_2$  and  $P_4E_2$  induce KLF4 and KLF15 reciprocally in uterine epithelial cells. KLF4 stimulates and KLF15 inhibits MCM2 transcription through modulating histone acetylation and methylation, and thereby RNA Pol II binding. The increased MCM2 as a result of KLF4 action in the estrogenized uterine epithelium allows for prereplication complex formation and thereby DNA synthesis initiation. In contrast, KLF15 inhibits *Mcm2* expression with a reduction in *Mcm2* mRNA and MCM2 protein, and thus DNA synthesis is inhibited. KLF15 expression alone is sufficient to block  $E_2$ -induced epithelial DNA synthesis both in vivo and in vitro, indicating that this TF is a key mediator of  $P_4E_2$  physiological action.

In their regulation of physiological events in the uterus, these sex steroid hormones act both cell autonomously and nonautonomously through their TF receptors (1, 10). In either case they induce the expression of downstream TFs in the target cell that propagate the initial signal. For example,  $P_4$  regulates epithelial synthesis of Indian Hedgehog (IHH). This IHH acts via the smoothed receptor expressed on stromal cells to induce the TF Coup-TF11, which in turn mediates downstream signaling, including the expression of bone morphogenic protein 2 that is required for stromal cell proliferation during the preimplantation period (41, 42). Meanwhile, over the same physiological period,  $P_4$  induces the helix-loop-helix TF, HAND2, in stromal cells to negatively regulate the stromal expression of FGFs that can induce epithelial proliferation (9). Similarly in the epithelium,  $E_2$ -induced cell proliferation is regulated by stromal synthesized IGF-1, which binds to the IGF-1 receptor (IGF-1R), resulting in the engagement of the canonical cell cycle machinery that causes phosphorylation of the Rb family of transcriptional repressors (8, 31, 43). In this study we identified two additional transcription





many cancers including hormonally regulated ones such as breast and prostate (26). In other diseases of the endometrium that display abnormal proliferation and  $P_4$  resistance such as in endometriosis, the regulation of MCMs by  $P_4$  is also lost (47).  $P_4$  represents a rare example in vivo of a well-characterized physiological negative regulator of cell division. Our results establish that KLF15 inhibits endometrial and breast epithelial DNA synthesis. KLF15 therefore represents an important factor to be considered therapeutically for the inhibition of  $E_2$  proliferation action in the uterus and breast.

## Materials and Methods

In brief, CD1 mice were ovariectomized and treated with hormonal regimens (31). At different times after the last  $E_2$  injection they were killed and their uteri fixed for IHC, or the uterine epithelium prepared and processed for

Western blotting or ChIP as described (48). Ishikawa and T47D cells were cultured and transfected with *Mcm2* promoter luciferase constructs and luminescence measure. Mice or cells were infected with Adenovirus expressing KLF15 or cells with a cDNA expressing KLF4 (48). All data were repeated at least three times and statistical analysis was performed using Prism 5. For details on all the above, see *SI Materials and Methods*.

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