Regulation of *u-PAR* gene expression by H2A.Z is modulated by the MEK–ERK/AP-1 pathway

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ABSTRACT

The urokinase receptor (u-PAR) which is largely regulated at the transcriptional level has been implicated in tumor progression. In this study, we explored the epigenetic regulation of *u-PAR* and showed that the histone variant H2A.Z negatively regulates its expression in multiple cell lines. Chromatin immunoprecipitation assays revealed that H2A.Z was enriched at previously characterized *u-PAR*-regulatory regions (promoter and a downstream enhancer) and dissociates upon activation of gene expression by phorbol ester (PMA). Using specific chemical and dominant negative expression constructs, we show that the MEK–ERK signaling pathway terminating at AP-1 transcription factors intersects with the epigenetic control of *u-PAR* expression by H2A.Z. Furthermore, we demonstrate that two other AP-1 targets (*MMP9* gene and miR-21 microRNA) are also H2A.Z regulated. In conclusion, our work demonstrates that (i) the expression of two genes and a microRNA all implicated in tumor progression are directly regulated by H2A.Z and (ii) MEK–ERK signaling terminating at AP-1 intersects with the epigenetic control of target gene expression by H2A.Z.

INTRODUCTION

The urokinase receptor (u-PAR), a 55–60 kDa heavily glycosylated disulfide-linked protein connected to the cell surface via a glycolipid moiety (1), promotes tumor progression. U-PAR is overexpressed in divergent tumors types (2–6) and potentially represents a promising therapeutic target (7–13). This cell surface receptor promotes tumor progression via multiple mechanisms. First, u-PAR interacts with the extracellular domain of integrins to regulate cell proliferation, cell attachment and tumor dormancy (14–18). Second, high affinity binding of the serine protease urokinase (u-PA) with u-PAR converts the inert plasminogen into the broadly acting plasmin at a higher rate than fluid phase reactants (19). Focusing of proteolysis (via plasmin generation) at the cell surface results in extracellular matrix degradation (14) thereby facilitating tumor cell migration/invasion. Finally, it has been shown that the seven *trans*-membrane receptor FPR-like receptor-1/lipoxin A4 receptor, a G protein-coupled receptor directly interacts with a soluble cleaved form of u-PAR to induce chemotaxis (20).

u-PAR expression is, to a large extent, regulated at the level of transcription (21–23) although mRNA stability (22,24), protein translational efficiency (25) and turnover (26) also contribute to the final amount of protein product. In regard to transcriptional control, the promoter *cis*-elements and *trans*-acting factors regulating u-PAR expression have been well-studied by our and other groups (21,27–33). Of the transcription factors previously reported to be regulatory for u-PAR expression, AP-1 and NF-κB modulate inducible expression by diverse stimuli including epidermal growth factor (EGF), transforming growth factor β type 1 (TGF-β1), phorbol-12-myristate-13-acetate (PMA), interferons and a mutation-activated K-Ras (21,27,31,32). In addition, we recently reported on the role of an AP-1-harboring intragenic enhancer in the regulation of both constitutive and inducible *u-PAR* expression (33).

Although a substantial amount of work has been done to identify the *cis*- and *trans*-acting factors regulating *u-PAR* expression (21,27–33), the epigenetic regulation of this gene is poorly understood. Previous studies have identified a multitude of genes involved in tumor progression whose expression is regulated epigenetically (34–37). Understanding the molecular mechanism of epigenetic regulation of genes involved in cancer and metastasis might, ultimately, lead to the development of drugs that correct the expression of epigenetically dysregulated genes. In light of the overwhelming evidence implicating u-PAR in cancer progression, we undertook a study to determine if, and how, u-PAR expression is epigenetically regulated.

We describe here a new mechanism of epigenetic regulation of *u-PAR* and two other AP-1-regulated targets (*MMP-9*, oncomiR-21) mediated by H2A.Z, a highly

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conserved histone variant of the canonical histone H2A. Histone variants differ from their canonical counterparts in that the former are synthesized throughout the cell cycle and deposited on DNA outside of S-phase (38). In this study, we report the novel finding that H2A.Z is repressive for the expression of u-PAR and two other AP-1-regulated targets (*MMP-9*, oncomiR-21). Moreover, induced expression of these three AP-1-regulated targets requires H2A.Z eviction from the corresponding regulatory regions, an event driven by an activated MEK–ERK signaling module.

MATERIALS AND METHODS

Materials

All the chemicals, antibodies and siRNA used in this study are listed in Supplementary Table S1. Primers and Taqman probes (Applied Biosystem) used in real time PCR are listed in Supplementary Table S2. The Tam-67 vector, encoding a c-Jun protein lacking the transactivation domain (amino acids 3–122) has been described elsewhere (39). The pBABE puro K-Ras V12 plasmid encoding a constitutively active mutant K-Ras protein (G12V mutation) was procured from Addgene (Catalog No. 9052).

Tissue culture

All cancer cell lines were maintained in McCoy's 5A media containing 10% FBS and antibiotics (as required) in a humidified incubator at 37°C with 5% CO_2. For ChIP assays, cultures were grown in 15-cm diameter plates to 60–70% confluency. For inducing gene expression, PMA was used in cultures at 100 nM concentration.

Chromatin immunoprecipitation assay and quantitative real-time PCR

The chromatin immunoprecipitation (ChIP) experiments were performed using the ChIP-IT-Express kit from Active Motif (Catalog No. 53009) according to the manufacturer's instructions. Briefly, cells were fixed, DNA–proteins cross-linked using 1% formaldehyde at room temperature for 10 min. Fixation was stopped by adding glycine and the cells lysed. The DNA was subjected to enzymatic digestion (using a micrococcal nuclease-containing enzymatic cocktail from Active Motif Catalog No. 53009) for 20–25 min at 37°C to obtain mostly mononucleosomes. The resulting chromatin preparation was incubated at 4°C with the appropriate antibody (3–8 μg) and precipitated complexes washed four times. Cross-linking was reversed by incubation at 65°C (4 h). After digestion with Proteinase K (2 h at 42°C), DNA was purified by ethanol extraction, air dried and re-dissolved in H_2O. The retrieved DNA was then subjected to real-time RT–PCR amplification using specific primers (amplicon size 90–110 bp) listed in Supplementary Table S2 and a SYBR green qPCR master mix (Applied Biosytem). Input (in each qRT–PCR reaction) was used to normalize the values. All ChIP assays were repeated twice and individual qPCR reactions performed in triplicates with results presented as average values ± SD. The relative enrichment was calculated as $2^{\Delta Ct}$ where $\Delta C_t = C_t \, (\text{Input}) - C_t \, (\text{ChIP})$.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from cell culture using Trizol as per the manufacturer's instruction (Invitrogen). Contaminating residual DNA was removed using the TURBO DNA-free kit (Ambion, Catalog No. AM1907). RNA (2 μg) was used to prepare cDNA using the high capacity cDNA reverse transcription kit as per the manufacturer's instruction (Applied Biosytem cat # 4368814). Taqman probes (Applied Biosystem) and real-time PCR master mix (Applied Biosystem) were employed for real-time PCR as per the manufacturer's instruction. All amplifications were carried out in triplicates. Data were normalized using GAPDH or Actin.

Western blotting

Cell lysis and western blotting was performed as described previously by us (26).

SiRNA knock downs and transient transfection

Lipofectamine 2000 was used for transfections with plasmid DNA and siRNA following the manufacturer's instructions (Invitrogen). Cells were harvested after 48 h for RNA isolation or ChIP assays.

RESULTS

H2A.Z occupies the regulatory regions of *u-PAR* and is evicted upon gene expression

In order to gain insight into the epigenetic regulation of *u-PAR* expression, ChIP assays were employed in an unbiased screen to identify histone modifications or exchange of histone variants at the *u-PAR* promoter and enhancer under conditions of gene activation. We elected to use GEO colon cancer cells which express low endogenous *u-PAR* levels but which can be rapidly induced for transcription of this gene (23) with PMA (Figure 1B). Quantitative real-time PCR (qPCR) was performed using chromatin generated from either unstimulated or PMA-stimulated GEO cells using primer sets corresponding to the promoter, enhancer and intron 3 (Figure 1A and C). While both the promoter and the enhancer were enriched in acetylated histone 3 (H3-Ac) and acetylated histone 4 (H4-Ac) as expected for a gene induced for expression, dimethylated histone 3 (at the arginine, H3R2me2) was lost from the promoter, enhancer and intron 3 of the *u-PAR* gene (Figure 1C).

However, the most striking observation was dissociation of the histone variant H2A.Z from the promoter and enhancer region but not from intron 3 upon PMA stimulation of *u-PAR* gene expression (Figure 1C). Note that a recent study indicates that there are two H2A.Z isoforms: H2A.Z-1 and H2A.Z-2 (40,41) with most studies to date evaluating the product of the H2A.Z-1 gene. The data presented here evaluate the role of H2A.Z-1 (H2A.Z) in u-PAR regulation.
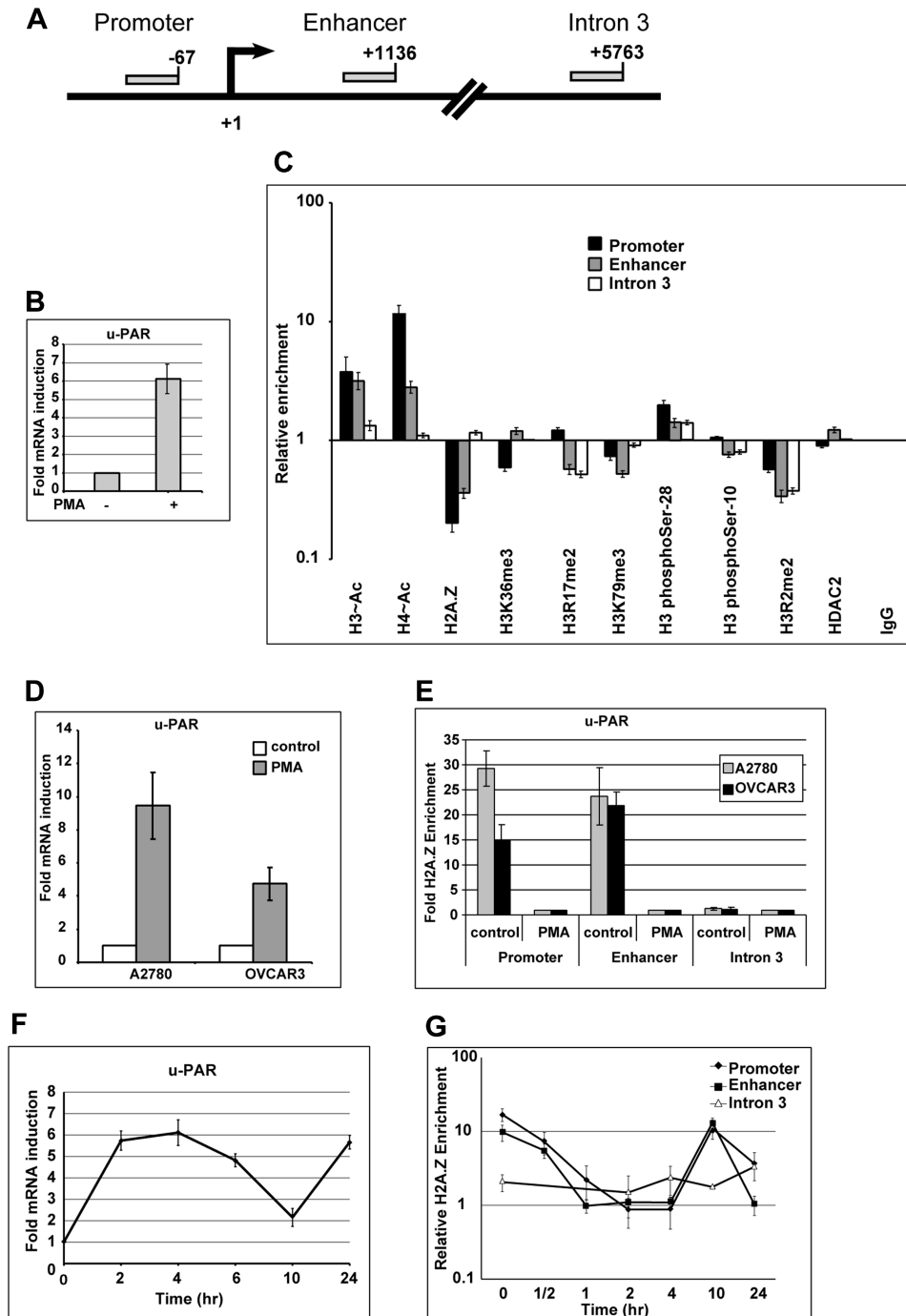
Figure 1. *u-PAR* transcription and H2A.Z deposition at the *u-PAR* promoter/enhancer are inversely correlated. (**A**) Schematic representation of the *u-PAR* gene indicating ChIP-Q-PCR amplicons. (**B**) qPCR showing *u-PAR* upregulation in response to PMA treatment (4 h) in GEO cells. (**C**) ChIP analysis showing the relative enrichment of various histone modifications and H2A.Z at the *u-PAR* promoter, enhancer and intron 3 upon PMA stimulation (4 h) compared to untreated samples. (**D**) qPCR showing *u-PAR* mRNA upregulation in response to PMA treatment (4 h) in A2780 and OVCAR3 cells. (**E**) ChIP assay of H2A.Z deposition at the *u-PAR* regulatory regions and intron 3 upon PMA induction (4 h) in A2780 and OVCAR3 cells. (**F** and **G**) qPCR (F) and ChIP assay (G) illustrates that *u-PAR* expression and H2A.Z deposition at the *u-PAR*-regulatory regions in response to PMA mirror each other but in the opposite direction. Data represent average (± SD) values of six separate determinations. The relative enrichment of protein in all ChIP assays was calculated as $2^{\Delta Ct}$ where $\Delta C_t = C_t$ (Input)-C_t (ChIP). All qRT–PCR data are normalized using GAPDH as an internal control.

To determine if H2A.Z loss associated with activation of *u-PAR* gene expression was a generalized response, or unique to GEO cells, we also performed ChIP with two other cell lines (A2780 and OVCAR3—both ovarian cancer derived). Like GEO cells, both A2780 and OVCAR3 showed a dramatic loss of H2A.Z from both *u-PAR* regulatory regions on induction of gene expression (Figure 1D and E). The possibility that the observed
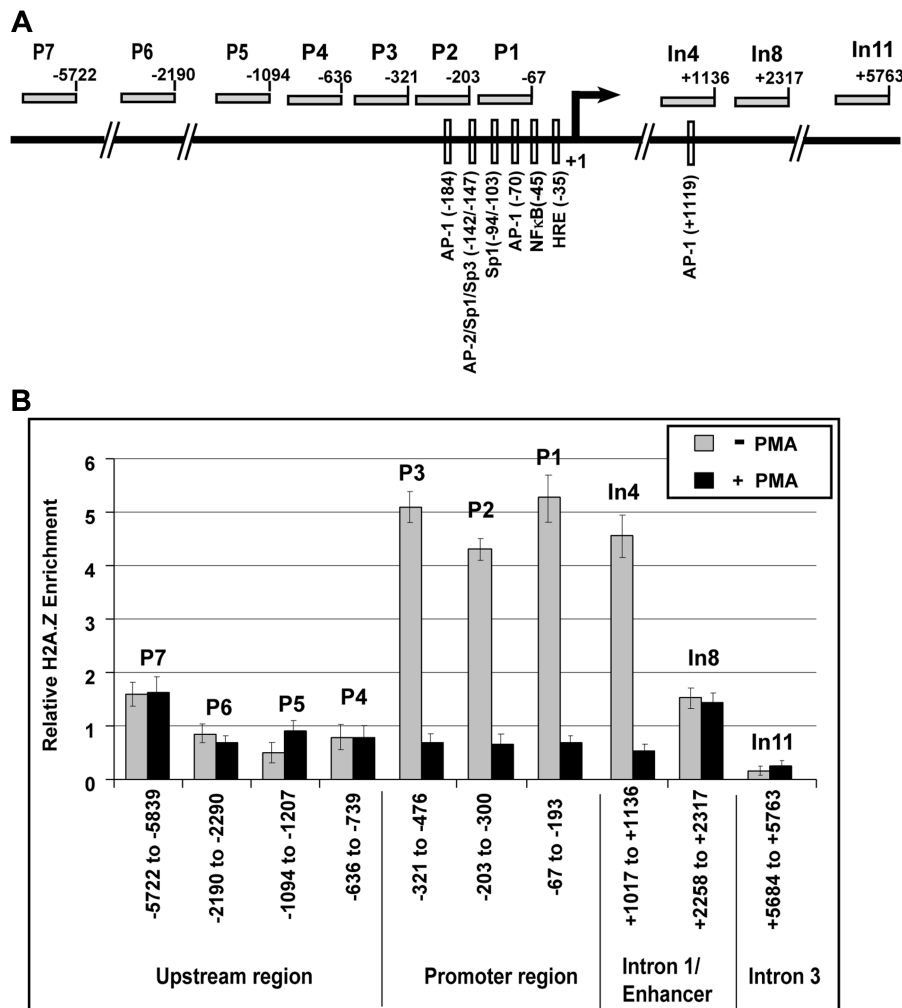
Figure 2. H2A.Z preferentially occupies the regulatory regions of *u-PAR*. (**A**) Schematic representation of the *u-PAR* promoter and intron 1 (which contains the enhancer) with ChIP-Q-PCR amplicons depicted. Various upstream transcription factor binding sites are shown. (**B**) Mapping of H2A.Z deposition in the absence, or presence, of PMA (4 h) at the various *u-PAR* regions. Various amplicons (e.g. P1, P2) and their genomic positions with reference to the transcription start site are marked in the figure. Data are shown as average values of six separate determinations ($\pm$SD). Relative enrichments are calculated as described in Figure 1.

changes of H2A.Z at the promoter/enhancer of the *u-PAR* gene was due to changes in nucleosome density is unlikely since the deposition of histone H3 at these regions showed negligible change upon PMA stimulation (Supplementary Figure S1A). Additionally, there was no change in the total amount of H2A.Z upon PMA induction (data not shown). Furthermore, the H2A.Z occupancy at a control (GAPDH) promoter was unaffected by PMA stimulation (Supplementary Figure S1B).

Next, to investigate the dynamics of the interaction of H2A.Z with the *u-PAR* promoter/enhancer and its relation to gene expression we performed a time course study with PMA. Modulation of *u-PAR* mRNA levels was cyclic with GEO cells peaking at 4 h and diminishing at 10 h before increasing again (Figure 1F). Interestingly, the occupancy of the *u-PAR* promoter/enhancer with H2A.Z was also cyclic although in the opposite direction (Figure 1G), such that maximal *u-PAR* mRNA levels at 4 h was associated with the greatest loss of H2A.Z. Conversely at 10 h post-PMA stimulation, a return of

u-PAR mRNA levels almost to baseline was associated with recruitment of H2A.Z to the promoter and enhancer. Taken together, the results suggest that the occupancy by H2A.Z is inversely correlated with *u-PAR* mRNA levels.

H2A.Z is preferentially enriched proximal to transcription factor-binding sites at the *u-PAR* promoter and enhancer

To map the regions of H2A.Z enrichment, we performed ChIP and tiled qPCR to interrogate various *u-PAR* genomic regions both upstream and downstream of the major transcriptional start site (Figure 2). Studies from our and other laboratories had previously identified several *cis*- and *trans*-acting factors regulatory for *u-PAR* transcription (Figure 2A) residing both upstream (e.g. AP-1, AP-2 and NFκB) of the transcriptional start site and downstream (AP-1) embedded in the intron 1 enhancer (21,30–33). Interestingly, H2A.Z was preferentially enriched proximal to these previously implicated
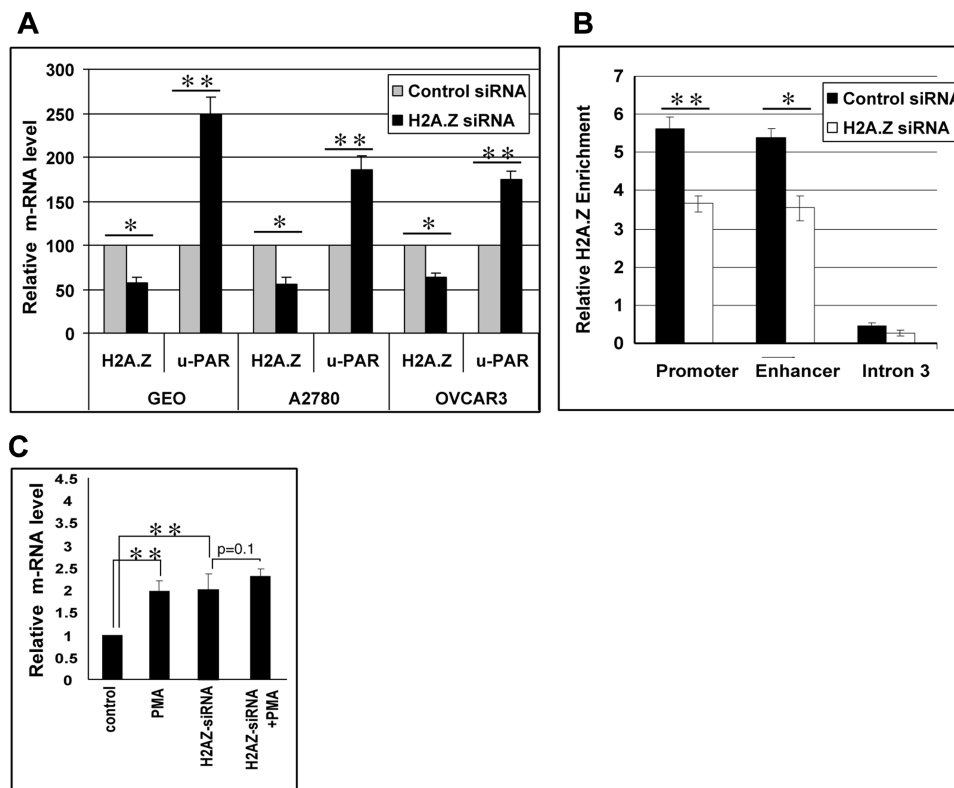
Figure 3. H2A.Z negatively regulates *u-PAR* expression. (**A**) qPCR using *H2A.Z* and *u-PAR* Taqman probes were performed using cDNA generated from three independent cell lines (GEO, A2780 and OVCAR3) transfected with 25 nM of a non-silencing siRNA (control) or an H2A.Z-specific siRNA for 48 h. Data are normalized using GAPDH as an internal control. (**B**) ChIP assay with H2A.Z-repressed GEO cells reveals reduced H2A.Z deposition at the *u-PAR* gene under the conditions specified in Panel A. (**C**) qPCR using *u-PAR* Taqman probe was performed using cDNA generated from GEO cells transfected with 25 nM of a non-silencing siRNA (control) or an H2A.Z-specific siRNA for 48 h before 30 min PMA treatment (as indicated). Data are shown as average values of six separate determinations ($\pm$ SD). Statistical significance was tested using Student's unpaired *t*-test and the level of significance was as follows: *$P < 0.010$, **$P < 0.005$.

transcription factor-binding sites within the *u-PAR* promoter (P1–P3 region) and enhancer (In4 region, Figure 2B and Supplementary Figure S2). At intron 1, H2A.Z was deposited around the enhancer region (at region In3 and In4) but not at the 3′-end of this intron (In5–In8, Supplementary Figure S2). Equally important, on PMA stimulation, H2A.Z specifically dissociated from both regulatory regions enriched for H2A.Z (P1–P3 and In4, Figure 2B) although levels were essentially unchanged in the regions either upstream (P4–P7) of the promoter or downstream of the enhancer region (In8 and In11, Figure 2B). The selective enrichment and loss (upon stimulation of gene expression) at these loci argue for a regulatory role of H2A.Z in *u-PAR* expression.

Depletion of cellular H2A.Z upregulates *u-PAR* expression

H2A.Z has been shown to regulate gene expression both positively and negatively (42–45). In order to determine the role of H2A.Z in *u-PAR* regulation, we knocked down H2A.Z in three independent cell lines (GEO, A2780 and OVCAR3) using siRNA (Figure 3A and Supplementary Figure S3). Interestingly, cellular depletion of H2A.Z resulted in upregulated *u-PAR* mRNA level in all three cell lines (Figure 3A). Data were normalized with GAPDH which itself was not affected by H2A.Z

knockdown. Western analysis done with GEO cells confirmed these data (Supplementary Figure S3). ChIP assays confirmed that reduced amounts of H2A.Z were deposited at these two regulatory regions in cells repressed for H2A.Z mRNA (Figure 3B). If PMA is inducing u-PAR expression by way of H2A.Z eviction then depletion of the latter should interfere with the ability of the phorbol ester to stimulate u-PAR expression. Indeed, while H2A.Z depletion of GEO cells induced ($P < 0.005$) u-PAR expression to the level observed with PMA alone (Figure 3C) there was no further enhancement of u-PAR expression when H2A.Z-depleted cells were treated with the phorbol ester (Figure 3C). Overall, these results suggest that H2A.Z negatively regulates *u-PAR* transcription.

MEK–ERK signaling intersects with the epigenetic control of *u-PAR* expression by H2A.Z

PMA mediates its effects by mimicking diacylglycerol (DAG), the endogenous activator of protein kinase C (PKC) the latter activating the MEK–ERK signaling pathway (Figure 4A). Since PMA treatment had resulted in the rapid eviction of H2A.Z from the *u-PAR* regulatory regions we speculated that the PKC–MEK–ERK signaling pathway (Figure 4A) intersects with the epigenetic control of *u-PAR* expression by H2A.Z. Pre-treatment of GEO cells with 10 μM Bisindolylmaleimide III
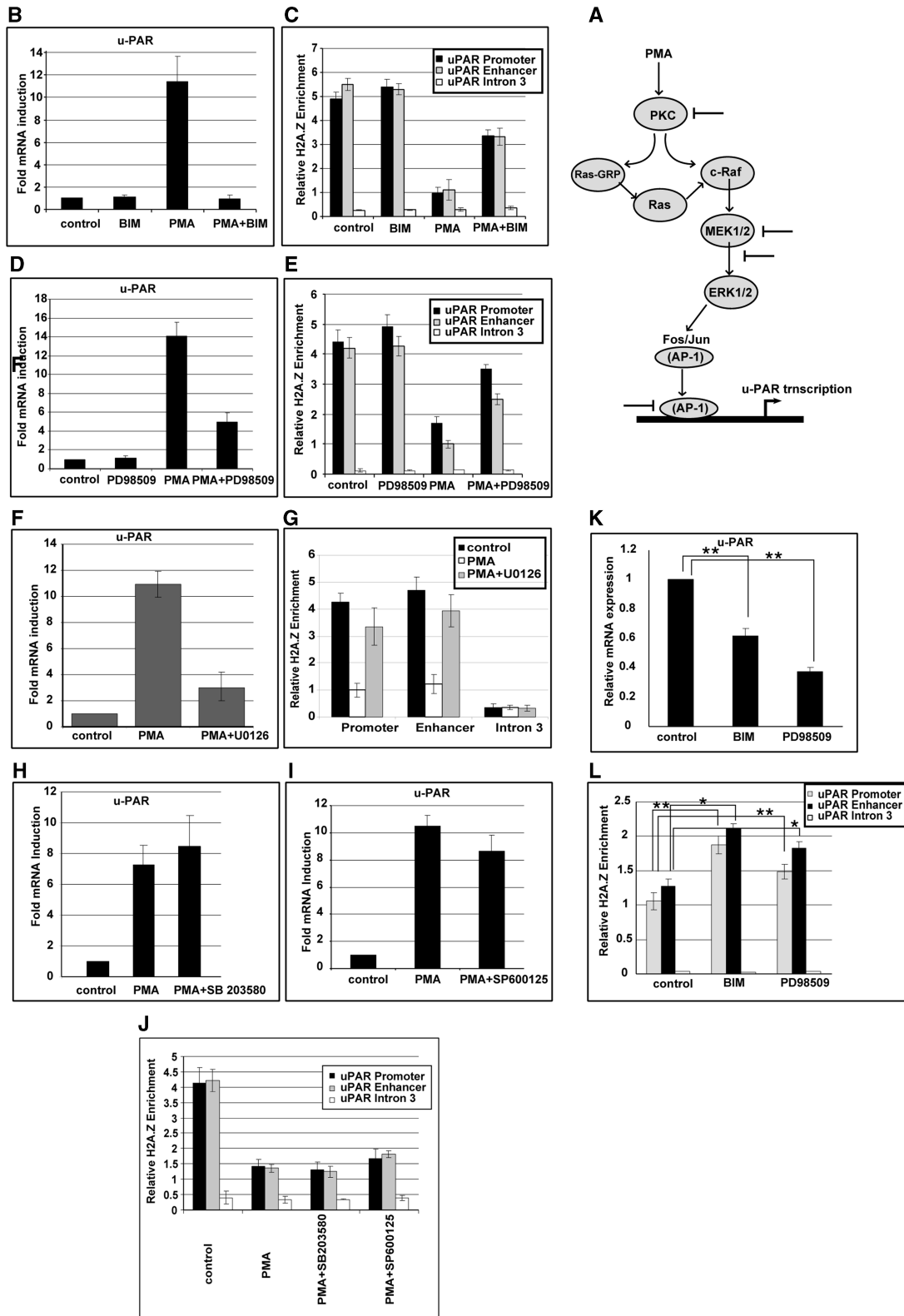
Figure 4. MEK–ERK signaling intersects with H2A.Z-mediated epigenetic control of u-PAR expression. (**A**) Schematic representation of the MEK–ERK signaling pathway. (**B–J**) qPCR analysis of *u-PAR* mRNA levels and ChIP analysis of H2A.Z deposition at the *u-PAR* promoter/enhancer and intron 3 from GEO cells pretreated for 2 h with the following inhibitors BIM (10 μM) (**B** and **C**), PD98509 (60 μM) (**D** and **E**), U0126 (20 μM) (**F** and **G**), SB203580 (2 μM) (**H** and **J**), SP600125 (20 μM) (**I, J**) before the addition of 100 nM PMA for 1 h. (**K** and **L**) qPCR analysis of *u-PAR* mRNA (**K**) and ChIP analysis for H2A.Z deposition at the *u-PAR* promoter/enhancer and intron 3 (**L**) from RKO cells treated for 4 h with BIM and PD98509 inhibitors using the conditions specified in Panels B and C. All q-RT–PCR data are normalized using GAPDH as an internal control. Data reflect average (± SD) values of at least six separate determinations. Statistical significance was tested using the Student's unpaired *t*-test and the level of significance was as follows: *$P < 0.05$, **$P < 0.005$.
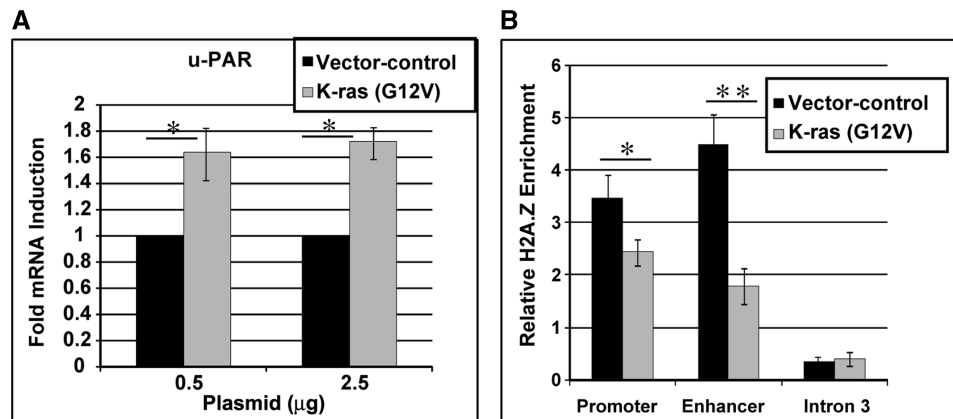
Figure 5. Overexpression of a constitutively active, mutation-activated K-Ras modulates H2A.Z deposition. (**A**) The indicated amount of the empty vector (vector-control) or the mutation-activated K-Ras (G12V) encoding vector was transiently transfected into GEO cells for 48 h and qPCR performed to measure *u-PAR* mRNA expression. (**B**) Under parallel conditions, ChIP analysis was performed to quantify the amount of H2A.Z deposited at the indicated *u-PAR* genomic regions. Data are shown as average values ($\pm$ SD) of six separate determinations. The Student's unpaired *t*-test and the level of significance were as follows: $*P < 0.05$; $**P < 0.005$. All qRT–PCR data were normalized with GAPDH as an internal control.

(BIM III), a highly selective cell-permeable inhibitor of PKC, completely blocked PMA-induced mRNA expression of *u-PAR* but had no effect on basal mRNA level (Figure 4B). Interestingly, ChIP assays indicated that BIM III treatment also diminished the PMA-driven dissociation of H2A.Z from the *u-PAR* promoter and enhancer (Figure 4C). Next, we used two highly specific inhibitors of MEK–ERK signaling, PD98059 (60 µM) and U0126 (20 µM). Pretreatment of GEO cells with either of these inhibitors blocked the PMA-induced *u-PAR* expression (Figure 4D and F) and considerably attenuated the PMA-mediated dissociation of H2A.Z from the *u-PAR* promoter and enhancer region (Figure 4E and G). Next, we queried the involvement of two other MAP-kinase pathways which can also be activated by PMA. SB203580 specifically blocks the p38 pathway and SP600125 targets the JNK pathway. Pre-treatment of GEO cells with either of these inhibitors had little effect on the ability of PMA to augment *u-PAR* mRNA levels or promote H2A.Z dissociation from the *u-PAR* promoter and enhancer regions (Figure 4H–J). In summary, our data are consistent with the idea that MEK–ERK signaling regulates H2A.Z deposition at the u-PAR regulatory regions.

We then determined the effect of BIM III and PD98059 on *u-PAR* mRNA levels/H2A.Z deposition in a colon cancer cell line (RKO) which intrinsically shows a high transcription rate of *u-PAR* (21). Consistent with our previous findings, both PKC and MEK1/2 inhibitors (BIM III and PD98059) attenuated *u-PAR* mRNA levels coincident with increased deposition of H2A.Z at the promoter/enhancer regions but not at intron 3 (Figure 4K and L).

Expression of a mutation-activated *K-Ras* (G12V) induces *u-PAR* expression and stimulates H2A.Z dissociation from the promoter/enhancer

K-Ras is activated in many cancers contributing to tumorigenesis and progression (46–48). Moreover

signaling of K-Ras through the MEK–ERK pathway is well established and our current and previous work demonstrated that *u-PAR* expression is increased by this oncogene (Figure 5A) (27,46–49). Consequently, using transient transfection assays, we then determined the effect of expressing an activated K-Ras (G12V) construct on *u-PAR* expression and H2A.Z deposition at the *u-PAR* regulatory regions in GEO cells. *u-PAR* expression was increased by overexpression of the mutation-activated *K-Ras* compared with the empty vector control (Figure 5A) concordant with reduced amounts of H2A.Z bound to the endogenous *u-PAR* promoter and enhancer (Figure 5B). This result further supports the notion that *u-PAR* transcription and H2A.Z deposition at the u-*PAR*-regulatory regions is modulated by the Ras-raf-MEK–ERK signaling pathway.

Trans-activation of the u-PAR gene by AP-1 is required for H2A.Z eviction

Our next goal was to determine how the MEK–ERK pathway regulated the loss of H2A.Z from the *u-PAR* regulatory regions. We speculated on a role for AP-1 since (i) the MEK–ERK signaling pathway regulates the activity/amount of AP-I family transcription factors (49,50) (ii) our previous studies showed that constitutive and PMA-inducible expression of *u-PAR* required two AP-1 motifs in the promoter region (21) and one in the downstream enhancer (33) (iii) of the transcription factors implicated in u-PAR expression, only AP-1 was common to both promoter and enhancer. Accordingly, we expressed the TAM-67 expression construct, encoding a c-Jun protein lacking its *trans*-activation domain (and therefore acting in a dominant negative fashion) in GEO cells and then treated the cells with PMA. Our data demonstrate that PMA-induced *u-PAR* mRNA level was effectively countered by expression of this construct compared to the empty vector control (Figure 6A). Equally important, in ChIP assays, expression of TAM-67 largely reversed the PMA-driven H2A.Z
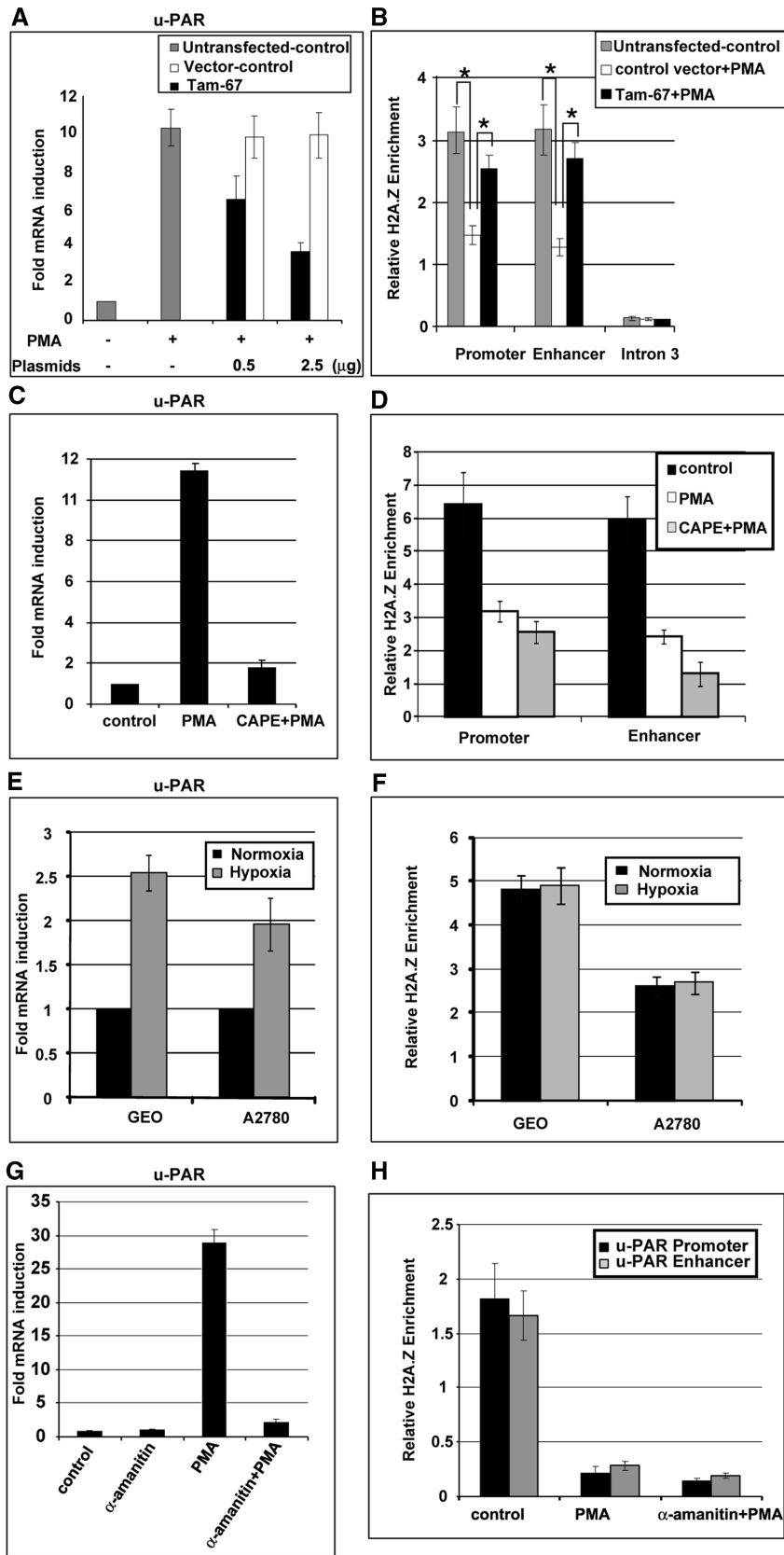
Figure 6. *Trans*-activation by AP-1 is a prerequisite for H2A.Z depletion at the *u-PAR* promoter/enhancer. (**A**) qPCR was performed to measure *u-PAR* mRNA expression in GEO cells transiently transfected with the indicated amount of the vector-control or Tam-67 for 24 h and then treated, where indicated, with PMA for 1 h. (**B**) Under parallel conditions, ChIP assays revealed that expression of Tam-67 attenuated PMA-induced H2A.Z eviction from the *u-PAR* promoter and enhancer. (**C**) qPCR quantitation of *u-PAR* mRNA level and (**D**) ChIP analysis of H2A.Z deposition at
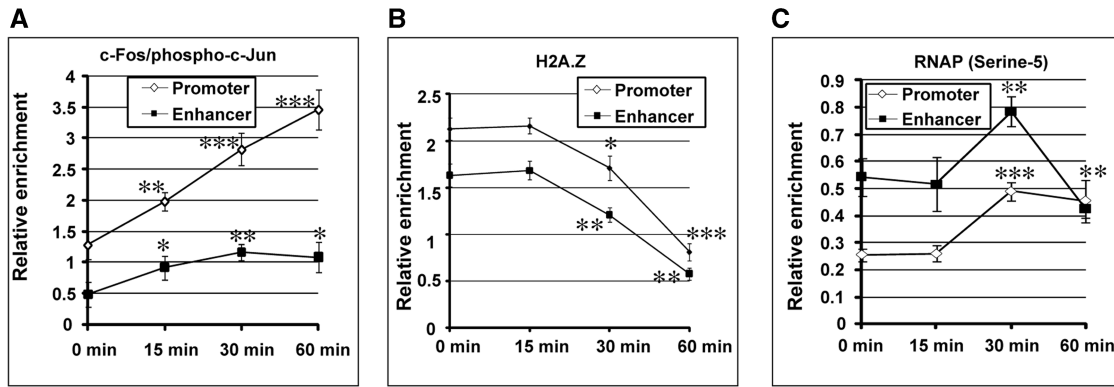
(continued)

Figure 7. AP-1 binding to the u-PAR gene precedes H2A.Z eviction. ChIP assay showing the kinetics of (**A**) AP-1 (using a mixture of pan antibodies directed against Fos and Jun family members) (**B**) H2A.Z (**C**) RNAP II (using an antibody against RNAP II phosphorylated at Serine 5) deposition at the *u-PAR* promoter and enhancer in response to 100 nm PMA for the indicated times. Data are shown as average values of six separate determinations ($\pm$ SD). The Student's unpaired *t*-test was used to test for the level of significance: *$P < 0.05$; **$P < 0.02$; ***$P < 0.0005$ using time 0 as reference.

dissociation from the *u-PAR* promoter and enhancer regions (Figure 6B). This result is consistent with the notion that AP-1-dependent *trans*-activation is required for H2A.Z eviction and *u-PAR* expression.

PMA has also been shown to induce NFκB (51,52), the latter implicated in *u-PAR* regulation (32). So next, we determined whether inhibition of NFκB affects the dynamics of H2A.Z interaction with the *u-PAR*-regulatory regions. Interestingly, Caffeic acid phenethyl ester (CAPE), a specific inhibitor of NFκB (but not AP-1) (53) while blocking PMA-induced *u-PAR* expression had no effect on the PMA-driven dissociation of H2A.Z (Figure 6C and D). This result indicates that NFκB is not involved in regulating H2A.Z deposition at the *u-PAR* promoter/enhancer.

Hypoxia also upregulates *u-PAR* expression via binding of HIF-α to the hypoxia response element (HRE) located within the *u-PAR* promoter (30). *u-PAR* expression was upregulated when GEO and A2780 cells were exposed to 24 h hypoxia (0.2%) (Figure 6E and F); however there was no accompanying change in H2A.Z occupancy at the *u-PAR* promoter (Figure 6E and F). Taken together, the data suggest that H2A.Z eviction is not a generalized requirement for transactivation of the *u-PAR* gene.

We also entertained the possibility that H2A.Z eviction was a consequence of increased transcription rather than a cause. To address this possibility we made use of α-amanitin which blocks transcription by RNA polymerase II (RNAP II). Treatment of GEO cells with 10 μM of α-amanitin almost completely inhibited PMA-induced expression of *u-PAR* (Figure 6G). However, strikingly, parallel ChIP assays revealed that inhibition of

PMA-induced transcription by α-amanitin did not affect the phorbol ester-driven loss of H2A.Z from the promoter or enhancer regions (Figure 6H). This result unambiguously shows that H2A.Z eviction is not secondary to RNAP II processivity.

AP-1 transactivation precedes H2A.Z remodeling

If AP-1 transactivation is required for H2A.Z eviction in turn leading to *u-PAR* gene expression then chip experiments should demonstrate such a temporal sequence. Accordingly, we performed ChIP assays using chromatin generated from GEO cells treated with PMA for varying times. These experiments revealed a statistically significant increase in binding of AP-1 (using a mixture of anti c-fos/phosopho-c-jun antibodies) at the *u-PAR* promoter and enhancer as early as 15-min post-PMA addition (Figure 7A). In contrast, H2A.Z dissociation (Figure 7B) and enrichment of processive RNA Polymerase II (Figure 7C) lagged being first evident after 30 min of phorbol ester treatment. Taken together, these results suggest that binding of AP-1 at the *u-PAR* promoter/enhancer precedes H2A.Z eviction and transcription initiation.

H2A.Z regulates *MMP-9* and oncomiR-21 transcription

To determine whether H2A.Z dissociation was also pertinent to the regulated expression of other AP-1 targets, we undertook studies with the *MMP-9* gene and miR-21 micro-RNA which are also regulated by PMA (54–58). OVCAR3 cells express low levels of *MMP9* mRNA but was induced 10- to 30-fold by PMA (Figure 8B).

Figure 6. Continued

the *u-PAR* promoter/enhancer and intron3 from GEO cells pretreated for 2 h with CAPE before the addition of 100 nM (final concentration) PMA for 1 h. (**E and F**) qPCR assay showing that hypoxia (24 h) induces *u-PAR* expression (E) but does not affect the H2A.Z deposition (F) at the *u-PAR* promoter in ChIP assays performed in two independent cell lines (GEO, A2780). (**G and H**) qPCR analysis showing α-amanitin (10 μM), inhibits PMA-inducible *u-PAR* expression (G); however PMA-driven eviction of H2A.Z from the u-PAR promoter/enhancer is not abrogated (H) as evidenced in ChIP assays undertaken with GEO cells. Data are shown as average ($\pm$ SD) values of six separate determinations. The Student's unpaired *t*-test and the level of significance were as follows: *$P < 0.01$. All qRT–PCR data are normalized with GAPDH as internal control except for hypoxic conditions where Actin was used.
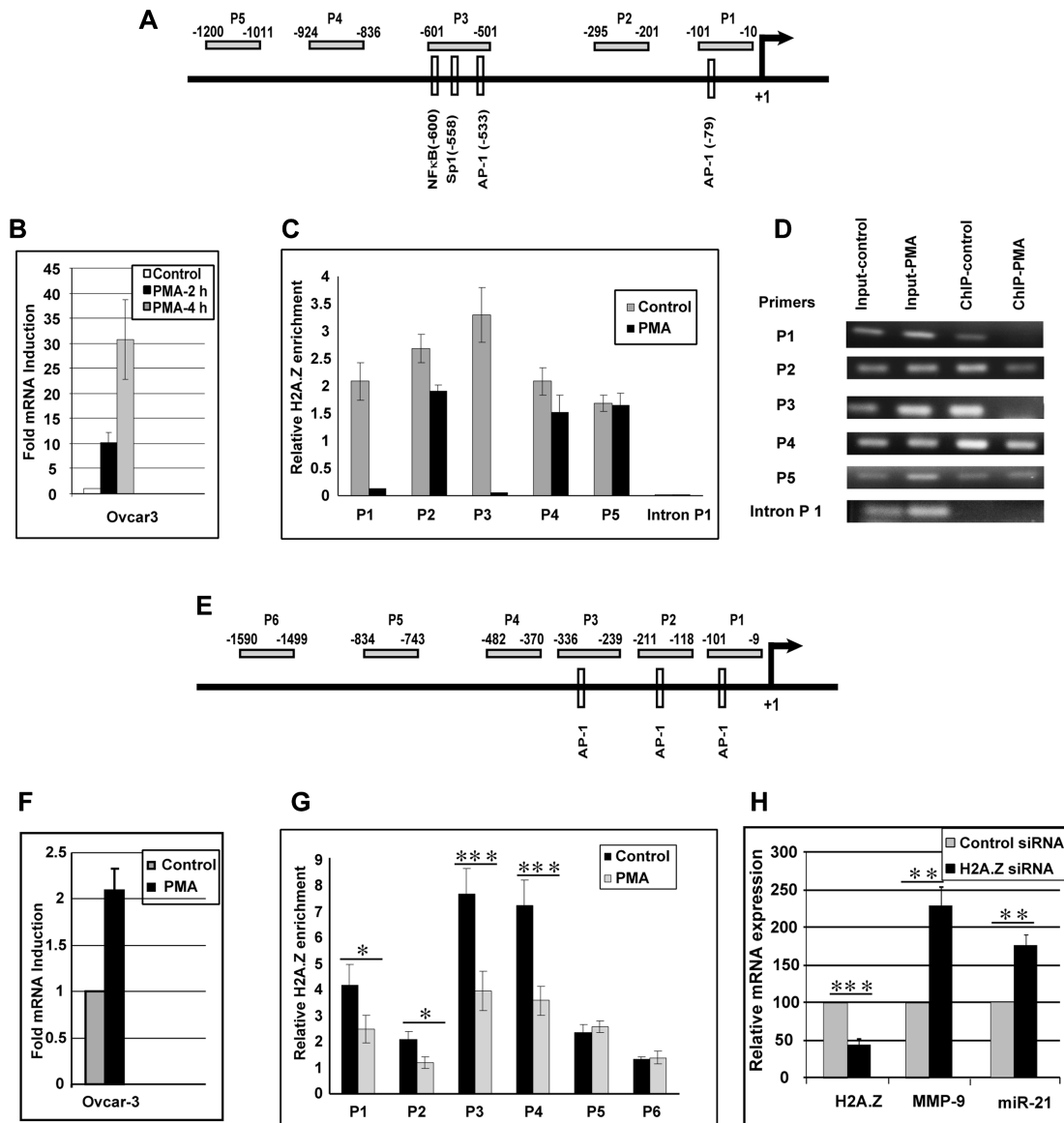
Figure 8. H2A.Z remodeling at other AP-1-regulated targets. (**A**) Schematic representation of the *MMP-9* upstream regulatory region with amplicons generated by qPCR. (**B**) qPCR showing *MMP-9* upregulation in response to PMA (4 h) in OVCAR3 cells. (**C**) Mapping of H2A.Z deposition in the absence, or presence, of PMA (4 h) at the *MMP-9* upstream region. (**D**) Image of the gel resolving amplification products generated as per Panel C (**E**) Schematic representation of the miR-21 promoter with Q-PCR amplicons. (**F**) qPCR showing miR-21 upregulation in response to PMA (4 h) in OVCAR3 cells. (**G**) Mapping of H2A.Z deposition in the absence, or presence, of PMA (4 h) at the miR-21 promoter. (**H**) qPCR data illustrating that H2A.Z knockdown (25 nM of siRNA) induces expression of both *MMP-9* and miR-21. Data are shown as average values ($\pm$SD) of six separate determinations. The Student's unpaired *t*-test was used to determine the level of significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$.

We mapped H2A.Z binding to the *MMP-9* promoter in PMA untreated/treated OVCAR3 cancer cells. H2A.Z was enriched upstream of the *MMP9* transcription start site with peak binding in the P3 region coincident with the distal AP-1 site shown previously by us to be regulatory for expression (Figure 8A and C). Moreover, on PMA treatment, the amount of H2A.Z bound to the P1 and P3 regions mapping to the proximal and distal AP-1 sites diminished to almost undetectable levels (Figure 8C and D).

Similarly on PMA treatment, transcription of miR-21 was induced 2-fold and again H2A.Z was depleted from the regions of the miR-21 promoter proximal to (P1–P4)

but not distal from (P5 and P6) the AP-1 sites (Figure 8E–G). Furthermore, depleting of cellular H2A.Z using siRNA in OVCAR3 cells induced the expression of both *MMP-9* and miR-21 (Figure 8H). Our results suggest that H2A.Z eviction from the regulatory regions is a key step in the control of, at least, a subset of AP-1-regulated targets.

DISCUSSION

Our study demonstrates a novel role of MEK–ERK signaling in the epigenetic regulation of three AP-1 targets, including *u-PAR*, via H2A.Z remodeling. H2A.Z is

enriched proximal to AP-1 motifs located within the regulatory regions of *u-PAR*, *MMP-9* and miR-21 and activation of MEK–ERK signaling (either genetically by a mutation-activated-*K-Ras* or chemically with phorbol ester) leads to its dissociation culminating in gene activation. To our knowledge, our study is the first to show regulation of a protease family member or, for that matter, any AP-1 target by H2A.Z.

H2A.Z has recently emerged as an epigenetic regulator of transcription, representing the terminus of diverse signaling systems including the glucocorticoid receptor (GR), estrogen receptor (ERα) and p53 (42,43, 59). Similar to our findings with AP-1, H2A.Z is enriched proximal to GR DNA-binding sites within the regulatory regions of target genes. Ligand binding to the corresponding receptor results in H2A.Z eviction at GR-inducible genes (59). In a similar vein, H2A.Z co-localizes with p53-binding sites at its downstream target, the p21 promoter, and again its dissociation is associated with gene activation (43). However, in some instances, H2A.Z accrual, rather than depletion, at target gene regulatory regions is correlated with augmented expression. Thus, deposition of this histone variant at ERα-targeted gene promoters including *TFF1* is coincident with activation of gene expression (43).

While our work demonstrated H2A.Z as repressive for, at least, a subset of AP-1-*trans*-activated genes, others have reported that macro H2A (another histone variant) is also targeted by this family of transcription factors culminating in altered gene expression. Thus, AP-1 recruits macroH2A to suppress gene transcription from the IL-6 promoter (60). Although the findings of histone variants as regulatory for a subset of AP-1-controlled genes is novel, modulation of AP-1-responsive genes through post-translational modification of canonical histones has been well studied. As one example, Miotta and co-workers (2006) reported that histone H4 acetylation enhances the ability of AP-1 to *trans*-activate target genes in *Drosophila* (61). Similarly, histone acetylation and phosphorylation associated with increased occupancy of AP-1 motifs with JunB, JunD and c-Fos culminates in the stimulation of *MMP-9* expression by PMA (62).

H2A.Z shows a preference for DNA-ase hypersensitive sites the latter often characteristic of regulatory regions i.e. promoters and enhancers (43, 59,63). In our study on the *u-PAR* gene, H2A.Z accrual or depletion at the promoter and enhancer were synchronized under conditions of gene activation or repression. Similarly, recruitment of GR to target genes resulted in H2A.Z eviction from both enhancers and promoters (59). It may be that under stimulatory/repressive conditions, both promoter and enhancer are juxtaposed by DNA looping bringing them into proximity with a common H2A.Z exchange machinery. However, this congruence in H2A.Z binding to promoter/enhancer is not universal and examples of asynchronous H2A.Z promoter/enhancer binding exist. For instance, while H2A.Z is enriched at ERα targeted gene enhancers, it is depleted from the ERα promoters of the same genes under repressive conditions. Conversely, gene activation is associated with depletion from the enhancer but enrichment at the promoter of these genes (e.g. *TFF1*) (43).

There is a paucity of studies linking signaling modules to the regulation of gene expression by non-canonical histones. To our knowledge, the only other study addressing this question implicated p38 MAPK signaling in the epigenetic regulation of muscle specific genes (64). In that study, p18[hamlet]/SRCAP-mediated H2A.Z incorporation at muscle gene promoters was controlled by p38 MAPK signaling. This epigenetic regulation of gene expression was the key to the expression of a muscle differentiation-specific gene program. This latter study parallels ours in establishing, for the first time, a direct link between MEK–ERK signaling and epigenetic regulation, via H2A.Z, of several AP-1 targets.

While the concept of non-canonical histones as targets of signaling pathways is relatively new, the link between MEK–ERK signaling and epigenetic control of gene expression via conventional histones (H1–H4) is well investigated (34,65,66). However, the variant and conventional histones show a significant difference in their modes of regulation. Thus, while exchange of variant histones represents the major mechanism for regulating gene expression, in contrast, post-transcriptional modification of S phase-synthesized (i.e. conventional) histones is the predominant mechanism by which gene expression is achieved. By way of example, MEK–ERK signaling terminates at MSK1/2 H3 kinase the latter mediating the phosphorylation of H3 histone at Serine 10 or 28 at immediate/early genes promoters to induce their expression (34,65,66). In another study, this signaling module was also reported to suppress tumor suppressor protein Cbp [C-terminal Src kinase (Csk)-binding protein] expression via modulating histone modifications (H4 acetylation, H3K27me3) (37). However, it is important to emphasize the possibility that these modes of regulation may not be mutually exclusive. It may be that post-translational modifications of canonical histones cooperate, positively or negatively, with the exchange of histone variants to effect a change in gene expression. Certainly, our previous work (33) demonstrated the *u-PAR* enhancer in u-PAR-expressing colon cancer cells to be engaged by acetylated/K4-methylated histone 3 under conditions of gene activation.

The MEK–ERK signaling pathway is involved in a multitude of biological events including cell proliferation, cell cycling and wound healing, physiological events commonly co-opted in malignancy/tumor progression (67,68). Moreover, this pathway is aberrantly activated in many cancers and sometimes at high frequency (47) and recent reports have documented H2A.Z depletion again associated cancer progression (69–72). Furthermore, a recent report found a gain of acetylated H2A.Z at the transcription start site despite an overall decrease in H2A.Z levels, in concert with oncogene activation in cancer cells (73). These reports together with our findings that the MEK–ERK pathway intersects with H2A.Z-mediated repression of a subset of AP-1-regulated genes raises the possibility that the node at which these two systems intersect represents a novel therapeutic target in cancer.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary tables 1–2, Supplementary figures 1–3.

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