the *gcvT* 5′-UTR of *B. subtilis* responds to glycine with characteristics that parallel those observed when conducting inline probing of the cooperative VC I-II RNA.

To assess whether glycine binding and in vitro transcription control correspond to genetic control events in vivo, we generated reporter constructs by fusing the IGR upstream of the *gcvT* operon from *B. subtilis* to a β-galactosidase reporter gene and integrated them into the bacterial genome (*17*). The reporter fusion construct carrying the wild-type IGR expresses a high amount of β-galactosidase when glycine is present in the growth medium, whereas a low amount of gene expression results when alanine is present (Fig. 4C). These results indicate that the *gcvT* motif is part of a glycine-responsive riboswitch with a default state that is off. Glycine binding is required to activate gene expression, as was also observed with the in vitro transcription assays (Fig. 4B).

The importance of several conserved features of the motif were examined by mutating the P1 and P2 stems of the first aptamer domain to disrupt (variants M1 and M3, respectively) and restore (M2 and M4, respectively) base pairing (Fig. 4A). Resulting gene expression levels from constructs carrying the mutant IGRs are consistent with base-paired elements predicted from phylogenetic analyses (*14*) (fig. S1). Furthermore, the introduction of mutations into the conserved cores of either aptamer I or aptamer II (variants M5 and M6, respectively) caused a complete loss of reporter gene activation. This latter result suggests that glycine binding to both aptamers is necessary to trigger gene activation, which is consistent with a model wherein cooperative glycine binding is important for riboswitch function.

The glycine-dependent riboswitch is a remarkable genetic control element for several reasons. First, glycine riboswitches form selective binding pockets for a ligand composed of only 10 atoms and thus bind the smallest organic compound among known natural and engineered RNA aptamers. This observation is consistent with the hypothesis that RNA has sufficient structural potential to selectively bind a wide range of biomolecules.

Second, the 5′-UTR of the *B. subtilis gcvT* operon is a genetic on switch, and thus joins the adenine riboswitch (*23*) as a rare type of RNA that has been proven to harness ligand binding and activate gene expression. In most instances, riboswitches cause repression of their associated genes, which is to be expected because many of these genes are involved in biosynthesis or import of the target metabolites. However, the glycine riboswitch from *B. subtilis* controls the expression of three genes required for glycine degradation. A ligand-activated riboswitch would be required to determine whether sufficient amino acid substrate is present to warrant production of the glycine cleavage system, thereby providing a rationale for why this rare on switch is used.

Third, this is the only known metabolite-binding riboswitch class that regularly makes use of a tandem aptamer configuration. In both *V. cholerae* and *B. subtilis*, the juxtaposition of aptamers enables the cooperative binding of two glycine molecules. For the *B. subtilis* riboswitch, this characteristic is expected to result in unusually rapid activation and repression of genes encoding the glycine cleavage system in response to rising and falling concentrations of glycine, respectively. Given the prevalence of the tandem architecture of glycine riboswitches, this more "digital" switch likely gives the bacterium an important selective advantage by controlling gene expression in response to small changes in glycine.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/306/5694/275/DC1
Materials and Methods
Figs. S1 to S5

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Human PAD4 Regulates Histone Arginine Methylation Levels via Demethylimination

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Methylation of arginine (Arg) and lysine residues in histones has been correlated with epigenetic forms of gene regulation. Although histone methyltransferases are known, enzymes that demethylate histones have not been identified. Here, we demonstrate that human peptidylarginine deiminase 4 (PAD4) regulates histone Arg methylation by converting methyl-Arg to citrulline and releasing methylamine. PAD4 targets multiple sites in histones H3 and H4, including those sites methylated by coactivators CARM1 (H3 Arg^{17}) and PRMT1 (H4 Arg^{3}). A decrease of histone Arg methylation, with a concomitant increase of citrullination, requires PAD4 activity in human HL-60 granulocytes. Moreover, PAD4 activity is linked with the transcriptional regulation of estrogen-responsive genes in MCF-7 cells. These data suggest that PAD4 mediates gene expression by regulating Arg methylation and citrullination in histones.

Posttranslational histone modifications, such as phosphorylation, acetylation, and methylation, regulate a broad range of DNA and chromatin-templated nuclear events, including transcription (*1–3*). Pairs of opposing enzymes, such as acetyltransferases-deacetylases

and kinases-phosphatases, regulate the steady-state balance of histone acetylation and phosphorylation, respectively. In contrast, although Arg- and Lys-specific methyltransferases have been identified (*3–5*), enzymes that remove methyl groups from histones or any other cellular proteins remain unknown (*6*).

Arg methylation has been identified on many nuclear and cytosolic proteins involved in various cellular processes, including transcription and cell signaling (*7–10*). The methylation of histones by PRMT1 and CARM1 facilitates transcription in association with nuclear hormone coactivators and p53 (*11–15*). Here, we demonstrate that peptidylarginine deiminase 4 (PAD4), an enzyme previously known to convert Arg to citrulline (Cit) in histones (*16–19*), can also demethyliminate histones in vitro and in vivo, thus regulating both histone Arg methylation and gene activity.

Multiple Arg residues in H3 and H4 can be methylated by CARM1 and PRMT1, respectively (fig. S1A). Free methyl-Arg amino acids (monomethyl-Arg and asymmetric dimethyl-Arg) can be converted to Cit by dimethylarginine dimethylaminohydrolyase (DDAH) (*20–21*). To identify enzymes that might catalyze a similar reaction on protein methyl-Arg substrates as that catalyzed by DDAH, we searched the Homologous Structure Alignment database for proteins homologous to DDAH and identified PAD4 (*22*) (fig. S1). Peptidylarginine deiminases are a family of enzymes known to convert protein Arg to Cit in a calcium- and dithiothreitol (DTT)-dependent reaction [reviewed in (*16*)]. These findings prompted us to test the hypothesis that PAD4 can convert histone methyl-Arg to Cit.

Previous studies have correlated PAD4 expression with histone citrullination (*17–18*). We purified a glutathione *S*-transferase (GST)–PAD4 (human) full-length fusion protein from *Escherichia coli* and tested it on reversed-phase high performance liquid chromatography (RP-HPLC)–purified cellular H3 and H4 as substrates. In the presence of calcium and DTT, GST-PAD4 generated Cit in H3 and H4 as detected by an antibody against a chemically modified form of Cit (Fig. 1, A and B). Cellular H3 and H4 either treated or untreated was probed with site-specific antibodies against methyl-H3 Arg^{17} and -H4 Arg^{3} residues (for antibody specificity, see fig. S2). A dramatic diminishment of H3 Arg^{17} and H4 Arg^{3} methylation was detected after PAD4 treatment (Fig. 1, A and B), suggesting that PAD4 targets select methyl-Arg sites in H3 and H4. Protein microsequencing showed that the N-terminal tail of PAD4-treated H3 and H4 was not being randomly degraded (table S1). To identify potential PAD4 target site(s) in the N-terminal tail of H3, we quantified the amount of Cit detected at cycles of microsequencing. As shown in table S1, PAD4 deiminated multiple Arg residues in H3 (e.g., ~93.6% of H3 Cit^{2} compared to ~98.9% of H3 Cit^{8}) in vitro. Cellular H4 is N-terminally acetylated, thus preventing direct microsequencing analyses. Therefore, we analyzed recombinant H4 after PAD4 treatment and found that its N terminus also remained intact and that ~99.6% of H4 Arg^{3} was citrullinated (table S1).

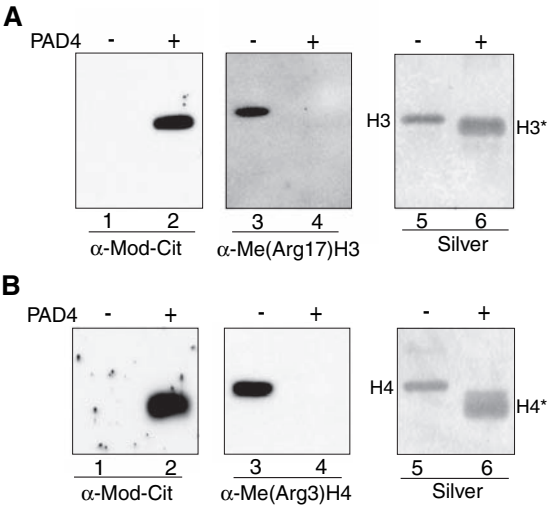

Fig. 1. PAD4 reduces Arg methylation levels and generates citrulline (Cit) in H3 (**A**) and H4 (**B**). (Left) Cit was detected in H3 or H4 when treated with PAD4. (Middle) After PAD4 treatment, the signal of H3 Arg^{17} or H4 Arg^{3} methylation was dramatically diminished (see fig. S2 for antibody specificity). (Right) Silver staining shows H3 and H4, as well as citrullinated H3 and H4 (H3* and H4*) in SDS-PAGE gels. Note the increased mobility of H3* and H4*.

Thus, PAD4 potently converts multiple Arg sites of H3 and H4 to Cit with low site preference in vitro. Neutralization of the positive charge of multiple Lys residues by acetylation alters the electrophoretic behavior of histones in SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gels (*23*). Because the positive charge of Arg is neutralized by citrullination, we postulate that the mass shift of histones observed on SDS-PAGE gels after PAD4 treatment is caused by deiminating multiple Arg residues in H3 and H4 and that the varying degrees of citrullination at different Arg residues caused the expansion of the band width of H3 and H4 (Fig. 1).

Two possible pathways can lead to the loss of methyl-Arg epitope (Fig. 2A). Either PAD4 removes the methylimine group from methyl-Arg, thus producing Cit and releasing methylamine (pathway 1), or the imine group is removed by PAD4 thereby producing methyl-Cit and releasing ammonium (pathway 2). To distinguish between these two pathways, we radioactively labeled recombinant H3 and H4 with CARM1 and PRMT1, respectively, and with [^{3}H]-S-adenosylmethionine as a methyl donor. The amount of [^{3}H]-methyl in H3 and H4 was then detected by fluorography. We found that the amounts of [^{3}H]-methyl in histones were dramatically decreased by PAD4 treatment (Fig. 2B). These results suggest that the methyl-group produced on H3 and H4 by CARM1 and PRMT1, respectively, is directly removed by PAD4.

We sought to analyze the biochemical nature of the released product. If PAD4 acts via pathway 1 (Fig. 2A), methylamine would be generated. To detect methylamine, we first took advantage of the solubility difference of methylamine in H_2O at different pH values (methylamine pK_a = 10.4) (*24*, *25*). After PAD4 treatment of recombinant H4 radioactively labeled by PRMT1, released volatile [^{3}H]-methyl radioactivity was detected from samples adjusted to a high pH (pH = 12, at which methylamine becomes volatile) but not from various control samples (Fig. 2C). The identity of methylamine as a methyl product was further confirmed by chromatography using an amino acid cation-exchange column (*26*). Radioactivity released from PAD4-treated [^{3}H]-methyl-H4 co-migrated with both an unlabeled monomethylamine standard (absorbance, 570 nm) and a [^{14}C]-dimethylamine standard, indicating that the volatile [^{3}H]-methylamine could be released in a monomethyl or dimethyl form (Fig. 2D) (*27*). In contrast, [^{3}H]-methylamine was not detected in the untreated [^{3}H]-methyl-H4 samples (Fig. 2E). These results support the hypothesis that PAD4 can convert methyl-Arg in histones to Cit and methylamine. Hereafter, we will

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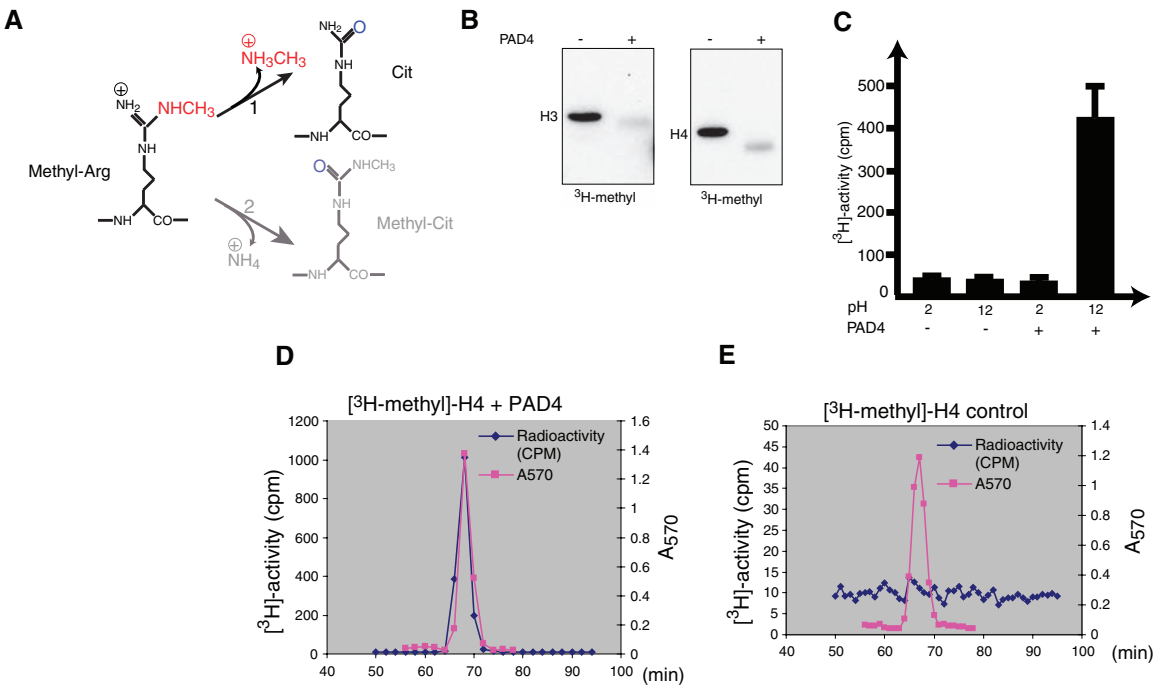

Fig. 2. PAD4 demethyliminates H3 and H4 and produces methylamine and Cit as reaction products. (**A**) Two possible mechanisms of PAD4 reaction on methyl-Arg in a protein substrate. (**B**) Recombinant H3 or H4 was first radioactively labeled by CARM1 or PRMT1, respectively. After PAD4 treatment, the [^{3}H]-methyl radioactivity in H3 and H4 dramatically decreased. (**C**) A volatility assay (*25*) to detect [^{3}H]-methylamine released from radioactively labeled H4 after PAD4 treatment. [^{3}H]-activity was found only from samples at a high pH (12) after-PAD4 treatment. Error bars indicate the means ± SD of three individual experiments. (**D**) A nonradioactive methylamine standard [10 μmol, detected by absorbance (*A*) at 570 nm] was co-eluted with the released radioactive products generated by PAD4 from radioactively labeled recombinant H4, suggesting that [^{3}H]-methylamine was produced. (**E**) [^{3}H]-methylamine was not detected in radioactively labeled recombinant H4 samples that were not treated with PAD4.

refer to this reaction as demethylimination to reflect these findings.

We next examined whether PAD4 modulates histone Arg methylation and citrullination in vivo. We chose to test this in HL-60 granulocytes where PAD4 expression can be induced by dimethyl sulfoxide (DMSO) and PAD4 can be activated by calcium ionophore (*17*, *18*) (Fig. 3A). When total histones were probed with site-specific antibodies against H3 methyl-Arg^{17} or H4 methyl-Arg^3, the signals were dramatically reduced after PAD4 activation (Fig. 3B). In addition, calcium ionophore treatment did not either increase histone citrullination (Fig. 3A) or decrease histone Arg methylation in undifferentiated HL-60 cells (*28*). These results correlate the activation of PAD4 with a loss of histone Arg methylation in a cellular context.

To further analyze the change of Arg methylation in individual cells, we carried out immunofluorescence analyses of HL-60 granulocytes. Before treatment, amounts of H3 Arg^{17} methylation in each cell were roughly comparable (Fig. 3C, top). In contrast, after 15 min of calcium ionophore treatment, H3 Arg^{17} methylation dramatically decreased in most of the cells (~57.3%, $n = 200$) (Fig. 3C, bottom). In contrast, amounts of H3 Lys^4 methylation were unchanged in calcium ionophore-treated cells (fig. S4), suggesting that the N terminus of H3 is intact and that PAD4 does not affect Lys methylation.

To directly demonstrate the conversion of particular H3 Arg residues to Cit in vivo, we

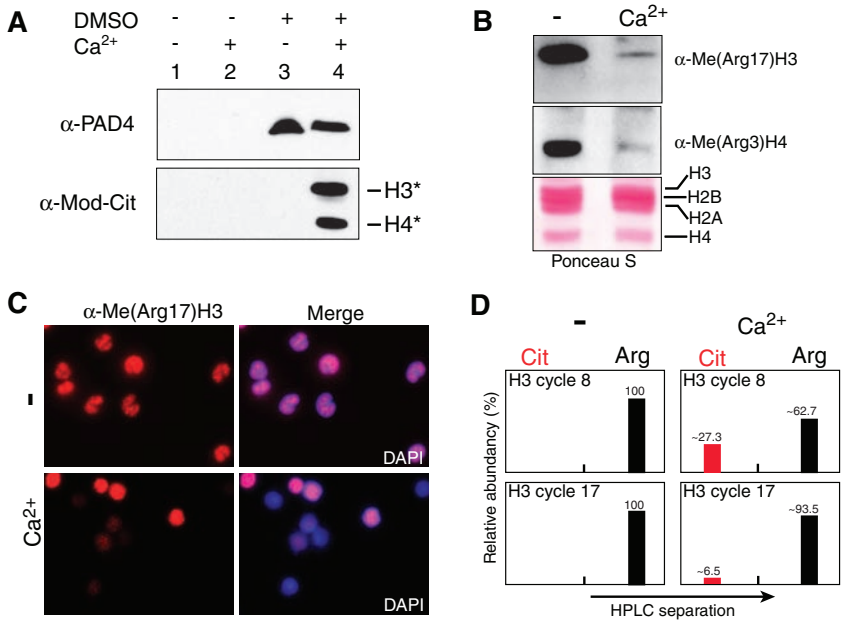

Fig. 3. Linking PAD4 activity with the regulation of H3 Arg^{17} methylation. (**A**) PAD4 protein was expressed in HL-60 granulocytes upon DMSO treatment (lanes 3 and 4). Citrullinated H3 and H4 (denoted by asterisks) were detected in histones purified from cells treated with both DMSO and calcium ionophore (lane 4). (**B**) Amounts H3 Arg^{17} methylation and H4 Arg^3 methylation decreased in HL-60 granulocytes after calcium ionophore treatment. (**C**) Before calcium ionophore treatment, H3 Arg^{17} methylation signals (red) are present at comparable levels in each HL-60 granulocyte. After 15 min of calcium ionophore treatment, methylation of H3 Arg^{17} strongly decreased in the majority of cells. (**D**) Protein microsequencing of H3 and citrullinated H3. Cit was not detected before calcium ionophore treatment. After PAD4 activation, ~27.3% of H3 Arg^8 is citrullinated (2.52 pmol of Cit versus 6.72 pmol of Arg), and ~6.5% of H3 Arg^{17} is citrullinated (0.21 pmol of Cit versus 3.02 pmol of Arg).

performed microsequencing with H3 isolated from HL-60 granulocytes. We found that H3 was only citrullinated after treatment with calcium ionophore and identified major PAD4 target sites at Arg[8] (~27.3% Cit) and Arg[17] (~6.5% Cit) (Fig. 3D). Although the H3 Arg[2] site was deiminated by PAD4 in vitro, its deimination was not detectable in HL-60 granulocytes. In addition, although only ~6.5% of H3 Cit[17] was detected, the majority of methyl-Arg[17] signal was lost (Fig. 3B), suggesting that methyl-Arg[17] was selectively targeted by PAD4. Furthermore, the high percentage of histone Cit[8] detected after calcium activation demonstrates that PAD4 can deiminate Arg in vivo.

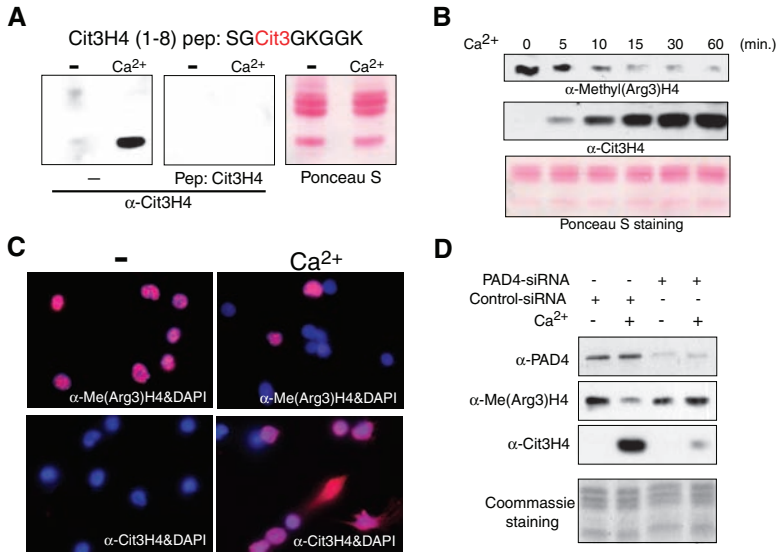

Fig. 4. PAD4 regulates H4 Arg[3] methylation and citrullination levels in HL-60 cells. (**A**) An antibody generated against an H4 Cit[3] peptide (amino acids 1 to 8 of H4) detects H4 after calcium ionophore treatment (left). This signal is specifically competed by the H4 Cit[3] peptide (middle). Equal loading of samples is shown by Ponceau S staining. (**B**) A dynamic decrease of H4 Arg[3] methylation mirrored by a concomitant increase in H4 Arg[3] citrullination after calcium ionophore treatment. (**C**). After calcium ionophore treatment, H4 Arg[3] methylation staining in the majority of cells was dramatically reduced (top). In contrast, a vast majority of cells became positively stained with the Cit3H4 antibody after calcium ionophore treatment (bottom). (**D**). PAD4 siRNA experiments in HL-60 cells. PAD4 protein amounts were dramatically reduced with PAD4 siRNA treatment (top). Cells treated with PAD4 siRNA had no obvious decrease in H4 Arg[3] methylation and little production of H4 Cit[3] after calcium ionophore treatment (middle). Equal protein loading was confirmed by Coomassie Blue staining (bottom).

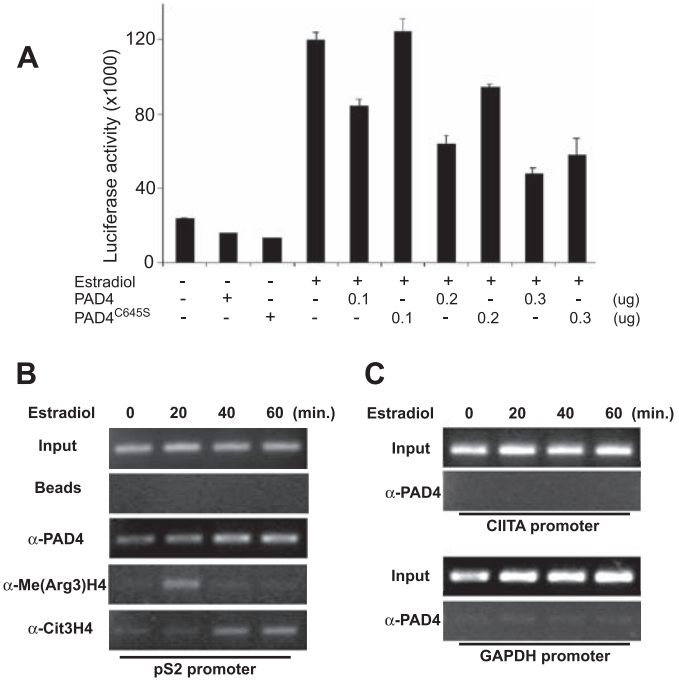

Fig. 5. PAD4 and the regulation of estrogen-responsive genes (**A**) Luciferase activity of an EREII-LUC reporter gene transfected into MCF-7 cells was dramatically increased in response to estradiol stimulation. Various amounts of plasmids (0.1 to 0.3 μg) expressing wild-type PAD4 efficiently inhibited the reporter gene activity in a dose-dependent manner. In contrast, a catalytic inactive form of PAD4 (C645S) displayed significantly reduced inhibitory effect. Error bars indicate the means ± SD of three individual experiments. (**B**) Association of PAD4 and the dynamic change of methylation and citrullination of H4 Arg[3] on the pS2 gene promoter in MCF-7 cells. (**C**) As controls, PAD4 was not associated with the promoter of CIITA gene (specific to immune cells). On the ubiquitously expressed GAPDH promoter, background levels of polymerase chain reaction signals were detected from PAD4 ChIP.

To investigate whether PAD4 can citrullinate H4 at Arg[3], we developed a specific antibody against H4 Cit[3] (α-Cit3H4). Western blot analyses showed that the Cit3H4 antibody strongly recognized H4 after treatment of HL-60 granulocytes with calcium ionophore (Fig. 4A). This reactivity was specifically decreased by the Cit3H4(1-8) peptide (Fig. 4A). These data suggest that PAD4 can target H4 Arg[3] site for citrullination.

To analyze the temporal changes in H4 Arg[3] methylation and citrullination, we performed Western blot experiments at different time points after calcium ionophore treatment. A gradual loss of H4 Arg[3] methylation was observed (Fig. 4B), which is directly correlated with a concomitant increase of H4 Cit[3] (Fig. 4B). The dynamic and complementary change of H4 Arg[3] methylation and citrullination in HL-60 granulocytes suggests that PAD4 either preferentially targets methyl-Arg[3] in vivo or reacts with both H4 methyl-Arg[3] and Arg[3] equally well.

As is the case of H3 Arg[17] methylation (Fig. 3), H4 methyl-Arg[3] antibody staining was greatly reduced in the majority of cells (~55.2%, n = 200) after 15 min of calcium ionophore treatment (Fig. 4C). By using an H2A/H4 phospho-Ser1 antibody (*29*), we found that this phosphorylation mark was not decreased after calcium ionophore treatment (fig. S4), suggesting that the extreme N terminus of H4 is unaltered. In contrast, although HL-60 granulocytes were not stained with the Cit3H4 antibody before calcium ionophore treatment (merged images in Fig. 4C), the majority of cells (~63.8%, n = 1178) were positively stained with the Cit3H4 antibody after 15 min of calcium ionophore treatment (Fig. 4C).

To address whether the observed decrease of H4 Arg[3] methylation and increase of H4 Cit[3] was dependent on PAD4 activity, we carried out PAD4 RNA interference experiments in HL-60 cells. As shown in Fig. 4D, the amount of PAD4 protein dramatically decreased after PAD4 small interfering RNA (siRNA) treatment but was not affected by a control siRNA (Fig. 4D). As expected, the ability of HL-60 granulocytes to decrease H4 Arg[3] methylation and to increase H4 Cit[3] was lost when PAD4 expression was inhibited (Fig. 4D). These data illustrate that PAD4 is the major, if not the only, enzyme that directly mediates the dy-

namic change of histone H4 Arg^3 methylation and citrullination in HL-60 granulocytes.

Histone Arg methylation at H3 Arg^{17} and H4 Arg^3 is known to regulate estrogen-responsive genes, such as the pS2 gene in MCF-7 cells (*11*, *30*). The observed demethylimination activity of PAD4 suggests it might regulate histone Arg methylation on specific promoters, leading to a change of gene expression. To test this idea, we first analyzed the effect of PAD4 and an enzymatically inactive form of PAD4 ($PAD4^{C645S}$) (fig. S3) on the activity of an EREII-luciferase reporter gene, which can be strongly induced by β-estradiol in MCF-7 cells (Fig. 5A). We found that the wild-type PAD4 effectively repressed the activity of the luciferase reporter in a dose-dependent manner (Fig. 5A), whereas the $PAD4^{C645S}$ mutant displayed weaker inhibitory effects. Intriguingly, the $PAD4^{C645S}$ mutant displays partial repressive activity when present at higher doses. Whether the mutant retains partial enzymatic activity, recruits additional cofactors, or heterodimerizes with endogenous PAD4 in MCF7 cells [as does wild-type PAD4 (*19*)] remains unclear.

The repressive activity of PAD4 on the EREII-luciferase reporter gene prompted us to test whether PAD4 plays a role in regulating the endogenous pS2 gene in MCF-7 cells after estradiol stimulation. We found both PAD4 expression and low amounts of H4 Cit^3 in MCF-7 cells (*28*). With chromatin immunoprecipitation (ChIP) analyses, we showed that PAD4 is associated with the pS2 gene promoter before the addition of estradiol and that PAD4 amounts increased ~twofold at 40 and 60 min after estradiol induction (Fig. 5B). We observed a strong increase of H4 Arg^3 methylation at 20 min and a decrease at subsequent time points, whereas H4 Cit^3 increased at 40 and 60 min. Therefore, the decrease of H4 Arg^3 methylation correlates with the increase of PAD4 protein and H4 Cit^3 levels on the pS2 gene promoter. In addition, PAD4 was not associated with the control CIITA gene and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene promoters before or after estradiol treatment (Fig. 5C). These data suggest that PAD4 acts specifically at the pS2 promoter and that its recruitment does not simply result from increased PAD4 expression upon hormone induction. Thus, our data support the conclusion that the demethylimination activity of PAD4 is likely involved in the subtle balance of the estrogen-inducible pS2 gene expression in MCF-7 cells.

Our finding that PAD4 can both deiminate and demethyliminate histones suggests that PAD4 may affect chromatin structure and function via two related but different mechanisms (fig. S5). Regarding demethylimination, histone Arg methylation mediated by secondary co-activators, such as CARM1 and PRMT1, has been correlated with gene activity (*11*–*15*) (fig. S5). Given the paradigm already established by reversible acetylation (*31*–*33*), it seems reasonable that Arg-directed methylation events, particularly those that lead to gene activation, would be reversible. In the case of estrogen-induced genes in MCF-7 cells, we favor the view that PAD4 also functions to remove histone Arg methylation marks, thereby reversing the transcriptional activation brought about by nuclear hormone receptor coactivators and histone arginine methyltransferases, likely in concert with other chromatin modifying activities (e.g., histone deacetylases) (fig. S5). It remains a formal possibility, however, that the repressive effect of PAD4 may be due to its deimination activity, which, in turn, prevents histone methylation by CARM1 and PRMT1. Because of the dual enzymatic activities of PAD4, deimination versus demethylimination, separating any observed transcriptional or other biological effects brought about by PAD4 at target Arg residues will represent a challenge for future studies.

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Supporting Online Material

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Materials and Methods
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References

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Carbonyl Sulfide–Mediated Prebiotic Formation of Peptides

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Almost all discussions of prebiotic chemistry assume that amino acids, nucleotides, and possibly other monomers were first formed on the Earth or brought to it in comets and meteorites, and then condensed nonenzymatically to form oligomeric products. However, attempts to demonstrate plausibly prebiotic polymerization reactions have met with limited success. We show that carbonyl sulfide (COS), a simple volcanic gas, brings about the formation of peptides from amino acids under mild conditions in aqueous solution. Depending on the reaction conditions and additives used, exposure of α-amino acids to COS generates peptides in yields of up to 80% in minutes to hours at room temperature.

The first suggestion that COS might be a prebiotic condensing agent appears in a footnote of a paper by Hirschmann and co-workers on peptide synthesis from 2,5-thiazolidinediones (*1*). The authors reported that traces of dipeptide are formed from phenylalanine