Supporting Online Materials

Materials and Methods

Protein purification and protein microsequencing
GST fusion proteins, including GST-PAD4 (human, 1-663), GST-PAD4<sup>C645S</sup>, GST-PRMT1 (rat, 1-353), and GST-CARM1 (mouse, 3-608) were expressed in <i>E.coli</i> and purified using glutathione agarose beads (Sigma-Aldrich) according to manufacturer's instructions (Amersham Pharmacia). The purity and integrity of the fusion proteins were analyzed by SDS-PAGE and commassie blue staining. Total histones were purified by acid extraction of tissue culture 293T cells with 0.4N H<sub>2</sub>SO<sub>4</sub> followed by precipitation with 20% trichloroacetic acid (TCA). Individual histones, H3 and H4, were further purified by RP-HPLC. For Edman protein microsequencing, RP-HPLC purified histones of various treatments were first separated by 15% SDS-PAGE and transferred to PVDF membrane. The sequencing was carried out by Dr. R. Cook at Baylor College of Medicine. The chromatographic property of Cit on the amino acid analysis column was determined using bulk citrulline amino acid as a standard.

Antibody development and western blots
To develop antibodies against histone H4 Cit3, a histone H4 peptide (SGCit3GKGGK) was chemically synthesized and used to immunize rabbits in collaboration with Upstate Biotech. Inc. The positive anti-sera were further purified by immuno-affinity purification using a H4 Cit3 peptide column. This antibody (α-Cit3H4) was diluted 1:2000 on western blots.

For western blots, histone proteins were separated in 15% SDS-PAGE gels, and PAD4 protein was separated in 10% SDS-PAGE gels. Proteins were electro transferred to nitrocellulose or PVDF membranes and then blocked for 30 min with 5% fat free dry milk in PBST (1xPBS with 0.1% Tween 20) before the primary antibodies were applied. All primary antibodies were purchased from Upstate Biotech Inc. unless otherwise mentioned. Antibody dilutions for western blots were, α-Me(Arg17)H3 (1:1000), α-Me(Arg3)H4 (1:1000), α-PAD4 (1:3000, a generous gift from Dr. Yamada), α-Me(Lys4)H3 (1:4000), α-Phos(Ser1)H2A/H4 (1:20000). After overnight incubation at 4°C and washing, corresponding HRP-conjugated secondary antibodies were incubated for 2 hr at room
temperature followed by washing. The HRP signals were detected by enhanced chemiluminescence reagents (Amersham Pharmacia). To detect protein bound citrulline with the α-Mod-Cit antibody, membranes were treated with a mixture of equal amounts of buffer A (0.025% FeCl₃ in a solution of 55% H₂O, 25% concentrated H₂SO₄, and 20% of H₃PO₄ (85%)) and buffer B (0.5% diacetyl monoxime, 0.25% antipyrine in 0.5M HAc) for 2 hr at 37°C with continuous and gentle mixing. After treatment, the modified citrulline was detected with α-Mod-Cit antibody (1:3000, a generous gift from Dr. Senshu) as described above.

**Histone methyltransferase assays and PAD assays**

Histones were labeled by methyltransferases under the following conditions. Recombinant histone H4 was labeled with PRMT1 for 30 min at 30°C in a 50µl reaction containing 50mM Tris.HCl, pH8.0, 0.5mM DTT, 0.5mM PMSF, 5µg recombinant histone H4, 2.2µCi [³H]-SAM, and 1µg GST-PRMT1. Recombinant histone H3 was labeled by GST-CARM1 under similar conditions except for that 5µg H3 and 0.5µg of CARM1 were used. After labeling, histones were precipitated with TCA and washed with acetone. For PAD4 treatment, about 2µg each of cellular histone H3, H4, [³H-methyl]-H3, or -H4 was treated with GST-PAD4 in 20µl PAD buffer containing 50mM Tris.HCl, pH7.6, 4mM DTT, 4mM CaCl₂, 1mM PMSF. After PAD4 treatment, the amount of Arg methylation in cellular histones was analyzed by western blot experiments, and the amount of [³H]-methyl associated with histone samples was analyzed by fluorography.

**Methylamine detection**

To detect methylamine, histone H4 was first radioactively labeled with PRMT1 and [³H]-SAM as described above. After treatment with PAD4, the reaction was split and the pH was adjusted to either ~2.0 or ~12.0 with HCl or NaOH, respectively. Samples were then spotted onto a piece of Whatman paper and stuck inside to the lid of a 15ml scintillation vial containing 5ml of scintillation solution. The released [³H]-methylamine was trapped in the scintillation solution and detected by a scintillation counter. A detailed description of a similar protocol can be found in Xie et al. (S1). Methylamine was further confirmed and analyzed with an amino acid cation exchange column as previously described (S2). 10 µmol
of cold methylamine was run together with \[^{3}\text{H}\]-methylamine through the column. Cold methylamine was detected by absorbance at OD\text{570} following a ninhydrin reaction, while \[^{3}\text{H}\]-methylamine was detected by scintillation counting. The measured results were analyzed and graphed using the Microsoft Excel program.

**HL-60 cell culture and treatments with DMSO and calcium ionophore**

Human HL-60 cells were grown in RPMI 1640 medium supplemented with 10% FBS and antibiotics. To differentiate the HL-60 cells into granulocytes, cells were treated in RPMI medium with 1.25% DMSO for 3 days at a starting density of 4x10\text{5} cells/ml. For calcium ionophore treatment, HL-60 cells or HL-60 granulocytes were resuspended in Locke's solution (10mM Heps.HCl, pH 7.3, 150mM NaCl, 5mM KCl, 2mM CaCl\text{2}, and 0.1% glucose) at a concentration of 2x10\text{6}/ml, and treated with 4µM calcium ionophore A23187 (Sigma-Aldrich) for 15 min at 37°C similarly as previously described \((S3)\). For the analyses of histone Arg3 methylation and citrullination dynamics, acid extracted histone samples were collected at different time points after the treatment of calcium ionophore.

**PAD4 siRNA treatment of HL-60 cells.**

For PAD4 loss of function analysis, 1.6 ×10\text{6} human HL-60 cells were transfected by electroporation at 230V (capacitance 1F) with 40 µl of 20pmol/µl solution of PADI4 SMARTpool siRNA duplexes from Dharmaco or with corresponding amount of the control siRNA duplex (directed at firefly luciferase \((S4)\); kind gift of Tom Tuschl laboratory). After electroporation cells were recovered overnight in RPMI 1640 medium supplemented with 10% FBS and antibiotics. The following day cells were treated in RPMI medium with 1.25% DMSO for 3 days at a starting density of 4x10\text{5} cells/ml. Subsequently, PAD4 siRNA and control siRNA transfected cells were divided each into two pools, spun down, and respectively, mock treated or treated with calcium ionophore for 15 minutes as described above. After treatment cells were counted, recovered by low speed centrifugation and lysed in Laemmli buffer. All samples were normalized by cell number and normalization was confirmed by Coomassie staining.

**Immunofluorescence**
HL-60 cells or HL-60 granulocytes were spun at 1200rpm for 5 min onto coverslips in a 6-well dish. Cells were fixed with a paraformaldehyde fixing solution (1xPBS, 0.1% Triton X-100, 0.2% NP-40, and 3.7% paraformaldehyde) for 10 min at room temperature. After 3 washes (10 min each) with PBST (1xPBS with 0.2% Triton X-100), cells were blocked with 2% BSA in PBST for at least 1 hr at room temperature. The cells were stained overnight at 4°C with antibodies diluted in PBST containing 2% BSA using the following antibody dilutions: α-Me(Arg17)H3 (1:100), α-Me(Arg3)H4 (1:100), α-PAD4 (1:300, a generous gift from Dr. Yamada), α-Me(Lys4)H3 (1:400), α-Phos(Ser1)H2A/H4 (1:1500), α-Cit3H4 (1:200). Primary antibodies were detected with appropriate Cy3-conjugated secondary antibodies (Jackson Immuno Research Lab Inc.). DNA was stained with DAPI (4,6-diamidino-2-phenylindole) before mounting. Images were collected using Zeiss Axiovert 200M microscope and processed in the Adobe Photoshop and Illustrator programs. The signal collection time was automatically determined using the strongest cells in the field to saturate the curve of data collection.

**Reporter assays and chromatin immunoprecipitation (ChIP)**

Reporter gene activity assay was performed by transient transfection of MCF-7 cells. Before transfection, MCF-7 cells were maintained in phenol-red free DMEM medium with 5% charcoal-dextran-treated FBS for at least two days. After transfection of 1.0ug of EREII-Luc (GL45) plasmid with combination of various amount of PAD4 and PAD4C645S plasmids (pSG5), MCF-7 cells were treated with 20nM β-estradiol for 48 hr before the reporter activity was measured. The data shown are representative of 4 independent experiments.

ChIP assay of the pS2 gene promoter in MCF-7 cells was carried out as previously described (S5). Briefly, MCF-7 cells were maintained in phenol-red free DMEM medium with 5% charcoal-dextran-treated FBS for three days before β-estradiol (100nM) treatment. The induction of β-estradiol was terminated by the addition of 1% formaldehyde at different time points following the addition of β-estradiol. The cells were then washed and sonicated to produce DNA fragments primarily in the range of 400-1000bp. The cell lysate was precleared by salmon sperm treated protein-G agarose (UBI). For each immunoprecipitation, 5µl of affinity purified α-Me(Arg3)H4, α-Cit3H4, or 4µl of α-PAD4 antibodies were
incubated with chromatin samples from $2 \times 10^6$ cells for overnight at 4°C. 40µl of protein agarose beads (50% slurry) was used to collect the immunocomplexes for 1 hr at 4°C. The DNA samples were purified after reverse cross-linking, phenol-chloroform extraction, and precipitation. The PCR primers used to amplified pS2 promoter are -167 forward (CAAGATGACCTCACCACATG) and -30 reverse (GAGCGTTAGATAACATTTGCC) (S6). Amplified DNA signals were monitored between cycles 28 to 34 of the PCR reaction. The PCR results shown are representative of three repeatable PCR reactions.
### Table S1: Edman protein microsequencing to detect the conversion of histone Arg to Cit by PAD4 in vitro

<table>
<thead>
<tr>
<th>PAD4</th>
<th>H3</th>
<th>H3</th>
<th>H3</th>
<th>H3</th>
<th>H3</th>
<th>H4</th>
<th>H4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Arg2, Cit2)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cit2</td>
<td>(Arg8, Cit8)</td>
<td>Cit8</td>
<td>(Arg17, Cit17)</td>
<td>Cit17</td>
<td>(Arg3, Cit3)</td>
</tr>
<tr>
<td>(pmol)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(pmol)</td>
<td>(%)</td>
<td>(pmol)</td>
<td>(%)</td>
<td>(pmol)</td>
<td>(%)</td>
</tr>
<tr>
<td>-</td>
<td>(6.93, N.A.)&lt;sup&gt;d&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0</td>
<td>(2.94, N.A.)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0</td>
<td>(0.42, N.A.)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0</td>
<td>(2.36, N.A.)</td>
</tr>
<tr>
<td>+</td>
<td>(1.93, 28.27)</td>
<td>~93.6</td>
<td>(0.22, 19.64)</td>
<td>~98.9</td>
<td>(-0.01, 2.94)</td>
<td>~100</td>
<td>(0.02, 5.24)</td>
</tr>
</tbody>
</table>

- Except for conversion of Arg to Cit, the rest of histone sequences were not found to be changed following PAD4 treatment.
- The amount of Arg and Cit was determined by Edman microsequencing, the background value was subtracted.
- The percentage of Cit was calculated using the formula 100xCit/(Arg+Cit).
- N.A. stands for not applicable.
- Data derived from the same run of H3 sample untreated by PAD4, note that the decrease of the amount of amino acid detected in the later cycles of microsequencing because of the sequencing efficiency.
Figures and figure legends:

(A) Schematic drawing of H3 N-terminal (1-28) and H4 N-terminal (1-20) residues. Major (Me) and minor (denoted by red dots) known Arg methylation sites are indicated.

(B) Bar graphs of human PAD4 and *Pseudomonas aureus* DDAH (dimethylarginine dimethylaminohydrolase). DDAH (residues 1-254) can be aligned with the C-terminus of PAD4 (residues 423-663) by homology comparison using the Fugue program. This homology defines a DHD (DDAH homology domain) domain in PAD4.

(C) Sequence alignment of DDAH (residues 1-254) and the C-terminus of PAD4 (residues 423-663). Conserved residues are highlighted by red color. Similar residues are highlighted by blue color. The conserved Cys residues (Cys249 of DDAH and Cys645 of PAD4) important for the catalytic activity of both DDAH and PAD4 are indicated by an arrow.

Fig. S1: The homology of PAD4 and DDAH

(A) Schematic drawing of H3 N-terminal (1-28) and H4 N-terminal (1-20) residues. Major (Me) and minor (denoted by red dots) known Arg methylation sites are indicated.

(B) Bar graphs of human PAD4 and *Pseudomonas aureus* DDAH (dimethylarginine dimethylaminohydrolase). DDAH (residues 1-254) can be aligned with the C-terminus of PAD4 (residues 423-663) by homology comparison using the Fugue program. This homology defines a DHD (DDAH homology domain) domain in PAD4.

(C) Sequence alignment of DDAH (residues 1-254) and the C-terminus of PAD4 (residues 423-663). Conserved residues are highlighted by red color. Similar residues are highlighted by blue color. The conserved Cys residues (Cys249 of DDAH and Cys645 of PAD4) important for the catalytic activity of both DDAH and PAD4 are indicated by an arrow.
Fig. S2: Specificity of the Me(Arg3)H4 antibody

The α-Me(Arg3)H4 antibody was originally generated using an H4 synthetic peptide with Arg3 asymmetrically dimethylated. H4 Arg3 was previously shown to be monomethylated mass spectrometry analysis in 293T cells (S7). To test the specificity of the α-Me(Arg3)H4 antibody, we carried out dot blot assays of this antibody with a series of H4 (1-16) peptides, including unmodified, monomethyl-Arg3, asymmetric dimethyl-Arg3, and symmetric dimethyl-Arg3. The α-Me(Arg3)H4 antibody strongly reacted with the asymmetric dimethyl-Arg3 peptide, significantly reacted with the monomethyl-Arg3 peptide, barely detected the unmodified, or the symmetric dimethyl-Arg3 peptides. These data suggest that the antibody applied in our experiments can recognize both monomethyl-Arg3 and dimethyl-Arg3 modifications.
Fig. S3: The H4 N-terminal (1-16) peptides are poor substrates of PAD4.

(A) Purified GST-PAD4 and an enzymatically inactive PAD4 mutant, GST-PAD4<sup>C645S</sup> were separated by SDS-PAGE and visualized by coomassie blue staining.

(B) As also shown in Fig. 2, the wild type GST-PAD4 dramatically reduced [3H]-methyl activity from H4 radioactively labeled with PRMT1. The C645S point mutation diminished the PAD4 activity.

(C) In contrast, the wild type PAD4 was unable to significantly reduce the [3H]-methyl activity from an H4 peptide (1-16) under the current experimental conditions.
Fig. S4
Fig. S4: PAD4 activation in HL-60 granulocytes did not affect histone H3 Lys4 methylation levels or the histone H4 Ser1 phosphorylation levels.

(A) As a control, no difference in the level of H3 Lys4 methylation was observed before (upper panels) and after (lower panels) calcium ionophore treatment.

(B) No obvious difference in the level of H4 Ser1 phosphorylation was observed before (upper panels) and after (lower panels) calcium ionophore treatment.

Fig. S5: Biochemical reactions and biological links of Arg methylation

AT = aminotransferase
(A) Protein level. Arg or methyl-Arg residues in histones can be converted to Cit by a PAD4 therefore generating various modification states.

(B) Chromatin level. Arg methylation and Lys acetylation of histones are correlated with gene expression (step I). It is an attractive possibility that PAD4 activity in concert with histone deacetylase (HDAC) would convert methyl-Arg to Cit and Ac-K to K, respectively (Step II). A putative aminotransferase (AT?) activity could convert Cit to Arg to counteract the effect of citrullination (step III). Alternatively, citrullinated histones in chromatin could be replaced by histone variants (step III).

References: