Protein arginine deiminase 4 (PAD4) facilitates the post-translational citrullination of the core histones H3 and H4. While the precise epigenetic function of this modification has not been resolved, it has been shown to associate with general chromatin decompaction and compete with arginine methylation. Recently, we found that histones are subjected to methylglyoxal (MGO)-induced glycation on nucleophilic side chains, particularly arginines, under metabolic stress conditions. These non-enzymatic adducts change chromatin architecture and the epigenetic landscape by competing with enzymatic modifications, as well as changing the overall biophysical properties of the fiber. Here, we report that PAD4 antagonizes histone MGO-glycation by protecting the reactive arginine sites, as well as by converting already-glycated arginine residues into citrulline. Moreover, we show that similar to the deglycase DJ-1, PAD4 is overexpressed and histone citrullination is upregulated in breast cancer tumors, suggesting an additional mechanistic link to PAD4’s oncogenic properties.
In eukaryotes, nucleosomes are the fundamental unit of chromatin, composed of DNA and histone proteins. Post-translational modifications (PTMs) on histones, including acetylation and methylation, are important in regulating chromatin structure and function during replication, transcription, and DNA damage. It is speculated that the combinations of specific PTMs affect the transcriptional state of a specific genomic locus. Citrullination, which occurs on arginine residues and involves the deamination of the guanidino group, reduces the net charge of the side chain and was generally shown to promote chromatin fiber decompaction, although specific sites have also been associated with gene repression. It was previously demonstrated that histone H3 arginine citrullination antagonizes methylation at the same residues by both blocking the modification sites and preventing the recruitment of the methyltransferases. Histone arginine citrullination is executed by the calcium-dependent enzyme, protein arginine deiminase 4 (PAD4). PAD4 substrates include specific sites on both core and linker histones, and it was suggested to play a role in determining cellular pluripotency as well as in the DNA damage response. Although PAD4 (PAD4) is a documented oncogene, the regulatory function of histone citrullination in pathological and physiological processes is still poorly understood.

Whereas the most well-characterized histone modifications are enzymatic, in the past few years it has become clear that histones are also prime substrates of nonenzymatic covalent modifications that induce changes in chromatin structure and function. We recently found that core histones are subjected to MGO glycation and that these adducts change chromatin architecture, the epigenetic landscape, and transcription, and particularly accumulate in disease states. We determined that short or low-concentration exposure to MGO induces chromatin decompaction by compromising the electrostatic interactions of the histone tails with DNA, in a mechanism similar to acetylation. These initial adducts can rearrange and undergo crosslinking, which ultimately leads to chromatin fiber hyper-compaction. While MGO reacts with histones nonenzymatically, we found that DJ-1/PARK7 is a potent histone deglycase, preventing the accumulation of histone glycation in vitro and in cells. Since MGO rapidly reacts with the guanidino group of arginines, we tested the effect of MGO glycation on cellular H3R8 methylation mark and found that MGO induces a reduction in H3R8me2 levels. As multiple arginine residues on histones, including H3R8, were shown to be substrates of PAD4, we hypothesized that citrullination, which alters the charge of the amino-acid side chains and reduces their reactivity against electrophiles, blocks MGO glycation on arginines and vice versa. In this study, we add address this hypothesis and find that beyond the direct competition between citrullination and MGO glycation, PAD4 itself acts as a deglycase, mediating the conversion of arginine glycation adducts into citrulline (Fig. 1).

**Results**

**PAD4-mediated citrullination prevent histone MGO glycation.** First, we aimed to analyze the biochemical mechanism governing the crosstalk between histone citrullination and glycation. To do so, we purified recombinant PAD4 and tested its activity in vitro on a range of substrates with increasing complexities, including free histone H3, nucleosome core particles (NCPs), and homododecameric (12-mer) nucleosomal arrays, which mimic the minimal chromatin fold. Our results indicate that PAD4 has increased reactivity towards more physiological substrates, with highest detected activity on 12-mer arrays (Supplementary Fig. 2), in a Ca2+-dependent manner (Supplementary Fig. 3). Next, we utilized unmodified and PAD4-citrullinated NCPs as substrates of MGO glycation to test the direct competition between these modifications. Our results indicate that citrullination protects NCPs from undergoing MGO glycation, since after a 12-h MGO treatment the citrullinated NCPs were substantially less glycated compared to unmodified NCPs (Fig. 2a). Moreover, this protective effect of PAD4-mediated citrullination against MGO glycation is dose dependent (Supplementary Fig. 4).

**PAD4 converts MGO-glycated histone arginines to citrullines.** Next, we tested the reciprocal competition—that is, whether MGO glycation protects NCPs from undergoing PAD4-mediated citrullination. To do so, we pretreated NCPs with MGO and, after removing the excess MGO, used the glycated-NCPs as substrates for PAD4 enzymatic reaction. The results unexpectedly indicated that PAD4 is still able to modify the glycated nucleosomes and that the citrullination is added at the expense of glycation, as evident by the decrease in MGO glycation and increase in citrullination signals (Fig. 2b). To test this newly identified histone deglycase activity of PAD4 and compare it to DJ-1, which we recently identified as a histone deglycase, we either added the enzymes to NCPs concurrently with MGO (C), or after a short (S) or a long (L) treatment with MGO (in both cases unreacted MGO was removed prior to enzymes addition). The results indicate that DJ-1 is capable of removing MGO glycation from NCPs when added concurrently or after a short exposure to MGO. However, glycation was persistent following a long exposure, which allows the rearrangement of the glycation adducts into late-stage products (Figs. 1 and 2b). In contrast to DJ-1 and the no-enzyme control, PAD4 was able to remove MGO-adducts and install citrulline, regardless of the incubation time (Fig. 2b), suggesting it is active on both early- (aminocarbinol) and late-stage (carboxyethyl arginine, CEA) MGO adducts. Indeed, applying both DJ-1 and PAD4 resulted in the almost complete abolishment of the glycation (Fig. 2b). To further validate the direct deglycase activity of PAD4, we performed a deglycation assay on biotinylated H3 and H4 N-terminal peptide substrates. The peptides were incubated with MGO, immobilized, washed and finally treated with PAD4. The reactions were analyzed by both dot blot (Fig. 2d) and LC-MS (Fig. 2e, Supplementary Figs. 5 and 6), confirming that PAD4 directly removes MGO-glycation adducts from both H3 and H4 tails in vitro. In contrast, a similar histone arginine modifying enzyme, protein arginine N-methyltransferase 1 (PRMT1), was not capable of converting the MGO-glycated H4-R3 to methylated arginine (Supplementary Fig. 7). Finally, to examine the effect of PAD4 activity on chromatin compaction, we performed a Mg2+ precipitation analysis on 12-mer arrays substrate. This assay relies on magnesium inducing the aggregation and precipitation of the 12-mer fibers, so the less compacted the arrays are, the more Mg2+ is required to precipitate them. Our results demonstrate that histone citrullination decompacts the chromatin arrays to a lesser degree than MGO treatment, and that PAD4 rescues the majority of MGO-dependent chromatin decompaction for all incubation conditions (Fig. 2c). This is in contrast to the deglycase DJ-1, which rescues glycation-induced decompaction only under short or co-incubation conditions (Supplementary Fig. 8).
As expected, PAD4 overexpression in 293 T cells induces an increase in histone citrullination at the expense of arginine methylation (Fig. 3a and Supplementary Fig. 9)\(^9,10\). In analogy to our in vitro results, treating these cells with increasing amounts of MGO induced the accumulation of H3 and H4 glycation (in addition to crosslinking), which was partially suppressed by PAD4 overexpression (Fig. 3a). To dissect the deglycation function of PAD4, we performed a pulse-chase experiment where cells were first pulsed with a gradient of MGO concentrations and then washed with fresh media. After a 6-h recovery, cells were transfected with PAD4 and grown for additional 12 h before final harvesting. Analysis of the histone samples from these cells revealed that overexpression of PAD4 resulted in a decreased MGO-glycation signal, suggesting PAD4 is actively removing MGO adducts from histones in live cells (Supplementary Fig. 10).

In a complementary analysis, similar assays were performed with an alkynyl methylglyoxal probe and further confirmed the deglycation activity of PAD4\(^26\). Moreover, a global chromatin compaction analysis by micrococcal nuclease (MNase) digestion revealed that PAD4 rescues MGO-induced chromatin...
decompaction (Supplementary Fig. 11). Finally, to demonstrate the direct binding of glycated histone tail to PAD4, we utilized a biotin-H3 peptide (residues 1–18), either glycated or non-glycated, to demonstrate PAD4 binding regardless of its catalytic activity (Supplementary Fig. 12). Together, these results establish that PAD4 directly affects chromatin compaction by regulating the epigenetic crosstalk between histone MGO glycation, citrullination, and methylation.

PAD4 is overexpressed in breast cancer. Previous studies revealed the overexpression of PAD4 in a variety of cancers and suggested it could drive tumor pathogenesis through multiple potential mechanisms. Since we recently reported high levels of both histone MGO glycation and DJ-1 expression in breast cancer, we utilized the same samples to analyze PAD4 and histone citrullination levels. As presented in Fig. 3b, all patient tumor samples display substantial overexpression of PAD4 and increased levels of histone citrullination. In addition, xenograft tumor samples show diverse levels of PAD4 expression that correlate with degree of histone citrullination. In support, 293 T cells treated with increasing amounts of MGO, which mimics the metabolic stress that exists in cancer cells, show better survival when overexpressing PAD4 but not the catalytically dead mutant C645S (Supplementary Fig. 13). To investigate the impact of PAD4 on histone PTM crosstalk in breast cancer cells, MCF7 cells were pretreated with the PAD4 inhibitor GSK484 followed by a treatment with increasing concentrations of MGO. The analysis, presented in Supplementary Fig. 14, shows that inhibition of PAD4 increases histone glycation and arginine methyltransferase activity. Together, these data suggest a role for PAD4 in cancer proliferation through the regulation of chromatin structure and function.

Discussion

This study revealed a new crosstalk between histone glycation and citrullination, which is mediated by PAD4. Our data (Fig. 2 and Supplementary Fig. 15) support a dual-functional model whereby PAD4 is capable of not only protecting arginine via deamination but rewriting MGO-adduct intermediates into citrulline, which is currently speculated to be a terminal product (Fig. 1, Supplementary Figs. 16 and 17). Although it was reported that free citrullines can be converted to arginines by argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) in the citrulline-NO cycle, to date there is no report of peptidyl citrulline being reverted back to arginine. Therefore, citrullination is likely to accumulate in long-lived proteins, such as histones, and prevent the target residues from undergoing electrophilic damage such as glycation. Based on this new deglycation activity of PAD4, it could potentially play a regulatory role in rescuing nonenzymatic damage and downstream changes in chromatin structure and function.

There are several implications for this newly identified rewriting function of PAD4 on MGO-glycated histones. First, it provides an additional pathway for the repair of...
pathophysiological histone glycation. We have previously shown that histone glycation is a modification that accumulates under metabolic stress, such as in highly-proliferating breast cancer tumors, and that it changes patterns of transcription and chromatin architecture. In that regard, both DJ-1 and PAD4 are proposed oncoproteins and targets of cancer therapy although the mechanisms are not fully understood. Indeed, we have shown that breast cancer patient samples contain massive over-expression of DJ-1 and PAD4 (Fig. 3). There are several efficient DJ-1 and PAD4 inhibitors reported, some of which are in pre-clinical trials, raising the potential of combinational therapy targeting these enzymes simultaneously.

Counterintuitively, our breast cancer patients’ samples also have high levels of glycation, suggesting that the overexpression of DJ-1 and PAD4 is not sufficient to remove all the glycation adducts. The results we present here indicate that this could be due to the fact that DJ-1 and PAD4 also work through distinct catalytic mechanisms. While DJ-1 erases early MGO-glycation adducts from both lysines and arginines, PAD4 is only active on arginines due to the fact that DJ-1 and PAD4 also work through distinct mechanisms. The results we present here indicate that this could be due to the fact that DJ-1 and PAD4 also work through distinct catalytic mechanisms.

Another important implication of this finding is the three-way metabolic crosstalk between glycation, citrullination, and methylation that compete for the same arginine sites on histones (Fig. 4). All these modifications contribute to chromatin architecture regulation. Both histone glycation at its early stages and citrullination induce chromatin decompaction. Indeed, the synergistic activity between PAD4 and DJ-1 leaves the treated glycated and non-glycated chromatin less compacted, with the newly added citrullination inducing chromatin fiber decompaction compared with untreated one (Figs. 2c, S8, and S11). These changes in the chromatin landscape directly correlate with the accessibility of the metabolites generated from the associated pathways: glycation with sugar glycolysis, citrullination with calcium homeostasis, and methylation with S-adenosyl methionine (SAM) metabolism, suggesting that this crosstalk is influenced by diet, metabolic state, and the cellular microenvironment. The balance between MGO, Ca²⁺, and SAM can be regulated through multiple processes including endoplasmic reticulum (ER) stress, reactive oxygen species (ROS), and mTOR signaling, providing an additional potential link to changes in gene expression. Together with our previous identification of the interrelationship between glycation and methylation, this work suggests a three-way crosstalk (Fig. 4) and new insights into the link between metabolism, epigenetics, and human disease.

Conclusions
Metabolic syndromes and diabetes increase the risk for certain diseases such as cancer. However, the mechanism behind this correlation is poorly understood. Methyglyoxal, a reactive dicarbonyl sugar metabolite found in cells under metabolic stress, can nonenzymatically modify arginine and lysine residues in histone proteins, making it a new epigenetic marker linking metabolism and disease. Histone MGO glycation induces changes in chromatin architecture and the epigenetic landscape, abrogating gene transcription. In this study, we found that PAD4 exhibits a dual function of antagonizing histone MGO glycation by both removing glycation adducts from arginines and converting the unmodified side chains into citrulline, protecting them from undergoing glycation. PAD4 induces changes in chromatin architecture by regulating not only charges of arginine residues but also the MGO-glycation levels on histones. This unique function together with the overexpression of PAD4 in breast tumors provide insights into a potential mechanism for its function in cancer cells and understandings of the correlation between metabolism and cancer epigenetics. Overall, these findings expand our understanding of PAD4 biochemistry and its pathophysiological function in human health.

Methods

General materials and methods. UV spectroscopy was performed on NanoDrop 2000c (Thermo Scientific). Biochemicals and media were purchased from Fisher Scientific or Sigma–Aldrich Corporation unless otherwise stated. T4 DNA ligase, DNA polymerase, and restriction enzymes were obtained from New England BioLabs. PCR amplifications were performed on an Applied Biosystems Veriti Thermal Cycler using either Taq DNA polymerase (Vazyme Biotech) or routine genoype verification or Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech) for routine high-fidelity amplification. Site-specific mutagenesis was performed according to the standard procedure of the QuickChange Site-Directed Mutagenesis Kit purchased from Stratagene (GE Healthcare) or Mut Express II (Vazyme Biotech). Primer synthesis and DNA sequencing were performed by Integrated DNA Technologies and Geneviz, respectively. PCR amplifications were performed on a Bio-Rad T100™ Thermal Cycler. Centrifugal filtration units were purchased from Millipore, and MINI dialysis units purchased from Pierce. Size exclusion chromatography was performed on an AKTA FPLC system from GE Healthcare equipped with a P-920 pump and UPC-900 monitor. Sephacyrl S-200 columns were obtained from GE Healthcare. All the western blots were performed using the primary antibodies annotated in Supplementary Table 1 and fluorophore-labeled secondary antibodies annotated in Supplementary Table 2 following the protocol for staining of histone arginine methylation, citrullination, and glycation.

**Fig. 4 Glycation, citrullination, and methylation have an interaction network.** A proposed model showing the three-way crosstalk of histone glycation, citrullination, and methylation.
recommended by the manufacturer. Blots were imaged on Odyssey CLX Imaging System (LI-COR). Amino-acid derivatives and coupling reagents were purchased from Advanced Bioscience (AB-Chemicals, DCM), and tris-isopropylsilane (TIS) was purchased from Fisher Scientific and used without further purification. Hydroxybenzotriazole (HOBt) and O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluoro phosphate (HBTU) were purchased from Fisher Scientific. Trifluoroacetic acid (TFA) was purchased from Fisher Scientific. N,N-Diisopropylethylamine (DIEPA) was purchased from Wako Pure Chemical Industries. Analytical reversed-phase HPLC (RP-HPLC) was performed on an Agilent 1200 series instrument with an Agilent C18 column (5 μm, 4 × 150 mm), employing 0.1% TFA in water (HPLC solvent A), and 90% acetonitrile, 0.1% TFA in water (HPLC solvent B) as the mobile phases. Analytical gradients were injected into a buffer 8–45 min at a flow rate of 0.5 mL/min, unless stated otherwise. Preparative scale purifications were conducted on an Agilent LC system. An Agilent C18 preparative column (15–20 μm, 2 × 250 mm) or a semi-preparative column (12 μm, 10 × 250 mm) was employed at a flow rate of 20 mL/min or 4 mL/min, respectively. HPLC Electrospray ionization MS (HPLC-ESI-MS) analysis was performed on an Agilent 6120 Quadrupole LC/MS spectrometer (Agilent Technologies). All the immunoblotting experiments in this research were done at least in triplicates. In the error bars in all the figures the standard deviation from three different experiments.

Recombinant histone expression and purification. Recombinant human histones H2A, H2B, H3.2, and H4 were expressed in Rosetta (DE3) cells with an overnight IPTG induction. For free histone citrullination, 50 μM PAD4 at 37°C for 2 h, and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot analysis. For nucleosome core particle (NCP) citrullination, 1 μM NCPs were treated with 0.1 μM PAD4 at 37°C for 2 h. The citrullinated NCPs were analyzed by SDS-PAGE or western dot blot analysis. Nucleosomal array citrullination assays were similarly prepared using 1 μM dodecameric arrays and 0.1 μM PAD4 with slight modification, that is, the concentration of CaCl2 was reduced to 100 μM to prevent the arrays from precipitation. For the NCP citrullination-glycation assays, the wild-type or citrullinated NCPs (1 μM) were treated with 5 μM MGO (Sigma) in 1 × PBS buffer (pH 7.4) at 37°C for 6 h (short treatment) or overnight (long treatment)18. The buffer exchange between citrullination and glycation assays was performed using 0.5 mL Centrifugal Filter (3 K, Millipore) with a 120-fold v/v for the removal of MGO or CaCl2 from the old reaction buffer systems. The co-incubation of NCPs, MGO and PAD4 was performed at 37°C overnight. The modified NCPs were analyzed by SDS-PAGE (without boiling or lyophilizing the sample) or native gel followed by western blot analysis. For the SDS-PAGE analysis, H3 was used as loading control, while ‘601 DNA was used as loading control (by ethidium bromide staining) in the native gel analysis.

For peptide deylation assays, 2 μM of the peptide substrate was incubated with 10 mM MGO in 1 x PBS buffer (pH 7.4) at 37°C for 30 min and then enriched by magnetic streptavidin beads (THERMO Fisher Scientific, 65602). After being washed by 1 x PBS buffer, the glycated peptide was eluted with 100 mM glycine buffer (pH 2.5) and then diluted by using citrullination buffer (Tri-HCl), followed by boiling the mixture at 37°C for 2 h. The elution and re-bound buffers in peptide deylation were made with H3O+ (Sigma-Aldrich, 329878). The reactions were analyzed by dot blot and LC-MS. For PRMT1 methylation assays, 30 μM full-length histone H4 was first incubated with (or without) 1 μM MGO in 1 x PBS buffer at 37°C for 2 h, and then treated with human recombinant 5′-M PAD4 at 37°C for 2 h. The elution and reaction was incubated on ice for 10 min, followed by a 10-min 17,000 rcf spin at 4°C. The A260 of the supernatant was measured and used to evaluate the fraction of soluble arrays.

Expression of PAD4 in 293 T cells. The PAD4 gene was amplified from pGEX-PAD4 plasmid by PCR. The primers used were 5′-ATATGGGCGCCGTCTACATCAGTGGCAGATTATGGCCCAGGGGACATTG-3′ (M1) and 5′-ATATGGTCATCTGAGAACCACTATGTCTCACCACACCC-3′ (M2). The amplified PCR product was inserted into pCMV plasmid by restriction endonuclease cloning. The catalytically dead mutant PAD4-C64S plasmid was constructed by site-directed mutagenesis using 5′-GGAGGTCAGACAGCGGCACCAACG-3′ and 5′-GGTTGGTGCCGCTG-3′ as primers. The HA-tagged PAD4 was overexpressed in HEK 293 T cells using Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer’s protocol. HEK 293 T cells (ATCC) were cultured at 37 °C with 5% CO₂ in DMEM medium supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), 2 mM L-glutamine and 500 units mL⁻¹ penicillin and streptomycin. Cells were stimulated with 2 μM calcium ionophore (Sigma-Aldrich, A23187) for 60 min at 37 °C before lysis, and then the PAD4 expression was detected by western blot analysis with anti-PAD4 and anti-HA antibodies.

Immunoprecipitation and pull down. The glycated and unglycated N-terminal biotinylated H3 peptides (residues 1–18) were used in the IP assays as previously described19. The glycated peptides were purified by a treatment of MGO in a 1:3 (peptide:MGO) molar ratio at 37°C for 30 min, as described above. Recombinant PAD4-C64S or total 293 T cell lysate containing overexpressed HA-PAD4-C64S were incubated with the peptides in 4°C for 2 h after which the peptide was used.
pulled down by BSA-blocked Streptavidin Magnetic Beads (Thermo Scientific).

Next, beads were washed three times with 1× PBS buffer (pH 7.4), boiled, separated on SDS-PAGE and analyzed by western blot with anti-PAD4 or anti-HA.

Salt extraction of histones from cells. The extraction of histones from cells was performed according to the previously described high-salt extraction method. Briefly, the cell lysis solution was prepared using extraction buffer (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34% sucrose, 10% glycerol, 0.2% NP40, protease and phosphatase inhibitors to 1× from stock). After spinning down, the pellet was extracted using a no-salt buffer (3 mM EDTA, 0.2 mM EGTA). After discarding the supernatant, the final pellet was extracted by using high-salt buffer (50 mM Tris pH 8.0, 2.5 mM NaCl, 0.05% NP40) in 4°C cold room for 1 h. After spinning down, the supernatant containing extracted histones was collected for further analyses.

Tumor samples. All cell-culture reagents were obtained from Thermo Fisher Scientific unless otherwise indicated. The cell lines for tumor xenografts were maintained at 37°C and 5% CO2 in humidified atmosphere. MCF7 was obtained from DSmZ, while T47D, BT474, ZR75-1, and Cama-1 cell lines used in this study were all sourced from ATCC. The cells were grown in DMEM/F12 supplemented with 10% FBS, 100 µg/ml penicillin, 100 µg/ml streptomycin, and 4 mMol/L-glutamine. All the cell lines tested negative for Mycoplasma and authenticated by short-tandem repeat (STR) analysis. Six to eight-week-old nu/nu athymic BALB/c female mice were obtained from Harlan Laboratories, Inc., and maintained in pressurized ventilated caging. All the studies were performed in compliance with institutional guidelines under an Institutional Animal Care and Use Committee-approved protocol (IRB#12-010). Xenograft tumors were established in nude mice by subcutaneously implanting 0.18 mg sustained release 17B-estradiol in Matrigel (Collaborative Research) on the opposite side 3 days afterward.

The clinical samples (MSKCC set) used in this study were obtained from the Biobank of MSKCC. The patients with breast cancer and either recurrence of disease after receiving adjuvant therapy or WHO-defined progression of metastatic disease on therapy were prospectively enrolled on an IRB approved tissue collection protocol (IRB#06-163). Informed consent was obtained from all patients. All patients underwent biopsy of at least a single site to document progressive disease. Mutational analysis of the metastatic biopsy was performed on fresh frozen specimens. Formalin fixed paraffin embedded (FFPE) blocks of the pretreatment primary tumor was obtained where possible for comparison. The presence of tumor, in both fresh samples and FFPE tissue sections, was confirmed by the study pathologist. Western blot analyses of PAD4 expression and histone citrullination were performed on fresh frozen specimens.

Cell fractionation. The cytosolic and nuclear fractions were prepared using NEPER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) according to the manufacturer’s protocol. Histones were extracted from the pellet using high-salt extraction protocol as described above. Histone extraction from tumor xenografts and patient samples followed a similar protocol, with slight variation, where tumors homogenized by mild sonication prior to extraction. Purity of fractionation was evaluated using the following antibodies: anti-Actin (cytosol), anti-MEK ½ (nucleoplasm) and anti-H3 (chromatin).

Pulse-chase experiments. 293 T cells were treated with a gradient of MGO for 12 h before the medium was changed to MGO-free DMEM. Cells were cultured for an additional 6 h, after which they were transfected with pCMV-PAD4 plasmid. After overnight incubation, the cells were harvested and cytosolic and histone fractions were prepared as described above. Samples were separated on a single SDS-PAGE, transferred to a PVDF membrane and blotted with the indicated antibodies.

Micrococcal nuclease (MNase) digestion assay. The MNase digestion assay was performed according to the previously described method with slight modifications. Cells were lysed in a hypotonic buffer (10 mM Tris-HCl pH 7.4, 10 mM KCl, 15 mM MgCl2) on ice for 10 min. Nuclei were pelleted by centrifugation and resuspended in MNase digestion buffer (50 mM Tris-HCl pH 7.9, 5 mM CaCl2) supplemented with RNase and incubated at 37°C for 30 min. The DNA was then pelleted again by centrifugation and resuspended in MNase digestion buffer supplemented with 100 µg/ml RSA and 40 IU MNase and incubated at room temperature for varying periods of time (5, 10, and 20 min). The MNase reaction was quenched with quenching buffer (0.4 M NaCl, 0.2% (w/v) SDS, 20 mM EDTA) followed by centrifugation. DNA was extracted and purified by standard procedures, and then analyzed by Tris-Borate-EDTA (TBE) gel electrophoresis.

Cell viability assay. Untreated and 24-h PAD4-transfected (WT or C645S) 293 T cells were cultured in a 96-well plate and treated with the indicated MGO concentrations for 12 h. Following the incubation, cell viability was evaluated using the Cell Counting Kit-8 (CCK-8, Sigma) according to the manufacturer’s protocol. The relative cell viabilities were given by detecting the absorbance at 450 nm at each well. Each experiment was performed in triplicate.

Inhibitor treatment to breast cancer cell lines. The PAD4 inhibitor GSK484 (Sigma, SML1658; 10 µM) was added to the MCF7 cell’s media 6 h prior to adding the corresponding concentrations of MGO. Cells were incubated for additional 12 h after which they were harvested and histones were extracted and analyzed as described above. Samples were separated on a single SDS-PAGE, transferred to a PVDF membrane and blotted with the indicated antibodies.

Statistics and reproducibility. For nucleosomial array collection and cell survival assays, data are presented as the mean ± S.E.M. of three independent experiments. All the western blotting and mass spectrometry data were repeated independently three times with similar results. Statistical analyses were performed in Microsoft Excel, GraphPad Prism 7 with ANOVA, the Student’s t-test, or q2 test.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All the data are available within the article and its Supplementary Information files or from the corresponding author upon reasonable request. The source data underlying Figs. 2, 3 and Supplementary Figs. 2, 3, 4, 7, 8, 10, 12, 13, 14, 15 are provided as a Source Data file. A reporting summary for this article is available as a Supplementary Information file.

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Author contributions

Q.Z. designed and performed all the experiments with A.O.’s help; Q.Z. and Y.D. analyzed the data; Q.Z. and Y.D. wrote the manuscript with A.O.’s help; Y.D. directed the research.

Competing interests

The authors declare no competing interests.

Additional information

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