Detection of histone modifications at specific gene loci in single cells in histological sections

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Chromatin immunoprecipitation assays have contributed greatly to our understanding of the role of histone modifications in gene regulation. However, they do not permit analysis with single-cell resolution, thus confounding analyses of heterogeneous cell populations. Here we present a method that permits visualization of histone modifications of single genomic loci with single-cell resolution in formaldehyde-fixed paraffin-embedded tissue sections based on combined use of in situ hybridization and proximity ligation assays. We show that dimethylation of lysine 4 of histone H3 (H3K4me2) at the MYH11 locus is restricted to the smooth muscle cell (SMC) lineage in human and mouse tissue sections and that the mark persists even in phenotypically modulated SMC in atherosclerotic lesions that show no detectable expression of SMC marker genes. This methodology has promise for broad applications in the study of epigenetic mechanisms in complex multicellular tissues in development and disease.

Epigenetics is defined as a heritable code other than the genomic sequence and includes histone post-translational modifications, DNA methylation and ATP-dependent chromatin remodeling1–3. Differentiation from embryonic stem cells (ESCs) to multiple differentiated cell types requires that the spatiotemporal expression of gene patterns be established4,5. As one example out of many6–9, we have previously shown that an SMC-specific epigenetic repertoire of histone modifications are acquired during development of SMC from ESC, including H3K4me2 at SMC marker genes such as ACTA2 and MYH11, the latter being the most specific marker of SMC lineage10–12. Notably, results show that H3K4me2 of these genes is restricted to SMCs and is absent in non-SMCs. Moreover, H3K4me2 enrichment of these genes persists when cultured SMCs are induced to undergo phenotypic switching to a less differentiated state13, a process dependent on activation of the ESC pluripotency factor KLF4 both in vivo14 and in cultured cells15. These results suggest that H3K4me2 of the MYH11 and other SMC marker gene loci represent stable epigenetic markers of SMC lineage. However, a major limitation in studies thus far is that data are derived nearly exclusively from studies in cultured SMCs, which are poorly differentiated because cell culture systems do not recapitulate complex environmental cues that regulate SMC differentiation in vivo14,15 under normal as well as pathological conditions. Moreover, it is impossible to obtain chromatin derived exclusively from SMCs from tissue sources because all SMC-containing tissues contain multiple other cell types. Conversely, all non-SMC tissues contain large numbers of SMCs because all tissues are vascularized. As such, conventional chromatin immunoprecipitation (ChIP)16,17 analyses cannot be used to rigorously test whether H3K4me2 of SMC gene loci are an exclusive epigenetic signature of SMC lineage in vivo. Indeed, an important general limitation of ChIP assays is that they do not permit analysis of histone modifications at a given gene locus in individual cells, thus confounding interpretation of analyses of heterogeneous cell populations and precluding direct assessment of the role of specific histone modifications within individual cell types in complex multicellular tissues, including disease specimens. For example, ChIP analyses on a tumor biopsy or atherosclerotic tissue specimen represent a composite signal derived from the many different cell types present in that tissue sample. Although one may perform ChIP assays on a given cell population obtained by cell sorting, such analyses result in the loss of critical information regarding the spatial orientation of cells within tissues and may be subject to epigenetic changes that occur during the tissue dissociation and/or sorting procedures.

Here we describe a method that permits visualization of histone modifications at a single genomic locus in human and mouse formalin-fixed paraffin-embedded tissue sections that is the equivalent of a single-cell ChIP assay, combining in situ hybridization (ISH) and proximity ligation assay (PLA) methods. We demonstrate that H3K4me2 of the MYH11 gene locus is a highly specific marker of SMC lineage in vivo that persists even in phenotypically modulated SMCs in atherosclerotic lesions that lack detectable expression of endogenous SMC marker genes.

RESULTS

Our overall strategy made use of the PLA18–20 to detect proximity between a biotin-labeled probe targeting the MYH11 promoter and H3K4me2 at this locus (Fig. 1a). PLA is widely performed for detection of protein-protein interaction or protein post-translational modifications in both cultured cells and tissue sections20,21, but it has not, to our knowledge, been used for

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assessing histone modifications at specific gene loci. We sought to combine PLA with ISH methods using two primary antibodies targeting (i) histone modifications and (ii) biotin residues included in a probe annealing to the genomic locus of interest. We first assessed the feasibility of the approach by estimating intermolecular distances between DNA and histone tails to ascertain the compatibility with the PLA range of detection of approximately 40 nm (Supplementary Fig. 1). The major methodological steps included (i) immunostaining of human or mouse formalin-fixed paraffin-embedded tissue sections with antibodies to SMC or non-SMC markers, (ii) ISH with a biotinylated DNA probe targeting the \( MYH11 \) locus and (iii) a PLA including incubation with anti-biotin (rabbit) and H3K4me2 (mouse) primary antibodies. We verified that nuclear detection of H3K4me2 was preserved after the ISH procedure (Supplementary Fig. 2) and mapped H3K4me2 enrichment of the \( MYH11 \) promoter to identify boundaries for our ISH probe (Supplementary Fig. 3). Moreover, for validation of our ISH procedure, we used a 5-TAMRA-dUTP–labeled Y chromosome probe in human samples from male patients, and we observed similar hybridization efficiencies of the Y chromosome in SMCs and non-SMCs cell types (Supplementary Fig. 4).

We performed ISH-PLA detection of H3K4me2 at the \( MYH11 \) locus in human coronary arteries because these are highly relevant to atherosclerotic disease. These vessels contain three distinct cell layers: the intima, consisting of endothelial cells (ECs); the media, composed primarily of smooth muscle \( \alpha \)-actin\(^+\) (ACTA2\(^+\)) SMCs; and the adventitia, composed mainly of fibroblasts negative for SMC markers but with abundant small blood vessels (Fig. 1b). We initially focused on small arteries within the adventitia of coronary arteries, which have a well-defined SMC layer. 

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**Figure 1** | ISH-PLA: a new method of detection of histone modifications at a single genomic locus in tissue sections. (a) Schematic of the ISH-PLA method for detecting H3K4me2 of the \( MYH11 \) promoter. (b) Immunostaining of 5-µm-thick sections of human carotid artery for ACTA2 (green) and DAPI (blue). M, media; A, adventitia. Arrows point to small vessels in the adventitia that contain ACTA2\(^+\) SMCs. Scale bar, 100 µm. (c) ISH-PLA detection in adventitial small arteries (\( n = 5 \)). Nuclear (DAPI), \( MYH11 \) H3K4me2 (PLA amplification), ACTA2 and CD31 staining are shown. Scale bars, 50 µm. Arrows in the merged image point to \( MYH11 \) H3K4me2 PLA\(^+\) SMCs; note its absence in CD31\(^+\) ECs (asterisks) and adventitial fibroblasts (arrowheads). (c, i–iii) High magnification with (i) ACTA2, (ii) PLA and (iii) merge. Scale bar, 10 µm. (d) ISH-PLA negative control in an adventitial vessel of human carotid artery sections. Scale bar, 50 µm. (e) Conventional ChIP assays showing enrichment of H3K4me2 of \( MYH11 \) in SMCs but not in ECs (mean ± s.d.; \( n = 3 \); *P < 0.05). IP, immunoprecipitated DNA. (f) ISH-PLA in cultured SMCs with arrows indicating \( MYH11 \) H3K4me2 PLA\(^+\) cells (\( n = 3 \)). (g) ISH-PLA in cultured ECs (\( n = 3 \)). Scale bars (f,g), 10 µm.
within ACTA2+ medial SMCs (Fig. 1c), whereas adventitial cells and ECs (CD31+) were PLA−. We used an empty biotinylated vector as a negative control, and it showed no PLA amplification in SMCs and non-SMCs, demonstrating that the MYH11 locus was observed exclusively in SMCs by both ChIP and ISH-PLA analyses.

To provide a model system for testing the stability of the MYH11 H3K4me2 mark in phenotypically modulated SMCs in vivo, we developed an SMC lineage–tracing system by crossing Myh11-CreERT2 mice with ROSA26 STOP-flox MYH11 biotinylated vector in SMC−EYFP+ mice (SMC-EYFP+/− mice) or in SMC−EYFP+/+ mice treated with tamoxifen (Fig. 2c), whereas >95% of medial cells within SMC-EYFP+/− mice were EYFP+ (Fig. 2d), indicating high efficacy of SMC-specific recombinated. Moreover, a substantial fraction of EYFP+ SMCs in large arteries (such as the aorta) of these SMC−EYFP+/+ mice showed complete concordance in that H3K4me2 enrichment of MYH11+ medial SMCs (Fig. 2a–c and Supplementary Fig. 5). However, adventitial cells including ECs and fibroblasts were negative (Fig. 2d and Supplementary Fig. 10). We did not observe any PLA amplification when we used an irrelevant probe (Fig. 3e). Medial SMCs were negative for H3K27me3 of the MYH11 locus, whereas ECs (CD31+ cells) (Fig. 3d) and adventitial fibroblasts (ACTA2− cells) (data not shown) were positive for this silencing mark on the basis of PLA analyses. Consistent with these results in human coronary arteries, PLA analyses of human brain sections (Fig. 3c and Supplementary Fig. 11) showed exclusive MYH11 H3K4me2 PLA labeling of SMCs, whereas neuronal cells were MYH11 H3K4me2 PLA− but MYH11 H3K27me3 PLA+. These results suggest that our ISH-PLA method can reliably and specifically detect histone modifications at specific gene loci in single cells in human and mouse tissue sections, and they are consistent with results of our previous studies using conventional ChIP assays on the MYH11 locus.
However, the studies here are, to our knowledge, the first to identify a cell type- and locus-specific histone modification in cells in vivo within intact tissue sections in a complex multicellular tissue specimen. Furthermore, we provide evidence that H3K4me2 of the \( \text{MYH11} \) gene locus represents a unique and specific epigenetic signature of cells of the SMC lineage in vivo. Finally, we show that our PLA methodology is readily adaptable to multiple gene loci and histone modifications.

Mature SMCs retain remarkable plasticity and can undergo phenotypic switching characterized by markedly decreased SMC marker gene expression and increased proliferation and migration under pathological conditions such as atherosclerosis\(^1\). However, because phenotypically modulated SMCs downregulate expression of SMC markers (including \( \text{ACTA2} \) and \( \text{MYH11} \)), there are major ambiguities as to which cells within lesions are derived from SMCs\(^2\). Previous studies demonstrated that PDGF-BB–induced phenotypic switching of cultured SMCs was associated with marked reductions in SMC marker expression and reduced H4 acetylation but no changes in H3K4me2 enrichment of the \( \text{ACTA2} \) or \( \text{MYH11} \) promoters\(^3\). We extended these studies herein by showing that induction of SMC phenotypic switching with the pro-atherogenic oxidized phospholipid PVOPC had similar effects, including reduced \( \text{MYH11} \) expression but no change in \( \text{MYH11} \) H3K4me2 enrichment (Fig. 4a–d).

To determine whether \( \text{Myh11} \) H3K4me2 persists during SMC phenotypic switching in vivo, we generated advanced atherosclerotic lesions by crossing our SMC-\( \text{EYFP}^{+/+} \) mice with \( \text{ApoE}^{-/-} \) mice and feeding them a Western diet for 18 weeks (Online Methods). We identified large numbers of phenotypically modulated SMCs in lesions that showed no detectible expression of \( \text{MYH11} \) (Fig. 4c) or \( \text{ACTA2} \) (Fig. 4d) and were identifiable as being of SMC origin only through detection of the SMC-specific EYFP lineage-tracing gene product. In contrast, the majority of medial SMCs were \( \text{ACTA2}^{+} \) (Fig. 4d,e). Notably, our SMC...
lineage–tracing model is conditionally activated through an ERT2-Cre system, allowing us to permanently lineage–tag mature SMCs that were expressing MYH11 during the tamoxifen treatment period (6–8 weeks of age). These cells and their progeny constitutively express EYFP, allowing us to lineage-trace these cells independently of the expression of traditional SMC markers, which can be downregulated. In contrast, any SMC derived from a non-SMC source at a later time point will not express EYFP. Results of our studies with this highly rigorous SMC lineage–tracing system showed that >95% of SMC-derived cells in atherosclerotic lesions in brachiocephalic arteries of SMC−/− mice were MYH11+ and MYH11+ SMCs on the basis of observations in vivo in the present studies and our previous studies in cultured SMCs11 (Fig. 5d).

DISCUSSION
Although several groups29,30 have used PLA to detect protein–nucleic acid interactions in vitro, our studies are the first, to our knowledge, to describe a method that permits detection of specific histone modifications at a given gene locus in single cells in fixed histological specimens. We believe this technique will have tremendous utility for investigating the role of epigenetic changes in development and disease. Indeed, there is a large body of research studies that have investigated the role of epigenetic regulation and chromatin signatures during stem cell differentiation or development of specific types of cancer31. Nevertheless, the technical limitations of ChIP assays do not allow the in vivo characterization of such epigenetic signatures except in the study of hematopoietic stem cells32 or leukemia33, in which cell sorting can be performed without extensive tissue dissociation before ChIP analyses. Thus, we believe that ISH–PLA will be a powerful tool for the investigation of epigenetic regulation in solid tumors, in which it is virtually impossible to ascertain whether a given epigenetic change is present within tumor or stromal cells. Similarly, our ISH–PLA method will have utility in investigating the role of specific histone modifications at selective gene loci of complex multicellular tissues during development. Moreover, the method’s applicability to paraformaldehyde–fixed paraffin–embedded tissue specimens, the standard protocol for archiving human biopsy specimens, will allow unprecedented studies of the role of epigenetic changes in the pathogenesis of human diseases using existing tissue banks. Finally, we believe that our ISH–PLA method may be adapted for the detection and visualization of DNA methylation or transcription factor binding on specific promoters with single-cell resolution, which will further expand our ability to investigate
transcriptional regulation of a given gene locus within its native context in complex multicellular tissues in vivo.

Our results show persistence of a cell-specific epigenetic signature when cells undergo phenotypic transitions to a less differentiated or altered phenotypic state in vivo, wherein the cell origin cannot be identified by detection of endogenous cell-selective marker genes. Evidence of appearance and maintenance of cell-specific epigenetic signature (other than the SMC lineage) have been previously described but with the limitation that results were based on studies in cultured cells under conditions that fail to recapitulate complex cell-cell, cell-matrix and other critical environmental cues that exist in vivo. In addition to having important implications for lineage tracing of cells in complex tissues, including human biopsy specimens, these observations may also have critical implications for the propagation of epigenetic mechanisms that control cell-lineage memory and cell-specific bookmarking during cell mitosis, normal development and disease progression. However, it remains to be determined whether persistence of H3K4me2 of SMC gene loci is important in the ability of SMCs to undergo reversible phenotypic switching during repair of vascular injury. That is, our studies have provided no evidence as to whether this histone modification plays a causal role in controlling SMC lineage and phenotype. Further mechanistic studies are needed in this important area but at present are not possible because of the lack of an approach to selectively remove this mark exclusively at SMC gene loci.

Our results indicate that numerous studies of atherosclerosis that relied on identification of intimal SMC as determined by detection of ACTA2 have grossly underestimated the frequency of SMCs within lesions and likely failed to detect possible transition of these cells to alternative phenotypes that may be critical in the pathogenesis of the disease. Until now, lineage tracing of lesion cells in vivo in human studies has been restricted to studying the possible role of hematopoietic cells in lesions on the basis of analyses of cross-gender bone marrow transplant specimens combined with Y chromosome detection by in situ hybridization. However, these approaches are highly limited because such samples are extremely rare, the approach does not allow rigorous

Figure 5 | Identification of epigenetic regulation of phenotypically modulated SMCs in human coronary atherosclerotic lesions by ISH-PLA. (a) Immunostaining of 5-µm-thick sections of human coronary arteries ACTA2, MYH11 and DAPI. Scale bar, 100 µm. (b) Immunostaining combined with MYH11 H3K4me2 ISH-PLA with ACTA2, PLA and DAPI (blue). Arrows in the merged images show MYH11 H3K4me2 PLA+ lesion cells that are ACTA2−. Right, higher-magnification images. Scale bars, 10 µm. (c) MYH11 H3K27me3 ISH-PLA in human coronary atherosclerotic lesions. Top, media of the coronary artery with MYH11 H3K27me3 PLA+ ACTA2+ SMCs. Bottom, lesion area showing MYH11 H3K27me3 PLA+ ACTA2− cells (white arrows) and MYH11 H3K27me3 PLA+ ACTA2− ECs (yellow arrows). Scale bars, 50 µm. (d) Cartoon summarizing the epigenetic regulation on the MYH11 promoter in mature SMCs, phenotypically modulated SMCs and non-SMCs in vivo.
lineage tracing of nonhematopoietic cells, and results are confounded by development of transplant atherosclerosis, which has a different etiology than normal atherosclerosis. We believe that ISH-PLA will greatly advance studies of human atherosclerosis by facilitating novel studies of the mechanisms and factors that regulate phenotypic transitions in SMCs in atherosclerotic lesions and their possible functional roles in plaque development, progression and end-stage events leading to clinical complications such as myocardial infarction or stroke.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. NCBI Reference Sequence: MYH11, NG_009299.1 and NM_002474.2; Myh11, NM_013607.2.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

G.K.O. supervised this study; D.G. and G.K.O. conceived of the ISH-PLA strategies, designed studies and wrote the paper; D.G. generated labeled DNA probes, performed immunostaining and all ISH-PLA experiments and analyzed data; D.G. performed in vitro experiments, CHIP and quantitative PCR; L.S.S. generated Myh11CreERT2 ROSA STOP-Flox EVFYflox/flox mice; L.S.S. and A.T.N. performed immunostaining on mouse sections; and B.G., L.S.S. and A.T.N. performed image acquisition and analysis.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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**ONLINE METHODS**

**Human tissues.** Coronary and carotid arteries specimens from atherosclerotic patients \((n = 10)\) were collected during coronary artery bypass graft (CABG) and carotid endarterectomy surgery as well as during autopsy. These specimens were processed and fixed in paraformaldehyde, and paraffin-embedded blocks were cut into 5-µm sections. Human temporal lobe sections were from autopsy cases from the University of Virginia, Department of Pathology. Brain tissue was immersion fixed in 10% zinc-buffered formalin and embedded in paraffin using standard procedures. The institutional review board at University of Virginia approved the use of all autopsy specimens.

**Mice.** Animal protocols were reviewed and approved by the University of Virginia Animal Care and Use Committee. *Myh11-CreERT2* mice were generously provided by S. Offermanns (Max Planck Institute)\(^2\); ROSA26 STOP-flox *EYFP*\(^{+/+}\) and *ApoE*\(^{-/-}\) mice were obtained from Jackson Laboratories. All mouse strains have been backcrossed to a C57BL/6J for greater than six generations. *Myh11-CreERT2* mice were genotyped by PCR as previously described\(^2\); ROSA26 STOP-flox *EYFP* mice were genotyped using the following primers: oIMR0316 5’-GGAGCCGGGAGA ATGGATATG-3’, oIMR0872 5’-AATTTGTCGACCGAC CG-3’, oIMR1416 5’-TCTTGAAGAAGATGGTGC-3’ and oIMR3621 5’-CGTGAATCGAATCGTCGTA-3’. Cre recombinase was activated with a series of ten 1-mg tamoxifen intra-peritoneal injections (Sigma-Aldrich) from 6 to 8 weeks of age for a total of 10 mg of tamoxifen per 25 g of mouse. Mice were fed a high-fat (Western-type) diet containing 21% milk fat and 0.15% cholesterol (Harlan Teklad) for 18 weeks starting at 8 weeks of age. Mice were euthanized by CO\(_2\) asphyxiation and then perfused via the left ventricle with 5 mL PBS followed by 10 mL 4% paraformaldehyde and an additional 5 mL PBS. Brachiocephalic arteries and various organs were carefully dissected and fixed for 30 min in 4% paraformaldehyde before they were embedded in paraffin.

**Cell culture and harvesting.** Mouse SMCs were cultured in growth medium (DMEM/F12, Gibco) supplemented with fetal bovine serum (10%), l-glutamine (1.6 mM, Gibco) and penicillin-streptomycin (100 U/mL, Gibco). Cells were starved for 3 d in serum-free medium and treated with 10 µg/mL POV-PC (1-((palmitoyl)-2-((5-oxovaleroyl))-sn-glycero-3-phosphatidylcholine, Cayman Chemical). Human coronary SMCs, human coronary ECs, U937 (human monocyte) and RAW264.7 (human macrophage) cell lines were purchased from Lonza and ATCC. Cells were grown in corresponding medium in the presence of serum to confluence. For ISH-PLA, SMCs and ECs were harvested and fixed in 10% phosphate-buffered formalin (PB, Fisher). After formalin fixation, cells were pelleted and suspended in 1% agarose (Sigma). Cell pellets were processed, and paraffin-embedded blocks were cut into 5-µm sections.

**Immunofluorescence staining.** Sections were de-paraffinized and rehydrated in xylene and ethanol series. After antigen retrieval (antigen retrieval solution, Vector), sections were blocked with fish skin gelatin–PBS (6 g/L) containing 10% goat or horse serum for 1 h at room temperature. Endogenous mouse IgG in mouse tissue sections was blocked by incubation with unconjugated Fab-fragment goat anti-mouse IgG (H+L) (Jackson Immunoresearch) for 1 h at room temperature. Slides were incubated with the following antibodies: mouse monoclonal SM α-actin-FITC (ACTA2) (4.4 µg/mL, clone 1A4, Sigma Aldrich), rat monoclonal SM myosin heavy chain (MYH11) (1 µg/mL, clone KM3669, Kamiya Biomedical), goat polyclonal anti-PECAM-1 (CD31) (2 µg/mL, #sc-1506, Santa Cruz), mouse monoclonal H3K4me2 (4 µg/mL, clone CMA303, Millipore) and goat polyclonal anti-GFP (4 µg/mL, ab6673, Abcam) for detection of EYFP. The secondary antibodies were donkey anti-rat conjugated to Alexa 555 (5 µg/mL, Abcam), donkey anti-goat conjugated to Alexa 647 (4 µg/mL, Invitrogen) and donkey anti-mouse conjugated to Alexa 555 (4 µg/mL, Invitrogen).

**Probe preparation and hybridization.** Human *MYH11*, mouse *Myh11*, human *CDH5*, mouse *Cdhl* and mouse *Tagln* biotin-labeled probes were generated. The proximal 2 kb of these promoters were amplified by PCR (see primers in **Supplementary Table** 1). PCR products were cloned into pcCR2.1 vector for amplification (TOPO cloning kit, Invitrogen). Probes were generated by Nick Translation (Roche) using biotin-14-dATP (Invitrogen). Nick translation efficiency was assessed by migration on 1% agarose gel. Labeled-DNA probes (40 ng per slide) undergo denaturation in hybridization buffer (2× SSC, 50% high-grade formamide, 10% dextran sulfate, 1 µg of human or mouse Cot-1 DNA) for 5 min at 80 °C. Directly after immunostaining, slides were dehydrated in ethanol series and incubated in 1 mM EDTA (pH 8.0) for 20 min. Then samples were incubated with pepsin (0.5%) in buffer (0.05 M Tris, 2 mM CaCl\(_2\), 0.01 M EDTA, 0.01 M NaCl) at 37 °C for 20 min, as previously described\(^3\). Hybridization mixture containing biotinylated probes or 5-TAMRA-dUTP-labeled Y chromosome probe (Clone RP11-88F4, Empire Genomics) was applied on sections. Sections were incubated at 80 °C for 5 min followed by 16–24 h at 37 °C. Hybridization was followed by multiple washes in 2× SSC, 0.1% NP-40 buffer.

**Proximity ligation assay.** PLA was performed directly after ISH according to the manufacturer’s instructions (Olink). After the blocking step, sections were incubated with mouse H3K4me2 (5 µg/mL, clone CMA303, Millipore), mouse H3K2me3 (5 µg/mL, ab6002, Abcam), mouse H4ac (5 µg/mL, clone 3HH4-4C10, Millipore) and rabbit biotin (5 µg/mL, ab53494, Abcam) antibodies overnight at 4 °C, followed by incubation with secondary antibodies conjugated with PLA probe at 37 °C for 1 h as recommended by the manufacturers. Then ligation and amplification were performed (Duolink detection kit Orange, 555 nm). Finally, mounting medium with 4,6-diamidino-2-phenylindole (DAPI) was used.

**Image acquisition and analysis.** Images were acquired with an Olympus BX41 fitted with a Q imaging Retiga 2000R camera. Image acquisition was performed with the Q Capture Pro software (Media Cybernetics and QImaging). Settings were fixed at the beginning of both acquisition and analysis steps and were unchanged. Brightness and contrast were lightly adjusted after merging. Image analysis was performed with ImageJ. Confocal images were acquired using a Zeiss LSM700 scanning confocal microscope with 405-nm, 488-nm, 555-nm and 637-nm solid-state lasers. Analysis of confocal images was completed using Zeiss Zen 2009 software.
**Chromatin immunoprecipitation.** ChIP was performed on cultured cells as previously described\(^1\). Cells were fixed with 1% paraformaldehyde for 10 min at room temperature. Cross-linking was stopped by addition of 125 mM glycine for 10 min. The cross-linked chromatin was sonicated to shear chromatin into fragments of 200–600 base pairs. The sheared chromatin was immunoprecipitated with 2 µg of H3K4me2 antibody (clone CMA303, Millipore), negative control was incubated with mouse IgG and input DNA without antibody, and immune complexes were recovered with magnetic beads (Millipore).

**Quantitative PCR.** Total RNA was prepared from cultured cells using Trizol (Invitrogen) according to the manufacturer’s protocol. One microgram of RNA was used for reverse transcription with iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed (iQ SYBR Green Supermix, Bio-Rad) on cDNA or DNA extracted after ChIP using primers listed in **Supplementary Table 1**.

**Statistics.** Values are expressed as mean ± s.d. For ChIP, three independent experiments were performed. Each experiment was repeated in triplicate. Comparison between groups was tested using ANOVA. A value of \( P \leq 0.05 \) was considered significant.