nature structural & molecular biology

An assessment of histone-modification antibody quality

Thea A Egelhofer[1,16], Aki Minoda[2,3,16], Sarit Klugman[4,5,16], Kyungjoon Lee[6], Paulina Kolasinska-Zwierz[7,8], Artyom A Alekseyenko[9,10], Ming-Sin Cheung[7,8], Daniel S Day[6], Sarah Gadel[11], Andrey A Gorchakov[9,10], Tingting Gu[11], Peter V Kharchenko[6], Samantha Kuan[4,5], Isabel Latorre[7,8], Daniela Linder-Basso[12], Ying Luu[4,5], Queminh Ngo[4,5], Marc Perry[13], Andreas Rechtsteiner[1], Nicole C Riddle[11], Yuri B Schwartz[12], Gregory A Shanower[12], Anne Vielle[7,8], Julie Ahringer[7,8], Sarah C R Elgin[11], Mitzi I Kuroda[9,10], Vincenzo Pirrotta[12], Bing Ren[4,5], Susan Strome[1], Peter J Park[6], Gary H Karpen[2,3], R David Hawkins[4,5] & Jason D Lieb[14,15]

We have tested the specificity and utility of more than 200 antibodies raised against 57 different histone modifications in *Drosophila melanogaster*, *Caenorhabditis elegans* and human cells. Although most antibodies performed well, more than 25% failed specificity tests by dot blot or western blot. Among specific antibodies, more than 20% failed in chromatin immunoprecipitation experiments. We advise rigorous testing of histone-modification antibodies before use, and we provide a website for posting new test results (http://compbio.med.harvard.edu/antibodies/).

This December, we celebrate the 100th anniversary of Albrecht Kossel's 1910 Nobel Prize in Physiology or Medicine, which was awarded in part for his discovery of histone proteins[1]. In 1964, soon after elucidation of the DNA-RNA-protein 'Central Dogma', came strong experimental evidence that histones are acetylated and methylated after completion of the polypeptide chain, and that these histone modifications "affect the capacity of the histones to inhibit ribonucleic acid synthesis *in vivo*"[2]. This work foreshadowed a very active period since the early 1990s, which has brought an explosion of insight regarding how DNA is packaged into chromatin, the multitude of enzymes that modify key histone residues in eukaryotic cells, and how those marks are associated with diverse functional states of chromatin[3].

Key to these recent advances has been the availability of antibodies to dozens of specific post-translational modifications on histones, coupled with the advent of chromatin immunoprecipitation (ChIP), DNA microarrays (ChIP-chip) and highly parallel DNA sequencing (ChIP-seq). This combination of antibodies and technology has enabled investigators to determine the genomic distributions of histone modifications and to connect them with biological functions[3]. However, the reproducibility and biological relevance of histone-modification landscapes depends on the specificity and performance of the antibodies, most of which are now provided commercially. The validity of results could be affected by recognition of unmodified histones, nontarget modifications and nonhistone proteins. In addition, antibodies might be highly specific, but be ineffective ChIP reagents.

Here we set out to assess the quality of histone-modification antibodies by western blot, dot blot and ChIP-chip or ChIP-seq analysis.

RESULTS

As part of our activities in the NIH modENCODE[4] and Roadmap Reference Epigenome[5] initiatives, we performed three types of characterization on 246 antibodies directed against 3 unmodified histones and 57 distinct histone modifications (**Supplementary Table 1**). We used western blot analysis to test for cross-reactivity of the antibodies with unmodified histones or with nonhistone proteins in nuclear or whole-cell extracts. We used dot blots with a panel of modified peptides to test for cross-reactivity with other modifications. We also used ChIP-chip or ChIP-seq[6] to test the ability of the antibodies to reproducibly immunoprecipitate discrete DNA regions. The results are summarized below, with the details provided in **Supplementary Table 1**.

Western blot analysis

For western blot analysis of samples from *D. melanogaster* and *C. elegans*, we electrophoresed a threefold dilution series of both total

[1]Department of Molecular, Cell and Developmental Biology, University of California Santa Cruz, Santa Cruz, California, USA. [2]Department of Molecular and Cell Biology, University of California at Berkeley, Berkeley, California, USA. [3]Department of Genome Dynamics, Lawrence Berkeley National Lab, Berkeley, California, USA. [4]Ludwig Institute for Cancer Research, La Jolla, California, USA. [5]Department of Cellular and Molecular Medicine, University of California, San Diego School of Medicine, La Jolla, California, USA. [6]Center for Biomedical Informatics, Harvard Medical School, Boston, Massachusetts, USA. [7]The Gurdon Institute, University of Cambridge, Cambridge, UK. [8]Department of Genetics, University of Cambridge, Cambridge, UK. [9]Division of Genetics, Department of Medicine, Brigham & Women's Hospital, Boston, Massachusetts, USA. [10]Department of Genetics, Harvard Medical School, Boston, Massachusetts, USA. [11]Department of Biology, Washington University in St. Louis, St. Louis, Missouri, USA. [12]Department of Molecular Biology & Biochemistry, Rutgers University, Piscataway, New Jersey, USA. [13]Ontario Institute for Cancer Research, Toronto, Ontario, Canada. [14]Department of Biology, Carolina Center for Genome Sciences, Chapel Hill, North Carolina, USA. [15]Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA. [16]These authors contributed equally to this work. Correspondence should be addressed to J.D.L. (jlieb@bio.unc.edu; for *C. elegans* and editorial issues), R.D.H. (d1hawkins@ucsd.edu; for human), G.H.K. (karpen@fruitfly.org; for *D. melanogaster*) or P.J.P. (peter_park@harvard.edu; for website).

Received 7 July; accepted 9 November; published online 5 December 2010; doi:10.1038/nsmb.1972

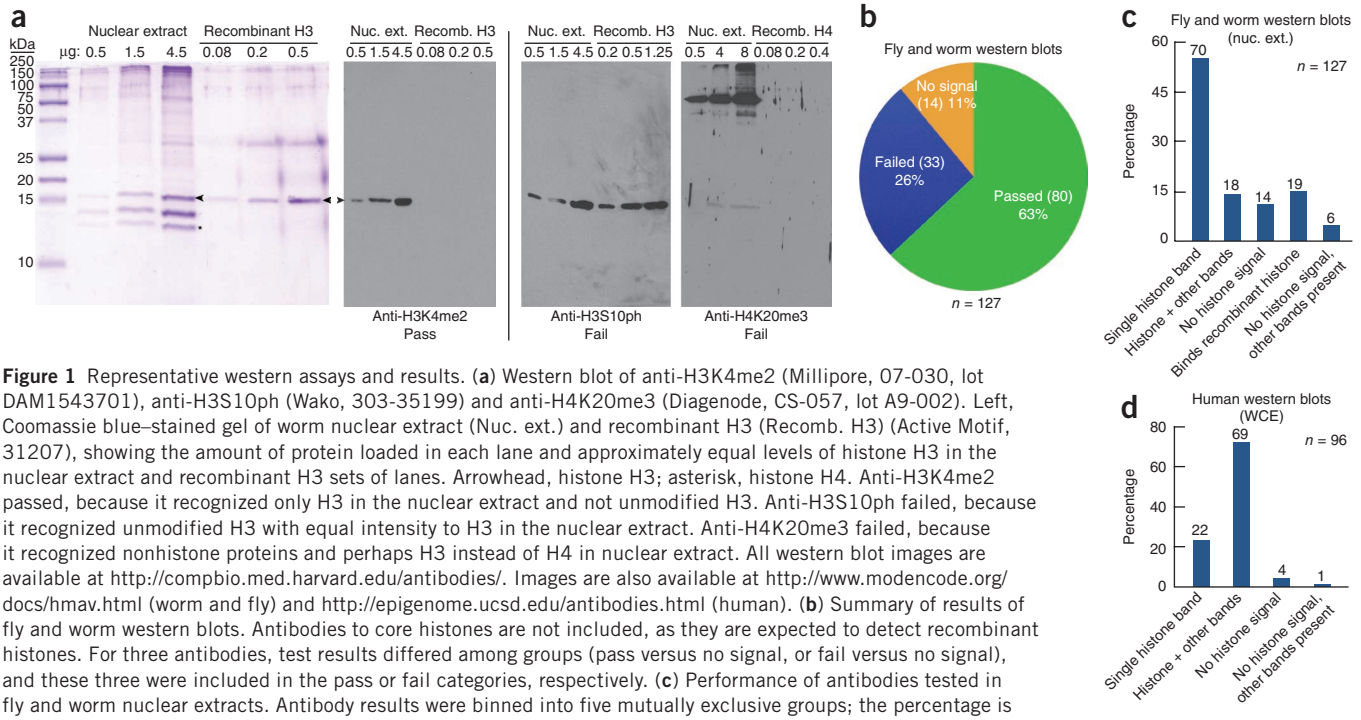


Figure 1 Representative western assays and results. (**a**) Western blot of anti-H3K4me2 (Millipore, 07-030, lot DAM1543701), anti-H3S10ph (Wako, 303-35199) and anti-H4K20me3 (Diagenode, CS-057, lot A9-002). Left, Coomassie blue–stained gel of worm nuclear extract (Nuc. ext.) and recombinant H3 (Recomb. H3) (Active Motif, 31207), showing the amount of protein loaded in each lane and approximately equal levels of histone H3 in the nuclear extract and recombinant H3 sets of lanes. Arrowhead, histone H3; asterisk, histone H4. Anti-H3K4me2 passed, because it recognized only H3 in the nuclear extract and not unmodified H3. Anti-H3S10ph failed, because it recognized unmodified H3 with equal intensity to H3 in the nuclear extract. Anti-H4K20me3 failed, because it recognized nonhistone proteins and perhaps H3 instead of H4 in nuclear extract. All western blot images are available at http://compbio.med.harvard.edu/antibodies/. Images are also available at http://www.modencode.org/docs/hmav.html (worm and fly) and http://epigenome.ucsd.edu/antibodies.html (human). (**b**) Summary of results of fly and worm western blots. Antibodies to core histones are not included, as they are expected to detect recombinant histones. For three antibodies, test results differed among groups (pass versus no signal, or fail versus no signal), and these three were included in the pass or fail categories, respectively. (**c**) Performance of antibodies tested in fly and worm nuclear extracts. Antibody results were binned into five mutually exclusive groups; the percentage is plotted, with the number of antibodies shown above each bar. The same exceptions were applied as in **b**. (**d**) Performance of antibodies tested in human whole-cell extracts (WCE). Many antibodies classified as 'Histone + other bands' passed ChIP tests.

nuclear extract from wild-type embryos and unmodified recombinant histone on an SDS polyacrylamide gel, using an amount of recombinant histone that was comparable to the corresponding histone level in the nuclear extract (**Fig. 1a**). We set the following criteria for an antibody to 'pass': the histone band constituted at least 50% of the total nuclear signal; was at least ten-fold more intense than any other single nuclear band; and was at least ten-fold more intense than recombinant, unmodified histone. By these criteria, 80 of the 127 histone-modification antibodies tested (63%) passed, whereas 33 (26%) failed, and 14 (11%) produced no signal (**Fig. 1b**,**c**). For western blots of human samples, we used whole-cell extract instead of nuclear extract. This resulted in a higher frequency of cross-reacting bands, many of which are likely to be irrelevant to assays performed on nuclear proteins. Therefore, we did not classify these as 'pass' or 'fail' but described their behavior as shown in **Figure 1d**.

Dot blot analysis

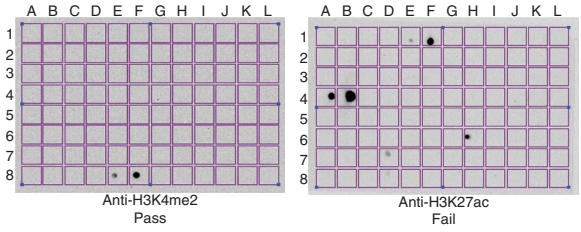
Dot blots were performed using a matrix of 43 peptides (**Fig. 2a**) or on one of the arrays of peptides shown in **Supplementary Figure 1a**,**b**. For an antibody to pass, we required that at least 75% of the total signal be specific to the cognate peptide. According to this criterion, 109 of the 149 antibodies tested (73%) passed, of which 56% (61/109) showed 100% specificity. By contrast, 20 (13%) of the antibodies produced signal on the dot blot but did not meet our specificity criterion, 16 (11%) yielded no signal and 4 (3%) had low signal (**Fig. 2b**). A particularly dangerous class of failure was defined by four antibodies (3%) that showed 100% specificity, but for the wrong peptide (**Supplementary Table 1**).

ChIP-chip and ChIP-seq analysis

We performed ChIP-chip or ChIP-seq experiments using 147 of the histone-modification antibodies, and we judged them to have passed if they achieved a correlation of more than 0.8 on any pair of ChIPs

a

	0.04 μg	0.4 μg	0.04 μg	0.4 μg	0.04 μg	0.4 μg	0.04 μg	0.4 μg	0.04 μg	0.4 μg	0.04 μg	0.4 μg
	A	B	C	D	E	F	G	H	I	J	K	L
1	H3K79me3		H3K9me3		H3K9ac		H4R3me1		H3K27me3		H3K27me2	
2	H4K20me3		H2A.Z		H4K91ac		H2AK5ac		H4K8ac		H4R3me2s	
3	H3K36me3		H3R17me2as		H3K9me2		H2AS129ph		H3K4ac		H3K4me3	
4	H3K27ac		H4K20me1		H2BK5me1		H3K79me2		H3K14ac			
5	H3K4me1		H3S10ph		H1.2		H3K36me2		H3K23ac			
6	H3K36me1		H3K9me1		H3T11ph		H3K56ac		H3K27me1			
7	H3K23me2		H3K18ac		H2BK15ac		H4K5ac		H3R17me2s			
8	H3K79me1		H2BK5ac		H3K4me2		H4K12ac		H3R2me2as			



b

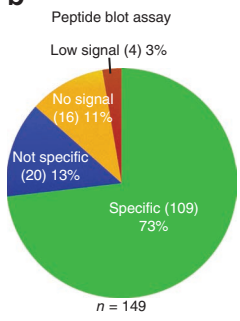


Figure 2 Representative dot blot assays and results. (**a**) Dot blot characterization of anti-H3K4me2 (Abcam, ab32356, lot 577702) and anti-H3K27ac (Abcam, ab4729, lot 726657). Top, positions of histone tail peptides spotted on membranes. Anti-H3K4me2 passed. Anti-H3K27ac failed owing to detection of multiple peptides. Human, fly and worm dot blot images are available at the websites listed in the legend to **Figure 1**. (**b**) Summary of peptide blot results. We classified 149 antibodies as described in the text. Low signal indicates that only the highest peptide concentration was detected by the antibody. See **Supplementary Figure 1** for a description of the peptide array used for each antibody and **Supplementary Table 1** for enumeration of cross-reacting peptides.

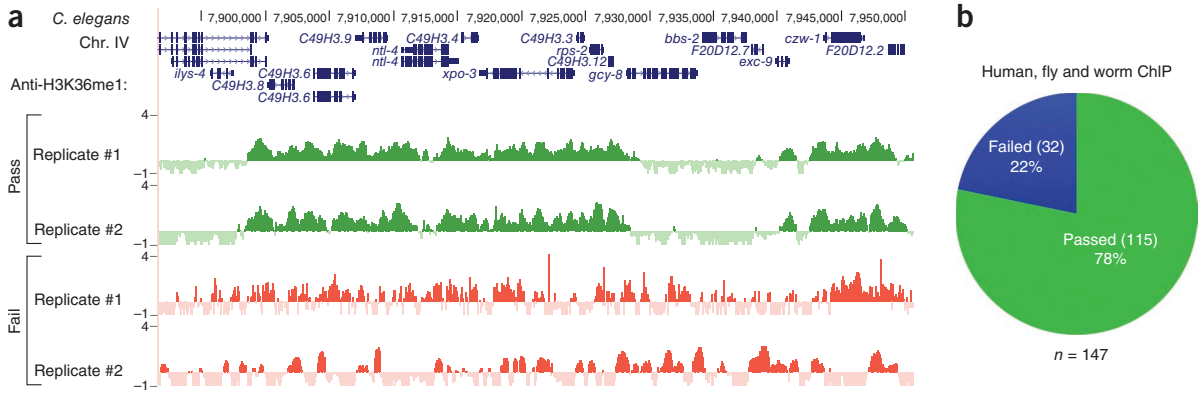


Figure 3 ChIP-chip and ChIP-seq. (**a**) Representative ChIP-chip characterization with anti-H3K36me1 from two different sources (Abcam, ab9048 lot no. 18882, and H. Kimura, 1H1). A ~60-kb region of *C. elegans* chromosome IV is shown, with annotated genes (*x* axis) and ChIP-chip *z* scores (standardized $\log_2$ ratios of ChIP/input signals; *y* axis) plotted for biological replicates using both antibodies. The replicates were highly correlated using the Abcam antibody (passed), but not using the 1H1 antibody (failed). (**b**) Summary of results. Antibodies to core histones are not included in the summary.

performed from independent preparations matched for stage, cell type or biological tissue (**Fig. 3a**). This criterion evaluates only the effectiveness of the antibody to generate reproducible ChIP results, and it does not measure whether the resulting distributions are biologically accurate. In the case of well-studied modifications, we were able to confirm that the signal conformed to previously established patterns: for example, anti-H3K4me3 (histone H3 trimethyl lysine 4) precipitating chromatin near gene promoters[3]. In all, 115 of the 147 antibodies tested by ChIP (78%) passed, and 32 (22%) failed (**Fig. 3b** and **Supplementary Table 1**). Of the failures, 23 were marketed as ChIP-grade.

DISCUSSION

Our results show that most commercially available histone-modification antibodies perform well, but that at least 25% have substantial problems of specificity or utility, suggesting that users should independently test purchased antibodies. Failure in one assay does not necessarily predict failure in another, indicating that antibodies should be tested in multiple assays regardless of initial success or failure in a given assay. Manufacturers often provide peptide blot data, but assessment of cross-reactivity with non-histone proteins is usually restricted to one species, and the data presented are often based on lots that are no longer available for purchase. Substantial lot-to-lot variation (**Supplementary Table 1**) mandates that lots be tested separately using extracts from the species under study. Development of monoclonal antibodies to histone modifications may alleviate many of these concerns[7]. The high rate of specificity problems raises concerns about the validity of ChIP data that have been generated and published without independent characterization.

To help to address issues of antibody quality in the community, we have developed an Antibody Validation Database website (http://compbio.med.harvard.edu/antibodies/) that allows researchers to post their assay results. This will provide up-to-date validation information, including tests of lot-to-lot variability. The website currently contains all histone-modification validation data described in this paper as well as data for other chromosomal proteins tested in the modENCODE project. The database can be searched by the modification or protein name, and it lists antibody details (source, catalog number, lot number and so on), links to the validation data including images, and other information such as the species and the laboratory in which testing was performed. Researchers who publish data generated using histone-modification antibodies are encouraged to upload their validation information to this site.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nsmb/.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

ACKNOWLEDGMENTS

This work was supported by US National Institutes of Health (NIH) modENCODE grants U01HG004270 to J.D.L. and U01HG004258 to G.H.K., and by the Reference Epigenome Roadmap project grant U01ES017166 to B.R. We thank H. Kimura (Kyoto University) and T. Jenuwein (Max Planck Institute of Immunobiology) for providing the noncommercial antibodies indicated in **Supplementary Table 1** at no cost.

AUTHOR CONTRIBUTIONS

J.A., A.A.A., M.-S.C., D.S.D., T.A.E., S.C.R.E., S.G., A.A.G., T.G., R.D.H., G.H.K., P.V.K., S. Klugman, P.K.-Z., S. Kuan, M.I.K., I.L., K.L., J.D.L., D.L.-B., Y.L., A.M., Q.N., P.J.P., M.P., V.P., A.R., B.R., N.C.R., Y.B.S., G.A.S., S.S. and A.V. designed, executed, and analyzed the experiments. T.A.E., R.D.H., G.H.K., J.D.L., A.M. and S.S. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/nsmb/.
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/.

1. Sourkes, T.L. *1901–1921 Nobel Lectures. Including Presentation Speeches and Laureates' Biographies* (Elsevier, Amsterdam, 1967).
2. Allfrey, V.G., Faulkner, R. & Mirsky, A.E. Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. *Proc. Natl. Acad. Sci. USA* **51**, 786–794 (1964).
3. Kouzarides, T. Chromatin modifications and their function. *Cell* **128**, 693–705 (2007).
4. Celniker, S.E. *et al.* Unlocking the secrets of the genome. *Nature* **459**, 927–930 (2009).
5. Bernstein, B.E. *et al.* The NIH Roadmap Epigenomics Mapping Consortium. *Nat. Biotechnol.* **28**, 1045–1048 (2010).
6. Park, P.J. ChIP-seq: advantages and challenges of a maturing technology. *Nat. Rev. Genet.* **10**, 669–680 (2009).
7. Kimura, H., Hayashi-Takanaka, Y., Goto, Y., Takizawa, N. & Nozaki, N. The organization of histone H3 modifications as revealed by a panel of specific monoclonal antibodies. *Cell Struct. Funct.* **33**, 61–73 (2008).

ONLINE METHODS

In addition to the websites below, all information about the antibodies is also listed at http://compbio.med.harvard.edu/antibodies/.

***C. elegans* nuclear extracts and western blotting.** *C. elegans* embryos, obtained by dissolving adult worms with bleach, were washed and dounce-homogenized 50 times using a tight pestle. Nuclei were collected by centrifugation and sonicated twice for 30 min each time using a Branson sonicator to prepare extract. Samples in sample buffer were boiled, and three-fold dilution series of both nuclear extract and recombinant histone (Active Motif) were electrophoresed on a 12.5% (w/v) SDS-polyacrylamide gel. The gel was stained with Coomassie blue to verify that approximately equal levels of recombinant histone and the corresponding histone were loaded. Samples were transferred to a nitrocellulose membrane. The membrane was blocked in nonfat milk, incubated with primary antibody, washed, incubated with secondary antibody, washed and developed with ECL (Pierce). Western blot images are available at http://www.modencode.org/docs/hmav.html.

***D. melanogaster* nuclear extracts and western blotting.** *D. melanogaster* embryo nuclear extracts were prepared[8]. Three different dilutions of nuclear extract and recombinant histone (expressed in *E. coli*) were separated on an SDS-polyacrylamide gel. Western blot analysis was performed as described above.

Human western blotting. Whole cell extracts (WCE) were made from HCT116 cells. Samples were run on precast 4–20% gels and transferred to membrane. Western blot analysis was performed as described above. Detailed protocols and images of all blots can be downloaded from the San Diego Epigenome Center website: http://epigenome.ucsd.edu/.

Dot blots (group 1). Single-modification peptides were obtained from Abcam and Active Motif. The purity of peptides was 70–95%. Peptides were spotted onto nitrocellulose membrane in the pattern shown in **Figure 2a**. The membrane was blocked in nonfat milk, incubated with primary antibody, washed, incubated with secondary antibody, washed, developed with ECL (Pierce), exposed in an imager and analyzed. Illuminated spots were encircled and quantified. Percent specificity is relative to total intensity of all illuminated modified-peptide spots normalized to background. Detailed protocols and images of all blots can be downloaded from the San Diego Epigenome Center website: http://epigenome.ucsd.edu/.

Dot blots (group 2). Slot/dot blot analysis was carried out as described[9] using nitrocellulose membrane and peptide (Diagenode) amounts from 100 to 3 pmol. Diagenode states that the purity of peptides is >70%. The intensities of the bands were analyzed by Image J, and percent specificity was calculated relative to total intensity of all spots. For peptide array, see **Supplementary Figure 1a**.

Dot blots (group 3). PVDF membranes (0.45 µm pore size) were prewashed in 100% methanol, rinsed three times in PBS and spotted with 100, 25 and 10 pmol of each peptide (Diagenode) in a 28-peptide matrix (**Supplementary Fig. 1b**). The membrane was allowed to dry, washed in 100% methanol, rinsed three times in PBS, blocked in 5% (w/v) milk for 3 h and then incubated with antibody.

***C. elegans* ChIP-chip.** ChIP-chip experiments were performed as described for early embryos[10] and L3 worms[11].

***D. melanogaster* ChIP-chip.** ChIP experiments were performed as described[12], with some changes. S2 *Drosophila* cultured cells were fixed in formaldehyde (Sigma) at a final concentration of 1.8% (v/v) for 10 min. After several washes, the cells were homogenized using a dounce homogenizer, pelleted and resuspended in cold buffer, and SDS was added to a final concentration of 1% (w/v). Cells were again pelleted, washed and finally resuspended at a final concentration of 1×10^8 nuclei per ml with 0.1% (w/v) SDS. Cells were sonicated using a Bioruptor sonicator. All lysates were combined, after which Triton-X 100 and deoxycholate were added. After centrifugation, the final supernatant contained soluble chromatin. Input chromatin was treated with RNase, followed by proteinase K, and cross-linking was reversed. The average size of the DNA fragments was 400–1,000 bp. For ChIP, chromatin was precleared by incubating with protein A–Sepharose beads. After the beads were removed, chromatin was incubated with the antibody for immunoprecipitation, and then protein A–Sepharose beads were added. After washing, sample attached to beads was treated with RNase A, followed by proteinase K, and cross-linking was reversed. Half of each ChIP sample and 50 ng of input DNA were amplified using a WGA Kit (Sigma no. WGA2). Samples were purified using a QIAquick PCR purification column (Qiagen). The amplified DNA was fragmented using RNase-free DNase I, after which the peak of bulk DNA was at 50–100 bp. The fragmented DNA library was labeled with biotin by a terminal deoxynucleotidyl transferase reaction, and hybridization cocktail was added. Genomic DNA Tiling Arrays v2.0 (Affymetrix) were prehybridized and then hybridized to ChIP sample or input DNA for 18 h, followed by washing and staining in a fluidics station EukGE-WS2v4 (Affymetrix). Enrichment *P* values were calculated using a sliding window (default size 1 kb) moved in steps across the genome (default step size 30 bp). A *P*-value enrichment score was calculated at each step using a one-sided *t*-test on the normalized log intensity ratios of probes that fell within the window. To capture both significant enrichment and significant depletion, *P* values for enrichment test (e*P*v) and depletion test (d*P*v) were calculated, and the score was given as $-\log_{10}(\min(ePv,dPv))$. The score was multiplied by −1 if d*P*v was smaller than e*P*v.

Human ChIP-chip and ChIP-seq. Procedures for ChIP-chip and ChIP-seq have been described[13]. For ChIP-chip, enrichment was defined as a >2-fold change and $P < 0.001$. For ChIP-seq, enrichment was defined as described, and biological replicates were correlated[13].

Equipment and settings. Coomassie staining and western blots (**Fig. 1a**) were scanned and then scaled and labeled using Photoshop. Dot blot images (**Fig. 2a**) were captured, processed and analyzed using the Alpha Innotec FC2 imager. The FC2 software was used to grid images and enumerate spots for dot blots.

8. Pazin, M. Preparation of nuclear extracts from *Drosophila embryos* and *in vitro* transcription analysis. in *Drosophila Protocols* (W. Sullivan, M. Ashburner & R. Hawley, eds.) 553–557 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, 2000).
9. Sambrook, J. & Russell, D. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, 2001).
10. Rechtsteiner, A. *et al.* The histone H3K36 methyltransferase MES-4 acts epigenetically to transmit the memory of germline gene expression to progeny. *PLoS Genet.* **6**, e1001091 (2010).
11. Kolasinska-Zwierz, P. *et al.* Differential chromatin marking of introns and expressed exons by H3K36me3. *Nat. Genet.* **41**, 376–381 (2009).
12. Schwartz, Y.B. *et al.* Genome-wide analysis of polycomb targets in *Drosophila melanogaster*. *Nat. Genet.* **38**, 700–705 (2006).
13. Hawkins, R.D. *et al.* Distinct epigenomic landscapes of pluripotent and lineage-committed human cells. *Cell Stem Cell* **6**, 479–491 (2010).

doi:10.1038/nsmb.1972