SUPPLEMENTAL INFORMATION

Figure S1, related to Figure 1. High-resolution MS/MS spectra of propionylmethylated peptide at lysine 57 (prQVHPDTGISSK$_{pm}$) in H2B (**A**), at lysine 77 (prDAVTYTEHAK$_{pm}$) in H4 (**B**), di-methylated peptide at lysine 79 (prEIAQDFK$_{me2}$TDLR) in H3 (**C**), formylated peptide at lysine 83 (IK$_{fo}$LGLK) in H1.1 (orH1.2 or H1.4) (**D**), formylated peptide at lysine 122 (RVTIMPK$_{fo}$DIQLAR) in H3 (**E**), acetylated peptide at lysine 122 (prVTIM$_{ox}$PK$_{ac}$DIQLAR) in H3 (**F**) and MS/MS/MS (**G**), monomethylated peptide (**H**) in H4 at arginine 55 (prISALIYEETR$_{me}$), hydroxylated peptide (**I**) in H2B at tyrosine 83 (prLAHY$_{oh}$NK$_{pr}$), at tyrosine 83 (LAHY$_{oh}$NK$_{pr}$R) (**J**) in H2B, propionylmethylated peptides at lysine 54 (prAVAASK$_{pm}$) in H1.1-H1.5 (**K**) and at lysine 50 (prK$_{pr}$AVTK$_{pm}$) in H2B (**L**). “pr” indicates a propionylated N-terminal residue or lysine residue while “pm” indicates a propionylmethylated residue. (**M**) Localization of the novel histone monomethyllysine, formyl, dimethyl and acetyllysine, monomethylarginine and hydroxyltyrosine sites in nucleosome. The 3D structure was obtained from the MMDB (MMDB id 48931) and viewed by Cn3D (v4.1). Novel sites, DNA-histone binding sites, histone-histone interaction sites and nucleosome face sites are indicated by red, yellow, orange and pink arrows, respectively.

Figure S2, related to Figure 4. (**A**) Chemical structures of vinylacetyllysine (3-butenoyllysine), methacryllysine, and cyclopropanecarboxyllysine. (**B**) Specificity of pan anti-acetyllysine antibody revealed dot-spot assay. The dot-spot assay was carried out

using pan anti-acetyllysine antibody and five peptide libraries with indicated amount. Each peptide library contains 13 residues CXXXXXKXXXXXX, where X is a mixture of 19 amino acids (excluding cysteine), C is cysteine, the 7th residue as a fixed lysine residue: unmodified lysine (K), acetyllysine (Kac), propionyllysine (Kpr), butyryllysine (Kbu), and crotonyllysine (Kcr). (**C**) Specificity of pan anti-crotonyllysine antibody revealed by Western blotting with BSA derivatives. Western blotting was carried out using pan anti-crotonyllysine antibody against 100 ng of SDS-PAGE resolved bovine serum albumin (BSA) derivatives including: unmodified BSA, vinylacetyl-K BSA, methacryl-K BSA, and crotonyl-K BSA. (**D, E**) Specificity of pan anti-Kcr antibody revealed by Western blotting and competition experiment. Western blotting analysis was carried out to detect histone crotonylation signals from purified human histones using anti-crotonyllysine pan antibody with competition of an equal amount of peptide library bearing a nonmodified lysine (K), metharcryllysine (metharcryl-K), or crotonyllysine (Kcr) (**D**), or peptide library bearing an unmodified lysine (K), acetyllysine (Kac), propionyllysine (Kpr), butyryllysine (Kbu), and crotonyllysine (Kcr) (**E**). About one μg of core histones was loaded for each line. (**F**) Kcr signals analyzed by immunofluorescenceof HeLa cells. The Kcr proteins are mainly co-located with chromatin in human HeLa cells. Kcr signal was detected by immonstaining using pan anti-Kcr antibody in nuclei in interphase cells (red solid arrow), and in chromatin structure in mitotic cells (white dash arrow) in cultured HeLa cells. (**G**) Isotopic distribution of D_4-crotonic acid. Sample was analyzed in negative mode Zoom scan in LTQ mass spectrometer. D_4-crotonic acid sample contains D_3-crotonic acid and D_2-crotonic acid. This characteristic isotopic distribution was used for D_4-crotonyllysine identification. (**H**) CBP

and p300 do not function as lysine crotonyltransferases. Overexpression of p300 (left) or CBP (right) elevated histone lysine acetylation but not crotonylation level *in vivo.* Dose-dependent amount of Flag-p300 or CBP-HA was transfected into 293T cells for 48 hrs. Core histones were isolated from the cells and subjected to Western blotting with pan anti-crotonyllysine or pan anti-acetyllysine antibody respectively. Transfected p300 or CBP were blotted using anti-Flag antibody or anti-HA antibody, respectively. The equal sample loadings were indicated by Western blotting analysis of Histone H3. (**I**) *In vitro* lysine decrotonylation activities among HDACs by fluorometric assay. *In vitro* lysine decrotonylation (left) and lysine deacetylation assays (right) were carried out among 11 HDACs.

Figure S3, related to Figure 5, 6, and 7. (**A**) Lysine crotonylation (Kcr) marks similar locations as acetylation (Kac) in IMR90 cells. Venn diagram shows that a significant portion of Kac peaks overlap with Kcr peaks identified from ChIP-seq assay. (**B**) ChIP-seq snapshot of histone Kac and Kcr in sperm cells. ChIP-seq revealed many lysine crotonylation marked histones that lack acetylation in sperm cells. “Spc_Kac” and “Spc_Kcr” designate ChIP-seq for mouse spermatocyte (Spc) using anti-Kac and anti-Kcr antibodies, respectively. “RS_Kac” and “RS_Kcr” designate ChIP-seq for mouse round spermatids (RS). (C) Peak signals around known TSS. Signals were calculated by the

sequencing depth within each 50bp window in the range of +/- 2000 bp around TSS, and were normalized between different ChIP-seq experiments. (**D**) The numbers of overlapped and non-overlapped peaks from ChIP-seq experiments in spermatocytes and round spermatids. The pan anti-Kcr or pan anti-Kac antibody was used for chromatin immunoprecipitation, whose peaks were indicated with red or blue, respectively. (**E**) Crotonylation enrichment of sex-chromosomes in round spermatids and hyper-crotonylation wave in elongating spermatids. Crotonylation was detected on paraffin mouse testis tubule sections representing different stages of spermatogenesis (as indicated in upper left corner of each panel) by immunohistochemistry. Scale bar: 20 μm. This experiment used anti-crotonyl antibody, as in Figure 6A but without cellular counterstaining, so that the crotonylation staining is easier to visualize. (**F**) Kcr and Kac signals analyzed by immunofluorescence of male germ cells. Kcr signals were detected in green, which were shown in co-staining with Kac signals in red fluorescence. Scale bar: 5 μm.

Figure S4, related to Figure 1. Annotated MS/MS spectra of crotonylated peptides identified from human HeLa histones. The MS/MS spectra were arranged from H2A, H2B,

H3, H4, and H1. The fragment ions were labeled by Mascot algorithm. Not all the ions were labeled.

Figure S5, related to Figure 1. Annotated MS/MS spectra of histone crotonylated peptides identified from HeLa cells treated with D_4-crotonate. The MS/MS spectra were arranged from H2A, H2B, H3, H4, and H1. The fragment ions were labeled by Mascot algorithm. Not all the ions were labeled.

Figure S6, related to Figure 1. Annotated MS/MS spectra of mouse histone crotonylated peptides. The MS/MS spectra were arranged from H2A, H2B, H3, H4, and H1. The fragment ions were labeled by Mascot algorithm. Not all the ions were labeled.

Figure S7, related to Figure 1. Annotated MS/MS spectra of all the modified peptides bearing a PTM other than Kcr in core and linker histones by Mascot or PTMap software. The MS/MS spectra were arranged from H2A, H2B, H3, H4, and H1. Not all the fragment ions analyzed by Mascot algorithm were labeled.

Table S1, related to Figure 1. A list of identified tryptic PTM peptides in linker histones.

Table S2, related to Figure 1. A list of identified tryptic PTM peptides in core histones.

Table S1. A list of identified tryptic PTM peptides in linker histones

No	Variant	Modification[a]	Peptide[b]	P[c]	Sequence	Observed Mass	Calculated Mass	Error (ppm)	Ref.[d]
1	H1.2;H1.4	S1ac$^{N\text{-term}}$	1-16	Y*	Ac-SETAPAAPAAAPPAEK	1519.75589	1519.75187	-2.64	(Happel and Doenecke, 2009; Wisniewski et al., 2007)
2	H1.5	S1ac$^{N\text{-term}}$	1-16	Y*	Ac-SETAPAETATPAPVEK	1639.78860	1639.79413	3.37	(Happel and Doenecke, 2009; Wisniewski et al., 2007)
3	H1.5	S17p	1-20	N*	SETAPAETATPAPVEKS(+80)PAK	2060.95926	2060.96677	3.65	(Wisniewski et al., 2007)
4	H1.2;H1.3;H1.4	K36me1	36-48	N*	K(+14)ASGPPVSELITK	1339.76646	1339.77116	3.51	(Bienvenut et al., 2008)
5	H1.2;H1.3;H1.4	K36me1	36-56	Y*	K(+70)ASGPPVSELITK(+56)AV AASK(+56)ER	2320.30237	2320.30027	-0.90	(Bienvenut et al., 2008)
6	H1.2;H1.3;H1.4	K36fo	36-48	N*	K(+28)ASGPPVSELITK	1353.74673	1353.75042	2.73	(Wisniewski et al., 2008)
7	H1.2;H1.3;H1.4	K48fo	37-54	N	ASGPPVSELITK(+28)AVAASK	1752.95882	1752.96220	1.93	(Happel and Doenecke, 2009; Wisniewski et al., 2008)
8	H1.2;H1.3;H1.4	K48fo	37-54	N*	ASGPPVSELITK(+28)AVAASK	1752.95816	1752.96220	2.31	(Happel and Doenecke, 2009; Wisniewski et al., 2008)
9	H1.5	K48ac	36-56	Y*	K(+56)ATGPPVSELITK(+42)AV AASK(+56)ER	2306.29536	2306.28461	-4.66	(Happel and Doenecke, 2009; Wisniewski et al., 2007)
10	H1.2-H1.5	**K54me1**	49-54	Y	prAVAASK(+70)	671.38060	671.38544	7.21	
11	H1.2;H1.3;H1.4	K65me1	57-65	Y	prSGVSLAALK(+70)	970.56645	970.56995	3.61	(Wisniewski et al., 2007)
12	H1.2;H1.3;H1.4	K65fo	57-66	N	SGVSLAALK(+28)K	1000.59212	1000.59173	-0.39	(Happel and Doenecke, 2009; Wisniewski et al., 2008)
13	H1.2;H1.3;H1.4	K65fo	57-66	N*	SGVSLAALK(+28)K	1000.58841	1000.59173	3.32	(Happel and Doenecke, 2009; Wisniewski et al., 2008)
14	H1.2;H1.3;H1.4	**K65ac**K66me1	57-81	Y*	SGVSLAALK(+42)K(+70)ALAAA GYDVEK(+56)NNSR	2700.45258	2700.44469	-2.92	(Happel and Doenecke, 2009; Wisniewski et al., 2007)
15	H1.1;H1.3;H1.4	K66fo	66-77	N	K(+28)ALAAAGYDVEK	1262.64596	1262.65070	3.75	(Happel and Doenecke, 2009; Wisniewski et al., 2008)
16	H1.2;H1.3;H1.4	**Y73oh**	67-77	N	ALAAAGY(+16)DVEK	1122.55514	1122.55575	0.54	
17	H1.1-H1.4	**Y73oh**	66-77	N*	KALAAAGY(+16)DVEK	1250.64761	1250.65071	2.48	
18	H1.1	**K83fo**	82-87	N	IK(+28)LGIK	698.46762	698.46910	2.12	

	H1.2-H1.5	**K83fo**	82-87	N	IK(+28)LGLK	698.46762	698.46910	2.12	
19	H1.1-H1.5	K87fo	82-92	N*	IKLGLK(+28)SLVSK	1212.77805	1212.78060	2.10	(Wisniewski et al., 2008)
20	H1.5	K83me1K87ac	82-99	Y*	IK(+70)LGLK(+42)SLVSK(+56) GTLVQTK	2080.28663	2080.28717	0.26	(Happel and Doenecke, 2009; Wisniewski et al., 2007)
21	H1.1-H1.5	**K92me1**	88-92	Y	prSLVSK(+70)	658.38951	658.39020	1.05	
22	H1.1;H1.2;H1.4; H1.5	K92fo	88-99	N	SLVSK(+28)GTLVQTK	1287.73824	1287.73985	1.25	(Happel and Doenecke, 2009; Wisniewski et al., 2007)
23	H1.1-H1.3;H1.5	K92ac	88-99	N	SLVSK(+42)GTLVQTK	1301.74930	1301.75550	4.76	(Happel and Doenecke, 2009; Wisniewski et al., 2007)
24	H1.1-H1.5	K92ac	88-99	N*	SLVSK(+42)GTLVQTK	1301.75027	1301.75550	4.02	(Happel and Doenecke, 2009; Wisniewski et al., 2007)
25	H1.1-H1.5	K99me1	93-99	Y	prGTLVQTK(+70)	871.49644	871.50154	5.85	(Lu et al., 2009)
26	H1.1-H1.5	K108me1	100-108	Y	prGTGASGSFK(+70)	936.45482	936.45531	0.52	(Lu et al., 2009; Wisniewski et al., 2007)
27	H1.4;H1.5	**K129me1**	124-129	Y	prK(+56)AGAAK(+70)	726.42265	726.42765	6.88	
28	H1.2	**K131me1**	125-131	Y	prAGGTK(+56)PK(+70)	839.46916	839.47533	7.35	
29	H1.3;H1.4	**K138me1**	132-138	Y	prK(+56)PAGAAK(+70)	823.47545	823.48041	6.02	
30	H1.2	**K150me1**	143-150	Y	prAAGGATPK(+70)	797.42106	797.42837	9.17	
31	H1.5	**K150me1**	145-150	Y	prAAGAK(+70)	542.30388	542.30647	4.78	
32	H1.2	K170me1	162-170	Y	prK(+56)PAAATVTK(+70)	1067.62413	1067.62272	-1.32	(Lu et al., 2009)
	H1.4	K170me1	162-170	Y	prK(+56)PAAAAGAK(+70)	965.55449	965.55464	0.16	(Wisniewski et al., 2007)
33	H1.3;H1.5	**K174me1**	171-174	Y	prK(+56)VAK(+70)	626.39696	626.40037	5.44	
34	H1.1;	**K190me1**	187-190	Y	prK(+56)VAK(+70)	626.39696	626.40037	5.44	
35	H1.2	**K186me1**	182-186	Y	prVAK(+56)PK(+70)	723.44914	723.45314	5.53	
36	H1.5	**K184me1**	180-184	Y	prK(+56)AAAK(+70)	669.40208	669.40619	6.14	
	H1.4;H1.5	**K226me1**	222-226	Y	prK(+56)AAAK(+70)	669.40208	669.40619	6.14	
37	H1.1;H1.3	**K196me1**	191-196	Y	prSPAKAK(+70)	726.42253	726.42764	7.03	
38	H1.1;H1.2;H1.4; H1.5	**K201me1**	197-201	Y	prAVK(+56)PK(+70)	723.44981	723.45314	4.60	
39	H1.3;H1.4;H1.5	**K206me1**	202-207	Y	prAAK(+56)PK(+70)	695.42265	695.42184	-1.16	
40	H1.1;H1.4	**K221me1**	211-215, 221	Y	prTAK(+56)PK(+70)	725.42955	725.43240	3.93	
41	H1.1;H1.2;H1.3	**K226me1**	222-226	Y	prK(+56)AAPK(+70)	695.41917	695.42184	3.84	

[a] Novel PTM sites are bolded and underlined.

[b] The modification sites in protein sequence among linker histone variants (H1.1, H1.2, H1.3, H1.4 and H1.5) were aligned by CLUSTAL 2.0.12 (details in the Extended Experimental Procedures) .

[c] Chemical derivatization states: N, non-propionylation; N*, SDS gel separation; Y, propionylation after digestion; Y*, propionylation before digestion.

[d] The modified sites were verified using the human originated database.

Table S2. A list of identified tryptic PTM peptides in core histones

No	Variant	Modification[a]	Position	P[b]	Sequence	Observed Mass	Calculated Mass	Error (ppm)	Ref.
1	H2A	K5ac	4-11	Y*	GK(+42)QGGK(+56)AR	898.49758	898.49851	1.04	(Kouzarides, 2007; Peterson and Laniel, 2004)
2	H2A	K5ac**K9me1**	4-9	Y	prGK(+42)QGGK(+70)	741.40056	741.40215	2.14	(Kouzarides, 2007; Peterson and Laniel, 2004)
3	H2A	**Y39oh**	36-42	Y*	K(+56)GNY(+16)SER	924.42812	924.43016	2.21	
4	H2A	**R42me1**	36-42	Y	prKGNYSER(+14)	922.45206	922.45089	-1.27	
5	H2A	**R42me1**	37-42	Y	prGNYSER(+14)	794.35624	794.35593	-0.39	
6	H2A	**R88me1**	82-88	Y	prHLQLAIR(+14)	919.55434	919.56038	6.57	
7	H2A	**K118me1**	100-118	Y	prVTIAQGGVLPNIQAVLLPK(+70)	2056.23416	2056.22966	-2.19	
8	H2A	K118fo	100-119	N*	VTIAQGGVLPNIQAVLLPK(+28)K	2086.24586	2086.25144	2.90	(Wisniewski et al., 2008)
9	H2A	**K125me1**	119-125	Y	prK(+56)TESHHK(+70)	1047.53366	1047.53497	1.25	
10	H2A	**K125me1**	120-125	Y	prTESHHK(+70)	863.41244	863.41378	1.55	
11	H2B	K5fo	1-11	N*	PEPAK(+28)SAPAPK	1119.59073	1119.59246	1.55	(Wisniewski et al., 2008)
12	H2B	K5ac	1-11	N	PEPAK(+42)SAPAPK	1133.60940	1133.60811	-1.14	(Peterson and Laniel, 2004)
13	H2B	K5ac	1-11	N*	PEPAK(+42)SAPAPK	1133.60538	1133.60811	2.41	(Peterson and Laniel, 2004)
14	H2B	K11acK12acK15acK16acK20ac	6-23	N*	SAPAPK(+42)K(+42)GSK(+42)K(+42)AVTK(+42)AQK	2034.14566	2034.14736	0.84	(Kim et al., 2006)
15	H2B	**K11me1**	6-11	Y	prSAPAPK(+70)	695.37968	695.38544	8.28	
16	H2B	K12acK15acK16acK20ac	12-20	N*	K(+42)GSK(+42)K(+42)AVTK(+42)AQK	1440.82608	1440.83005	2.76	(Kim et al., 2006)
17	H2B	K12acK15acK16acK20acK23ac	12-21	N*	K(+42)GSK(+42)K(+42)AVTK(+42)AQK(+42)K	1610.93033	1610.93557	3.26	(Kim et al., 2006)
18	H2B	**K15me1**	12-15	Y	prK(+56)GSK(+70)	600.34764	600.34834	1.17	
19	H2B	K16acK20ac	16-23	N	K(+42)AVTK(+42)AQK	956.56630	956.56551	-0.83	(Kim et al., 2006)
20	H2B	K16acK20ac	16-23	N*	K(+42)AVTK(+42)AQK	956.56214	956.56551	3.53	(Kim et al., 2006)
21	H2B	**K20me1**	16-20	Y	prK(+56)AVTK(+70)	727.44835	727.44805	-0.41	
22	H2B	K20acK23me1	16-23	Y	prK(+56)AVTK(+42)AQK(+70)	1096.64206	1096.64926	6.57	(abcam, 2010; Peterson and Laniel, 2004)
23	H2B	K20acK23me1	17-23	Y	prAVTK(+42)AQK(+70)	912.52272	912.52808	5.87	(abcam, 2010; Peterson and

									Laniel, 2004)
24	H2B	K34fo	34-43	N*	K(+28)ESYSVYVYK	1292.627346	1292.62891	1.21	(Wisniewski et al., 2008)
25	H2B	**Y37oh**	35-43	N	ESY(+16)SVYVYK	1152.53134	1152.53395	2.26	
26	H2B	**Y37oh**	34-43	N*	KESY(+16)SVYVYK	1280.624906	1280.62892	3.13	
27	H2B	K46fo	43-57	N*	VLK(+28)QVHPDTGISSK	1535.826316	1535.83079	2.91	(Wisniewski et al., 2008)
28	H2B	**K57me1**	47-57	Y	prQVHPDTGISSK(+70)	1293.6498	1293.65653	5.20	
29	H2B	**R79me1**	73-79	Y	prIAGEASR(+14)	772.40794	772.40797	0.04	
30	H2B	**Y83oh**	80-85	Y	prLAHY(+16)NK(+56)	872.43467	872.43928	5.28	
31	H2B	**Y83oh**	80-85	Y*	LAHY(+16)NK(+56)R	972.51212	972.51417	2.11	
32	H2B	**Y83oh**	80-85	N	LAHY(+16)NK	760.38572	760.38684	1.47	
33	H2B	**Y83oh**	80-85	N*	LAHY(+16)NK	760.387296	760.38684	-0.60	
34	H2B	**K85me1**	80-85	Y	prLAHYNK(+70)	870.45882	870.46001	1.37	
35	H2B	R99me1	93-99	Y	prEIQTAVR(+14)	885.48922	885.49203	3.17	(Mersfelder and Parthun, 2006)
36	H2B	K108fo	100-108	N	LLLPGELAK(+28)	980.58780	980.59067	2.93	(Wisniewski et al., 2008)
37	H2B	K108fo	100-116	N	LLLPGELAK(+28)HAVSEGTK	1789.99366	1789.99383	0.09	(Wisniewski et al., 2008)
38	H2B	K108fo	100-116	N*	LLLPGELAK(+28)HAVSEGTK	1789.989776	1789.99383	2.26	(Wisniewski et al., 2008)
39	H2B	**K116me1**	109-116	Y	prHAVSEGTK(+70)	953.48253	953.48186	-0.70	
40	H2B	**K116fo**	109-120	Y	prHAVSEGTK(+28)AVTK(+56)	1366.71251	1366.70929	-2.36	
41	H2B	**K116fo**	109-120	N	HAVSEGTK(+28)AVTK	1254.65572	1254.65685	0.90	
42	H2B	**K116fo**	109-120	N*	HAVSEGTK(+28)AVTK	1254.654936	1254.65685	1.53	
43	H2B	**K120fo**	117-125	N	AVTK(+28)YTSSK	1011.52158	1011.52371	2.11	
44	H3	K4me1	3-8	Y	prTK(+70)QTAR	829.46622	829.46582	-0.48	(Allis et al., 2007; Kouzarides, 2007; Peterson and Laniel, 2004)
45	H3	K4me1	3-8	Y*	TK(+70)QTAR	773.43880	773.43960	1.03	(Allis et al., 2007; Kouzarides, 2007; Peterson and Laniel, 2004)
46	H3	K9me1	9-17	Y*	K(+70)STGGK(+56)APR	1026.58260	1026.58225	-0.34	(Kouzarides, 2007; Peterson and Laniel, 2004)
47	H3	K9me1K14me1	9-14	Y	prK(+70)STGGK(+70)	772.42851	772.43313	5.98	(Kouzarides, 2007; McKittrick et al., 2004; Peterson and Laniel, 2004)
48	H3	K9me2	9-14	Y	prK(+28)STGGK(+56)	716.40751	716.40661	-1.26	(Allis et al., 2007)
49	H3	K9me1K14ac	9-17	Y	prK(+70)STGGK(+42)APR	1068.59170	1068.59281	1.04	(Kouzarides, 2007; Peterson and Laniel, 2004)
50	H3	K9acK14ac	9-17	N	prK(+42)STGGK(+42)APR	984.5353	984.5357	0.4	(Kouzarides, 2007; Peterson and

									Laniel, 2004)
50	H3	K14ac	9-17	Y*	K(+56)STGGK(+42)APR	998.54812	998.55094	2.82	(Kouzarides, 2007; Peterson and Laniel, 2004)
51	H3	K18me1	18-28	Y*	K(+70)QLATK(+56)AAR	1111.67184	1111.67140	-0.40	(Zee et al.)
52	H3	K18ac	18-23	N	K(+42)QLATK	729.43657	729.43853	2.69	(Kouzarides, 2007; Peterson and Laniel, 2004)
53	H3	K18acK23ac	18-26	Y	prK(+42)QLATK(+42)AAR	1125.64516	1125.65065	4.88	(Kouzarides, 2007; Peterson and Laniel, 2004)
54	H3	K18acK23ac	18-26	N	K(+42)QLATK(+42)AAR	1069.62160	1069.62443	2.65	(Kouzarides, 2007; Peterson and Laniel, 2004)
55	H3	K18acK23ac	18-26	N*	K(+42)QLATK(+42)AAR	1069.620386	1069.62443	3.78	(Kouzarides, 2007; Peterson and Laniel, 2004)
56	H3	K18acK23ac	18-26	Y*	K(+42)QLATK(+42)AAR	1069.62436	1069.62443	0.07	(Kouzarides, 2007; Peterson and Laniel, 2004)
57	H3	K23me1	18-23	Y	prK(+56)QLATK(+70)	869.51799	869.52228	4.93	(Kim et al., 2006)
58	H3	K23ac	18-26	N*	KQLATK(+42)AAR	1027.610986	1027.61387	2.81	(Peterson and Laniel, 2004)
59	H3	K23ac	19-26	Y	prQLATK(+42)AAR	955.54568	955.54512	-0.59	(Peterson and Laniel, 2004)
60	H3	K27me1	27-40	Y*	K(+70)SAPATGGVK(+56)K(+56)PHR	1614.92061	1614.92063	0.01	(Kouzarides, 2007)
61	H3	K27me1	27-36	N*	K(+14)SAPATGGVK	928.53207	928.53422	2.32	(Kouzarides, 2007)
62	H3	K27me1K36me1	27-36	Y	prK(+70)SAPATGGVK(+70)	1110.62130	1110.62853	6.51	(Kouzarides, 2007)
63	H3	K27me1K36me1	27-40	N*	K(+14)SAPATGGVK(+14)KPHR	1460.85513	1460.85762	1.71	(Kouzarides, 2007)
64	H3	K27me1K36me2	27-40	N*	K(+14)SAPATGGVK(+28)KPHR	1474.87015	1474.87297	1.91	(Garcia et al., 2005; Kouzarides, 2007)
65	H3	K27me2	27-36	Y	prK(+28)SAPATGGVK(+56)	1054.60338	1054.60201	-1.30	(Allis et al., 2007)
66	H3	K27me2	27-36	N*	K(+28)SAPATGGVK	942.54825	942.54957	1.40	(Allis et al., 2007)
67	H3	K27me2K36me2	27-40	N*	K(+28)SAPATGGVK(+28)KPHR	1488.88589	1488.88832	1.63	(Allis et al., 2007; Garcia et al., 2005)
68	H3	K27me3	27-36	Y	prK(+42)SAPATGGVK(+56)	1068.61386	1068.61801	3.88	(Peterson and Laniel, 2004)
69	H3	K27me3K36me1	27-36	Y	prK(+42)SAPATGGVK(+70)	1082.63186	1082.63366	1.66	(Kouzarides, 2007; Peterson and Laniel, 2004)
70	H3	K27ac	27-36	N*	K(+42)SAPATGGVK	956.52743	956.52913	1.78	(Peterson and Laniel, 2004)
71	H3	K36me1	28-36	Y	prSAPATGGVK(+70)	912.49016	912.49170	1.69	(Allis et al., 2007; Kouzarides, 2007; Peterson and Laniel, 2004)
72	H3	K36me2	28-40	N*	SAPATGGVK(+28)KPHR	1332.76102	1332.76236	1.01	(Garcia et al., 2005)
73	H3	**K56fo**	54-63	N	YQK(+28)STELLIR	1277.69332	1277.69799	3.66	
74	H3	**K56fo**	54-63	N*	YQK(+28)STELLIR	1277.69693	1277.69799	0.83	
75	H3	K56ac	54-63	N*	YQK(+42)STELLIR	1291.71402	1291.71364	-0.29	(Allis et al., 2007)

76	H3	**R63me1**	57-63	Y	prSTELLIR(+14)	900.52769	900.52808	0.43	
77	H3	K79me1	73-79	N	EIAQDFK(+14)	863.43583	863.43893	3.59	(Kouzarides, 2007; Peterson and Laniel, 2004)
78	H3	K79me1	73-83	N	EIAQDFK(+14)TDLR	1348.69668	1348.69872	1.51	(Kouzarides, 2007; Peterson and Laniel, 2004)
79	H3	K79me1	73-83	Y*	EIAQDFK(+70)TDLR	1404.71970	1404.72494	3.73	(Kouzarides, 2007; Peterson and Laniel, 2004)
80	H3	K79me2	73-83	Y	prEIAQDFK(+28)TDLR	1418.74068	1418.74029	-0.27	(Garcia et al., 2007a)
81	H3	K79fo	73-83	N	EIAQDFK(+28)TDLR	1362.67440	1362.67798	2.63	(Wisniewski et al., 2008)
82	H3	K79ac	73-83	N*	EIAQDFK(+42)TDLR	1376.69376	1376.69363	-0.09	(Garcia et al., 2007a)
83	H3	K122me1	117-122	Y	prVTIMPK(+70)	813.46477	813.46707	2.83	(Garcia et al., 2007a)
84	H3	K122fo	116-128	N	RVTIMPK(+28)DIQLAR	1567.88667	1567.88687	0.13	(Wisniewski et al., 2008)
85	H3	K122fo	118-122	N*	VTIMPK(+28)DIQLAR	1411.78445	1411.78576	0.93	(Wisniewski et al., 2008)
86	H3	**K122ac**	117-128	Y	prVTIM(+16)PK(+42)DIQLAR	1481.82094	1481.827631	4.52	
87	H3	**R128me1**	123-128	Y	prDIQLAR(+14)	784.43986	784.44435	5.72	
88	H4	K5acK8ac	4-12	N*	GK(+42)GGK(+42)GLGK	884.50534	884.50800	3.01	(Kouzarides, 2007; Peterson and Laniel, 2004; Young et al., 2009)
89	H4	K5acK8acK12ac	4-16	N*	GK(+42)GGK(+42)GLGK(+42)GGAK	1239.69009	1239.69356	2.80	(Kouzarides, 2007; Peterson and Laniel, 2004; Young et al., 2009)
90	H4	K5acK8acK12ac K16ac	4-17	N*	GK(+42)GGK(+42)GLGK(+42)GGAK(+42)R	1437.80228	1437.80523	2.05	(Kouzarides, 2007; Peterson and Laniel, 2004; Young et al., 2009)
91	H4	K5acK8ac K12acK16ac	4-17	Y	prGK(+42)GGK(+42)GLGK(+42)GGAK(+42)R	1493.82400	1493.83145	4.99	(Kouzarides, 2007; Peterson and Laniel, 2004; Young et al., 2009)
92	H4	K5acK8ac K12acK16ac	4-17	N	GK(+42)GGK(+42)GLGK(+42)GGAK(+42)R	1437.79874	1437.80523	4.51	(Kouzarides, 2007; Peterson and Laniel, 2004; Young et al., 2009)
93	H4	K8acK12acK16ac	6-17	N	GGK(+42)GLGK(+42)GGAK(+42)R	1210.67556	1210.67825	2.22	(Kouzarides, 2007; Peterson and Laniel, 2004; Young et al., 2009)
94	H4	K8acK12acK16ac	4-17	N*	GKGGK(+42)GLGK(+42)GGAK(+42)R	1395.79031	1395.79467	3.13	(Kouzarides, 2007; Peterson and Laniel, 2004; Young et al., 2009)
95	H4	K8acK12ac	4-17	Y*	GK(+56)GGK(+42)GLGK(+42)GGAK(+56)R	1465.83518	1465.83655	0.93	(Kouzarides, 2007; Peterson and Laniel, 2004; Young et al., 2009)
96	H4	K12foK16ac	9-17	N*	GLGK(+28)GGAK(+42)R	912.50961	912.51415	4.98	(Kouzarides, 2007; Wisniewski et al., 2008)
97	H4	K12ac	4-17	Y*	GK(+56)GGK(+56)GLGK(+42)GGAK(+56)R	1479.85250	1479.85221	-0.20	(Kouzarides, 2007; Peterson and Laniel, 2004; Young et al., 2009)
98	H4	K16ac	4-17	Y*	GK(+56)GGK(+56)GLGK(+56)GGAK(+42)R	1479.85404	1479.85221	-1.24	(Kouzarides, 2007; Peterson and Laniel, 2004; Young et al., 2009)
99	H4	K12acK16ac	9-17	Y	prGLGK(+42)GGAK(+42)R	982.55167	982.55602	4.43	(Kouzarides, 2007; Peterson and Laniel, 2004; Young et al., 2009)
100	H4	K12ac**K16me1**	9-16	Y	prGLGK(+42)GGAK(+70)	854.48198	854.48622	4.96	(Kouzarides, 2007; Young et al., 2009)

101	H4	K20me1	20-23	Y	prK(+70)VLR	640.42644	640.42725	1.26	(Kouzarides, 2007; Peterson and Laniel, 2004; Young et al., 2009)
102	H4	K20me1	20-35	Y*	K(+70)VLRDNIQGITK(+56)PAIR	1947.16512	1947.16298	-1.10	(Kouzarides, 2007; Peterson and Laniel, 2004; Young et al., 2009)
103	H4	K31fo	24-35	N	DNIQGITK(+28)PAIR	1352.73788	1352.74125	2.49	(Wisniewski et al., 2008)
104	H4	K31fo	24-35	N*	DNIQGITK(+28)PAIR	1352.73636	1352.74125	3.62	(Wisniewski et al., 2008)
105	H4	**R35me1**	24-35	Y	prDNIQGITK(+56)PAIR(+14)	1450.81406	1450.81443	0.26	
106	H4	**Y51oh**	46-55	N	ISGLIY(+16)EETR	1195.60446	1195.60851	3.39	
107	H4	**R55me1**	46-55	Y	prISGLIYEETR(+14)	1249.65128	1249.65546	3.34	
108	H4	K59me1	56-59	Y	prGVLK(+70)	541.34666	541.34760	1.74	(Peterson and Laniel, 2004; Zhang et al., 2003)
109	H4	K59fo	56-67	N*	GVLK(+28)VFLENVIR	1413.83450	1413.83442	-0.05	(Wisniewski et al., 2008)
110	H4	**K59me2**	56-67	Y	prGVLK(+28)VFLENVIR	1469.86571	1469.86064	-3.45	
111	H4	**R67me1**	60-67	Y	prVFLENVIR(+14)	1058.61722	1058.61248	-4.48	
112	H4	**K77me1**	68-77	Y	prDAVTYTEHAK(+70)	1259.60607	1259.60343	-2.10	
113	H4	K79fo	79-91	N*	K(+28)TVTAMDVVYALK	1465.78165	1465.78509	2.35	(Wisniewski et al., 2008)
114	H4	**Y88oh**	80-91	N	TVTAM(+16)DVVY(+16)ALK	1341.67354	1341.68506	8.59	
115	H4	K91fo	80-92	N	TVTAMDVVYALK(+28)R	1493.79160	1493.79124	-0.24	(Wisniewski et al., 2008)
116	H4	K91ac	80-92	N*	TVTAMDVVYALK(+42)R	1507.80484	1507.80689	1.36	(Yan et al., 2009)

[a] Novel PTM sites are bolded and underlined.
[b] Chemical derivatization states: N, non-propionylation; N*, SDS gel separation; Y, propionylation after digestion; Y*, propionylation before digestion.
[C] The PTM sites were verified using the human originated database

EXTENDED EXPERIMENTAL PROCEDURES

Materials

All peptides used in this study were synthesized through customer synthesis using Fmoc-Lysine (crotonyl)-OH. All chemicals of the highest purity available or analytical grade, and Flag M2 antibody were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). HA antibody was purchased from Roche Diagnostics (Indianapolis, IN). Anti-CBP and anti-p300 antibodies were purchased from Bethyl Laboratory (Montgomery, TX). The histones were extracted from *S. cerevisiae* cells, *C. elegans* cells, S2 cells, mouse embolic fibroblast (MEF) cells, human Caucasian fetal lung fibroblast (IMR90) cells, and HeLa cells using procedures previously described (Shechter et al., 2007; Tateishi et al., 2009). 4,4,4,3-D_4-crotonic acid was prepared using D_4-acetaldehyde (Cambridge Isotope Laboratories, Andover, MA) and malonic acid. The plasmids HA-CBP and Flag-p300 used in this study were described previously (Chen et al., 2007). Polyclonal pan anti-Kcr and anti-Kac were generated in house using a procedure described below.

Preparation of histones from HeLa cells

HeLa cell histones were prepared as previously described (Zhang et al., 2010). Briefly, HeLa cells were grown in DMEM culture medium supplemented with 10% fetal bovine serum. The cells were then harvested and washed twice with ice-cold PBS containing 5 mM sodium butyrate. The cells were lysed in Triton extraction buffer (TEB; PBS containing 0.5% (v/v) Triton X-100, 2 mM PMSF, and 0.02 % (w/v) NaN_3). After centrifugation, the supernatant was removed. The pellet was washed, centrifuged, and resuspended in 0.4 N H_2SO_4 overnight at 4 °C. After centrifugation, the supernatant was removed; histones in the supernatant were precipitated by the addition of 20% (v/v, final concentration) TCA to the protein solution. The suspension was incubated at -20 °C for 4

hrs. The protein precipitate was spun down, collected, and washed with acidified acetone (0.1% (v/v) HCl), followed by two washes with ice-cold acetone. After being dried at room temperature, the pellets were dissolved in water.

In-solution proteolytic digestion and chemical derivatization of histone proteins

Histone tryptic peptides were generated by three methods: (i) Generation of histone peptides without *in-vitro* lysine propionylation. The histone pellet obtained above was suspended in 50 mM ammonium bicarbonate solution (pH 8.4) and was digested using a protocol previously described (Kim et al., 2006; Luo et al., 2008). (ii) *In vitro* lysine propionylation after histone tryptic digestion. The *in vitro* chemical reaction was performed as previously described (Garcia et al., 2007b). To generate derivatized histone peptides, 3 mg histone tryptic digests obtained above were dissolved in 25 µl of 100 mM ammonium bicarbonate buffer (pH 8.0), and 600 µl of 50% propionic anhydride in methanol (v/v) was added into the solution. The pH of the solution was quickly adjusted to pH 8.0 with ammonium hydroxide. The mixture was then incubated at 51 °C for 20 min and dried in a SpeedVac. The procedure was repeated once to ensure completion of the chemical reaction. (iii) *In vitro* lysine propionylation of core histones prior to tryptic digestion. Histones were derivatized by propionylation reaction as described above, and the derivatized histones were subjected to in-solution tryptic digestion overnight.

HPLC/MS/MS analysis and protein sequence database searching

The dried peptide extracts were dissolved in 3 µl HPLC solvent A (0.1% formic acid in water, v/v). 1 µl sample was injected into a NanoLC-1D plus HPLC system (Eksigent Technologies, Dublin, CA), which was connected to a home-made capillary Jupiter C_{12} column (10 cm length x 75 µm ID, 4 µm particle size, 90 Å pore size; Phenomenex, St. Torrance, CA). Peptides were eluted with a 2-hour gradient of 2% to 80% HPLC solvent B (0.1% formic acid in acetonitrile, v/v) in solvent A at a flow rate of 200 nl/min. Peptides were then ionized and analyzed by an LTQ Orbitrap Velos mass spectrometer (ThermoFisher Scientific, San Jose, CA) using a nano-spray source. High-resolution full scan MS spectra (from *m/z* 350 – 1400) were acquired in the Orbitrap with resolution R = 60,000 at *m/z* 400 and lockmass enabled (*m/z* at 445.120025), followed by MS/MS fragmentation of the twenty most intense ions in the linear ion trap with collisionally activated dissociation (CAD) energy of 35%. The exclusion duration for the data-dependant scan was 36 seconds, and the exclusion window was set at ± 0.01% *m/z*.

The MS/MS data were analyzed by both non-restrictive sequence alignment by PTMap algorithm (Chen et al., 2009) and sequence alignment using limited, pre-specified PTMs by Mascot algorithm. The specific parameters for protein sequence database searching included lysine mono-, di- and tri-methylation, formylation and acetylation, arginine mono-methylation and di-methylation, tyrosine hydroxylation, methionine oxidation, and lysine crotonylation (K + 68.02621 Da) as variable modifications for non-propionylated histones. For histone samples generated by tryptic digestion of propionylated histones, the specific parameters included lysine propionylmethylation (+ 70.04187 Da) and lysine propionylation as variable modifications. For histone samples propionylated after trypsin digestion, N-terminal propionylation was

included as a fixed modification. Other parameters used in data analysis were: six allowed missing cleavages; mass error of 10 ppm for precursor ions, and 0.6 Da for fragment ions. Charge states of +1, +2, and +3 were considered for parent ions. If more than one spectrum was assigned to a peptide, only the spectrum with the highest Mascot or PTMap score was selected for manual analysis. All peptides identified with peptide scores of PTMap > 0.8 and Mascot > 20 were manually examined using rules described previously (Chen et al., 2005).

Verification of lysine crotonylated peptides by HPLC/MS/MS analysis

The lysine crotonylated peptide in tryptic digest of histones, its synthetic counterpart, and their mixture were injected into nano-HPLC system and analyzed by high-resolution MS and MS/MS in the Orbitrap mass spectrometer, respectively. Full MS scans were acquired with resolution R = 30,000 at *m/z* 400 with lockmass enabled (*m/z* at 445.120025), and targeted MS/MS spectra were acquired at a resolution of 7,500 at *m/z* 400.

Synthesis of bovine serum albumin (BSA) derivatives

Five mg of but-3-enonylic acid, crotonic acid, or metharylic acid was mixed with 5 mg of BSA in 4 ml of PBS buffer, followed by the addition of 25 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). The mixture was stirred at room temperature for 4 hrs to generate vinylacetyl-BSA, crotonyl-BSA, and methacryl-BSA, respectively. The unreacted EDC and other small molecules were removed from BSA derivatives by gel filtration. The modified BSAs were confirmed by SDS-PAGE.

Conjugation of crotonyllysine-immobilized agarose beads

The crotonyllysine residue was conjugated to AminoLink Plus Coupling Resin (Pierce Biotechnology, Rockford, IL) following the manufacturer's protocol. Two mL of resin were washed with PBS and then suspended in 6 mL PBS. The beads were then mixed with 2 mg of the crotonyllysine (pre-solubilized in 2 mL PBS) and then $NaCNBH_3$ (to a final concentration of 50 mM) was added. After incubation for 6 hrs at room temperature with agitation, the beads were washed by 4 mL of PBS and then blocked by 2 mL of 1.0 M Tris•HCl, pH 7.4 for 30 min at room temperature. The beads were sequentially washed with 10 mL of 1.0 M NaCl and 4 mL of PBS.

Generation of pan anti-Kac and anti-Kcr antibodies

The anti-crotonyllysine IgG was co-developed with PTM BioLab, Co., Ltd (Chicago, IL) by immunizing 10 rabbits with lysine-crotonylated BSA. The rabbits were immunized with four injections. Five batches of serums were collected from each rabbit. The serum with the highest ELSA titer was used for enriching anti-crotonyllysine antibody.

The pan anti-Kcr antibody was enriched using the crotonyllysine-conjugated agarose beads. The serums were centrifuged at 20,000 g to remove possible protein particles. About 10 mL of serum were incubated overnight with 2 mL of the crotonyllysine-conjugated agarose beads in a column. The beads were then sequentially washed with 20 mL of PBSN buffer (PBS containing 0.5% NP40), 20 mL of PBSS buffer (PBS containing 0.1% SDS), 6 mL of PBSS (PBS containing 0.8 M NaCl), and 6 mL of PBS. The bound antibodies were eluted from the beads with 0.1 M glycine (pH 3.0) and immediately neutralized with 1.0 M Tris-HCl (pH 8.5). The antibodies were dialyzed against in cold PBS overnight. Both dot-spot assay and Western blotting were performed to check quality of the antibody.

The pan anti-Kac antibodies were developed likewise using lysine-acetylated BSA as an antigen. The antibody was purified using acetyllysine-conjugated agarose using the above procedure.

Western blotting with competition with a peptide library

One µg of histone protein extracts were resolved in SDS-PAGE. Crotonylation signal was detected by pan anti-crotonyllysine antibody with competition by a peptide library bearing a non-modified, acetyl, propionyl, butyryl, methacrylyl, or crotonyl lysine.

Affinity enrichment of crotonyllysine peptides using anti-Kcr antibody

The affinity-purified anti-Kcr antibody was immobilized to protein A agarose beads (GE Healthcare Biosciences, Pittsburgh, PA) by incubation at 4 °C for 4 hrs. The supernatant was removed and the beads were washed three times with NETN buffer (50 mM Tris·HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% NP40). The histone tryptic peptides were resolubilized in NETN buffer. Affinity purification was carried out by incubating the peptides with 20 µL of anti-crotonyllysine antibody-immobilized protein A beads at 4°C overnight with gentle shaking. The beads were washed three times with NETN buffer and twice with ETN buffer (50 mM Tris·HCl pH 8.0, 100 mM NaCl, 1 mM EDTA). The bound peptides were eluted from the beads by washing three times with 50 µL of 0.1% TFA. The elutes were combined and dried in a SpeedVac.

The synthesis of *tert*-butoxycarbonyl-lysine(crotonyl)-7-amino-4-methylcoumarin (Boc-Lys(crotonyl)-AMC) and *tert*-butoxycarbonyl-lysinse(acetyl)-7-amino-4-methylcoumarin (Boc-Lys(ac)-AMC)

Boc-Lys(Crotonyl)-AMC (0.1 mmol, 40.3 mg), 4-Dimethylaminopyridine (DMAP) (0.1 mmol, 12.2 mg), crotonic acid (0.12 mmol,10.3 mg) and EDC·HCl (0.12 mmol, 23.0 mg)

were dissolved in 4 mL of tetrahydrofuran (THF). The mixture was stirred at room temperature for 5 hrs. Then the solution was evaporated and the residue was purified using flash chromatography (eluted with CH_2Cl_2/MeOH (v/v = 15/1)). A yield of about 24 mg (51%) of Boc-Lys(Crotonyl)-AMC was obtained. The reaction product was confirmed by HRMS and NMR.

Boc-Lys(ac)-AMC was synthesized likewise except that acetic acid instead of crotonic acid was used. The compound was confirmed by HRMS and NMR.

***In vitro* lysine decrotonylation and lysine deacetylation reaction determined using fluorometric assays**

Reactions were performed in a final volume of 50 µL per well in a 96-well microplate. Briefly, 1 µL of Boc-Lys(crotonyl)-AMC stocking solution (10 mM) and 200 ng HDACs (1~11) were added to the reaction buffer (25 mM Tris·HCl, 130 mM NaCl, 3.0 mM KCl, 1 mM $MgCl_2$, 0.1% PEG8000, pH=8.0) and incubated at 37 °C for 3 hrs. The reaction was then stopped by adding 25 µL of trypsin solution (25 mM Tris·HCl, 130 mM NaCl, 3.0 mM KCl, 1 mM MgCl, 30% isopropanol, 0.01 mg/mL trypsin, pH =8.0). The resulting solvent was mixed and incubated at 37 °C for another 1 hr. The fluorescence was analyzed by a fluorescence plate reader (The Wallac 1420 Workstation, IET Ltd, Vernon Hills, IL) with excitation and emission wavelengths at 355 nm and 460 nm, respectively. The lysine deacetylation was assayed likewise with Boc-Lys(ac)-AMC using the same experimental procedure.

Transient transfection

One million of 293T cells were spitted into a 6-well plate the day before transfection. HA-CBP or Flag-p300 plasmids with different dosage (0, 0.8, or 1.6 µg) were transfected into

293T cells with lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The protein whole-cell lysates were extracted using SDS buffer (20 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% mercaptethonal) 48 hours after transfection. The lysates were separated by SDS-PAGE for Western blotting.

In vivo D_4-crotonate labeling of histones

Human cervical cancer HeLa cells were grown in DMEM medium with 10% FBS and 25 mM of D_4-crotonate for 24 hours before harvesting. Histones were extracted from sodium D_4-crotonate labeled HeLa cells.

Immunofluorescence of HeLa cells using anti-Kcr antibody

One million HeLa cells were seeded on coverslips and maintained in DMEM medium supplemented with 10% FBS overnight prior to experiment. Cells were pre-washed with ice-cold PBS three times and fixed in 3.7 % paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in PBS for 10 min at room temperature. After brief rinse with PBS three times, cells were permeablized with 0.5 % Triton X-100 in PBS for 5 min on ice followed by PBS rinse again. Permeablized cells were then blocked with 5% BSA in PBS for 30 min at room temperature. After blocking, cells were incubated with pan anti-crotonyllysine antibodies with final concentration 1 μg/mL at 4 °C overnight, followed by incubation with FITC-goat anti-rabbit IgG (Invitrogen, Camarillo, CA) for 20 min at 37 °C. Finally, cells were counterstained with DAPI and mounted to glass slides with Vectashield mounting medium (Vector Laboratries, Inc., Burlingame, CA). Microscopic images were captured by Olympus DP72 (Tokyo, Japan).

Immunofluorescence of germ cells using anti-Kcr antibody

Tubules of mice were prepared as previous reported (Kotaja et al., 2004). Cells were

fixed in 90% ethanol for 10 min at room temperature. They were permeabilized with 0.2% Triton X-100 and 0.5% saponine in PBS for 15 min at room temperature and blocked with 5% milk in PBS. They were incubated with pan anti-crotonyllysine 1/250, pan anti-acetyllysine 1/50, anti-HP1γ (Euromedex, France) 1/500 antibodies with milk 1% in PBS at 4 °C overnight, followed by incubation with Alexa 488 or Alexa 546 conjugated secondary antibodies for 30 min at 37 °C. Finally, cells were counterstained with DAPI and mounted onto glass slides with Vectashield. Microscopic images were captured.

Immunohistochemistry of testis tubule cross-sections using anti-Kcr antibody

Immunohistochemistry was performed on testis paraffin sections similarly as previously described by our group (the Khochbin's laboratory) (Hazzouri et al., 2000). Briefly, Bouin's fixed, paraffin-embedded testicular sections (10 µm) were deparaffinized using toluene and hydrated through graded series of ethanol (100%, 90%, 70%; 3 X3 min each). The sections were then incubated in sodium citrate (10 mM, pH 6.0) at 100 °C for 20 min and then at room temperature for 20 min for antigen retrieval. They were then blocked by incubation with PBS containing 5% milk for 30 min. Immunostaining was performed by three sequential experimental steps: incubation with pan anti-Kcr antibody (diluted at 1/250) in TBS containing 0.5% milk for 2 hours, washes using TBS three times, and then incubation with a biotinylated secondary antibody (diluted 1/2000 in PBS containing 0.5% milk) for 30 min. After the slides were then washed with TBS, the final detection was performed using the ABC Elite kit Vectastain and the DAB peroxidase Substrate kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. The slides were then washed and stained with PAS (Periodic Acid Schiff; Sigma-Aldrich) and hematoxylin eosin, dehydrated and mounted in Eukitt (Sigma-

Aldrich). The testis tubules sections were observed with a transmission light microscope and staged according to the criteria previously described (Russell et al., 1990).

Mouse spermatogenic cell fractionation

Spermatogenic cell fractionation was carried out by sedimentation of total germ cells on a BSA gradient as previously described (Pivot-Pajot et al., 2003). Cell fractions were controlled by visual inspections for the enrichment assessment. Each sedimentation experiment used 4 testes and the respective fractions corresponding to spermatocytes and round spermatids from the 10 fractionation experiments were pooled for subsequent experiments. The enrichments in spermatocytes and round spermatids obtained in the corresponding fractions were 79% and 84%, respectively (data not shown).

Chromatin immunoprecipitation in fractionated germ cells

ChIP-seq for histone crotonylation was carried out as previously described with 100 µg of fractionated germ cells chromatin and 5 µg pan anti-crotonyllysine antibody or 5 µg pan anti-acetyllysine (Hawkins et al., 2010), except that chromatin was prepared by MNAse digestion (Delaval et al., 2007). Digestion was performed to obtain chromatin fragments of approximately 200 bp. A chromatin input sample was also sequenced as negative control.

Expression analyses

The .CEL files with the raw transcriptomic data corresponding to various mouse tissues and male germinal cells were downloaded the GEO website (http://www.ncbi.nlm.nih.gov/geo/, data from studies GSE10744, GSE21749 and GSE 4193) and analyzed with the GeneSpring GX software (version 11, Agilent Technologies)

using the RMA algorithm for summarization and a quantile normalization. The heatmaps were obtained by representation of the normalized values of expression of these genes in the indicated color scale (green: low expression; red: high expression) using the Permutmatrix software (version 1.9.3).

Multiple sequence alignment for linker histones (H1.1, H1.2, H1.3, H1.4 and H1.5) by CLUSTAL 2.0.12

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gi|4885373|ref|NP_005316.1|       SETVPPAPAASAAPEKPLAGKKAKKPAKAAAASKKKPAGPSVSELIVQAA 50
gi|197692471|dbj|BAG70199.1|      SETAPAAPAAAPPAEKAPVKKKAAKKAG-GTP--RKASGPPVSELITKAV 47
gi|4885377|ref|NP_005311.1|       SETAPLAPTIPAPAEKTPVKKK-AKKAG-ATAGKRKASGPPVSELITKAV 48
gi|4885379|ref|NP_005312.1|       SETAPAAPAAPAPAEKTPVKKKARKS---AGAAKRKASGPPVSELITKAV 47
gi|4885381|ref|NP_005313.1|       SETAPAETATPAPVEKSPAKKKATKKAAGAGAAKRKATGPPVSELITKAV 50
                                  ***.*  .: ... **. . **  *    . .  :*.:**.*****.:*.

gi|4885373|ref|NP_005316.1|       SSSKERGGVSLAALKKALAAAGYDVEKNNSRIKLGIKSLVSKGTLVQTKG 100
gi|197692471|dbj|BAG70199.1|      AASKERSGVSLAALKKALAAAGYDVEKNNSRIKLGLKSLVSKGTLVQTKG 97
gi|4885377|ref|NP_005311.1|       AASKERSGVSLAALKKALAAAGYDVEKNNSRIKLGLKSLVSKGTLVQTKG 98
gi|4885379|ref|NP_005312.1|       AASKERSGVSLAALKKALAAAGYDVEKNNSRIKLGLKSLVSKGTLVQTKG 97
gi|4885381|ref|NP_005313.1|       AASKERNGLSLAALKKALAAGGYDVEKNNSRIKLGLKSLVSKGTLVQTKG 100
                                  ::****.*:***********.**************:***************

gi|4885373|ref|NP_005316.1|       TGASGSFKLNKKASSVETKPGASKV--ATKTKATGASKKLKKATGAS--K 146
gi|197692471|dbj|BAG70199.1|      TGASGSFKLNKKAASGEAKPKVKKAGGTKPKKPVGAAKKPKKAAGGATPK 147
gi|4885377|ref|NP_005311.1|       TGASGSFKLNKKAASGEGKPKAKKAGAAKPRKPAGAAKKPKKVAGAATPK 148
gi|4885379|ref|NP_005312.1|       TGASGSFKLNKKAASGEAKPKAKKAGAAKAKKPAGAAKKPKKATGAATPK 147
gi|4885381|ref|NP_005313.1|       TGASGSFKLNKKAASGEAKPKAKKAGAAKAKKPAGAT--PKKAKKAAGAK 148
                                  **************:* * ** ..*.  :.  *..**:   **.  .:  *

gi|4885373|ref|NP_005316.1|       KSVK-TPKKAKKPAATRK---SSKNPKKPK-TVKPKKVAKSPAKAKAVKP 191
gi|197692471|dbj|BAG70199.1|      KSAKKTPKKAKKPAAATVTKKVAKSPKKAK-VAKPKKAAKS--AAKAVKP 194
gi|4885377|ref|NP_005311.1|       KSIKKTPKKVKKPATAAGTKKVAKSAKKVK-TPQPKKAAKSPAKAKAPKP 197
gi|4885379|ref|NP_005312.1|       KSAKKTPKKAKKPAAAAG-AKKAKSPKKAK-AAKPKKAPKSPAKAKAVKP 195
gi|4885381|ref|NP_005313.1|       KAVKKTPKKAKKPAAAGV-KKVAKSPKKAKAAAKPKKATKSPAKPKAVKP 197
                                  *: * ****.****::      :*..** * . :***..**   .** **

gi|4885373|ref|NP_005316.1|       KAAKARVTKPKTAKP-----KKAAPKKK 214
gi|197692471|dbj|BAG70199.1|      KAAKP-----KVVKP-----KKAAPKKK 212
gi|4885377|ref|NP_005311.1|       KAAKPKSGKPKVTKA-----KKAAPKKK 220
gi|4885379|ref|NP_005312.1|       KAAKPKTAKPKAAKP-----KKAAAKKK 218
gi|4885381|ref|NP_005313.1|       KAAKPKAAKPKAAKPKAAKAKKAAAKKK 225
                                  ****.     *..*.     ****.***
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Identification of mono-, di-, and trimethylated lysine residues

Lysine monomethylated peptides were identified by a mass shift of 14.01565 Da in non-propionylated samples and by a mass shift of 70.04187 Da in propionylated samples,

the latter being generated by *in vitro* propionylation of the monomethylated lysine residue (Garcia et al., 2007b). To our knowledge, 10 and 21 monomethylation sites have been previously reported in linker histones and core histones, respectively (Allis et al., 2007; Kouzarides, 2007; Lu et al., 2009; Peterson and Laniel, 2004; Wisniewski et al., 2007). Here, we identified 22 unique lysine monomethylation sites in linker histones, among which 15 sites were identified for the first time, including 2 sites in globular domains and 13 sites in C-terminal domains (Table S1).

In the core histones, we found 3 lysine monomethylation sites in H2A, 7 in H2B, 8 in H3, and 4 in H4 (Table S2). In H2A, 3 new sites are located at K9 in N-terminal tail and K118 and K125 in the core body (Figure 1D). The H2AK9 can be acetylated or biotinylated, while H2AK125 biotinylated (Chew et al., 2006), suggesting the possible interplay among the three PTMs. In H2B, 2 novel lysine monomethylation sites were mapped in the N-terminal tail and 4 novel lysine monomethylation sites in the core domain (Figure 1D). Among the 6 H2B new monomethylation sites, K15me and K20me are located at N-terminal sites, which were also reported to be sites of acetylation and have known roles in transcriptional activation (Craig L Petersona, 2004; Freitas et al., 2004). We identified 8 lysine monomethylation sites in H3 and 4 lysine monomethylation sites were found in H4, of which H4K16 and K77 had not been previously reported (Figure 1D).

The mass shift induced by lysine dimethylation is 28.03130 Da and that by trimethylation is 42.04695 Da. High-resolution MS enables to distinguish dimethylation from formyllysine (mass shift = 27.99491 Da) and trimethylation from acetyllysine (mass shift = 42.01056 Da). We identified 5 dimethyllysine sites and 1 trimethyllysine site in this study, among which H4K59me2 is novel site (Figure 1D).

Identification of N^{ε}-formylated and acetylated lysine residues

N^{ε}-formylation at lysine residues has been described in the past (Jiang et al., 2007; Wisniewski et al., 2008). In this study, we identified 21 lysine formylation sites, including 6 sites in linker histones and 15 in core histones. Among these sites, 4 are new sites, which are H1.2K83 in linker histone and H2BK116, H2BK120 and H3K56 in core histones (Figure 1D). Among the 3 novel sites in core histones, 2 are known to be acetylated or ubiqutinylated (H2BK116 and H2BK120) and 1 is known to be acetylated or methylated (H3K56), which play a role in DNA damage repair (Freitas et al., 2004; Kouzarides, 2007; Mersfelder and Parthun, 2006).

We identified 5 and 19 lysine acetylation sites in linker and core histones, respectively (Tables S1 and S2). Among these 24 lysine acetylation sites, 2 were novel (H1.2K65ac and K122ac) (Figure 1D).

Identification of monomethylated arginine residues

Because the guanidine group of the arginine residue cannot react as easily with propionic anhydride as does the ε-amino group of lysine, monomethylation of arginine can easily be detected by a mass shift of 14.01565 Da. In this study, 8 novel arginine monomethylation sites (H2AR42me, H2AR88me, H2BR79me, H3R63me, H3R128me, H4R35me, H4R55me, and H4R67me) were mapped in core histones (Figure 1D and Table S2).

Identification of hydroxylated tyrosine residues

Oxidation of a tyrosine residue to the protein-bound 3,4-dihydroxyphenylalanine residue (PB-DOPA, 3-hydroxytyrosine) can be caused either by an enzyme-catalyzed reaction mediated by tyrosinase or tyrosine oxidase, or by radical reaction (Nelson et al., 2007). A mass shift of 15.99492 Da at tyrosine residues can be assigned to tyrosine oxidation

using high-resolution MS (Tables S1 and S2). We found 1 and 5 novel hydroxyltyrosine site in linker and core histones, respectively (Figure 1D). To our knowledge, this is the first study demonstrating that tyrosine can be hydroxylated in histones.

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