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Detection and characterization of ubiquitylated H2B in mammalian cells

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Abstract

Histone H2B ubiquitylation was shown to be associated with actively transcribed genes in mammalian cells and has been suggested to be involved in transcriptional regulation. Despite the limited applicability of genetic tools to analyze H2B ubiquitylation in mammals, several biochemical and immunological approaches have been successfully implemented to study this modification. Here we describe several techniques to detect ubiquitylated H2B in mammalian cells and to dissect its genomic localization.

Keywords

Ubiquitylation; H2B; chromatin; hBre1; RNF20

1. Introduction

While the identification of histone H2B ubiquitylation in mammals [1] preceded the identification of histone ubiquitylation in yeast [2] by almost two decades, our understanding of mammalian H2B ubiquitylation has evolved much less rapidly. This is partly due to the fact that mammals have numerous copies of histone genes [3] versus a single copy in yeast. Thus, in yeast, mutating the ubiquitylation site of the single H2B gene can yield important insights about ubiquitylated H2B, such as its distribution throughout the genome and its effects on gene expression (for an elaborate description of analysis of H2B ubiquitylation in yeast see Trujillo et al. and Chandrasekharan et al. in this issue of “Methods”). In mammals, the site-specific mutation of numerous H2B genes (e.g. at least 17 in humans, ref. 3) cannot be implemented. However, other methods can be successfully utilized to study H2Bub in mammalian cells as described below.

In spite of lacking genetic tools comparable to those available for yeast studies, valuable information has accumulated about H2B ubiquitylation in mammalian cells over the years. The ubiquitylation site was determined to be lysine 120 of human H2B [4] and the enzymes responsible for this ubiquitylation were identified as Rad6 (the H2B E2, ref. 5) and hBre1/RNF20 (the H2B E3, ref. 6). H2B deubiquitylating enzymes were described as well (e.g. ref.

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7). However, an association of H2Bub with specific genomic loci could not be demonstrated for many years. More recently, the generation of specific anti-H2Bub antibodies paved the way to demonstrating H2Bub association with the transcribed regions of transcriptionally active genes in mammalian cells [8]. Notably, while H2Bub seems to be associated with a broad spectrum of genes, its depletion (by siRNAs to hBre1) affects the expression of only a subset of genes [9], similarly to what has been found in yeast [10]. Importantly, studies of mammalian chromatin templates *in vitro*, as well as *in vivo* studies, demonstrated transcription-dependent H2B ubiquitylation and the dependency of H3K4 and H3K79 methylation on H2B ubiquitylation [5].

In this chapter we describe methods for the detection of H2Bub in mammalian cells and for the characterization of its genomic localization.

2. Production of H2Bub-specific monoclonal antibodies

To date, H2Bub is the only reported ubiquitin-protein conjugate to which monoclonal antibodies were intentionally generated. It would thus be of interest to detail the procedure of generating these antibodies. While the immunization and hybridoma generation procedures follow similar lines to reported protocols, there may be additional considerations involved in the choice of immunogen and the hybridoma screening steps as detailed below. Therefore, in the sections concerning immunization and hybridoma generation, we focused on particular issues relevant to the production of H2Bub-specific antibodies. The detailed protocol used for generation of mouse monoclonal antibodies is as previously described [11].

2.1 Immunization

2.1.1 Choice of immunogen—Antibodies prepared by immunization with denatured peptides or proteins may not react with native proteins. However, in the case of H2Bub, using the full length native ubiquitylated H2B for immunization will likely shift the immune response towards private epitopes of either ubiquitin or H2B, located far from the ubiquitylation site. We therefore chose to use a short branched peptide comprising sequences of both mammalian H2B and mammalian ubiquitin together, derived from the vicinity of the ubiquitin conjugation site on human H2B [8] (Figure 1). We chose the peptide arm length around the conjugated lysine to be 4 amino acids, theoretically allowing the complementarity determining regions of an antibody to recognize up to 9 amino acid residues (5 residues of H2B, including the conjugated lysine itself, and 4 last residues of ubiquitin). We did not check additional peptides with different arm lengths, though they may constitute successful immunogens as well (e.g. [12]). A cysteine was added to the N-terminus of the H2B sequence to facilitate conjugation to KLH (keyhole limpet hemocyanin, see section 2.1.4).

2.1.2. Synthesis of immunogen—Solid-phase peptide synthesis was used throughout chain assembly of the peptide [13] using an automated solid-phase multiple peptide synthesizer (AMS422; Abimed Analyzer-Technik GmbH) and following the commercial protocols of the company.

The amino side-chain of the lysine building block was masked by a protecting group which is capable of selective removal under mild conditions, while leaving other protecting groups intact. Following removal of the protective group of the amino side chain of the conjugated lysine, the sequence derived from the ubiquitin sequence (LRGG) was assembled using the same synthesis protocol. The fully synthesized branched peptide was removed from the polymeric carrier, along with the protecting groups, by acidolysis with trifluoroacetic acid.

The crude peptide was purified by preparative HPLC and analyzed by mass spectrometry, amino acid analysis and analytical HPLC.

2.1.3 Choice of animal species—While we discuss here the generation of mouse monoclonal antibodies against mammalian H2Bub, it is important to note that polyclonal antibodies against H2Bub or other ubiquitin conjugates may also be generated in a variety of mammalian hosts. Indeed, rabbit polyclonal antibodies targeting yeast H2Bub were recently described [14].

2.1.4 Immunization procedure

1. Peptide should first be conjugated to keyhole limpet hemocyanin (KLH) and should then be prepared in complete Freund's adjuvant (CFA) according to standard procedures [11], to increase the immunogenicity of the peptide.
2. Four three-month old BALB/c female mice were immunized. Mice should be injected subcutaneously with 50–100 μ g of the conjugated preparations in CFA (approximately 50–100 μ L).
3. Repeat injections as indicated in (2) several times at intervals of two weeks. Immune reaction should be monitored by bleeding and analysis of serum reactivity (see section 2.4.1 below).
4. Three weeks following the last injection as in (3), mice should receive two injections on two consecutive days.

2.2 Fusion of mouse cells with Polyethylene Glycol

1. Spleens are removed three days after the last injection and spleen lymphocytes are collected. NS0/1 myeloma cells [15] in logarithmic growth phase are used as fusion partner.
2. 100 10^6 cells from each individual spleen are mixed with 20 10^6 NS0/1 myeloma cells, centrifuged, and resuspended for 1 minute in 2ml of 37 C 41% Polyethylene Glycol (PEG 1500, Serva), followed by one minute incubation at 37 C.
3. Slowly and carefully wash fused cells with serum free Dulbecco's Modified Eagle's Medium (DMEM, Gibco, catalog number 11995) and resuspend in DMEM-HAT (DMEM supplemented with 1mM pyruvate, 2mM glutamine, 10U/ml penicillin, 20ug/ml streptomycin, 15% heat inactivated horse serum, 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine).
4. Following fusion, distribute cells into 96-well microtiter plates at a concentration of 2×10^4 viable myeloma cells per well and maintain in a humidified incubator with 8% CO₂ in air.

2.3 Hybridoma maintenance and cloning

1. Medium should be replaced at least once a week after onset of fusion.
2. 10–14 days after fusion DMEM-HAT medium is replaced by DMEM-HT medium (same as DMEM-HAT but without the aminopterin), and 10 days later by regular serum-containing DMEM.
3. When confluent, hybridomas can be transferred to 24 well plates.
4. We used cloning under conditions of limiting dilutions rather than soft agar cloning, by diluting the hybrid cells to less than one cell/well. Cloning was repeated for a few cycles to ensure secretion of the monoclonal antibody.

2.4 Screening of immune reactivity

2.4.1 Screening of mouse serum reactivity—Following repeated immunization, mice were bled and serum was used to probe individual strips of SDS-PAGE-separated U2OS whole cell lysates (prepared by the method described in section 3.1.2) blotted onto a nitrocellulose membrane. As H2Bub is ubiquitous in mammalian cells, other mammalian cell lines can be used as well (e.g. HEK293, H1299). While other methods were considered for screening (e.g. ELISA), it is noteworthy that a part of the immune response was directed against H2B (and to a lesser degree against ubiquitin), and thus it was necessary to resolve the lysate proteins by SDS-PAGE according to their size. Use of polyclonal serum from mice showing strong H2Bub-specific immune response showed a preference for H2Bub (~24kDa) compared to H2B (~17kDa) in Western blots. Specifically, the 1:100–2:100 ratio between H2Bub and H2B signals commonly seen in western blots of mammalian cell extracts was shifted in favor of H2Bub to a ratio of 10:100–20:100 or more. A standard can be generated by probing a strip with anti-H2B antibodies and comparing the resulting H2Bub/H2B signal ratio to that observed when using the mouse serum for probing.

2.4.2 Screening of hybridoma clones—Hybridoma supernatants can be collected for analysis as early as 10–14 days after fusion, as soon as vigorous growth and change of medium color are observed. Hybridoma supernatants should be used to probe mammalian cell extracts as described for mouse serum above.

Positive clones should show reactivity with a 24kDa band but not with a 17kDa band or other bands. As potentially hundreds of clones should be screened, one can use a miniblotted (e.g. http://www.bio-world.com/productinfo/2_26_192/125218/Miniblotted-Channels.html) enabling the performance of multiple antibody incubations on a single membrane. Specificity of positive clones can be further validated by comparing in western blots the reactivity of their supernatants with untreated whole cell extracts to their reactivity with whole cell extracts depleted of H2Bub (e.g. extracts from cells treated with proteasome inhibitors or knocked down for hBre1/RNF20). Once an H2Bub-specific antibody is identified, it can be tested in additional applications like immunofluorescence, chromatin immunoprecipitation etc.

3. Detection of H2B ubiquitylation by western blot

The following is a standard protocol suitable for a wide range of human and mouse cell lines. It allows the detection of either endogenous H2Bub, using the H2Bub specific antibodies described above, or ubiquitylated transfected H2B.

3.1 Detecting H2B ubiquitylation in whole cell extracts using H2Bub specific antibodies

This simple protocol allows the detection of global H2Bub levels by western blot. The first step of protein extraction can be performed either in NP-40 buffer, as described in 3.1.1, or directly in protein sample buffer (PSB), described in 3.1.2. The main advantage of protein extraction in NP-40 buffer is that it allows quantification of total protein content in each sample using the Bradford assay or an equivalent assay, thus ensuring equal loading in each lane. The advantages of direct extraction of proteins in PSB are that it is quicker and simpler, and reduces the chance of deubiquitylation during the incubation in NP-40 buffer.

3.1.1 Protein extraction and quantification in NP-40 buffer

1. Collect approximately 10^6 cells by centrifugation in a table-top centrifuge at 1500rpm for 3 minutes.
2. Wash cells once in PBS.

3. Suspend pellet in 100µl of NP-40 buffer in a 1.5ml eppendorf tube (50mM Tris pH 8.0, 150 mM NaCl, 1.0% NP40, and protease inhibitors).
4. Incubate ~1 hour at 4°C. Alternatively, samples can be sonicated (Bioruptor) for 3 consecutive cycles of 30 seconds on and 1 minute off.
5. Centrifuge in a 1.5ml eppendorf microfuge for 10 minutes at 4°C, maximal speed.
6. Analyze the sample (supernatant) for total protein content via a Bradford assay or BCA protein assay kit (PIERCE, #23227) according to the manufacturer's protocol. It is recommended not to discard the pellet at this stage, since it may contain residual histones that were not properly released into the supernatant.
7. Add appropriate amount of X4.5 protein sample buffer to the 1.5ml eppendorf tube (30% Glycerol, 15% 2-mercaptoethanol, 10% SDS, 180mM Tris pH 6.8, 0.05% bromophenol blue). If cells were not sonicated, it is crucial to perform at least two rounds of the following: (1) extensive vortexing, (2) 5 minutes boiling of samples, in order to ensure release of histones from the chromatin.
8. Centrifuge in a 1.5ml eppendorf microfuge for 10 minutes at maximal speed.

3.1.2 Direct protein extraction in protein sample buffer

1. Collect approximately 10^6 cells by centrifugation in a table-top centrifuge at 1500rpm for 3 minutes.
2. Wash cells once in PBS.
3. Resuspend cells in 150µl of X1.5 protein sample buffer in a 1.5ml eppendorf tube (10% Glycerol, 5% 2-mercaptoethanol, 3% SDS, 60mM Tris pH 6.8, 0.02% bromophenol blue).
4. Perform at least two rounds of the following: (1) extensive vortexing, (2) 5 minutes boiling of samples.
5. Centrifuge for 10 minutes at maximal speed in a 1.5ml eppendorf microfuge.

3.1.3 Western blot analysis of protein extracts

1. Load 15–30µg onto an SDS polyacrylamide gel. As histones are small acidic proteins, typically 15% PAGE gels are used.
2. Following electrophoresis, transfer proteins onto a nitrocellulose membrane in Tris-glycine buffer. It is recommended to perform a transfer of 1–1.5 hours.
3. Block the membrane in PBS-Tween (PBS + 0.02% Tween-20) with 3–5% milk for 30–60 minutes.
4. Incubate the membrane with the H2Bub specific monoclonal antibodies, in PBS-Tween with 3% BSA overnight at 4°C with constant rotation. Alternatively, it is possible to perform incubation at room temperature for ~2 hours; however, overnight incubation produces a stronger signal. The recommended antibody concentration is approximately 1µg/ml, but this depends on cell type, treatments and amount of extract loaded, and therefore should be calibrated. At present, suppliers of H2Bub antibodies include Medimabs and Millipore.
5. Wash the membrane 3 times, 5 minutes each, with PBS-Tween.
6. Incubate the membrane with anti-mouse HRP conjugated secondary antibody (Jackson ImmunoResearch 115-035-003) diluted 1:10,000 in PBS-Tween for 30 minutes at room temperature.

7. Wash the membrane 3 times, 5 minutes each, with PBS-Tween.
8. Develop using Amersham ECL™ Western Blotting Detection Reagents (GE-healthcare, RPN2209), according to manufacturer's protocol. H2Bub runs on the gel at a position corresponding to approximately 24kD, while H2B runs at approximately 17kD.

Figure 2 shows a Western blot analysis of H2Bub and total H2B 48 hours after transient transfection of HeLa cells with RNF20 siRNA (siRNF20) or LacZ siRNA (siLacZ).

3.2 Detecting transfected H2B ubiquitylation in whole cell extracts using anti-flag antibodies

This protocol describes the detection of ubiquitylated transfected H2B. The main advantages in monitoring transfected H2B, rather than the endogenous protein, lies in the ability to mutate the transfected H2B sequence at will. For example, it is possible to transfect cells with a H2B-flag construct in which lysine 120 is replaced by alanine, therefore abolishing the ubiquitylation site.

3.2.1 Transfection of cells with H2B-flag constructs—This protocol is used in our lab to transfect a variety of mammalian cell lines. Some cell lines, such as HeLa, 293, HCT116 and H1299 can be transfected with high efficiency, while other cell lines, such as WI-38 and MCF10A, are usually less transfectable.

1. Seed approximately 10^6 cells in a 10cm dish, and allow growth overnight (exact number of cells may differ among different cell lines).
2. Transfect H2B-flag expression plasmid, as well as control empty vector, using jetPEI DNA transfection reagent (101-10, Polyplus transfection), according to the manufacturer's instructions. Other methods of transfection can be used as well, depending on cell type and previous experience.
3. 24 hours after transfection, add antibiotics as appropriate, in order to select for transfected cells (optional).
4. When selection is complete, harvest cells for western blot analysis (section 3.2.2) or immunoprecipitation (section 3.2.3).

3.2.2 Protein extraction and western blot analysis

1. Extract proteins according to the protocol described above in section 3.1.1.
2. Load 15–30µg of protein onto a 15% SDS polyacrylamide gel, and follow the protocol described in 2.1.3, using at stage 4 anti-flag specific monoclonal antibodies (Sigma, F3165) at 1:1000 dilution.
3. Following development of the membrane with the ECL kit, it should be possible to discriminate the H2B-flag from the ubiquitylated H2B-flag by their size: Ubiquitylated H2B-flag should migrate on the gel with an apparent molecular mass of approximately 29kD, while H2B-flag should migrate at approximately 20kD.

3.2.3 Protein extraction and immunoprecipitation of H2B-flag under denaturing conditions—While it is possible to discriminate H2B-flag and ubiquitylated H2B-flag in whole cell extracts by their size, one can reduce background and simplify interpretation of the results by immunoprecipitating H2B-flag with anti-Flag antibodies. Doing so under denaturing conditions as described below should further reduce background

resulting from H2B-associated proteins, as the binding of H2B to other proteins is largely disrupted under these conditions.

1. Following transfection (as described in section 3.2.1) collect cells from approximately one 10cm dish in 200 l of 1% SDS in phosphate-buffered saline (PBS) in a 1.5ml eppendorf tube.
2. Boil for 5 minutes and vortex vigorously. Repeat 3 more times.
3. Add 400 µl of 1.5% Triton X-100 in Tris-buffered saline and centrifuge at 15000g for 10 minutes at room temperature in a 1.5ml eppendorf microfuge.
4. Collect supernatant and incubate with 20 µl of anti-FLAG M2-agarose beads (Sigma A2220) for 1 hour at 4°C in a 1.5ml eppendorf tube.
5. Wash beads three times in NP-40 buffer (120 mM NaCl, 1 mM EDTA, 20 mM Tris pH 8.0, 0.5% NP-40).
6. Elute immunoprecipitates by boiling in PSB and follow section 3.2.2 for detection of H2B-flag by western blot analysis.

Figure 3 shows a Western blot analysis of anti-FLAG immunoprecipitates from HEK293 cells transfected with FLAG-H2B.

4. Analyzing H2B ubiquitylation pattern and dynamics in the human genome by chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation is a powerful tool to study the pattern of H2B ubiquitylation on a specific genomic locus or genome-wide, either under basal conditions or in response to various experimental manipulations or physiological signals. This method allowed the identification of H2Bub distribution pattern and dynamics in the human genome [8, 9].

4.1 Fixation and isolation of nuclei

1. Grow cells to a confluence of 70–90% (calculate the amount of cells needed: approximately $5\text{--}20 \times 10^6$ cells per ChIP reaction).
2. Fixation: to each plate add 0.1 of total volume of 11% formaldehyde solution.
3. Rock for 10min at RT.
4. Add 0.06 of total volume of 2.5M glycine. Rock for 5min in RT.
5. Wash cells once with PBS.
6. Add Trypsin solution C (0.05% Trypsin and EDTA 1:5000, in DPBS, Biological industries, 03-054-1B) diluted 1:5 from a stock in PBS.
7. Incubate at 37°C for 10 min.
8. Add to each plate 500µl of fetal bovine serum to stop trypsin activity.
9. Wash cells once with PBS.
10. Add PBS and harvest cells in a 1.5ml eppendorf tube.
11. Centrifuge for 3min at 1500rpm in a 1.5ml eppendorf microfuge at room temperature.

12. Resuspend cells in 1ml cold cell lysis buffer in a 1.5ml eppendorf tube (5mM PIPES pH8.0, 85mM KCL, 0.5% NP-40 + fresh protease inhibitors (Sigma, P8340; 1:500). From this step on, everything is done on ice or at 4°C.
13. Rock for 10min at 4°C.
14. Centrifuge for 8min at 4000rpm (4°C) in a 1.5ml eppendorf minifuge.
15. Resuspend cells in 100µl nuclei lysis buffer (50mM Tris-Cl pH8.0, 10mM EDTA, 1% SDS + protease inhibitors 1:500).
16. Incubate for 10 min at 4°C.
17. Add 1ml of dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl pH8.0, 167mM NaCl + protease inhibitors 1:500).

4.2 Sonication and calibrations

1. Sonicate samples (Bioruptor) by repeated cycles of 30 seconds on and 1 minute off, for a total of about 15 minutes, in order to shear DNA into fragments of average size of 500–1000bp. It is recommended to calibrate the number of sonication cycles needed for each cell type, by withdrawing samples after sonication and running them on an agarose gel with suitable size markers.
2. Centrifuge for 5 minutes, maximal speed, at 4°C. Transfer supernatant to a new tube for pre-clearing.

4.3 Immunoprecipitation of chromatin

1. Pre-clearing of chromatin: Prior to use, block beads by overnight incubation with TE containing 1mg/ml sonicated salmon sperm DNA and 1mg/ml BSA at 4°C, followed by 3 washes with TE and resuspension in dilution buffer. Add to each sample 150µl of 50% slurry blocked protein A Sepharose beads (Sigma, P9424) and incubate at 4°C for 2hr.
2. Spin down beads at 4°C. Take supernatant for another round of pre-clearing (optional) or transfer to a new tube.
3. Take out ~3% of the sample and store at –20°C for input analysis. To the rest of the sample add 30µl of 50% slurry blocked protein A Sepharose beads and 5µl of H2Bub antibody. Incubate at 4°C overnight with rocking in a 1.5ml eppendorf tube.

4.4 Washing of immunoprecipitates and elution of bound proteins

1. Centrifuge samples to spin down the beads (4°C) in a 1.5ml eppendorf minifuge. Wash immunoprecipitates twice as follows: add 1 ml of dilution buffer, rotate 3 minutes, spin down beads and remove supernatant.
2. Perform two washes as described above, with wash buffer (100mM Tris-Cl pH 8.0, 500mM LiCl, 1% NP-40, 1% deoxycholic acid + protease inhibitors 1:500).
3. Finally, resuspend beads (and also the stored input samples) in 150µl of TE pH8.0 and 150µl of elution buffer (50mM NaHCO₃, 1% SDS).

4.5 Reversal of cross-links and DNA purification

1. Add to each 1.5ml eppendorf tube (ChIP and input) DNase-free RNase to a final concentration of 50µg/ml. Incubate for 30 minutes at 37°C.
2. To get rid of proteins in the sample and clean up the immunoprecipitated DNA, add 200µg/ml proteinase K. Incubate for 3 hours at 45°C.

3. To complete the reversal of cross-links, incubate samples overnight at 65°C.
4. To isolate DNA, spin down the beads, transfer supernatant (about 300µl) into a new 1.5ml eppendorf tube. Do same for the input samples. Continue with PCR purification kit (Qiagene, 28104) according to the manufacturer's protocol.

The purified DNA obtained from the ChIP can be further used to test by RT-PCR the association of H2Bub with specific genomic regions. In addition, it is possible to analyze the genome-wide patterns of H2Bub localization by subjecting the DNA to massively parallel sequencing. Figure 4 shows the pattern of H2Bub distribution in HeLa cells, as determined by ChIP-seq.

5. Conclusions

In spite of lacking optimal genetic tools to explore H2B ubiquitylation in mammalian cells, it is possible to successfully utilize numerous biochemical and molecular biology methods as described above. The generation of anti-H2Bub antibodies now enables the analysis of site-specific histone ubiquitylation and will likely be instrumental in additional studies of dynamics of histone ubiquitylation during transcription and other processes. It can also be used for studying the association of H2Bub with other histone modifications at specific genomic loci. The application of these methods and reagents has already expanded our knowledge about this modification in the past years and will continue to be useful in future studies.

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The anti-ubiquityl-H2B antibody described in this paper is available from Millipore (Catalog number 05–1312) and from Medimabs (Catalog number MM-0029-P).

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Figure 1. Branched peptide used for immunization

The amino acid sequences in black and grey are derived from human H2B and ubiquitin, respectively. The underlined Cysteine was added to the sequence to facilitate conjugation to KLH.

Figure 2. Western blot analysis with H2Bub specific antibodies

HeLa cells were transiently transfected with RNF20 siRNA (siRNF20) or LacZ siRNA (siLacZ). 48 hours after transfection, cells were harvested according to the above protocol (3.1.1) and equal amounts were resolved by western blot with antibodies for RNF20, H2Bub and total H2B (Millipore, 07-371).

Figure 3. Ubiquitination analysis of transfected FLAG-H2B

HEK-293T cells were transfected with either wild-type (WT) FLAG-tagged H2B or a ubiquitination-deficient Flag-tagged H2B mutant (K120R). 48 hours after transfection, denatured cell extracts were subjected to immunoprecipitation using an anti-Flag antibody. The resulting pellets were resolved by SDS-PAGE followed by western blot analysis with the antibodies indicated above each panel. The lower left panel represents a short exposure of the upper panel. Positions of non-modified Flag-H2B and monoubiquitinated H2B are indicated. LC, antibody light chain.

Figure 4. Pattern of H2Bub distribution in HeLa cells, as determined by ChIP-seq
For each gene, the number of sequence reads mapped to the vicinity of the TSS (10 kb upstream or downstream) was smoothed with a moving average of 300 bp. The smoothed data was averaged over all genes in the genome. Position 0 denotes the TSS.