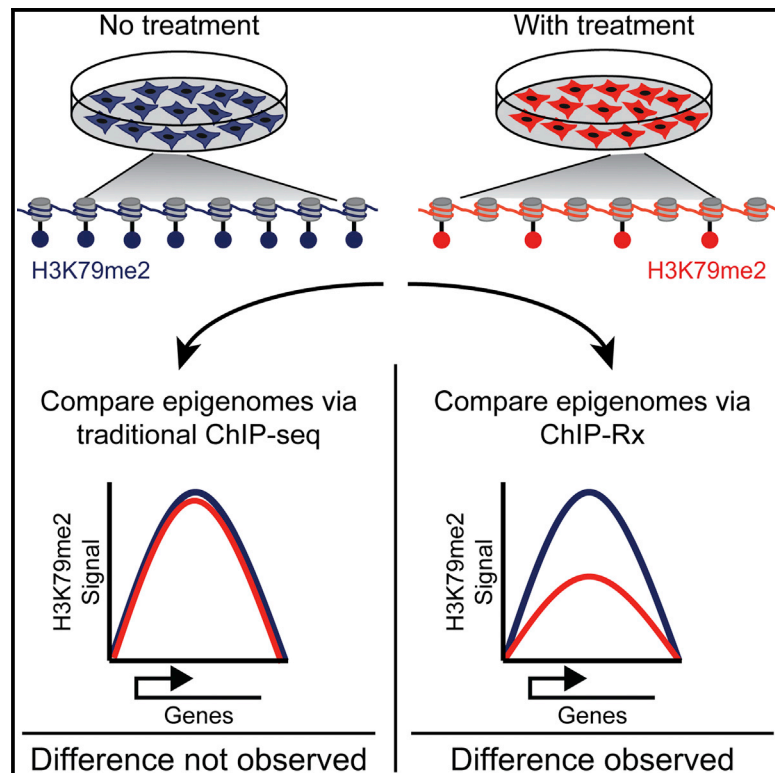


Quantitative ChIP-Seq Normalization Reveals Global Modulation of the Epigenome

Graphical Abstract



Highlights

ChIP-seq is a prevailing methodology to investigate and compare epigenomic states

Lack of an empirical normalization strategy has limited the usefulness of ChIP-seq

ChIP-Rx allows genome-wide quantitative comparisons of histone modification status

ChIP-Rx identifies graded epigenomic changes following chemical perturbations

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In Brief

The lack of an empirical methodology to enable normalization among chromatin immunoprecipitation coupled with massively parallel DNA sequencing (ChIP-seq) experiments has limited the precision and comparative utility of this technique. Orlando et al. describe a method, called ChIP with reference exogenous genome (ChIP-Rx), that allows one to perform genome-wide quantitative comparisons of histone modification status across cell populations using defined quantities of a reference epigenome. They use the method to detect disease-relevant epigenomic changes following drug treatment.

Accession Numbers

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Quantitative ChIP-Seq Normalization Reveals Global Modulation of the Epigenome

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SUMMARY

Epigenomic profiling by chromatin immunoprecipitation coupled with massively parallel DNA sequencing (ChIP-seq) is a prevailing methodology used to investigate chromatin-based regulation in biological systems such as human disease, but the lack of an empirical methodology to enable normalization among experiments has limited the precision and usefulness of this technique. Here, we describe a method called ChIP with reference exogenous genome (ChIP-Rx) that allows one to perform genome-wide quantitative comparisons of histone modification status across cell populations using defined quantities of a reference epigenome. ChIP-Rx enables the discovery and quantification of dynamic epigenomic profiles across mammalian cells that would otherwise remain hidden using traditional normalization methods. We demonstrate the utility of this method for measuring epigenomic changes following chemical perturbations and show how reference normalization of ChIP-seq experiments enables the discovery of disease-relevant changes in histone modification occupancy.

INTRODUCTION

The ability to map genomic occupancy of transcriptional regulators, histone posttranslational modifications, and DNA methylation (epigenomic modifications) has enabled the elucidation of transcriptional mechanisms, genome organization, mapping of functional regulatory elements, and discovery of disease-associated chromatin markers (Badeaux and Shi, 2013; Barski et al., 2007; Lee and Young, 2013; Rivera and Ren, 2013; Zhou et al., 2011). Such targeted and large-scale epigenome mapping efforts have revealed chromatin regulatory proteins that are therapeutic targets for a wide variety of human diseases (Azad et al., 2013; Dawson and Kouzarides, 2012; Deshpande et al., 2012; Wee et al., 2014). Many of these chromatin regulators exhibit cell-type-selective, gene-selective, or disease-relevant effects,

creating a critical need to study the chromatin modifications catalyzed by these regulators. Accurate quantification of both global and loci-specific chromatin modifications is needed to allow the discovery and characterization of epigenomic regulators and epigenome-modulating agents.

Traditional ChIP-seq methodologies are not inherently quantitative and therefore do not allow direct comparisons between samples derived from different cell types or between cells that have experienced a perturbation, such as a genomic alteration or chemical treatment. For example, if we employ the traditional reads per million (RPM) ChIP-seq normalization method, a cell population containing chromatin state “A” (a high level of histone posttranslational modification) will appear similar to a cell population containing chromatin state “B,” where 50% of the signal has been removed (Figure 1A), because the signal is quantified as a simple percentage of all mapped reads. Moreover, additional variables, such as variations in genome fragmentation, immunoprecipitation efficiency, or other experimental steps, frequently confound analysis. Efforts to correct for these variables have produced *in silico* normalization strategies, but an empirical method to enable direct and quantitative comparisons among epigenomic ChIP-seq data sets is still lacking (Bardet et al., 2012; Landt et al., 2012; Liang and Keleş, 2012; Liu et al., 2013; Nair et al., 2012). Because of the experimental and analytical restrictions of ChIP-seq, a robust normalization methodology is needed to quantify epigenome differences among varying cell populations, treatments, and genomic states.

RESULTS

Here we present a method, called ChIP with reference exogenous genome (ChIP-Rx), that utilizes a constant amount of reference or “spike-in” epigenome, added on a per-cell basis, to allow direct comparison between two or more ChIP-seq samples (Figure 1B). Analogous methodologies have been applied in areas of gene-expression analysis that have revealed global transcriptional amplification upon normalization and in MethyC-seq, where bisulfate conversion rates have been normalized (Kanno et al., 2006; Krueger et al., 2012; Lin et al., 2012; Lovén et al., 2012; van de Peppel et al., 2003). These advancements have allowed standardization, precision, and a mechanistic

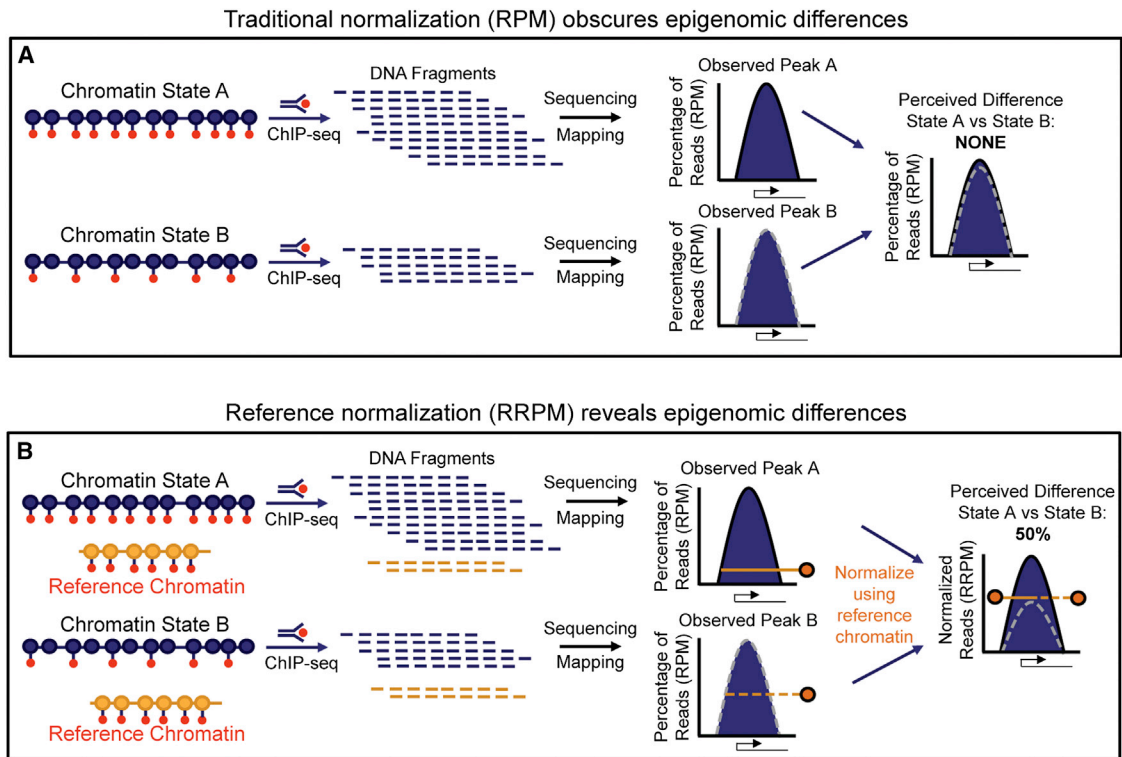


Figure 1. Normalization and Interpretation of ChIP-Seq Data

(A) Schematic representation of a typical ChIP-seq data workflow. Interrogation of a human epigenome (Blue circles, nucleosomes) with a full complement of histone modification (red circles, top) versus an epigenome with a half complement of histone modification (red circles, bottom). ChIP, sequencing, and mapping using reads per million (RPM) reveals ChIP-seq peaks (blue). A comparison of the peaks as a percentage of the total reads reveals little difference.

(B) Schematic representation of a ChIP-seq data workflow with reference genome normalization. Interrogation of a human epigenome (Blue circles, nucleosomes) with a full complement of histone modification (red circles, top) versus an epigenome with a half complement of histone modification (red circles, bottom). A fixed amount of reference epigenome (orange, nucleosomes; red, histone modifications) is added to human cells in each condition. After ChIP, sequencing, and mapping, the ChIP sequence reads are normalized to the percentage of reference genome reads in the sample (reference-adjusted RPM [RRPM]). A comparison of ChIP-seq signals using normalized reads reveals a 50% difference between peaks. This method is called ChIP with reference exogenous genome (ChIP-Rx).

understanding of RNA transcription (van Bakel and Holstege, 2004; Jiang et al., 2011; Li et al., 2013); however, no cell-count-normalized methods have been applied to global correction of histone posttranslational modifications. Since a vast array of histone modifications have been described in eukaryotic cells that play roles in organismal development, maintenance of cell state, differentiation, and disease, including those associated with transcriptional processes, genome organization, DNA repair, and cell-cycle progression (Calo and Wysocka, 2013; Pastor et al., 2013; Rinn and Chang, 2012; Rivera and Ren, 2013; Tan et al., 2011; Tian et al., 2012), a quantitative method for comparing these key marks is needed. We reasoned that the *Drosophila melanogaster* genome would be a desirable exogenous reference for mammalian cells because the *Drosophila* genome is well studied and has a high-quality sequence assembly, there is minimal mapping of the *Drosophila* genome sequence to human or mouse genomes (>0.05%; Table S1; Supplemental Experimental Procedures), *Drosophila* cells are readily available in large quantities, and the *Drosophila* epigenome displays nearly all of the key histone modification marks reported in humans. Moreover, histone proteins are among the most conserved proteins from humans to yeast, indicating that

available ChIP-quality antibodies would likely recognize both *Drosophila* and human chromatin (Sullivan et al., 2002; Wolffe and Pruss, 1996).

To determine the impact of mixing interspecies epigenomes, we tested whether the addition of a reference genome (*Drosophila* S2 cells) would inherently affect our ability to detect a histone modification within the test sample (human cells) using ChIP-seq. We compared Jurkat cells alone with Jurkat cells that had been mixed with *Drosophila* cells and analyzed the resulting histone H3 lysine-79 dimethyl (H3K79me2) ChIP-seq profiles (Figure S1; Table S2). We determined that mixing of *Drosophila* and human cells did not induce large-scale changes in the H3K79me2 profiles, as the profiles of these cell populations were highly correlated by total signal as well as enriched loci overlap (Pearson correlation = 0.96; Supplemental Experimental Procedures). Moreover, reads originating from human or *Drosophila* could be separated with 99% accuracy (Supplemental Experimental Procedures). Together, these results indicate that the addition of a reference genome did not impede our ability to detect histone mark occupancy.

We devised an experiment to test our ability to detect changes in histone modification occupancy throughout the human

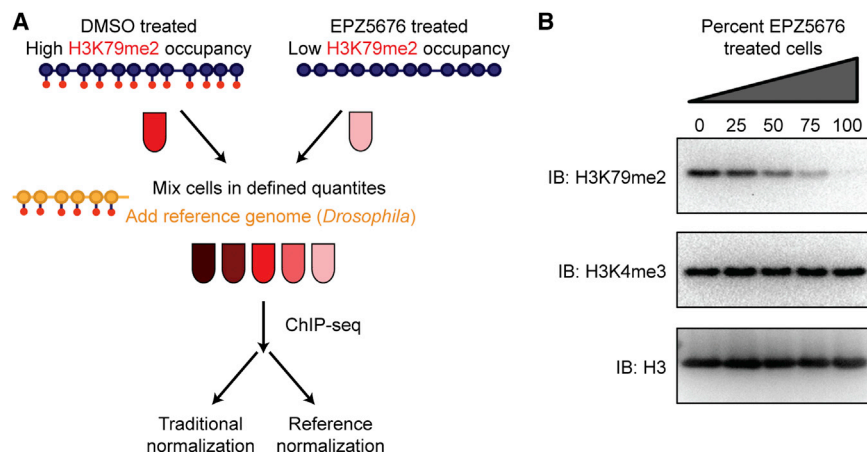


Figure 2. Experimental Design of Differential H3K79me2 Detection

(A) Schematic representation of differential H3K79me2 detection and normalization strategies. Two populations of cells were produced: a human epigenome (blue nucleosomes) with a full complement of H3K79me2 (red circles, top left) and a human epigenome (blue nucleosomes) with depleted H3K79me2 due to EPZ5676 exposure (top right). These cells were mixed in defined proportions in order to allow a dilution of total genomic histone modification (dark red to pink). Cell mixtures were subjected to ChIP-seq in the presence of the reference *Drosophila* epigenome (orange). ChIP-seq signals were calculated based on traditional or *Drosophila*-reference-normalized methods. See also Figure S1.

(B) Western blot validation of H3K79me2 depletion in Jurkat cells. Mixtures of 0%–100% EPZ5676-

treated cells (0:100; 25:75; 50:50; 75:25; 100:0 proportions of [DMSO-treated:EPZ5676-treated] cells) were measured by immunoblot (IB) for the presence of H3K79me2, H3K4me3, or total histone H3 (loading control). Treated cells were exposed to 20 μ M EPZ5676 for 4 days. See also Table S1.

epigenome using ChIP-Rx (Figure 2A). We reasoned that an initial test of ChIP-Rx normalization should feature an epigenomic modification that could be readily removed and was not essential for cell viability in the model cell line. Using the selective DOT1L inhibitor EPZ5676 (Daigle et al., 2013), we depleted the Histone H3 lysine-79 dimethyl (H3K79me2) modification from Jurkat cell bulk histones (Figure 2B). The H3K79me2 modification is catalyzed by the DOT1L protein and is associated with the release of paused RNA Polymerase II and licensing of transcriptional elongation (van Leeuwen et al., 2002; Ng et al., 2002; Shanower et al., 2005; Steger et al., 2008). This modification is typically deposited within the 5' regions of genes and its presence is not critical for Jurkat cell viability (Daigle et al., 2011, 2013; Schübeler et al., 2004; Steger et al., 2008). By mixing untreated cells (full H3K79me2) with EPZ5676-treated cells (H3K79me2 depleted), we created a set of cell populations with defined quantities of H3K79me2, as verified by immunodetection (Figure 2B). To each of these cell populations we added *Drosophila* cells at a ratio of one *Drosophila* cell per two human cells, which provided a constant “reference” amount of H3K79me2 per human cell. We performed ChIP-seq from samples consisting of 0:100, 25:75, 50:50, 75:25, and 100:0 proportions of EPZ5676 to DMSO-treated Jurkat cells. We then tested whether traditional ChIP-seq analysis methods would reveal the decrease in human per-cell H3K79me2 occupancy and, if not, whether the addition of the *Drosophila* epigenome would allow detection of H3K79me2 removal.

A key prediction of our normalization method is that as the global level of a histone modification is depleted in human cells, the percentage of total reads mapping to the reference *Drosophila* genome should increase. This is because the constant amount of reference genome added per human cell accounts for a greater percentage of total ChIP DNA fragments as human epitopes are lost (see the ratio of blue to orange DNA fragments in Figure 1B). To test this prediction, we interrogated cells with defined H3K79me2 levels (Figure 2B) by ChIP-Rx to measure genomic H3K79me2 occupancy. As a control, we also measured H3K4me3 occupancy, which is a histone

modification that is not appreciably changed within our test cell populations (Figure 2B). As predicted, H3K79me2 depletion in Jurkat cells both reduced ChIP-Rx reads mapping to the human genome and increased reads mapping to the *Drosophila* genome (Figure 3A). We did not observe a similar change in the mapping ratio for H3K4me3 in the same samples, consistent with the finding that H3K4me3 was not preferentially removed from the human genome (Figure 3B). These results demonstrate that a reference genome can internally normalize the read count.

We next used the reference *Drosophila* genome to quantitatively normalize across experiments. To make ChIP-seq data quantitative on a per-cell basis, it is necessary to introduce a reference signal that is constant per cell, from which a normalization factor can be derived. Our ChIP-Rx protocol uses the signal from a fixed amount of *Drosophila* genome per human cell as this reference. We derived a normalization factor (see the Supplemental Experimental Procedures) for each experiment, such that the resulting *Drosophila* signal was equilibrated across all experiments (Table S2; Figure S2). Using traditional RPM normalization, the loci-specific ChIP-seq profiles and metagene profiles for H3K79me2 and H3K4me3 appear unchanged for the majority of samples (Figures 3C–3F), despite evidence that the H3K79me2 modification is progressively depleted (Figure 2B). After normalization with the *Drosophila* reference (normalized reference-adjusted RPM [RRPM]), a striking and graded decrease in H3K79me2 signal across the samples is evident (Figures 3C, 3E, 3G, and S3). Normalization did not appreciably affect the metagene profiles of the control H3K4me3 experiments (Figures 3D, 3F, 3H, and S3). Repeat experiments produced the same result in all cases: normalization revealed a loss of H3K79me2 across the samples and H3K4me3 profiles were not significantly affected (Figure S4). These results indicate that normalization to a *Drosophila* reference is an effective method for quantitatively comparing multiple experiments and can reveal changes in histone modification that may not be apparent without proper normalization.

Having validated the ChIP-Rx methodology using standardized quantities of H3K79me2, we next tested our ability to

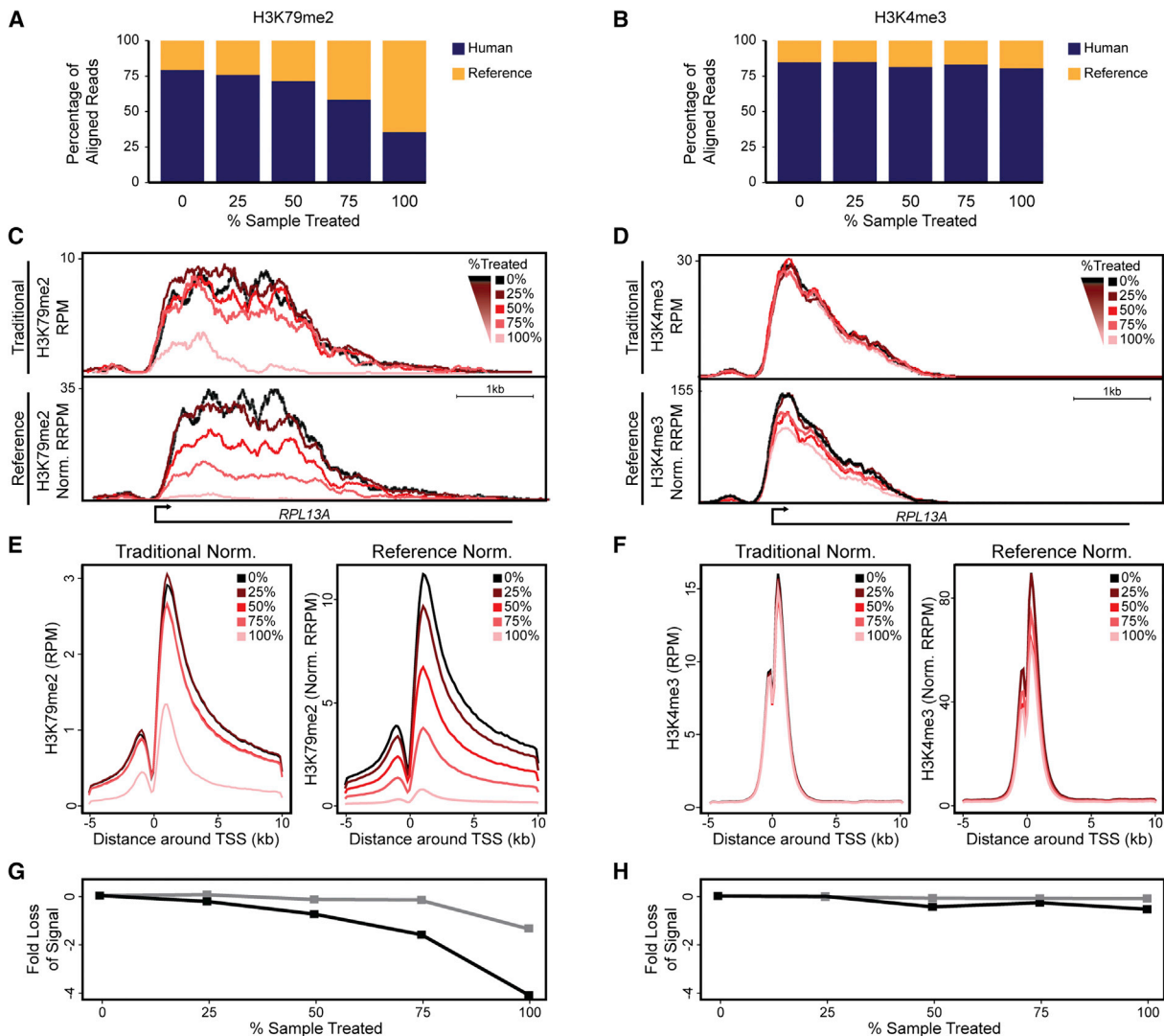


Figure 3. CHIP-Rx Reveals Quantitative Epigenome Changes

(A and B) Percentage of reads aligning to either test (human, blue) or *Drosophila* (reference, orange) genomes after H3K79me2 ChIP-Rx (A) or H3K4me3 ChIP-Rx (B). Samples containing 0%, 25%, 50%, 75%, or 100% EPZ5676 treated Jurkat cells were used as defined in Figure 2B.

(C and D) Sequenced reads from H3K79me2 (C) and H3K4me3 (D) immunoprecipitations at the RPL13A gene locus in traditional reads per million (RPM, top) or reference-adjusted reads per million (RRPM, bottom; see [Experimental Procedures](#)). Color indicates the percentage of sample treated with EPZ5676. The gene model is shown below the track.

(E) Meta-gene profile of H3K79me2-occupied genes in Jurkat cells. Meta-gene profiles were produced with traditional RPM (left) or RRPM (right). Color indicates the percentage of Jurkat cell sample treated with EPZ5676 as in Figure 2B. Region -5 to $+10$ kb around the transcription start site (TSS) is shown. Meta-gene profile was derived from top 5,000 protein-coding genes as defined by total H3K79me2 signal in the 0% treated (untreated with EPZ5676) sample. A meta-gene profile representing all genes is shown in [Figure S3](#).

(F) Meta-gene profile of H3K4me3-occupied genes in Jurkat cells. Meta-gene profiles were produced with traditional RPM (left) or RRPM (right). Color indicates the percentage of Jurkat cell sample treated with EPZ5676 as in Figure 2B. Region -5 to $+10$ kb around the transcription start site (TSS) is shown. Meta-gene profile was derived from top 5,000 protein-coding genes as defined by total H3K4me3 signal in the 0% treated (untreated with EPZ5676) sample. A meta-gene profile representing all genes is shown in [Figure S3](#).

(G and H) Line graphs display the observed fold-change difference in average meta-gene signal across the -5 to $+10$ kb window around the TSS for each H3K79me2 (G) or H3K4me3 (H) ChIP sample (x axis) relative to the signal from the 0% treated population using traditional (gray) or reference (black) normalization. See also [Figures S2–S4](#) and [Table S2](#).

normalize between ChIP-seq experiments in a disease-relevant system. MV4;11 acute myelomonocytic leukemia cells are a DOT1L-inhibitor sensitive model of human mixed-lineage-linked leukemia, a disease characterized by reciprocal translocations

of the mixed-lineage leukemia (MLL) gene ([Daigle et al., 2011, 2013](#); [Deshpande et al., 2012](#)). We treated MV4;11 cells with DOT1L inhibitor and measured changes in H3K79me2 occupancy in the presence or absence of the reference *Drosophila*

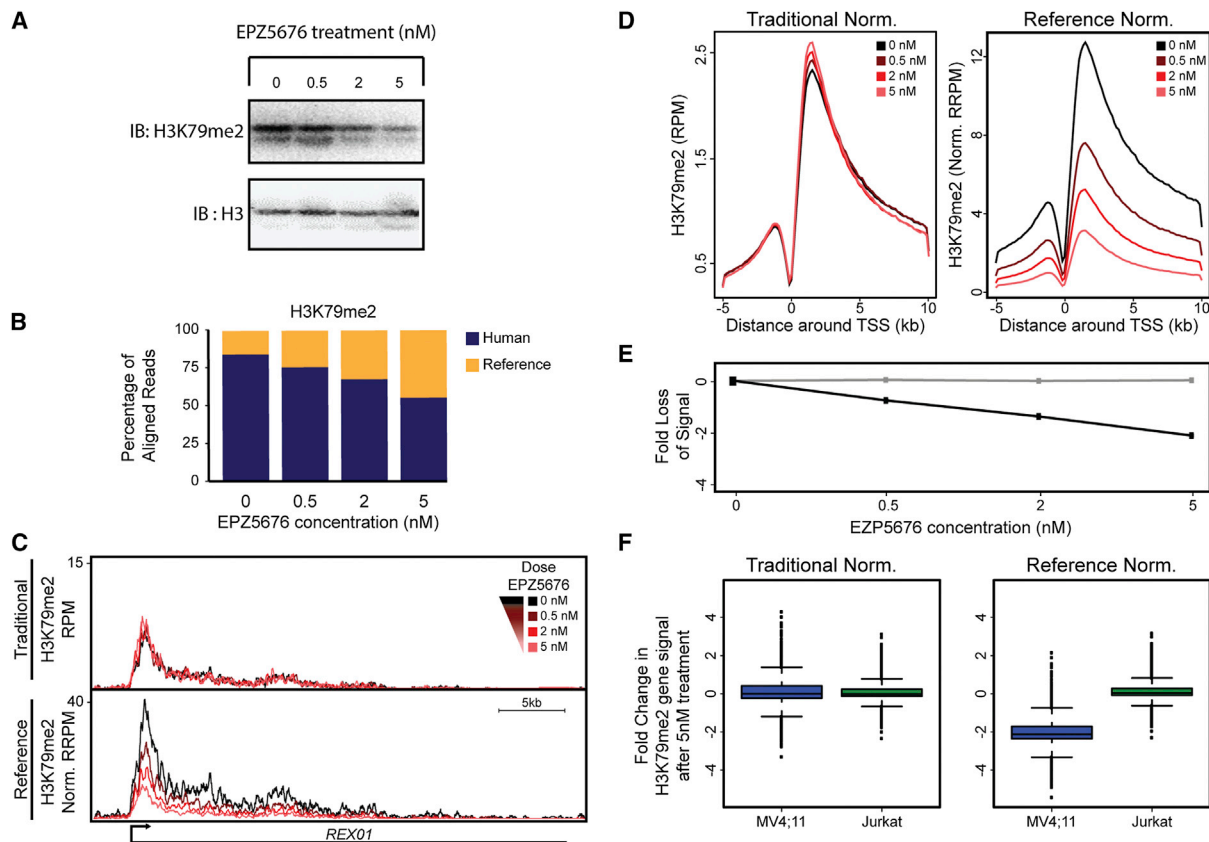


Figure 4. ChIP-Rx Reveals Epigenomic Alterations in Disease Cells that Respond to Drug Treatment

(A) Western blot showing the levels of H3K79me2 in MV4;11 cells after treatment for 4 days with increasing concentrations of EPZ5676. (B) Percentage of H3K79me2 ChIP-seq reads aligning to either test (human, blue) or *Drosophila* (reference, orange) genomes after H3K79me2 ChIP-Rx from MV4;11 cells treated as in (A). (C) Sequenced reads from H3K79me2 immunoprecipitations at the REXO1 gene locus in standard RPM (top) or RRPm (bottom) (see [Experimental Procedures](#)). Color indicates the concentration of EPZ5676 given to each sample. The gene model is shown below the track. (D) Meta-gene profile of H3K79me2-occupied genes in MV4;11 cells. Meta-gene profiles were produced with traditional Reads Per Million (RPM, left) or Reference-adjusted Reads Per Million (RRPM, right). Color indicates the concentration of EPZ5676 used in each sample. The region -5 kb to $+10$ kb around the TSS is shown. Meta-gene profile was derived from top 5,000 protein-coding genes as defined by total H3K79me2 signal in the 0nM treated (untreated with EPZ5676) sample. A meta-gene profile representing all genes is shown in [Figure S3](#). (E) Line graph displays the observed fold-change difference in average meta-gene signal across the -5 to $+10$ kb window around the TSS for each H3K79me2 ChIP sample (x axis) relative to the signal from the 0 nM treated population using standard (gray) or reference (black) normalization. (F) Box plots display the distribution of the observed fold change of H3K79me2 signal -5 kb to $+10$ kb around the TSS of all genes between the 0 nM and 5 nM treated samples (blue, MV4;11; green, Jurkat) for all genes using traditional (left) or reference-adjusted (right) normalization (see the [Supplemental Experimental Procedures](#)).

See also [Figures S3](#) and [S5](#) and [Table S2](#).

epigenome ([Figures 4A–4E](#), [S5A](#), and [S5B](#)). EPZ5676 induced a dose-dependent decrease in bulk H3K79me2 ([Figure 4A](#)), but this result was masked when we quantified H3K79me2 occupancy using traditional normalization ([Figures 4C](#) and [4E](#)). We observed a dose-dependent decrease in H3K79me2 genomic occupancy only after employing reference normalization ([Figures 4C–4E](#)). This unmasking of epigenomic effects may be critical for understanding the cell-type-selective effects of small-molecule epigenome modulators. For example, MV4;11 cells exhibit global H3K79me2 depletion at a low dose of EPZ5676, consistent with the known selectivity of the DOT1L inhibitor for leukemic cells carrying MLL translocations, but EPZ5676-insensitive Jurkat cells do not ([Figures 4A](#) and [S5C](#); [Daigle et al., 2013](#)).

Thus, normalizing to a reference exogenous genome rectifies the protein-level measurements and genome occupancy of modified histones, and reveals subtle epigenomic changes that may underlie or predict cellular responses to drugs ([Figure 4](#)). These results show that ChIP-Rx enables the discovery of epigenomic changes that can provide insight into disease and inform drug mechanisms.

DISCUSSION

In summary, we have demonstrated that ChIP-Rx allows the discovery and quantification of dynamic epigenomic profiles across mammalian cells that would otherwise remain hidden using

traditional normalization methods. A recent study employed a similar reference strategy for ChIP-seq normalization (Bonhoure et al., 2014); however, our method offers two crucial advantages that allow direct comparative epigenomic analysis. First, our method introduces the reference at the beginning of the experiment, thus normalizing for variation throughout the experiment, including chromatin fragmentation and immunoenrichment, both of which are critical for epitope and genome retrieval (Kidder et al., 2011; Meyer and Liu, 2014; Raha et al., 2010). Second, as was analogously shown for RNA expression correction (Lovén et al., 2012), our method introduces the reference “spike-in” on a per-cell basis as opposed to total chromatin, thus allowing the detection of unidirectional chromatin changes irrespective of variations in ploidy or gross chromatin. Thus, our method provides greater accuracy in determining epigenome changes that occur upon cell perturbation or exposure to small-molecule inhibitors as compared with current methods. Importantly, ChIP-Rx allows for the detection of subtle epigenomic changes, as opposed to qualitative occupancy calls, and thus advances the ChIP-seq methodology from a descriptive, binary readout to one that reveals graded epigenomic changes. This is particularly important for the dose-ranging characterization of chemical tools and therapeutics targeting chromatin-associated complexes via genome-wide approaches. Application of this methodology to additional model systems, including mouse, rat, and zebrafish (Table S1), as well as additional histone modifications, including repressive (i.e., H3K27me3) and activating (i.e., H3K27ac) histone modifications, will enable far-reaching studies of comparative epigenomics.

We recommend the implementation of ChIP-Rx whenever quantitative or comparative epigenomic changes are under investigation. The method described here will be critical for understanding the global and site-selective epigenomic changes that occur in human disease, during cell-state changes, and especially the action of small-molecule inhibitors of chromatin-modulating proteins.

EXPERIMENTAL PROCEDURES

Human Cell Lines, Growth, and Treatment

Jurkat cells were obtained from ATCC and maintained in RPMI (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Life Technologies) at 5% CO₂ in 37°C. MV4;11 cells were obtained from ATCC and maintained in RPMI (Life Technologies) supplemented with 10% FBS at 5% CO₂ in 37°C.

Jurkat cells were treated with DMSO or EPZ5676 (Selleck Chemicals, catalog number S7062) at 5 nM or 20 μM for 4 days, and MV4;11 cells were treated with DMSO or EPZ5676 at 0.5 nM, 2 nM, or 5 nM for 4 days. Live-cell numbers were quantified using the Countess cell counter (Life Technologies).

At harvest, cells were crosslinked with 1% formaldehyde by addition of 1/10 volume of fresh 11% formaldehyde solution (11% formaldehyde 0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM HEPES) and incubation at room temperature for 8 min. Crosslinking reactions were quenched with a 1/20 volume of 2.5 M glycine for 1–5 min and cells were pelleted. The cells were then washed three times with ice-cold PBS. Washed cell pellets were flash frozen and stored at –80°C.

Preparation of *Drosophila* S2 Cells

Drosophila S2 cells (ATCC catalog number CRL-1963; Biovest part number OO.763/OO.627) were cultured in Schneider's *Drosophila* media (Life Technologies catalog number 21720-024) supplemented with 10% FBS to attain a density of 0.5–0.6 × 10⁶ cells/ml. Cell culture and scale-up to 2 L was performed by Biovest International.

At harvest, cells were crosslinked with 1% formaldehyde by addition of a 1/10 volume of fresh 11% formaldehyde solution (11% formaldehyde 0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM HEPES) and incubation at room temperature for 8 min. Crosslinking reactions were quenched with a 1/20 volume of 2.5 M glycine for 1–5 min and cells were pelleted. The cells were then washed three times with ice-cold PBS. Washed cell pellets were flash frozen and stored at –80°C at 1 × 10⁸ cells per aliquot.

ChIP-Rx

For each ChIP-Rx experiment, a 2:1 ratio of human:*Drosophila* cells was used. This corresponds to 20 million crosslinked human cells and 10 million crosslinked S2 cells (Jurkat experiments) or 15 million crosslinked human cells and 7.5 million crosslinked S2 cells (MV4;11 experiments).

S2 cells were added to human cells at the beginning of the ChIP-Rx workflow (during nuclei isolation). Once *Drosophila* S2 and human cells were combined, the sample was treated as a single ChIP-seq sample throughout the experiment until completion of DNA sequencing.

Briefly, frozen, crosslinked human and *Drosophila* cells were resuspended in parallel in cold Lysis Buffer 1 (140 mM NaCl, 1 mM EDTA, 50 mM HEPES, 10% glycerol, 0.5% NP-40, 0.25% Triton-X-100), incubated 10 min at 4°C, and pelleted. Both human and *Drosophila* cell samples were resuspended in parallel in Lysis Buffer 2 (10 mM TRIS [pH 8.0], 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA), incubated for 10 min at 4°C, and combined to the desired cell number ratios (two human cells per one *Drosophila* cell). The composite cell nuclei was then pelleted and resuspended in sonication buffer (10 mM TRIS [pH 8.0], 1 mM EDTA, 0.1% SDS).

Composite samples (human + *Drosophila* S2) in sonication buffer were sonicated using a Covaris E220 sonication water bath for 5 min. Sheared chromatin was diluted 1:1 in 2× dilution buffer (300 mM NaCl, 2 mM EDTA, 50 mM TRIS [pH 8.0], 1.5% Triton-X, 0.1% SDS) and incubated with either H3K79me2 (Abcam 3594)- or H3K4me3 (Millipore 07-473)-conjugated Protein G Dynal beads (Invitrogen) overnight (8–16 hr, rotating) at 4°C, and then washed two times with wash buffer 1 (50 mM HEPES, 140 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.75% Triton-X, 0.1% SDS, 0.05% DOC), two times with high-salt wash buffer (50 mM HEPES, 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.75% Triton-X, 0.1% SDS, 0.05% DOC), and one time with TE-NaCl buffer (10 mM Tris [pH 8.0], 1 mM EDTA, 50 mM NaCl). Samples were eluted from beads for 1 hr at 65°C in elution buffer (50 mM TRIS [pH 8.0], 10 mM EDTA, 1% SDS) and supernatant reverse-crosslinked at 65°C for 6–16 hr. Samples were diluted 1:1 with TE buffer (50 mM TRIS [pH 8.0], 1 mM EDTA) and treated with RNase A (0.2 mg/ml) for 2 hr at 37°C and then Proteinase K (0.2 mg/ml) for 2 hr at 55°C. DNA was isolated by phenol-chloroform extraction and ethanol precipitation. For detailed protocols see the Supplemental Experimental Procedures and Guenther et al. (2008).

Library Construction, Sequencing, and Data Collection

Libraries were constructed with the Illumina Tru-Seq library preparation kit using a target fragment size of 200–400 bp and multiplexing barcodes. Libraries were sequenced using Illumina HiSeq 2000 with single-end reads for 40 cycles. Sequences were demultiplexed and aligned using Bowtie2 against a “genome” that combines the human hg19 genome and the *Drosophila* dm3 genome (see the Supplemental Experimental Procedures). Individual accession numbers and read statistics available in Table S2.

Western Blots

Cells were harvested from all treatment groups and lysed with Triton extraction buffer (PBS containing 0.5% Triton X-100 [v/v], cOmplete Protease Inhibitors [Roche]) for 10 min with rotation. Nuclei were collected and acid extracted with 0.2 N HCl overnight. Histone proteins were collected from the supernatant and immunoblotted for H3K4me3 (Millipore 07-473), H3K79me2 (Abcam 3594), and histone H3 (Abcam 1791).

Determination of the Normalization Factor

A complete description of the basis and derivation of the ChIP-Rx normalization factor is provided in the Supplemental Experimental Procedures. In brief, we derived a normalization constant, α , such that after normalization the signal

per-reference cell (β) is the same across all samples. The total ChIP-seq signal derived from reference cells is simply the count of reads (in millions) aligning to the *Drosophila* genome, which we represent as N_d . Because the percentage of reference cells as a fraction of the total number of cells is constant and we assume that the epigenome of the reference cells does not vary appreciably, we can derive α as

$$\alpha * N_d = \beta$$

Because β is a constant, we can simply rewrite this as

$$\alpha * N_d = 1$$

or

$$\alpha = \frac{1}{N_d}$$

multiplying the read counts by α produces a normalized read count in normalized RPPM.

ACCESSION NUMBERS

The raw sequencing data reported in this work have been deposited in the NCBI Gene Expression Omnibus under accession number GSE60104.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.10.018>.

AUTHOR CONTRIBUTIONS

D.A.O., M.G.G., J.E.B., C.C.F., and E.R.O. designed and analyzed the research. M.W.C. conducted ChIP-seq and ChIP-Rx experiments. All other experiments were performed by M.W.C., V.E.B., Y.J.C., S.S., and M.G.G. D.A.O. performed the computational analysis. M.G.G. and D.A.O. wrote the manuscript.

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