Overlapping chromatin-remodeling systems collaborate genome wide at dynamic chromatin transitions

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ATP-dependent chromatin remodeling is an essential process required for the dynamic organization of chromatin structure. Here we describe the genome-wide location and activity of three remodeler proteins with diverse physiological functions in the mouse genome: Brg1, Chd4 and Snf2h. The localization patterns of all three proteins substantially overlap with one another and with regions of accessible chromatin. Furthermore, using inducible mutant variants, we demonstrate that the catalytic activity of these proteins contributes to the remodeling of chromatin genome wide and that each of these remodelers can independently regulate chromatin reorganization at distinct sites. Many regions require the activity of more than one remodeler to regulate accessibility. These findings provide a dynamic view of chromatin organization and highlight the differential contributions of remodelers to chromatin maintenance in higher eukaryotes.

Organization of the eukaryotic genome into chromatin is essential for all DNA-templated processes. Packaging of DNA into nucleosomal arrays not only acts to condense the genome, allowing for efficient organization within the cell’s nucleus, but also is an important mechanism to regulate access to DNA-encoded information. Maintenance of a balance between efficient packaging and accessibility is achieved through the combined activities of multiple specialized proteins that are critical for the dynamic alteration of chromatin structure. ATP-dependent chromatin-remodeler enzymes have a key role in this process. Each member of this large family of enzymes is characterized by a highly conserved helicase-like ATPase domain used to generate energy from ATP hydrolysis to reposition, evict or otherwise modify nucleosomes. In terms of function, the outcome of remodeling is well understood to result in the regulation of chromatin accessibility and the exposure of DNA regulatory elements. Regions of accessible chromatin, often characterized as DNase I-hypersensitive sites, have been mapped genome wide in different cell types and shown to demarcate regulatory elements such as promoters, enhancers, silencers and locus-control regions. However, remodeler studies have focused predominantly on understanding of the mechanism of ATP-mediated catalysis of nucleosome movement in vitro. Less is known concerning their distribution throughout the genome and their individual roles in specific chromatin reorganization processes. In vitro activity analysis demonstrates that a common reaction mechanism is shared by remodeling complexes, suggesting that the functional differences seen between individual complexes in vivo may be due to regulatory differences. Indeed, the interaction of complexes with cofactors and the targeting of remodelers to specific modified regions of chromatin have been linked to distinct and, in some cases, opposing functions. In particular, the recruitment of complexes by either repressors or activators to areas of accessible and inaccessible chromatin, respectively, would contribute to region-specific activities of different complexes within the cell. The importance of these systems in cell-selective gene expression has attracted increasing attention.

To gain an understanding of the potential interplay between multiple remodeling systems and their functions in cells, we have begun to build a comprehensive map of remodeler localization and genome-wide function in mouse cells. Using mutant variants of Brg1, Chd4 and Snf2h, we directly assigned remodeling activity at individual sites, demonstrating that each remodeler contributes to chromatin accessibility. Unexpectedly, many regions of accessibility require the concerted actions of all three proteins. Thus, we propose a general mechanism wherein the genome-wide organization of nucleosomes is a dynamic process requiring the activity of multiple remodeling systems.

RESULTS

Localization of three chromatin-remodeler proteins genome wide

To expand the understanding of the interplay between remodeler proteins, we focused on the remodelers Brg1, Snf2h and Chd4, which are from the SWI-SNF, ISWI and CHD families, respectively. Recent reports suggest that these remodelers perform unique roles in the regulation of chromatin structure, thus making them ideal candidates for use in our studies. To begin our analysis of how these proteins function in vivo, we mapped their genome-wide locations in mouse mammary epithelial cells by chromatin immunoprecipitation sequencing (ChIP-seq), using both specific monoclonal and polyclonal.
antibodies (Supplementary Fig. 1a,b). Binding profiles of individual genomic regions demonstrate that each protein binds to defined locations within the genome (Fig. 1a and Supplementary Fig. 1c) and is characterized by a mix of binding events. In particular, we found two major site types composed of locally distributed regions characterized by single, defined peaks, nearly the size of a transcription-factor binding site (~150 bp)\textsuperscript{19,20}, as well as regions more broadly distributed. We found the average size of a remodeler site to be 638 bp (589 bp for Brg1, 719 bp for Chd4 and 605 bp for Snf2h), consistent with these two types of binding. In total, we identified 38,896, 37,525 and 46,614 Brg1, 719 bp for Chd4 and 605 bp for Snf2h), consistent with these two types of binding. In total, we identified 38,896, 37,525 and 46,614

When we characterized the distribution of these sites relative to annotated genes, we found similar localization patterns for each remodeler, in which ~60% of these sites were located in the promoters and bodies of genes, whereas ~40% of sites were in intergenic regions (Fig. 1b). However, analysis of binding-tag density values revealed differences between these remodelers at the level of enrichment (Supplementary Fig. 1d). Specifically, we found Brg1 enrichment to be higher at regions distal to promoters (\(P < 10^{-7}\)), whereas Chd4 and Snf2h were co-occupied by Snf2h (76%), and 65% of Snf2h sites were bound by Chd4 (Fig. 2a). In comparisons of all three remodelers' binding sites, a large proportion of sites for each remodeler were found to be shared by the other two remodelers (59%, 62% and 50% of Brg1, Chd4 and Snf2h sites, respectively) with binding-profile comparisons further supporting these findings (Fig. 2b,c and Supplementary Fig. 2).

The colocalization patterns could result from transient occupancy by separate remodelers in subsets of the cell populations or could be due to a direct interaction between these proteins. We performed coimmunoprecipitation experiments and found that although each remodeler was capable of interacting with previously identified complex members, an association between the remodelers as soluble proteins was not detectable (Supplementary Fig. 3a). We also examined potential interactions at template genomic regions by sequential ChIP (re-ChIP) analysis (Supplementary Fig. 3e–g). Very weak re-ChIP signals were present, but these signals were so low (0.00003% range) that they probably resulted from nonspecific trapping of two remodelers in the same cross-linked genomic co-occupancy by remodeler proteins

We wondered whether common binding sites were shared by these proteins, given the similar distribution patterns. To determine whether they colocalized to the same genomic regions, we performed pairwise comparisons of the genomic sites occupied by each remodeler. When we compared Brg1 and Chd4 sites, the majority of sites for each protein were shared, with 74% of Brg1 sites overlapping with 76% of Chd4 sites. Comparisons between Brg1 and Snf2h revealed that 68% of Brg1 sites were also bound by Snf2h, whereas 56% of Snf2h sites were occupied by Brg1. Similarly, the majority of Chd4 sites were co-occupied by Snf2h (76%), and 65% of Snf2h sites were bound by Chd4 (Fig. 2a). In comparisons of all three remodelers' binding sites, a large proportion of sites for each remodeler were found to be shared by the other two remodelers (59%, 62% and 50% of Brg1, Chd4 and Snf2h sites, respectively) with binding-profile comparisons further supporting these findings (Fig. 2b,c and Supplementary Fig. 2).

Figure 1 Remodeler proteins bind to distinct regions of chromatin. (a) Example ChIP-seq genome-browser views of occupancy of Brg1 (top, blue tracks), Chd4 (middle, green tracks) and Snf2h (bottom, brown tracks). Images represent tag densities (mapped sequence tags) relative to genome coordinates. For each remodeler, the lower browser image displays an expanded view of the selected region where examples of localized distributions (single peak, <500 bp) are highlighted by gray shading, and broad distributions (>500 bp) are highlighted by light-orange shading. Chr, chromosome. (b) Distributions of remodeler occupancy at annotated genic regions. Sites are classified as promoter (\(\pm 2.5\) kb from transcription start site (TSS)), exon (>2.5 kb downstream from TSS, to the last intron, not intron), distal upstream (>2.5 kb upstream from TSS), downstream (>2.5 kb downstream from TSS, not exon or intron) or intron.

- \(\pm 2.5\) kb from transcription start site (TSS): 25%
- Exon: 25%
- Distal upstream: 25%
- Downstream: 25%
- Promoter: 25%
- Intronic: 25%
Figure 2  Brg1, Snf2h and Chd4 tend to co-occupy the same genomic regions. (a) Venn diagrams displaying overlaps of binding-site occupancy between pairs of remodelers. (b) ChIP-seq genome-browser view of occupancy of Brg1 (blue track), Chd4 (green track) and Snf2h (brown track) at the same genomic coordinates on chromosome 6. Mapped sequence tags represented as tag density are indicated on the y axis. (c) An expanded view of the selected region in b. Displayed on the right is a three-way Venn diagram demonstrating the overlap between the binding sites of Brg1 (blue), Chd4 (dark yellow) and Snf2h (brown). (d) Distribution at annotated genic regions of shared and unique remodeler-binding sites. Promoter represents region ± 2.5 kb from TSS.

chromatin complex. This lack of direct interaction suggests that binding of remodelers to sites of colocalization occurs through transient, sequential binding events (described in Discussion).

To further characterize remodeler localization, we examined the tag density and distribution of both unique and shared sites to determine whether there were distinct features associated with each type of region. In analyzing average tag density values, we found that regions occupied by multiple remodelers and, in most cases, sites co-occupied by all three remodelers displayed higher enrichment levels than did sites occupied by a single remodeler (Supplementary Fig. 3b–d). Further, we found the distribution of these co-occupied sites to be very similar to the distribution patterns observed initially for individual remodeler-bound regions, with a slight increase in the number of sites at promoters (39% versus 29%–32%). In contrast, unique remodeler-binding sites (i.e., those bound by a single remodeler) tended to occur at regions distal to promoters (Fig. 2d). Taken together, our initial findings suggest that these remodelers may act in coordination at co-occupied regions. Additionally, these results indicate that the colocalization of these proteins is not exclusively a consequence of promoter occupancy, and hence of activity at promoters, but may be a feature associated with various DNA regulatory elements.

DNA-binding-factor motifs at remodeler-binding sites

A likely quality of these bound regions is their content of DNA sequence-specific protein-binding sites. Several reports indicate that chromatin-remodeler complexes interact with regulatory factors as a mechanism of targeting to chromatin21–23. To investigate this possibility, we used the de novo DNA motif–discovery program MEME24 to identify consensus-sequence motifs associated with the most enriched 2,000 overlapping and unique remodeler-bound regions. This analysis revealed significant enrichment of several motifs associated with each of these regions (P < 10^-4) (Fig. 3 and Supplementary Fig. 4). In sites co-occupied by Brg1, Chd4 and Snf2h, these included HEB, AML1, TEF and, most significantly, AP-1 (Fig. 3a). We found AP-1 to be the most enriched motif at Brg1-occupied sites, including regions uniquely bound by Brg1 (Fig. 3b and Supplementary Fig. 4a). These results are in line with previous reports of Brg1 binding to AP-1 sites and of AP-1 maintenance of baseline chromatin accessibility25,26.

Further, when we compared the binding sites of Brg1, Snf2h and Chd4 to available AP-1 ChIP-seq data27, we found that 57% of the sites shared by all three remodelers overlap with 45% of AP-1 sites (Fig. 3c and Supplementary Fig. 4b). Unlike results for Brg1, the most significantly enriched motif associated with both Snf2h and Chd4 sites, including unique sites, was CTCF (Fig. 3d and Supplementary Fig. 4c,d). Notably, the remodeler Chd8 has been shown to functionally interact with CTCF and is required for enhancer-blocking activity, but neither Snf2h nor Chd4 has been directly linked to CTCF association28. When we analyzed and compared the localization of CTCF with these remodelers, we found that 27% of Chd4 sites overlap with 25% of CTCF sites, whereas 35% of Snf2h sites overlap with 40% of CTCF sites (Fig. 3f and Supplementary Fig. 4e). Thus, the association of multiple DNA sequence-specific motifs with remodeler-bound sites may reflect the range of factors that can either interact with, recruit or be targeted by these remodelers, allowing for varied downstream effects on chromatin. Moreover, the predominance of the same factor at both highly enriched unique and shared regions emphasizes the importance of not only binding but also enrichment levels, which differ between the sites found for Brg1, Chd4 and Snf2h (Supplementary Fig. 1d).

Association of remodelers with accessible chromatin regions

Because we know that remodelers regulate the packaging of chromatin, it seemed likely that we would find these proteins at
accessible chromatin. To determine whether these proteins specifically localized to remodeled regions of chromatin, we identified genome-wide DNase I–hypersensitive sites (DHSs) by DNase I sequencing (DNase I–seq) and compared the locations of these sites to those bound by remodelers. Binding-profile comparisons between remodeler and DHSs revealed strikingly similar patterns (Fig. 4a). Global analysis of site overlap indicated that 88% of Brg1 sites, 85% of Chd4 sites and 75% of Snf2h sites correspond to sites of chromatin accessibility (Fig. 4b). Yet, for each, there are clear examples of sites not associated with DHS (Fig. 4a,b). When we compared DHSs bound by each remodeler, we discovered 21,129 sites to be co-occupied by all three proteins, indicating that the majority (91%) of the regions shared by Brg1, Chd4 and Snf2h occur at open chromatin (Fig. 4c and Supplementary Fig. 5a). In an examination of the average tag density values for remodelers bound at DHSs, we found enrichment levels to be significantly ($P < 2.2 	imes 10^{-16}$) higher at these regions than for remodeler binding away from DHS (Supplementary Fig. 5b–d). Of the sites away from DHSs, between 23% (Snf2h) and 27% (Brg1 and Chd4) were within 500 bp of a DHS, which may indicate that a small number are not truly bound to inaccessible regions but may, in fact, be the tail end of a larger remodeler site. Nonetheless, this does not undermine the finding that there are regions of remodeler binding not associated with DHSs. Results were similar for DHSs occupied by remodelers, indicating a strong correlation between the level of accessibility at these sites and binding by these specific remodelers (Supplementary Fig. 5e,f).

Analysis of genome-wide remodeling activity

Given the strong association of each remodeler with accessible chromatin and their known functions in remodeling, we next sought to determine the role of each in the regulation of chromatin accessibility. To analyze remodeling function, we took advantage of the high conservation of the catalytic ATPase domain and, for each protein, created a dominant-negative variant (denoted by ‘dn’ prefix) by mutating a conserved lysine in this region (Supplementary Fig. 6a). Similar mutations have been successfully used for these and other remodelers in yeast and mammalian cells29–34. Using these mutant constructs, we then created three stable congenic cell lines under control of the tetracycline (Tet)–off conditional system, which enabled expression of each dominant-negative variant in the absence of Tet (Supplementary Fig. 6b–d). A fourth cell line expressing the tetracycline transactivator (tTA) protein system alone acted as a control for genomic effects mediated by expression of the tTA regulatory protein in our cells. Induction of a given remodeler had no effect on expression levels for the other remodelers under consideration (Supplementary Fig. 6e–g).

To examine the genome-wide roles of these remodelers in the regulation of chromatin structure, we analyzed changes in DNase I hypersensitivity by performing DNase I–seq in the absence or presence of each dominant-negative remodeler protein. After expression of each of these variants, we found examples of chromatin where regions were unchanged (conserved), rendered inaccessible (lost) or newly opened, thus indicating that each remodeler is capable of opening and closing sites (Fig. 5 and Supplementary Fig. 7a–c). However, when we globally analyzed the effects of remodeler inhibition, we noted remodeler-specific trends in chromatin-structure regulation (Fig. 6, Supplementary Figs. 7d–f, 8 and 9 and Supplementary Table 1). In particular, we noted predominant trends in the effect on accessibility after the expression of dnBrg1 and dnChd4 (Fig. 5b–g and Supplementary Fig. 7d–f). After inhibition of Brg1 activity, 1,175 DHSs were lost, whereas approximately 3,247 DHSs were reduced at least two-fold in size, thus indicating a principal requirement for Brg1 maintenance of a subset of remodeled chromatin. In contrast, inhibition of Chd4 activity did not lead to substantial change in the size of conserved DHSs but did lead to the opening of chromatin at a subset of regions (4,688 sites), indicating a role for Chd4 in the maintenance of chromatin in a closed state. Expression of dnSnf2h also produced changes in chromatin accessibility, with
both loss and opening of a small number of sites, results consistent with a previous report of this remodeler’s involvement in controlling subtle changes in *Drosophila* nucleosome positioning. The examination of sites lost and gained in the presence of dnBrg1 and dnChd4, respectively, for DNA-binding factor–specific motifs, indicates that these sites may represent potential functional regulatory elements. Specifically, the remodelled regions that are newly opened indicates that these sites may represent potential functional regulatory elements. Specifically, the remodelled regions that are newly opened by multiple systems (Fig. 7). Some elements are primarily controlled by one remodeling system (Fig. 7a–d) and others by two systems functioning either collaboratively (Fig. 7e) or in opposition (Fig. 7f, g). There are also examples in which all three remodelers function at a single element (Fig. 7h–k). We tabulated occurrences frequencies for each of the 27 possible interaction classes for over 95,000 individual sites pooled from the three dominant-negative cell lines (Supplementary Table 1 and Supplementary Fig. 9). These values represent underestimates because of the following considerations: (i) There are likely to be cases in which the equilibrium result of multiple functional systems may lead to compensating effects. If one system is abrogated by activation of the dominant negative, the contribution of a second system may increase, thus masking the effect. (ii) Many members of the large class of sites that manifest no change with any of the remodelers could originate from other members of the large family of remodeling systems in mammalian cells.

We also examined effects of the dominant-negative remodelers on selective gene expression by global analysis of RNA expression patterns with activation of each dominant-negative remodeler (Supplementary Fig. 10a–e). Two examples are shown for genes that are strongly derepressed with dnChd4. In each case, a DHS element immediately upstream of the promoter is dramatically induced by activation of dnChd4. Importantly, induction of wild-type Chd4 has no effect on expression. In summary, more than 800 genes are deregulated by the three dominant-negative remodelers (Supplementary Fig. 10f).

**DISCUSSION**

Many multiprotein remodeling complexes have been characterized, but how these complexes interact with each other, and with site-specific DNA-binding proteins, to generate specific chromatin structures remains a conceptual puzzle, especially in higher eukaryotes. Through ChIP-seq analysis with specific antisera, coupled with the controlled expression of inducible dominant-negative
remodeling-system variants, we addressed this problem by assigning site-specific remodeling activities, genome wide, for three remodeling systems in mouse cells. We report that each system is capable of both opening and closing chromatin. Although remodeling systems are generally considered to be involved in the repositioning or disassembly of localized nucleosome structures, there is also a precedent for reversing access, or ‘closing’, localized structures, presumably by reassembly of nucleosome structures. Alternatively, repositioning of nucleosome(s) could modulate factor binding in a way that would lead to the return of localized chromatin structure to a closed state. Our data sets reveal the widespread, and unexpected, extent of this chromatin closing phenomenon (Fig. 5). Although Chd4 is the most frequent participant in chromatin closing events, the other two remodelers are also capable of catalyzing this process.

Current models regarding the mechanism of remodeling action suggest that a unique remodeling system is recruited to a given DHS element. However, of the approximately 90,000 DHS elements that we have characterized in this study, a substantial fraction are associated with multiple remodeling systems. Indeed, we identify

Figure 5 Remodeler-protein distribution at conserved and lost sites. (a) Browser-view examples of DHSs (DNase I–seq) in the absence (+Tet) or presence (–Tet) of each dominant-negative (dn) remodeler. Tag densities (y axis) are indicated for sites located at the displayed genomic coordinates. (b–g) Aggregate plot of average DHS tag density values over conserved and lost sites for dnBrg1 (b), gained sites for dnBrg1 (c), conserved and lost sites for dnChd4 (d), gained sites for dnChd4 (e), conserved and lost sites for dnSnf2h (f) and gained sites for dnSnf2h (g). The shaded areas are up to ±s.d. from the average profile.

Figure 6 Trends in remodeler-protein regulation of chromatin accessibility. DNase I hypersensitivity (DHS tag density) of DHSs after expression (–Tet, y axis) of each dominant-negative (dn) remodeler. Insets, expanded views of selected scatter-plot regions. Conserved (green) are sites that existed before and after the expression of dominant-negative remodeler; lost (red) are sites lost after expression of dominant-negative remodeler; and gained (blue) are newly opened sites. Solid red line, trend line used to indicate direction of DHS tag density change after negative remodeler expression. Dashed black line, diagonal line indicating position of trend line if there were no changes in hypersensitivity. Control –Tet/+Tet distributions for parental cells with no negative remodelers are shown in Supplementary Figure 8.
Figure 7  Multiple remodelers contribute to the regulation of individual DHSs. (a–k) Examples of DHSs affected by expression of each of the indicated dominant-negative remodelers. (a–d) Single remodeler effects. (e–g) Double synergism. (h–k) Triple synergism. For comparison, the −Tet induction of dominant-negative tracks are displaced down and to the right (black coordinates, +Tet; red coordinates, −Tet). ± dSnf2h (pink, −Tet; red, +Tet); ± Tet regulator control (Gray, −Tet; black, +Tet). Tet regulator control, cell line expressing only the tetracycline transactivator protein. Red arrows denote increases or decreases in accessibility; dashed horizontal line indicates no change. (I) Mechanism of dynamic transitions in chromatin structure mediated by transient recruitment of remodelers and their associated activity. Remodeler complexes are targeted to a nucleosomal region by specific DNA-bound factors. Both events—remodeler recruitment and factor binding—are transient. Transitions may involve a unique remodeler or multiple complexes acting sequentially (right). Furthermore, some reactions may lead to chromatin closing rather than opening (left). Thus, localized chromatin states monitored by current methodologies represent population averages of complex processes that sometimes involve multiple remodeling systems.

many DHSs bound by all three remodelers. Our studies also show that these redundant systems not only co-occupy the DHS elements but also function in many combinatorial modes involving both synergistic and antagonistic action (Fig. 7a–k). Verrijzer and colleagues recently reported little overlap in remodeler distributions genome wide in Drosophila. Yet, further support for the potential contributions of redundant remodelers comes from studies in yeast wherein deletions of multiple remodelers resulted in numerous alterations in chromatin structure, but individual deletions produced relatively minor effects. Given the much-higher complexity of mammalian genomes, it seems possible that more-complex mechanisms have evolved to provide control of chromatin remodeling in these systems.

Additionally, studies are emerging that support antagonistic remodeler-complex functions at individual sites. Given these findings, we propose a general mechanism of cyclical, targeted remodeler activity to maintain a fluid chromatin state (Fig. 7I). Under this model, chromatin remodelers and transcription factors both cycle dynamically through a complex series of states. The nature and sequence of these states would be dependent on both the chromatin context (histone and DNA marks) and the specific transcription factors that recruit remodelers to the site. An important consideration is the timescale limitations inherent to ChIP and DHS assays, which give the appearance of relatively static states because the signals are averaged across heterogeneous cell populations. However, many lines of evidence support highly transient interactions of remodelers and DNA-binding proteins during remodeling, consistent with the model discussed here.

How remodelers are targeted to specific sites in chromatin remains a central question. Motifs for transcription factors cluster at regulatory elements such as enhancers and promoters, thus providing binding sites for many factors that could, in turn, recruit multiple remodeling systems. It is also well established that a specific remodeling system can be required for function at selected elements, as is observed for neural development. In our analysis of recognition elements present at remodeler-bound sites, we identified several distinct motifs pointing to a subset of factors involved in selective recruitment. Regulatory-protein motifs most frequently associated with each remodeler (AP-1 for Brg1 and CTCF for Chd4 and Snf2h) represent constitutive nuclear proteins that have previously been linked to the regulation of chromatin accessibility and genomic organization. Specifically, we observed that blocking of AP-1 binding to chromatin reduced chromatin accessibility at glucocorticoid-receptor response elements in mammary cells. Presumably, this occurs through AP-1 targeting of a remodeling complex. Although transcription factors may recruit selected remodeling proteins by direct protein-protein contacts, it is also likely that specific histone modifications at a given site contribute to remodeler selectivity. Many of the remodeling proteins contain bromo-chromo domains, which recognize specific histone marks. Thus, selective recruitment is likely to be achieved through the combined action of site-specific DNA-binding proteins and histone modifications; however, the enzymes directing these marks are also recruited in turn by transcription factors.

The finding of a functional involvement of multiple remodeling systems at many DHSs implies a directed, or sequential, order of recruitment.
DHSs completely lost or gained after the suppression of a single remodeler are the simplest case. For Brg1, several sites completely lost in the presence of the mutant variant were, in fact, bound by multiple remodelers. In this case, it is likely that the activity of Brg1 is an initial event, whereas the functions of other remodelers bound to the same site are unclear. Reports of sites bound by Brg1–Chd4–NuRD demonstrated a NuRD-complex dependence on Brg1 binding and loss of a dependence for Brg1 on the NuRD complex.39,42 Thus, the activity of the initial recruited complex may affect the recruitment and activity of subsequent complexes. However, for the majority of elements characterized in our study, multiple systems are shown to be not only present but also functional. It will be very difficult to determine the order of events in these complex examples through whole-cell investigations. Biochemical reconstruction of these processes in vitro will eventually contribute to a detailed understanding of the sequence of events.

Given the large number of remodeler complexes in the mammalian genome, the potential number of remodeler interactions with chromatin is clearly extensive. The findings presented here demonstrate a further unanticipated complexity: A process that seems to move from one static state to another when examined by population-averaged and time-averaged methodologies is in fact highly dynamic. The localized reorganization of nucleosome structures will require intensive examination, both in vivo and in vitro, to develop a detailed understanding of these important processes.

METHODS

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

Accession codes. ChiP-seq data have been deposited in the Gene Expression Omnibus database under accession code GSE53585.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.A.M. and G.L.H. conceived of and designed the study. S.A.M., M.W., T.A.J. and R.L.S. performed the experiments. S.I. provided technical advice. S.B. and M.-H.S. conducted the bioinformatics analysis. S.A.M. performed the experimental analysis and data interpretation. S.A.M. and G.L.H. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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**ONLINE METHODS**

**Cell lines and culture conditions.** The 3134 mouse mammary epithelial cell line, originally derived from a subclone of 904.13 (ref. 37), was maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, and 5 mg/mL penicillin-streptomycin in a 37 °C incubator with 5% CO2. To generate cell lines expressing dominant-negative variants, the coding sequence of human SNF2H (hSNF2H) fused to the Flag tag sequence at the N terminus in the pCII-neo vector (a gift from D. Pickets (University of Ottawa)) was used. Using BamHI and SalI restriction-enzyme sites, Flag-hSNF2H was cloned into a tetracycline-inducible retroviral vector (pRevTRELink)59. This was followed by site-directed mutagenesis of Lys211 to arginine with the QuikChange XL Site Directed Mutagenesis Kit according to the manufacturer’s instructions (Stratagene, La Jolla, CA). This dominant-negative variant of Flag-hSNF2H (dnSnf2h) was then stably integrated into a cell line containing the tetracycline transactivator regulatory system (3134Tet, 7110), as described previously59. Similarly, the coding sequence of mouse Chd4 (a gift from J. Saven (University of Wisconsin)) was cloned into pRevTRELink containing a triple Flag tag sequence with NotI/SalI restriction-enzyme sites. This was followed by site-directed mutagenesis of Lys250 to cysteine and integration into the 3134Tet cell line. The tetracycline-inducible dnBrg1 cell line was described previously59. Constructs were fully sequenced to confirm accuracy before cell-line integrations. All tetracycline-regulated cell lines were maintained in DMEM and 5 μg/mL tetracycline to repress expression of the dominant-negative proteins. For experiments, cells were plated in DMEM supplemented with 10% charcoal-dextran–treated FBS with or without tetracycline for 48 h.

**Chromatin immunoprecipitation.** Chromatin immunoprecipitation (ChIP) experiments were performed per standard protocols (Millipore, Billerica, MA) with minor modifications. Briefly, cells were cross-linked for 10 min with 1% formaldehyde at 37 °C. This reaction was subsequently quenched with 150 mM glycine for 10 min. Each ChIP contained 400 μg of soluble, sonicated chromatin. DNA–protein complexes were immunoprecipitated with the following antibodies: anti-BRG1 (1 μg, EPNCIR11A, made in collaboration with Epitomics, Burlingame, CA), anti-Chd4 (7 μg, ab24718, Abcam, Cambridge, MA), anti-SNF2H (5 μg, ab27499, Abcam), and anti-CTCF (10 μL, 07-729, Millipore, Billerica, MA); validation of the antibodies is provided on the manufacturers’ websites. DNA isolated from ChIPs was purified and/or confirmed by real-time quantitative PCR amplification with SYBR green mix (Bio-Rad, Hercules, CA). Primer sequences were available upon request. Three samples from two biological replicates were pooled as a single sample before generation of sequencing libraries. Two replicates per condition were sequenced.

**Preparation of DNase I–digested DNA.** DNase I–digested DNA was prepared for sequencing as previously described with minor modifications5. Briefly, expression of dominant-negative–variant proteins was induced by removal of tetracycline for 48 h before harvest by trypsinization. Nuclei from harvested cells were isolated and digested with 60–80 U/mL DNase I (Roche, Indianapolis, IN) for 3 min at 37 °C. Digested DNA was incubated at 55 °C with 10 μg/mL RNase A (Roche) for a few hours to overnight. This was followed by addition of 25 μg/mL Proteinase K (Ambion, Austin, TX) and incubation at 55 °C for at least 4 h. DNA fragments were purified by phenol-chloroform extraction and ultracentrifugation through a sucrose gradient. After purification, fragments (between 100 and 500 bp in size) were then pooled, precipitated and assembled into libraries for sequencing.

**ChIP-seq and DNase I–seq data analysis.** Sequence reads (36-mer) were generated for ChIP-seq and DNase I–seq experiments on the Illumina Solexa genome-analyzer platform, and tags were uniquely aligned to the mouse reference genome (UCSC mm9 assembly). A description of the replicate data sets is presented in **Supplementary Table 2.** In total, 58.6 million, 69.3 million, 56.7 million, and 8.4 million uniquely aligned reads were obtained for Brg1, Chd4, Snf2h, and CTCF, respectively. For the DNase I–seq experiments, 39.7 million, 32.8 (39.3) million, 17.5 (40.2) million, 57.6 (52) million, and 54.8 (50.1) million uniquely aligned reads were obtained for 3134, 3134Tet +Tet (−Tet), dnBrg1 +Tet (−Tet), dnChd4 +Tet (−Tet), and dnSnf2h +Tet (−Tet), respectively. Regions of enriched tags known as ‘hotspots’ were called and determined significant with previously described algorithms and methods, with minor modifications60,61. Briefly, ChIP-seq and DNase I–seq data–set tag density values were normalized to 10 million reads to adjust for differences in sequencing depth and to allow for comparisons across data sets before hotspots were called at 0% FDR. Replicate concordance was then calculated between replicates. ChIP-seq data sets were additionally normalized by subtraction of tags found in the corresponding input data (sequenced sonicated genomic DNA after sequencing depth normalization). In all data, artifacts from sequencing (small regions of high-density tags) were filtered out, including satellites, long interspersed repetitive elements, and short single tandem repeats after extension of these regions on either side to 150 bp. To increase the robustness of called ChIP-seq hotspots, tag density thresholds were determined and applied on the basis of the calculated mode for each set. The final hotspots for each group were required to exceed this value and were 16 for Brg1, 8 for Chd4, and 9 for Snf2h. In comparisons of data sets, regions were considered to overlap if at least 2 bp were shared. Changes in chromatin accessibility in the presence or absence of dominant-negative variants (changes in DNase I–seq hotspot tag density values in the presence or absence (expression of dominant-negative variant) of tetracycline (Tet)) were determined by initial filtering of each set of hotspots against hotspots generated in the control 3134Tet cell line. Specifically, hotspots with −Tet/+Tet ratios ≥2 in the 3134Tet data were removed in the corresponding dominant-negative sets. Hotspots in these cell lines were defined as ‘lost’ if the site was found only in the +Tet (dominant-negative variant not expressed) DNase I–seq data, ‘gained’ if found only in the −Tet (dominant-negative variant expressed) DNase I–seq data, and ‘conserved’ if found in both the +Tet and −Tet DNase I–seq data sets. To rule out differences created by comparisons of very small hotspots (for example, the difference between 24/12 is the same as 4/2), tag density thresholds based on mode were applied to dominant-negative–variant DNase I–seq hotspots before classifications. These values were 16 for dnBrg1 +Tet, 9 for dnBrg1 −Tet, 8 for dnChd4 +Tet, 5 for dnChd4 −Tet, 8 for dnSnf2h +Tet, and 8 for dnSnf2h −Tet.

**Western blots.** Cells were grown with and without tetracycline (for dominant negative–variant expression) for 48 h. This was followed by two washes in cold PBS and suspension in PBS containing protease inhibitors (EDTA–free complete protease inhibitor cocktail, Roche). Cell pellets isolated by centrifugation were then suspended in buffer (50 mM Tris, pH 8.0, 250 mM NaCl, 5 mM EDTA, 20 mM Na2HPO4, and 0.1% NP-40) containing protease inhibitors and lysed by freeze thaw (3×). Protein concentrations of collected supernatants (whole cell lysates) were measured by Bradford assay, and 50 to 75 μg of cell lysates were separated by electrophoresis on 3–8% NuPAGE Novex Tri-acetate gels (Invitrogen) and transferred to PVDF membranes. After being blocked in TBST containing 5% milk, membranes were probed with the following primary antibodies: anti-Flag (1:1,000, F1804, Sigma-Aldrich, St. Louis, MO), anti-tetracycline transactivator (TET) (1:5,000, TET01, MobiTec, Boca Raton, FL), and anti-Actin (1:500, sc-1615, Santa Cruz, Santa Cruz, CA) in TBST with 5% milk overnight at 4 °C. Validation of these antibodies and reference citations are provided on the manufacturers’ websites. After several washes, membranes were probed with HRP-conjugated secondary antibodies, extensively washed, and visualized with Super Signal C-D Western blot detection reagent (Pierce (Thermo Scientific), Rockford, IL). Membranes were exposed to X-ray film to capture images.

**De novo DNA sequence motif-discovery analysis.** Analysis of de novo DNA sequence motifs was performed on ChIP and DNase I–seq hotspots with the MEME algorithm62. For ChIPs, 150–bp peaks derived from the top 2,000 hotspots (by tag density) were analyzed for each of the indicated groups, and the top 1,000 hotspots from the selected DNase I data sets were analyzed with a width of 150 bp. The minimum and maximum motif sizes were 8 bp and 40 bp, respectively, with a maximum of 50 motifs used for the search. After MEME analysis, motif comparisons and identification of enriched sequences in unknown motifs (MEME E values < 10−5) were performed with a TOMTOM search against the Transfac database of characterized transcription-factor motifs. Matches were considered significant if the majority of sequence nucleotides were shared and P values were <10−4.

**Preparation of nuclear extracts.** Nuclear extracts were prepared according to standard nuclear-fractionation protocols (Abcam). For chromatin immunoprecipitation experiments, 200 μg of nuclear extract were incubated overnight in a total volume of 1 ml at 4 °C with or without 1 μg of antibody (anti-BRG1 (Epitomics),
Preparation of RNA and quantitative real-time PCR analysis. RNA was extracted from cells, grown in media with or without tetracycline for 48 h, according to standard methods with TRizol reagent (Invitrogen) and purified with the RNeasy Mini Kit (Qiagen, Valencia, CA). All RNA samples were treated with RNase-free DNase (Qiagen). After purification, reverse transcription of total RNA was performed with the Bio-Rad cDNA Synthesis Kit via the manufacturer’s instructions and analyzed by real-time quantitative PCR with SYBR green (Bio-Rad). Primer sequences are available upon request.

Statistical analysis and calculation of P values. Significantly different P values between box-plot data sets were determined by calculation of means and analysis of these values with a Kolmogorov–Smirnov test (R function KS.test). For box plots with multiple comparisons, significant differences between means were determined for pairwise comparisons by variance analysis (one-way ANOVA) combined with Tukey’s honestly significant difference (HSD) test.

For Supplementary Figure 1d, we performed the nonparametric Kruskal–Wallis rank-sum test for the equality of tag density medians on peaks among the five groups. For all three chromatin remodelers (Brg1, Chd4 and Snf2h), the test results correspond to P values less than 2.2 × 10−16. Therefore, the null hypotheses were rejected, and we concluded that tag densities were not identically distributed among the groups. Next, Tukey’s HSD test was performed for the equality between all possible pairs of groups. For Brg1, the test showed that the density mean of promoter hotspots was significantly lower than those of the other groups (all four corresponding P values < 2.2 × 10−16). However, for Chd4, only two pairwise comparison tests between (intronic versus downstream and promoter versus downstream) have P values less than 0.001, adjusted for multiple comparisons with Bonferroni correction.

Sequential ChIP (re-ChIP) analysis. Re-ChIP experiments were performed as described previously59, with changes as follows. The first IP was performed

as described in John, et al.59 with the exception of the elution step, which was performed in 10 mM DTT at 37 °C for 30 min. DTT elutions were diluted 1:60 in ChIP dilution buffer, and antibodies for the second IP were then added for overnight incubation at 4 °C. The Re-ChIP IPs were then processed per ref. 59.

Analysis of cross-regulation by dn-remodeler proteins. For Supplementary Figure 6e–g, all cells were maintained in DMEM (Invitrogen) supplemented with 10% Tet-approved FBS (Clontech cat. no. 631101) and 10 μg/ml tetracycline. Cells were washed twice with PBS and trypsinized and 1 million cells plated in 10-cm dishes either in the presence or absence of Tet. Cells were collected 48 h later by trypsinization and pelleted in growth media. The cells were washed twice with PBS, and the pellet was lysed in 100 μl of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris, pH 8.0) supplemented with a protease-inhibitor cocktail (Sigma cat. no. P2714). The protein concentrations of the resulting cell extracts were measured with the Pierce BCA Protein Assay Kit (cat. no. 23227). 15 μg of each cell extract was loaded onto a 15-well 1-mm NuPAGE 4–12% bis-Tris gel (Invitrogen, cat. no. NP0323) along with 8 μl of Novex SeeBlue prestained marker (Invitrogen cat. no. 100006636) and run at 200 V in the MOPS NuPAGE buffer system. The proteins were transferred to a nitrocellulose membrane in Tris-glycine transfer buffer supplemented with 0.037% SDS at 400 mA for 2 h to facilitate transfer of high-molecular-weight proteins. The membranes were blocked with 5% nonfat dry milk (NFDM) and probed with the following antibodies diluted in NFDM: rabbit anti-Flag polyclonal 1:2,000 (Sigma, F7425, lot 068K4800); mouse anti-Chd4 monoclonal 3F214 1:2,000 (Abcam, ab70467, lot GR104037-3); rabbit anti-Snf2h/ISWI polyclonal 1:2,000 (Bethyl, A301-017A, lot A301-017A-1); rabbit anti Brg1 custom monoclonal antibody (described above) 1:20,000; mouse anti-GAPDH 6C5 monoclonal antibody (Abcam, ab8245, lot 917777). The blots were probed with anti-IgG antibodies for the appropriate species conjugated to HRP at 1:5,000 in NFDM (Jackson Labs goat anti-mouse, cat. no. 115-035-003 and goat anti-rabbit, cat. no. 111-035-144). Validation of these antibodies and reference citations are provided on the manufacturers’ websites. Protein bands were detected with Pierce SuperSignal West Pico Chemiluminescent Substrate (Pierce cat. no. 34080, lot MC154418) and imaged on a Bio-Rad ChemiDoc MP imaging system.

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**Corrigendum: Overlapping chromatin-remodeling systems collaborate genome wide at dynamic chromatin transitions**

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In the version of this article initially published, the ChIP-seq data were not available in a public repository. The error has been corrected in the HTML and PDF versions of the article.