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Molecular Architecture of Transcription Factor Hotspots in Early Adipogenesis

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SUMMARY

Transcription factors have recently been shown to colocalize in hotspot regions of the genome, which are further clustered into super-enhancers. However, the detailed molecular organization of transcription factors at hotspot regions is poorly defined. Here, we have used digital genomic footprinting to precisely define factor localization at a genome-wide level during the early phase of 3T3-L1 adipocyte differentiation, which allows us to obtain detailed molecular insight into how transcription factors target hotspots. We demonstrate the formation of ATF/C/EBP heterodimers at a composite motif on chromatin, and we suggest that this may be a general mechanism for integrating external signals on chromatin. Furthermore, we find evidence of extensive recruitment of transcription factors to hotspots through alternative mechanisms not involving their known motifs and demonstrate that these alternative binding events are functionally important for hotspot formation and activity. Taken together, these findings provide a framework for understanding transcription factor cooperativity in hotspots.

INTRODUCTION

The recent increase in the number of genome-wide maps of transcription factor binding has made it increasingly clear that transcription factors do not work alone but rather collaborate with other transcription factors at genomic target regions. Colocalization of two cooperating factors has been observed in several different systems, e.g., activating protein 1 (AP1) has been shown to regulate the chromatin accessibility at glucocorticoid receptor (GR)-binding sites in mammary cells (Biddie et al., 2011), FoxA1 regulates the estrogen receptor (ER)-activated gene program by directly targeting ER-binding sites (Carroll et al., 2005; Hurtado et al., 2011), PU.1 cooperates with several different factors at specific target sites including CCAAT/enhancer-binding protein β (C/EBPβ) (Heinz et al., 2010), and peroxisome proliferator-activated receptor γ and C/EBPα-binding profiles overlap extensively in mature adipocytes (Letterova et al., 2008; Nielsen et al., 2008) and cooperate in gaining access to chromatin (Madsen et al., 2014). In addition to cooperation between transcription factor pairs, we (BoergeSEN et al., 2012; Siersbæk et al., 2011; 2014, this issue of Cell Reports) and others (Chen et al., 2008; Gerstein et al., 2012; He et al., 2011) have demonstrated that multiple transcription factors can target the same genomic regions that we refer to as transcription factor hotspots (Siersbæk et al., 2011). Cooperative binding of transcription factors is also the basis for modeling of cis-regulatory modules determining cell specificity (Frith et al., 2003; Krivan and Wasserman, 2001). However, chromatin immunoprecipitation sequencing (ChIP-seq), which is the most widely used method to map transcription factor binding, has a relatively low resolution and does not discriminate between direct protein binding to DNA and indirect recruitment to chromatin through protein–protein interactions. The molecular architecture (i.e., the organization) of transcription factors at hotspots is therefore poorly understood.

Here, we have used digital genomic footprinting to precisely define protein localization for several adipogenic transcription factors at a genome-wide level. In combination with ChIP-seq data, these analyses reveal molecular insight into the organization of transcription factors at hotspot regions, which provides a framework for understanding transcription factor cooperativity on chromatin.

RESULTS

Digital Genomic Footprinting Reveals Precise Protein Footprints at a Genome-wide Level

To begin to understand how transcription factors communicate in hotspots, it is essential to know how the factors are organized at these regions, including which factors are engaged in direct DNA interactions. We therefore employed high-resolution digital genomic footprinting (Figure 1A), a recently developed method to identify protein footprints (i.e., areas of restricted nucleosome access) within DNase I hypersensitive (DHS) regions based on ultradepth sequencing (>100 M sequence tags) of DHS-seq.
libraries (Boyle et al., 2011; Hesselberth et al., 2009; Neph et al., 2012). We used an algorithm that we have recently developed to efficiently identify protein footprints at a genome-wide level with high sensitivity and specificity (M.-H.S., M. Guertin, S.B., and G.L.H., unpublished data). Based on analysis of 129,028 DHS regions detected 4 hr after induction of 3T3-L1 adipogenesis, we identify ~330,000 protein footprints (false discovery rate = 1%) using this algorithm. These footprints have a median size of 532 bp (Figure S1A). To assign the identified footprints to specific transcription factors, we combined motif analyses with ChIP-seq data for several transcription factors involved in the early phase of adipocyte differentiation (Siersbaek et al., 2011, 2014). Footprints were assigned to specific factors based on the presence of a ChIP-seq peak for that factor as well as a predicted binding site using the known position weight matrix for the same factor (unless otherwise noted, predicted sites discussed below will always refer to predictions made in this way—see Supplementary Experimental Procedures for more details). As illustrated in Figure 1, this approach revealed strong footprints for several factors. The localized decrease in DNase I digestion at footprints is highly correlated with a colocated peak in sequence conservation (Figures 1B–1E), indicating that generally these footprints occur at functionally important sites.

C/EBP-ATF Footprints at a Composite Motif Demonstrate Heterodimer Formation on Chromatin

The high resolution of genomic footprinting allows us to obtain information about protein localization within ChIP-seq peak regions (see Figure 1B for an example of the high resolution of footprinting analyses compared to ChIP-seq. Note the different scales used for visualization of DNase I cut counts and ChIP-seq data. Average DNase I cut counts and phastCons score in the vicinity of C/EBPβ footprints are shown at the bottom. (C–E) Average DNase I cut counts as in (B) for JunB (C), KLF5 (D), and PBX1 (E). See also Figure S1.)
where ATF and C/EBP footprints overlap (Figure 2B, left). C/EBP-ATF heterodimers have previously been shown to bind to this DNA sequence in vitro (Mann et al., 2013; Shuman et al., 1997; Vinson et al., 1993), and our data provide direct evidence that such heterodimers also bind to this element in a chromatin context in cells. Importantly, the composite DNA element is significantly enriched at shared ATF-C/EBP regions identified by ChIP-seq (Figure 2C), indicating that it specifically directs ATF-C/EBP heterodimer formation. Consistent with heterodimer formation on this composite DNA element, ATF7 and C/EBP simultaneously occupy all three investigated predicted sites in re-ChIP experiments (Figure S2). Taken together, this suggests that alternative heterodimer formation on composite binding sites may be an important mechanism through which C/EBPs and ATFs are targeted to specific genomic regions and thereby integrate different signaling pathways on chromatin.

**Extensive Transcription Factor Recruitment to Hotspots through Alternative Mechanisms**

Intriguingly, investigation of the GR motif occurrence at distal GR-binding sites (>2 kb away from the transcription start site [TSS]) revealed that the binding site prediction score (i.e., the degree of resemblance of a predicted site to the GR motif) decreased with the number of co-occupying factors (Figure 3A). This suggests that the sequence-specific constraints for GR binding are relaxed when many other factors bind to the site.
This is not due to a general relaxation of binding constraints to open chromatin in hotspots, because binding site prediction scores for STAT, PBX1, and ATF are unaffected and prediction scores for C/EBP and AP1 increase with the number of factors at these distal GR-binding sites. The fact that prediction scores for C/EBP and AP1 increase at these sites furthermore suggests that C/EBP and AP1 factors may play a direct role in facilitating GR binding. A similar pattern of prediction scores was observed for distal STAT- and PBX1-binding sites (Figures 3B and 3C), indicating that, also for these factors, binding is less constrained than their motifs indicate and more dependent on AP1 and C/EBP sites when binding in hotspots.

To further investigate the mechanisms of binding of transcription factors to hotspots, we took advantage of the information obtained from our footprinting analyses that allows us to identify direct binding events of transcription factors to DNA. Using this approach, we subdivided ChIP-seq peaks for each factor into three different categories (Figure 4A). First, peaks characterized by the presence of a footprint with the corresponding predicted transcription factor site could be classified as transcription factor footprints (i.e., regions where the factor binds directly to its predicted site). Second, peaks characterized by the presence of a predicted binding site for that factor but the absence of an associated footprint in the DNase I digestion pattern were classified as indeterminate binding events. Although the presence of a predicted binding site would indicate that the factor binds directly to DNA, the reason for the absence of a footprint could be many, including short residence time of the protein on DNA (Voss and Hager, 2014). Third, peaks characterized by the absence of a predicted binding site for the corresponding factor were classified as alternative binding events. Alternative binding events may stem from indirect binding to DNA via tethering to other factors or from direct binding of factors to alternative sites in the DNA possibly assisted by other DNA bound factors (Figure 4A). As expected, and consistent with previous findings (Neph et al., 2012), transcription factor footprints have a significantly higher ChIP-seq signal compared to alternative binding events (Figures 4B and S3A). Based on the
Figure 4. Extensive Recruitment of Transcription Factors to Hotspots through Alternative Mechanisms Not Involving Their Predicted Sites orCanonical Binding Preferences

(A) Illustration of the classification of ChIP-seq regions into three different types: (1) regions containing a footprint for the factor, (2) indeterminate binding events characterized by the presence of a predicted binding site that is not found in a footprint region, and (3) alternative mechanisms of recruitment, where no predicted binding site for the factor is found at all.

(B) Boxplots show the binding intensity (i.e., the number of ChIP-seq tags) for C/EBPβ and JunB at footprints and binding sites where these factors are recruited through alternative mechanisms. See also Figure S3A.

(C) Colocalization of footprints and alternative binding events for each transcription factor pair. The fraction of alternative binding events for the factors on the y axis that colocalizes with footprints for the factors on the x axis is shown.

(D) Percentage of DHS sites (black) and footprints for C/EBPs (green), AP1 factors (yellow), and KLFs (red) located in promoters of annotated genes. The proximal promoter is defined as the 1 kb region immediately upstream of the TSS. *p < 0.01 as determined by Fisher’s exact test.

(legend continued on next page)
classification of binding events for each factor described above, we can correlate these different mechanisms of transcription factor binding for all transcription factor pairs. Indeterminate binding events are omitted from these analyses as it is not clear whether or not the corresponding predicted binding site is involved in recruitment of the factor to chromatin. Interestingly, footprints for C/EBPs, KLFs, and AP1 factors (i.e., c-Jun, JunB, and FOSL2) are highly associated with alternative binding events for all other factors (Figure 4C). Taken together with the motif analyses described above (Figure 3), this indicates that these factors may function through direct interactions with their known binding sites to facilitate recruitment of other factors either through indirect binding (tethering) or through direct DNA binding to sites not corresponding to their canonical motifs by dynamic assisted loading (Voss et al., 2011).

Importantly, the footprints for C/EBPs, KLFs, and AP1 factors are significantly enriched in the vicinity of genes that are induced during the first 4 hr of differentiation (Figure S3B), strongly suggesting that these regions are in fact involved in regulating gene transcription. Interestingly, KLF footprints are significantly enriched in the proximal promoter region of annotated genes relative to all DHS sites, whereas footprints for the AP1 factors JunB, c-Jun, and FOSL2 are depleted in sites relative to DHS sites (Figure 4D). This indicates that KLF factors function as facilitating factors that preferentially bind directly at promoters in addition to distal elements, whereas AP1 factors almost exclusively bind directly to their predicted binding sites in distal enhancers. Interestingly, assigning genes that are induced during the first 4 hr of differentiation to hotspots where different factors form a footprint reveals that these hotspots are associated with distinct sets of Gene Ontology (GO) categories (Figure 4E). Thus, different facilitating factors appear to drive the formation of hotspots that control different gene programs.

Taken together, these detailed footprinting analyses suggest that alternative mechanisms of transcription factor recruitment not involving the known factor motifs are central for hotspot formation and that especially C/EBPs, AP1, and KLF proteins may be involved in facilitating alternative mechanisms of recruitment of other factors (Figure 4F).

**Alternative Mechanisms of C/EBPβ Recruitment Are Functionally As Important as Direct Binding to Predicted C/EBP Sites**

To investigate the relative functional importance of footprints and alternative binding events for hotspot formation, we first analyzed the effect of C/EBPβ depletion on recruitment of transcription factors to hotspots where C/EBPβ binds directly to its predicted binding sites in DNA relative to hotspots where C/EBPβ binds through alternative mechanisms. Surprisingly, alternative STAT5A and VDR recruitment to these two types of hotspots is impaired to the same extent by C/EBPβ depletion (Figure 5A). Importantly, STAT5A and VDR recruitment to sites without C/EBPβ binding is largely unaffected by C/EBPβ knockdown. This indicates that alternative mechanisms of C/EBPβ binding to hotspots are functionally as important as direct binding to its predicted sites for the recruitment of additional transcription factors. Consistent with a functionally important role of alternative binding mechanisms, MED1 recruitment is similarly affected at C/EBPβ footprints and alternative C/EBPβ-binding regions by knockdown of C/EBPβ, whereas MED1 recruitment to binding regions without C/EBPβ is unaffected (MED1 ChiP-seq data were obtained from Siersbaek et al., 2014) (Figure 5B). Furthermore, RNA-seq in 3T3-L1 cells depleted of C/EBPβ revealed that early induced genes in the vicinity of hotspots where C/EBPβ binds through alternative mechanisms are affected to the same extent by the C/EBPβ knockdown as induced genes in the vicinity of hotspots, where C/EBPβ binds directly to its predicted sites (Figure 5C). Interestingly, however, hotspots where C/EBPβ is recruited through alternative mechanisms are associated with a different gene program than the hotspots with a C/EBPβ footprint (Figure 5D), which is consistent with the observation that hotspots occupied by different facilitating factors are associated with distinct gene programs (Figure 4E). Taken together, this demonstrates that, despite the lower ChiP-seq signal, C/EBPβ recruitment to chromatin by alternative mechanisms is as important as direct C/EBPβ binding to its predicted sites for additional transcription factor recruitment, enhancer formation, and gene activation.

**DISCUSSION**

The functional importance of hotspots (Siersbaek et al., 2014) along with the intriguing finding that multiple transcription factors associate with a relatively small stretch of DNA of a few hundred base pairs at hotspots prompted us to investigate how transcription factors are organized at these regions.

We demonstrate that digital genomic footprinting combined with ChiP-seq and motif analyses can be used to identify binding of specific heterodimers to accessible chromatin. The findings that a specific composite ATF-C/EBP motif is found in footprints and enriched within shared C/EBP and ATF ChiP-seq peaks strongly suggest that these sites direct ATF-C/EBP heterodimer formation on chromatin. It has previously been demonstrated that ATF2 and ATF4 can form heterodimers with C/EBPβ that bind to the composite site also identified here (i.e., TGACGCAA) in vitro (Mann et al., 2013; Shuman et al., 1997; Vinson et al., 1993). Our data suggest that this is a general feature of C/EBPs and ATFs, and we provide direct evidence of ATF-C/EBP heterodimer formation in a chromatin context within cells. In addition to the composite ATF-C/EBP motif described here, it

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(E) Genes that were induced during the first 4 hr of differentiation (Siersbaek et al., 2014) were assigned to a given factor if a hotspot where that factor formed a footprint was located within 50 kb of their TSS. The genes assigned to C/EBP, KLF, and AP1 factors were then subjected to GO enrichment analysis, and the p values for the significant GO terms are illustrated in the heatmap. GO term-enrichment analysis was performed using GeneAnswers (Feng et al., 2010).

(F) Schematic model of the alternative mechanisms through which transcription factors can be recruited to hotspots. In all cases, alternative mechanisms of recruitment do not involve the factor’s canonical motif. Instead, transcription factors can be recruited indirectly to DNA through mechanisms involving protein–protein interactions that are facilitated by DNA-bound factors (e.g., C/EBPs and AP1 factors) or they can be directly recruited to DNA through alternative motifs, which may also be facilitated by other factors.

See also Figure S3.
knockdown as determined by RNA-seq, were identified. Induced genes were defined as genes that are significantly induced during the first 4 hr of 3T3-L1 differentiation (D). Different mechanisms of C/EBP motifs remain unclear.

Interestingly, we have observed that different factors have different abilities to recruit additional factors to hotspots through mechanisms not involving their predicted binding sites to all investigated hotspots. Binding sites where STAT5A or VDR bind without C/EBP cointegration are included as control sites (n = 9 for STAT5A and n = 8 for VDR). The red lines represent the medians of the data. *Student’s t test p < 0.05 for difference between fold change at control sites and the investigated hotspot group. Results are representative of two independent experiments.

(B) Loss of MED1 binding as determined by ChIP-seq upon depletion of C/EBP at binding sites where C/EBP binds directly to its predicted sites or through alternative mechanisms. The loss of MED1 binding sites without C/EBP (Siersbæk et al., 2014) is shown as a control. Error bars show the 95% confidence interval around the mean. *p < 2.2 x 10^-16, Student’s t test. MED1 ChIP-seq data were obtained from Siersbæk et al. (2014). (C) The induced genes that have a hotspot where C/EBP binds through alternative mechanisms or directly to its predicted sites within 50 kb of their TSS, which are significantly regulated by C/EBP isoforms.

Figure 5. Alternative Mechanisms of C/EBP Recruitment Are Functionally as Important as Direct Binding to Predicted C/EBP Sites

(A) Fold change in C/EBPβ, STAT5A, and VDR ChIP-seq upon depletion of C/EBPβ at hotspots, where C/EBPβ binds directly to its predicted sites in DNA (n = 12) or through alternative mechanisms (n = 11). STAT5A and VDR are recruited through alternative mechanisms not involving their predicted binding sites to all investigated hotspots. Binding sites where STAT5A or VDR bind without C/EBPβ cointegration are included as control sites (n = 9 for STAT5A and n = 8 for VDR). The red lines represent the medians of the data. *Student’s t test p < 0.05 for difference between fold change at control sites and the investigated hotspot group. Results are representative of two independent experiments.

(B) Loss of MED1 binding as determined by ChIP-seq upon depletion of C/EBPβ at binding sites where C/EBPβ binds directly to its predicted sites or through alternative mechanisms. The loss of MED1 binding at binding sites without C/EBPβ (Siersbæk et al., 2014) is shown as a control. Error bars show the 95% confidence interval around the mean. *p < 2.2 x 10^-16, Student’s t test. MED1 ChIP-seq data were obtained from Siersbæk et al. (2014).

(C) The induced genes that have a hotspot where C/EBPβ binds through alternative mechanisms or directly to its predicted sites within 50 kb of their TSS, which are significantly regulated by C/EBPβ isoforms.

is likely that there is an additional repertoire of motifs that can direct ATF-C/EBP heterodimer formation on chromatin, as has been described previously in vitro (Mann et al., 2013; Vallejo et al., 1993). Taken together, these findings indicate that the assembly of alternative dimers may be a general way to integrate external signals on the chromatin template.

Interestingly, our genomics analyses also revealed that alternative binding of transcription factors not involving their regular binding sites is a key mechanism through which transcription factors are recruited to hotspots. This is consistent with a recent study demonstrating that a large fraction of ChIP-seq peaks for several different factors is associated with a binding site for another factor, but not a site for the factor itself (Wang et al., 2012). Whether these alternative binding events occur via indirect binding or assisted loading to nonconsensus motifs remains unclear.

Our footprinting analyses demonstrate that C/EBPs, KLFs, and AP1 factors make many strong footprints that are highly associated with alternative mechanisms of binding of other factors, indicating that these factors may be involved in facilitating recruitment of additional factors to hotspots through mechanisms not involving their known motifs. Interestingly, however, we have observed that different factors have different abilities to make footprints, with nuclear receptors being the least efficient, consistent with a recent study (He et al., 2014). It is therefore likely that the other factors we have investigated, including nuclear receptors, can also bind directly to some hotspots and be involved in facilitating recruitment of additional factors through alternative mechanisms as described here for C/EBPs, AP1, and KLFs, even though we do not identify a footprint for these factors in this study. The basis for the varying efficiency of footprint formation by different factors is unclear but may relate to the molecular details of how factors bind to DNA or the dynamics of the protein-DNA interactions. In favor of the latter is the finding that nuclear receptors have a very short residence time on DNA (McNally et al., 2000; Voss et al., 2011) and a poor ability to make footprints (M.-H.S., M. Guertin, S.B., and G.L.H., unpublished data; this study; He et al., 2014).

Surprisingly, we find that alternative binding events for C/EBPβ appear to be functionally as important as direct binding to its predicted sites for the formation of hotspots, recruitment of coactivators, and gene activation, even though alternative binding events are associated with significantly lower ChIP-seq signal than footprints at predicted sites. Intriguingly, the finding that C/EBPβ footprints and alternative binding of C/EBPβ are associated with distinct gene programs suggests that it may...
be possible to design drugs to target specific gene programs by specifically targeting one, but not the other, of these binding mechanisms of a particular transcription factor.

In conclusion, we present molecular insight into how transcription factors are organized and function at shared target regions, thereby providing a framework for understanding transcription factor cooperativity on chromatin.

EXPERIMENTAL PROCEDURES

Cell Culture and Knockdown
3T3-L1 cells were grown and induced to differentiate as described in Siersbæk et al. (2014). Knockdown of C/EBPβ was done using the pSicoR PGK puro (Addgene; 12004) system as described in Siersbæk et al. (2014).

ChIP
ChIP was performed as described (Siersbæk et al., 2011) using antibodies against C/EBPβ (C-19, sc-150; Santa Cruz) and STAT5A (L-20, sc-1081; Santa Cruz). Purified DNA was analyzed by quantitative PCR (qPCR).

RNA-Seq
Cells were harvested in Isol-RNA lysis reagent (5′ PRIME) and purified according to the manufacturer. Purified RNA was then prepared for sequencing (Illumina) according to the instructions of the manufacturer. Sequence reads were mapped to the mouse genome (mm9) as described in Siersbæk et al. (2014). Mapped reads in exons were counted using HOMER (Heinz et al., 2010), and differentially expressed genes (adjusted p < 0.05) were identified using DESeq (Anders and Huber, 2010).

Digital Genomic Footprinting
Using ultra-deep sequencing of our previously published DHS-seq library (Siersbæk et al., 2011), we obtained 137 million mapped sequence tags. These were analyzed using a footprinting algorithm we developed, DNase2TF, to obtain footprints of 8–30 bp (M.-H.S., M. Guertin, S.B., and G.L.H., unpublished data). All footprints were scanned with JASPAR motifs (Mathelier et al., 2014; Sandelin et al., 2004) to predict binding sites for the investigated factors. See Supplemental Experimental Methods for more details.

Genomics Analyses
Mapped ChIP-seq (Siersbæk et al., 2011, 2014) and RNA-seq (this study; Siersbæk et al., 2014) were analyzed by HOMER (Heinz et al., 2010) and BEDTools (Quinlan and Hall, 2010).

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