Integrative Genomics Identifies the Corepressor SMRT as a Gatekeeper of Adipogenesis through the Transcription Factors C/EBP β and KAISO

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SUMMARY

The molecular role of corepressors is poorly understood. Here, we studied the transcriptional function of the corepressor SMRT during terminal adipogenesis. Genome-wide DNA-binding profiling revealed that this corepressor is predominantly located in active chromatin regions and that most distal SMRT binding events are lost after differentiation induction. Promoter-proximal tethering of SMRT in preadipocytes is primarily mediated by KAISO through the conserved TCTCGCGAGA motif. Further characterization revealed that KAISO, similar to SMRT, accelerates the cell cycle and increases fat accumulation upon knockdown, identifying KAISO as an adipogenic repressor that likely modulates the mitotic clonal expansion phase of this process. SMRT-bound promoter-distal sites tend to overlap with C/EBP β -bound regions, which become occupied by proadipogenic transcription factors after SMRT clearance. This reveals a role for SMRT in masking enhancers from proadipogenic factors in preadipocytes. Finally, we identified SMRT as an adipogenic gatekeeper as it directly fine-tunes transcription of pro- and antiadipogenic genes.

INTRODUCTION

Our knowledge about the role and importance of transcriptional corepressors is still in its infancy, despite the fact that several have already been implicated in a wide range of biological processes and diseases and that the majority yield developmental defects or other severe phenotypes when deleted in mice (Deplancke, 2009; Perissi et al., 2010). An interesting example is the *n*uclear receptor corepressor 2 (NCoR2), also known as silencing *m*ediator of *r*etinoic acid and *t*hyroid

hormone receptor (SMRT), which, among many other biological processes, has been implicated in terminal white fat cell differentiation (Perissi et al., 2010). For example, Yu et al. (2005) demonstrated that SMRT knockdown (KD) causes increased differentiation of 3T3-L1 preadipocytes as compared to control cells (Figure S1), likely because of the upregulation of adipogenic proteins, such as the master regulator PPAR γ . Recent in vivo studies have confirmed the role of SMRT in adipogenesis, demonstrating that heterozygous SMRT knockout mice (Sutanto et al., 2010) or mice harboring targeted disruptions in the protein interaction domain of SMRT (Fang et al., 2011) become super obese and exhibit adipocyte hypertrophy when challenged with a high-fat diet.

While the involvement of SMRT in adipogenesis is fairly well established, the precise underlying molecular mechanism is much less understood. Based on their observations, Yu et al. (2005) proposed that in the absence of ligand, SMRT binds to a nuclear receptor dimer consisting of RXR α and the master regulator of terminal adipogenesis PPAR γ , and as such represses the target genes of this dimer through its well-documented interaction with histone deacetylases (HDACs) (Feng et al., 2011; Haberland et al., 2010; Perissi et al., 2010). When ligand is present, SMRT dissociates from PPAR γ -RXR α , resulting in the activation of target genes and the induction of terminal adipogenesis. Thus, given its association with HDACs and its repressive function, SMRT was assumed to be predominantly located in deacetylated genomic regions enriched for repressive histone marks.

The lack of genome-wide DNA-binding profiling studies of SMRT within this biological system or even large-scale corepressor analyses in general makes it difficult to validate this hypothesis. We therefore set out to identify functional SMRT antibodies as well as to optimize the ChIP protocol to allow genome-wide ChIP coupled to high-throughput sequencing (ChIP-Seq) of SMRT during terminal adipogenesis. For this purpose, we used the mouse 3T3-L1 fibroblast cell line, given its status as a well-accepted model system of terminal white fat cell differentiation (Green and Kehinde, 1974; Rosen and MacDougald, 2006). This is also reflected by the wealth of



molecular, often genome-wide data already available for these cells (Mikkelsen et al., 2010; Nielsen et al., 2008; Rosen and MacDougald, 2006; Siersbæk et al., 2011), enabling us to exploit the power of an integrative genomics approach to characterize the molecular involvement of SMRT in terminal adipogenesis.

RESULTS

Genome-Wide DNA-Binding Profiling of SMRT during Terminal Adipogenesis

We first assessed genome-wide SMRT DNA-binding in undifferentiated 3T3-L1 cells (day 0 or D0), and initially identified >6,000 SMRT peaks. To evaluate the specificity of the SMRT antibody and the accuracy of our peak detection approach, we compared the ChIP-qPCR enrichment of several randomly selected peaks as well as negative control genomic sites in SMRT and control shRNA-treated 3T3-L1 cells (Figure S1). Fourteen out of the fifteen tested regions showed a decrease in SMRT binding (of which, 11 more than 2-fold), whereas no difference was observed for negative control regions (Figure S2A), providing confidence as to the specificity of the SMRT antibody as well as to the accuracy of peak detection.

We then performed SMRT ChIP-Seq at four time points after induction of differentiation, i.e., at 2 hr, day 1 (D1), day 4 (D4), and day 6 (D6), to explore the dynamic behavior of SMRT binding throughout terminal adipogenesis. These analyses revealed two principal temporal patterns featuring respectively SMRT-bound sites specific for D0 (2421) and those present at all-time points (2270) (Figure 1A) (see Supplemental Experimental Procedures for details, Table S1A for sequencing statistics, and Table S1B for all identified SMRT peaks). We validated the observed SMRT-DNA-binding dynamics by performing ChIP-qPCR on randomly selected D0-specific SMRT peaks at D0 and D1 in independent biological replicates. Out of 25 SMRT-bound regions, 18 peaks (72%) showed a drop in enrichment at D1 (Figure S2B), supporting our genome-wide observation of decreased SMRT binding after differentiation induction.

SMRT Exhibits Spatiotemporal DNA-Binding Properties

Further analysis of the D0-specific and all-time point peak groups revealed a striking, nonrandom, time-dependent, and bimodal pattern of DNA binding. Specifically, we found two dominant locations for SMRT binding, respectively ~100 bp and ~50 kb up- or downstream of the transcription start site (TSS) with a clear demarcation of the proximal and distal binding site groups (Figure 1B). The majority (70% versus 30%) of the D0-specific SMRT target sites were located in distal (i.e., >1 kb upstream/downstream of the TSS) as compared to proximal (i.e., <1 kb upstream/downstream of the TSS) regions, in sharp

contrast to all-time point peaks, which showed a more balanced (42% distal versus 58% proximal) SMRT-binding pattern (Figure 1C). Interestingly, this spatial DNA-binding pattern delineates distinct target gene functionalities: genes with proximal SMRT peaks are predominantly involved in generic biological processes, such as RNA metabolism, translation, transcription, cell cycle, or oxidative phosphorylation, whereas distal peaks were typically found around genes being involved in cell communication, cell structure and motility, mesoderm development, cell proliferation and differentiation, and ligand-mediated signaling (Table S2A). In addition, we found a significant enrichment (Q-value < 4.6E-15) among D0 SMRT targets for genes that are differentially expressed during terminal adipogenesis as annotated by the Molecular Signature Database (MSigDB, Table S2B). Many of these also have a well-characterized function in this differentiation process, including $Ppar\gamma$, Atf2, Prkab2 (Ampk1 catalytic activity regulating subunit), $Rxr\alpha$, $Rxr\beta$, $Rar\alpha$, Bmp2k, Klf3, Klf6, Klf9, Med1, Ncoa4, etc. Representative UCSC snapshots showing the dynamic binding pattern of SMRT on important adipogenic genes are shown in Figure 1D.

De Novo Motif Analysis Identifies Several Putative SMRT-Recruiting Factors

To better understand the molecular origin of this time-dependent and bimodal DNA-binding behavior, we analyzed distal and proximal SMRT DNA-binding sites for overrepresented motifs at both temporal groups (D0-specific and all-time point peaks). This analysis revealed a striking difference between motifs enriched at distal versus proximal sites and between motifs enriched at sites present in preadipocytes versus differentiating adipocytes (Figure 2A), indicating that distinct sets of TFs are likely responsible for mediating the spatiotemporal demarcation of SMRT DNA-binding sites. We also studied the positional distribution of motifs around the respective SMRT peak center, as this can provide further detail as to whether SMRT may be directly recruited to the DNA by the respective TFs or rather via more complex, indirect interactions. For SMRT peaks containing the TCTCGCGAGA, EBF1- or CTCF-associated motifs, we observed exact motif positioning around the peak center (Figure S3A), indicative of direct SMRT recruitment, whereas the other motifs showed a bimodal (SP1, ZFP143/RBPJ), multimodal (AP4, CWGSCWG), or broader distribution (GABPA, AP1; data not shown).

PPAR_γ and SMRT Exhibit Antagonistic Temporal DNA-Binding Patterns during Terminal Adipogenesis

To further examine the molecular mechanisms underlying the dynamic DNA-binding behavior of SMRT, we reanalyzed ChIP-Seq data of PPAR γ (Nielsen et al., 2008), given its hypothesized

Figure 1. Genome-wide Temporal Profiling of SMRT during Terminal Adipogenesis Reveals a Time-Dependent and Bimodal DNA-Binding Behavior

⁽A) Clustering of SMRT peaks at different time points of 3T3-L1 differentiation: preadipocyte or day 0 (D0), 2 hr, day 1 (D1), day 4 (D4), and day 6 (D6). (B) Histogram depicting the number of SMRT peaks with respect to their distance to the nearest transcription start site.

⁽C) Pie chart stratifying D0-specific and all-time point SMRT peaks with respect to their distance to the nearest transcription start site.

⁽D) Representative UCSC browser snapshots demonstrating SMRT binding dynamics at the adipogenic genes *Ebf1*, *Ppary*, *Med1*, and *Sirt4*. Black bars represent significant SMRT peak calls as determined by the software QuEST. Peak densities at respective time points of differentiation represent raw tag counts after library size adjustment.



importance in tethering SMRT to the DNA (Yu et al., 2005). We found that PPAR_Y and SMRT follow opposite DNA-binding dynamics, and have few overlapping binding regions. We also tested whether SMRT may mark future PPAR_Y sites in mature adipocytes (D6), but we found little overlap between SMRT-targeted regions and PPAR_Y sites (5% or 480 out of 9090 peaks, Figure 2B), together suggesting that PPAR_Y does not play a major role in tethering SMRT to the DNA, at least in 3T3-L1 cells. Importantly though, 33% (>1,100) of putative SMRT target genes are eventually regulated by PPAR_Y during adipogenesis, including well-characterized PPAR_Y target genes such as *Cav1*, *Cav2*, *Plin4*, and *AcsI1*, consistent with our observation that SMRT is targeting or marking critical adipogenic genes.

Given the low overlap between PPAR_Y and SMRT-marked sites, we examined whether RXR_α, the primary dimerization partner of PPAR_Y, is also absent from SMRT occupied regions. We observed that only 15% (584) of all SMRT sites at D0 are bound by RXR_α. However, in contrast to PPAR_Y, we found that half (2273) of all SMRT sites are targeted by RXR_α at different days of adipogenesis (Figure 2B). These results therefore suggest that SMRT targets many early adipogenic regulatory sites that eventually become bound by RXR_α as either a homodimer or a heterodimer involving partners other than PPAR_Y.

SMRT Colocalizes with C/EBPβ at Distal Regulatory Sites during Early Adipogenesis

It is well established that the TF C/EBP β has a prominent role during early adipogenesis (Rosen and MacDougald, 2006). We therefore analyzed the relationship between SMRT and C/EBPß using recently published C/EBPß ChIP-Seq data (Siersbæk et al., 2011), revealing a marked colocalization down to the level of peak maxima of C/EBP_β- and SMRT-binding events in preadipocytes, especially at distal (>60%) compared to proximal (<15%) SMRT-bound sites (Figures 2C-2E and Table S3A). We found that 1659 (89%) out of 1871 sites primed by C/EBP_β-SMRT in preadipocytes also showed stable or recurring C/EBP_β-binding during the first 2 days after differentiation induction (Figure 2E). These results suggest a so far unappreciated role for C/EBPß in mediating genome-wide tethering of SMRT to promoter-distal sites in preadipocytes. In addition, we observed that the majority of preadipocyte-marked C/EBP β -SMRT sites are targeted by either C/EBP β (86%), C/EBP_δ (66%), STAT5a (29%), glucocorticoid receptor (27%), or RXRa (20%) at 4 hr (Figures 2F and 2G), an early time point at which most SMRT DNA-binding at the respective sites has disappeared (Figure S3B). This observation is consistent with the finding that C/EBP^β acts as a pioneering TF by marking so-called TF "hotspots" prior to differentiation induction (Siersbæk et al., 2011). Interestingly, we found that SMRT-marked TF hotspots show a 2.8-fold higher tag density of C/EBPß at D0 as compared to TF hotspots which are not marked by SMRT and, moreover, that the average tag density of C/EBPß at SMRT-marked sites remains stable between D0 and 4 hr (Figure 2H). We also observed that 40% (119/299) of TF hotspots, characterized by stable C/EBP β DNA-binding between D0 and D2 (see Supplemental Experimental Procedures), are marked by SMRT, as opposed to only 10% of hotspots transiently induced at 4 hr (Table S3B). These results therefore indicate that SMRT and C/EBP^β together appear to mark regulatory sites in preadipocytes that are destined to become populated by multiple proadipogenic TFs at an early phase of terminal adipogenesis.

KAISO Targets SMRT to Promoter-Proximal DNA-Binding Sites in Preadipocytes

The striking enrichment of the palindromic TCTCGCGAGA DNA sequence in preadipocyte-specific promoter-proximal sites (Figure 2A) as well as its position close to the TSS directly underneath SMRT peak maxima (Figures 3A and S3A) prompted us to further characterize this putative DNA-binding site. This motif was previously identified as one of the top ten most strongly enriched and well-conserved motifs in human promoters (Xie et al., 2005; Pique-Regi et al., 2011). To complement recent, unfruitful efforts to deorphanize this motif (Mikula et al., 2010), we scanned publicly available ENCODE TF ChIP-Seq data sets for enriched DNA target sequences that resembled this motif. Interestingly, data from one ChIP-Seq experiment targeting the human BTB/POZ zinc finger TF KAISO in a lymphoblastoid cell line revealed a near identical palindromic motif as the one detected within SMRT peaks in the majority of human KAISO sites (Figure 3B), suggesting that the highly conserved mouse homolog of this TF, i.e., ZBTB33 or mouse KAISO, may mediate SMRT targeting to mouse gene promoters. When studying this palindromic site within human KAISO-bound regions in more detail, we coarsely observed four different motif classes with differential ChIP-Seq enrichment, including the full site

Figure 2. De Novo Motifs and Integrative Genomics Analyses Identify Several Putative SMRT-Recruiting Factors

(F) Co-occupancy of C/EBPß and C/EBPδ at proximal and distal SMRT sites in preadipocytes.

⁽A) Web logos of significantly enriched motifs predicted with MEME (see Supplemental Experimental Procedures). The occurrence frequencies and enrichment values are indicated as well as the corresponding TFs if known.

⁽B) Pie charts depicting the colocalization dynamics of PPAR_Y, RXR_α, and PPAR_Y-RXR_α peaks at all 2033 proximal and 2658 distal SMRT DNA-binding sites during terminal adipogenesis.

⁽C) SeqMINER-based clustering of all 4691 SMRT peaks sorted according to local DNA-binding enrichment of C/EBPβ in preadipocytes. Colors indicate C/EBPβ enrichment (blue high and white low).

⁽D) Histogram depicting the distance between C/EBP β and SMRT peak maxima in preadipocytes.

⁽E) C/EBP_β DNA-binding dynamics at proximal and distal SMRT peaks before (D0) and after differentiation induction (i.e., 2 hr, 4 hr, and D2).

⁽G) Histogram depicting DNA-binding co-operativity of the early adipogenic TFs C/EBP β , C/EBP δ , GR, RXR α , and STAT5a at sites marked by SMRT-C/EBP β in preadipocytes and bound by C/EBP β at 4 hr.

⁽H) Box plots depicting the DNA-binding dynamics of C/EBPβ during three early adipogenic time points (D0, 2 hr, and 4 hr) at SMRT positive (left subpanel) compared to SMRT negative TF hotspots (right subpanel).



Figure 3. KAISO Targets SMRT to Promoter-Proximal DNA-Binding Sites in Preadipocytes

(A) Histogram depicting the distance of KAISO motifs, enriched in D0-specific promoter-proximal SMRT peaks, to the nearest transcription start site (TSS).
(B) Comparison between the orphan TCTCGCGAGA motif enriched in D0-specific promoter-proximal SMRT peaks and a motif derived from the top 25% high-confidence peaks in human KAISO ChIP-Seq data.

(TCTCGCGAGA), intact half sites (TCTCGCG and CGCGAGA), and degenerate sites harboring either mutations in the core CGCG sequence or in both flanking sequences (Figure 3C and Table S4A).

Based on the latter observation, we expect strongest binding of mouse KAISO to regions containing the full motif, and we therefore may expect strongest binding of SMRT to these regions as well if, as we hypothesize, KAISO mediates SMRT DNA-binding. We found that SMRT peaks containing the full motif had indeed 1.5- and 2.5-fold more reads as compared to, respectively, the two half or degenerate motif-containing peaks (p < 1E-4, Mann-Whitney U test; Figure 3D). In addition, we observed that human syntenic regions of mouse SMRTbinding sites have a high likelihood of also having a KAISO peak containing either a palindromic or half motif site, consistent with the reported conserved nature of this motif (Xie et al., 2005) (Figures 3E and 3F).

To examine the relevance of the palindromic site in KAISO DNA binding, we used a microfluidics-based protein-DNA interaction detection technology, MITOMI (Maerkl and Quake, 2007), to study the relative DNA-binding affinity of mouse KAISO for the detected sites as well as for the previously reported KAISObinding sequence or KBS (TCCTGCNA; Daniel et al., 2002). We thereby also examined the DNA-binding capacity of this TF to a methylated version of the palindromic motif (TCTCGCGAGA) given the previously reported ability of human KAISO to bind to methylated CpG dinucleotides (Daniel et al., 2002; Yoon et al., 2003). We observed highest relative affinity to the methylated palindromic site, whereas the affinities for the unmethylated palindromic site were near background (i.e., negative control) (Figures 3G and S3C), thus providing direct evidence that the conserved orphan motif TCTCGCGAGA is bound by KAISO. Importantly, swapping the flanking sites around the methylated core motif CGCG clearly reduced the relative affinity, suggesting that, while DNA methylation is critical, the flanking sequences play an important role. We also observed binding to the KBS, revealing a higher affinity for this site than the core methylated CGCG motif. However, KAISO possesses an even higher affinity for the methylated palindromic motif than the KBS, indicating, together with the ChIP-Seq data, that the former site may be the principal binding motif.

Yoon et al. (2003) have previously shown that human KAISO can interact with the SMRT homolog NCoR1 in HeLa cells, suggesting that mouse KAISO may be able to bind to SMRT as well. As shown in Figure 3H, pull-down of SMRT revealed the presence of KAISO, indicating that SMRT and KAISO are components of the same protein complex as also observed for KAISO and NCoR1 in mouse cells (Figure S3E). Moreover, we detected less KAISO after a SMRT IP in a SMRT KD versus an NCoR1 or control (empty vector) context, lending support to the specificity of the uncovered interaction (Figure S3F). To confirm that KAISO is most likely tethering SMRT to DNA in 3T3-L1 cells, we employed SMRT ChIP-qPCR on 12 randomly selected KAISO motif-containing promoters in KAISO KD preadipocytes (Figure S3G) and compared the results against SMRT ChIP-qPCR enrichments in shRNA control and SMRT KD cells, respectively. These analyses revealed a decrease in SMRT enrichment in KAISO KD compared to control cells at all 12 promoters, similar to the decrease observed in SMRT KD cells. To further validate the involvement of KAISO in the binding of SMRT to the DNA, we performed KAISO ChIP-gPCR, using two different antibodies on 15 sites predicted to be bound by KAISO at D0, and found that 14 out of 15 analyzed regions are occupied by KAISO (Figures 3I and S3H). In contrast, none out of 15 SMRT-bound regions without a predicted KAISO motif and none out of two negative control regions were bound. Thus, our results strongly support the notion that KAISO is an important mediator of SMRT DNA-binding to promoter-proximal sites.

KAISO and SMRT Are Located in Semiaccessible and Active Chromatin Regions

Both KAISO and SMRT have been described as transcriptional repressors (Daniel and Reynolds, 1999; Perissi et al., 2010). Consistent with this observation is the detection of KAISO in a DNA region enriched for the repressive H3K9me2 mark (Yoon et al., 2003). However, ChIP-qPCR for H3K9me2 on 14 sites positive for KAISO binding in preadipocytes (Figure 3I) revealed that these DNA regions are not enriched for this mark, as opposed to other non-KAISO-bound regions (Figure 3J). For SMRT, it has been proposed that its repressive function is mediated by its interaction with HDACs, which establish a local,

⁽C) ChIP-Seq enrichment at KAISO peaks with full (TCTCGCGAGA), half (TCTCGCG or CGCGAGA), or degenerate KAISO motifs.

⁽D) Number of reads at SMRT peaks in preadipocytes with full (TCTCGCGAGA), half (TCTCGCG or CGCGAGA), or degenerate KAISO motifs.

⁽E) Browser snapshot showing the position of a KAISO peak near the TSS of the human HNRNPK gene based on human KAISO ChIP-Seq data.

⁽F) Browser snapshot showing the position of a SMRT peak near the TSS of the mouse *Hnrnpk* gene.

⁽G) The left panel shows raw MITOMI data revealing the extent of KAISO-eGFP binding (i.e., "DNA bound") to the methylated (top) as compared to the nonmethylated TCTCGCGAGA motif (bottom). The right panel shows the relative affinity of KAISO to an unmethylated control sequence, to the previously reported KAISO binding site (KBS) (TCCTGCCA; Daniel et al. [2002]), to the methylated and unmethylated palindromic TCTCGCGAGA motif, and to a methylated or unmethylated core CGCG motif. Relative affinities are expressed in arbitrary units (a.u.) and scaled based on the extent of KAISO binding to the methylated palindromic motif ("100" value, see also Supplemental Experimental Procedures). Error bars represent the standard deviation of three replicate MITOMI experiments.

⁽H) SDS-PAGE followed by western blotting for SMRT or KAISO proteins in SMRT antibody immunoprecipitated samples. The presented data constitute a representative blot of three replicate experiments.

⁽I) KAISO ChIP-qPCR at SMRT-bound promoter-proximal regions containing a predicted KAISO motif (KAISO [+]) as compared to SMRT-bound promoterproximal and promoter-distal regions lacking a discernible KAISO motif (KAISO [-]). Error bars represent the standard deviation from three replicate experiments. (J) ChIP-qPCR enrichment analysis of the H3K9me2 histone modification mark in SMRT-bound regions with (+) or without (-) a predicted KAISO motif. Ctl1 and Ctl2 are control regions selected because they are positive for H3K27me3 in preadipocytes (Mikkelsen et al., 2010). Error bars represent the standard deviation from three replicate experiments.

repressed chromatin state, as indicated above. However, to our surprise, we found that SMRT is predominantly located within seemingly open and active chromatin regions based on DHS, H3K27ac, H3K4me1, H3K4me3, and H3K27me3 data (Figures 4A–4E and S4A–S4E). Interestingly, however, when examining the change in chromatin "accessibility" (as determined by the peak height of DHS regions) of SMRT-bound sites before and after differentiation induction, we found a general rise in the extent of chromatin accessibility, especially after 2 hr, suggesting that a loss of SMRT binding correlates with an increased accessibility of these sites (Figure S5A). A similar trend was observed when only KAISO motif-containing SMRT-binding sites were considered (Figure S5B). A representative summary of all the observed SMRT DNA-binding properties can be observed at the *Tle3* gene locus (Figure 4F).

Preadipocyte-Specific SMRT-Bound Genes Show Increased Gene Promoter and Gene Body RNA Polymerase II Tag Density after Differentiation Induction

To explore the significance of these findings with respect to transcription, we integrated our SMRT peak with ChIP-Seq data representing the genome-wide mapping of RNA pol II over the course of 3T3-L1 terminal differentiation (Nielsen et al., 2008). These analyses revealed that RNA pol II occupancy on the promoters of SMRT target genes is significantly greater than that of other genes, indicating that SMRT primarily associates with transcriptionally active or at least primed genes, consistent with the histone modification and DHS data (Figure S6). Interestingly, we also found that the median promoter occupancy of genes with proximal SMRT peaks at D0 is almost double that of genes featuring only distal peaks in contrast to gene body RNA pol II occupancy, which is similar for both categories (Figures 5A-5D and S6). This result may be reflective of increased RNA pol II pausing at the promoters of genes with proximal SMRT sites. Finally, we observed that preadipocytespecific SMRT target genes typically exhibited increased RNA pol II binding at both their promoters and gene bodies after differentiation induction, in contrast to their all-time point counterparts (Figures 5E-5H). Thus, genes with continuous SMRT binding tend to remain in a silenced or at least more repressed state, consistent with the repressive properties of SMRT.

KAISO Is a Repressor of Terminal Adipogenesis Likely by Controlling the Mitotic Clonal Expansion of Preadipocytes through SMRT

D0-specific SMRT peaks that are located close to promoters are significantly enriched for the KAISO motif (i.e., 393 out of 709 sites). Thus, we specifically determined whether genes with promoter-proximal SMRT sites containing a KAISO motif could be responsible for the observed RNA pol II-based transcriptional activity pattern when considering all proximal SMRT sites. For this purpose, we plotted promoter versus gene body RNA pol II occupancy, revealing a striking dynamic relationship between these two parameters (Figure 5I). Specifically, we found that the promoters of SMRT/KAISO-positive genes exhibit high RNA pol II occupancy at D0 in contrast to their respective gene bodies and that loss of SMRT (D1) correlates with an important RNA pol II occupancy increase at the promoters and especially in gene bodies. This trend is reversed at D3–4 to the point where, at D6, respective RNA pol II occupancy values are lower than those at D0 for both gene promoters and bodies, indicating that many genes may have entered a transcriptionally silent state. We also performed a similar analysis for SMRT peaks containing the ZFP143/RBPJ motif, which is enriched in alltime point proximal SMRT sites, and observed low promoter and gene body RNA pol II occupancy throughout differentiation (Figure 5J), indicating that all-time proximal SMRT peaks tend to be associated with transcriptionally silent genes. Together, these results suggest that SMRT controls the transcriptional activity of genes by mediating RNA pol II pausing and release through KAISO.

As indicated earlier, the list of proximal SMRT targets is significantly enriched for genes involved in cell cycle, transcription, and translation. As expected, a similar observation was made for SMRT/KAISO targets (Tables S4B and S4C). Together with their transcriptional upregulation after differentiation induction, this prompted us to speculate that genes targeted by SMRT/ KAISO may mediate the mitotic clonal expansion (MCE) phase of terminal adipogenesis, during which growth-arrested cells re-enter the cell cycle and undergo at least one round of clonal expansion as a prerequisite to express adipogenic proteins and thus to generate terminally differentiated cells (Tang et al., 2003). To test this hypothesis, we profiled the cell cycle in SMRT KD and shRNA control cells during the first 24 hr of terminal differentiation. The underlying reasoning is that the absence of SMRT may accelerate the expression of critical MCE genes and hence prime the cells for a faster cell-cycle progression upon exposure to differentiation medium. Using propidium iodide-based FACS, we observed that SMRT KD cells reached the G2/M phase significantly faster than control cells (Figures 6A-6E), suggesting that SMRT indeed plays a role in cell-cycle control. Finally, we tested whether KAISO KD would have similar phenotypic consequences to SMRT KD with respect to the cell cycle and the ability to differentiate. This may be expected if SMRT and KAISO indeed functionally interact. As shown in Figures S7A-S7E, KAISO KD accelerates the cell cycle and enhances fat cell differentiation as compared to control cells (Figure 7A). Thus, these findings suggest that KAISO is a repressor of terminal adipogenesis, likely by controlling the initial MCE phase during terminal adipogenesis through SMRT.

SMRT Is a Gatekeeper of Terminal Adipocyte Differentiation

To further explore the transcriptional function of SMRT in terminal adipogenesis, we used RNA pol II ChIP-Seq to specifically investigate the genome-wide effect of SMRT KD on gene transcription in preadipocytes. We identified 417 genes that are differentially transcribed between SMRT KD and shRNA control cells (fold-change > 1.5 and FDR < 1%; see Table S5A for all differentially transcribed genes). We also examined whether these transcriptional changes were apparent at the expression (mRNA) level and based on 29 tested genes, the vast majority (79%) were found to exhibit a similar differential expression trend (Table S5B). The majority (234 or 59%) of the



Figure 4. Chromatin Regions Bound by SMRT are Semiaccessible and Contain Active Chromatin Marks

(A) Correlation of SMRT-bound regions with DNase I hypersensitive sites (DHSs) during terminal adipogenesis.

(B–E) Enrichment of the H3K27ac (B), H3K27me3 (C), H3K4me3 (D), and H3K4me1 (E) histone modification marks in SMRT-bound DNA regions. (F) Overview of SMRT DNA-binding properties as illustrated by its binding pattern and overlap with histone modification marks, DHSs, C/EBPβ, and C/EBPδbinding sites at the *TIe3* gene locus.

Pol II load at promoter (RPKM)

Genes with proximal SMRT peaks Genes with distal SMRT peaks Α В 25 25 8 Pol II load at promoter (RPKM) 20 Pol II load at promoter (RPKM) 20 000 15 15 10 10 Preadipocyte-specific Preadipocyte-specific 5 5 0 0 D0 D1 D2 D3 D4 D6 D1 D2 D3 D4 D6 D0 С D . 5 000 000 8 00000 8 1 Pol II transcription (RPKM) 4 Pol II transcription (RPKM) 8 3 3 2 2 1 4 0 0 D0 D1 D2 D3 D4 D6 D1 D2 D3 D0 D4 D6 25 Ε 25 F 0 0 0 00 8 Pol II load at promoter (RPKM) 20 8 00 Pol II load at promoter (RPKM) 20 000 8 15 15 10 10 5 5 All-time point All-time point 0 0 D0 D1 D2 D3 D4 D6 D0 D1 D2 D3 D4 D6 G Н 0 Pol II transcription (RPKM) Pol II transcription (RPKM) 4 4 3 3 2 2 1 0 0 D0 D1 D2 D3 D4 D6 D0 D1 D2 D3 D4 D6 ZFP143/RBPJ-motif associated genes I KAISO-motif associated genes J D0 D1 D2 D0 D1 D2 Pol II transcription (RPKM) Pol II transcription (RPKM) D3 D4 D6 D3 D4 D6

Pol II load at promoter (RPKM)

Molecular Cell SMRT Regulates Adipogenesis via C/EBPβ and KAISO



Figure 6. SMRT KD Cells Exhibit Accelerated Cell Cycle Progression during the Early Mitotic Clonal Expansion Phase of Terminal Adipogenesis

(A–B) Propidium iodide (PI) staining-based FACS analysis of SMRT KD (A) and shRNA control (B) cells 3 days before and during the first 24 hr after differentiation induction.

(C–E) Histograms indicating the percentage of SMRT KD and shRNA control cells at each cell-cycle phase (G0/G1, S, and G2/M) during the first 24 hr after differentiation induction (**p < 0.01, *p < 0.05; t test). Error bars show the standard error of the mean from three replicate experiments.

differentially transcribed genes were upregulated, consistent with the role of SMRT as a corepressor. Importantly, 71 (30%) of these 234 genes were direct SMRT targets, which represents a significant enrichment over randomly selected genes (p < 0.01). Nevertheless, the transcriptional activity of the majority of SMRT target genes was not altered upon SMRT KD (Figure 7B). While a similar effect (or lack thereof) has been observed for many TFs (Farnham, 2009), one explanation could be that this may be due to compensatory mechanisms by the SMRT homolog NCoR1. To investigate this, we determined the DNA-binding profiles of NCoR1 pre (D0)- and post (D4)-differentiation.

As displayed in Figure 7C, we found that around two thirds of all SMRT-bound regions are also enriched for NCoR1 at D0, indicating that the DNA-binding profiles of these two corepressors largely overlap. In addition, overall DNA-binding enrichment of NCoR1 pre- and postdifferentiation decreased, together suggesting that, similar to SMRT, DNA-binding in preadipocytes is an important functional property of NCoR1 and that SMRT and NCoR1 may act cooperatively to control transcription.

Of note, several of the upregulated genes in SMRT KD cells are involved in terminal adipogenesis such as $C/ebp\alpha$, Ebf1, Stard4, Plin4, and $Ppar\gamma2$. The upregulation of both $C/ebp\alpha$ and $Ppar\gamma2$,

Figure 5. Transcriptional Dynamics of SMRT Target Genes

(A–H) RNA pol II occupancy at the promoters (-30 to 300 bp relative to TSS) and bodies of D0-specific (A–D) and all-time point (E–H) SMRT-bound genes over the course of terminal adipogenesis.

(I–J) Transcriptional dynamics of genes with promoter-proximal SMRT peaks containing KAISO (I) or ZNF143/RBPJ (J) motifs over the course of terminal adipogenesis. Colors indicate the density of genes (red low and pink high).





which is also apparent at the mRNA and protein levels (Figures 7D and 7E), is of major interest given their status as master regulators of terminal adipogenesis. This is, however, not sufficient to trigger terminal adipogenesis, as SMRT KD cells do not spontaneously differentiate, suggesting that PPAR_Y2 may not be transcriptionally active. To test this, we exposed SMRT KD preadipocytes to rosiglitazone, a synthetic PPAR_Y ligand and activator, and monitored the extent of differentiation. As shown in Figures 7F and 7G, the addition of this ligand, but not of either insulin or dexamethasone, was sufficient to induce differentiation in SMRT KD but not in shRNA control cells, indicating that, as suggested above, SMRT KD cells are primed for differentiation, but require a critical activating signal to pass a differentiation threshold.

DISCUSSION

This study used an integrative genomics approach to uncover several findings regarding how SMRT may transcriptionally control terminal adipogenesis. First, we found that SMRT exhibits a time-dependent, bimodal pattern of DNA binding, which may be directly mediated by KAISO and C/EBP β . This is because we (this study) and others (Rosen and MacDougald, 2006) have shown that both TFs affect the terminal differentiation process. In addition, these TFs are linked to the majority of respectively proximal and distal SMRT DNA-binding sites, and their binding motifs (TCTCGCGAGA for KAISO) or peaks (C/ EBP_β) are highly enriched at SMRT peak maxima, suggestive of direct interactions. The latter observation is supported by our (KAISO) as well as other published protein interaction data (C/EBP_β; Ki et al. [2005]), even though further biochemical experiments will be required to elucidate the precise molecular context of these interactions. In contrast to KAISO, which is widely known as a transcriptional repressor (Daniel and Reynolds, 1999), the association between C/EBP β and SMRT is not very intuitive, given that this TF has been shown to promote terminal adipogenesis by directly inducing the adipogenic master regulators PPAR_Y and C/EBPa (Rosen and MacDougald, 2006). This suggests that C/EBP β either acts by itself as an activator or acts within an activating and not a repressing complex. In addition, it has long been believed that C/EBP^β DNA binding only occurs after differentiation induction. However, recent genome-wide DNA-binding profiling of this TF demonstrated substantial DNA-binding activity in preadipocytes, challenging this earlier view (Siersbæk et al., 2011). Together with the extensive overlap between C/EBP_β- and SMRT-bound regions and with our observation that many of these regions are targeted by early adipogenic TFs within 2-4 hr of differentiation induction accompanied by an increased accessibility of the surrounding chromatin, these findings suggest a model in which SMRT functions to block the adipogenic regulatory potential of C/EBPβ in preadipocytes until the appropriate differentiation stimuli are present. SMRT may do so by restraining the accessibility of C/EBPβ-bound sites through its interaction with HDACs, thereby preventing the binding of early activating TFs (e.g., GR or $RXR\alpha$) and the subsequent recruitment of a coactivator complex. Indeed, docking of GR along with other activating TFs to C/EBP_β-bound distal regions has previously been shown to result in recruitment of a coactivator complex leading to activation of adipogenic genes such as PPARy (Steger et al., 2010). Moreover, about two thirds of all SMRT-C/EBP_β-bound sites in preadipocytes were classified as D0-specific, indicating that the inverse correlation between SMRT binding and early proadipogenic TF recruitment appears to be an important regulatory aspect of terminal adipogenesis (Figure S8).

A second major finding is that, in contrast to what was so far predicted (Rosenfeld et al., 2006; Yu et al., 2005), SMRT is predominantly tethered to DNA regions enriched for active histone marks. In addition, many SMRT-bound promoters also exhibited relatively high RNA pol II occupancy levels, reflective of primed gene transcription. These findings are however consistent with recent work demonstrating that the promoters of several genes activated by lipopolysaccharide through Toll-like Receptor 4 signaling tend to be occupied by both stalled RNA pol II and the SMRT homolog NCoR1 under basal conditions in macrophages (Hargreaves et al., 2009). Interestingly, several of these LPS-responsive genes were previously shown to exhibit de-repression in macrophages lacking NCoR1 (Ogawa et al., 2004). Thus, these findings suggest a possible link between NCoR1 and RNA pol II stalling and imply that active removal of

Figure 7. KAISO KD Enhances Fat Cell Differentiation, NCoR1 and SMRT DNA-Binding Profiles Overlap to a Large Extent, and SMRT KD Preadipocytes Exhibit Increased Expression of Adipogenic Genes and Differentiate upon Exposure to the PPAR_γ Ligand Rosiglitazone (A) Oil red O staining of shRNA control and KAISO KD cells after 6 days of differentiation. The presented images constitute a representative result of three replicate experiments.

⁽B) Dot plot showing differential changes in RNA pol II transcription of annotated transcripts in SMRT KD as compared to control shRNA treated 3T3-L1 cells at D0. Red dots show transcripts with significant differential transcription between both cell lines (FDR < 1%; fold-change > 1.5; see Supplemental Experimental Procedures for more details). Blue circles indicate differentially transcribed transcripts annotated as putative SMRT targets (Table S1B). Several marker genes known to be involved in terminal adipogenesis are highlighted.

⁽C) NCoR1 ChIP-Seq enrichment at all 4691 SMRT sites in preadipocytes (D0) and after differentiation induction (D4). Peaks are sorted based on SeqMINER clustering.

⁽D) Fold-change in mRNA levels of adipogenic genes in SMRT KD versus shRNA control cells over the course of terminal adipogenesis as measured by qPCR. Error bars depict the standard error of the mean from three replicate experiments.

⁽E) Changes in SMRT, PPAR γ , and C/EBP α nuclear protein levels in SMRT KD and shRNA control cells over the course of terminal adipogenesis as determined by SDS-PAGE followed by western blotting. The data shown are representative of three replicate experiments. PCNA is used as the loading control. Note that additional Ponceau S staining confirmed equal sample amount loading (data not shown).

⁽F and G) Oil red O staining of shRNA control and SMRT KD cells at D6 cultured in distinct differentiation media: well1, uninduced cells; well2, normal differentiation medium; well3, 100 nM rosiglitazone; well4, 250 nM rosiglitazone; well5, 167 nM insulin; well 6, 1 µM dexamethasone. The presented data constitute a representative result of three replicate experiments.

NCoR1-containing complexes may be a prerequisite for RNA pol II release and hence gene activation as recently proposed (Glass and Saijo, 2010). Here, we provide evidence on a genome-wide level that SMRT may function in similar fashion during terminal adipogenesis, in that we found that SMRT tends to be associated with genes with high RNA pol II promoter, but low gene body, occupancy and that loss of SMRT correlates with RNA pol II release. This trend was most prominent for proximal SMRT peaks, particularly those containing KAISO-binding sites. Together with the increasing recognition that proximal binding complexes may regulate transcription by directly interacting or stabilizing general TFs (Farnham, 2009), our findings suggest that SMRT may have a much more active role in controlling transcriptional dynamics than is currently appreciated.

A third major finding is the observation that both KAISO and SMRT have an impact on the cell cycle and that they as such may control the MCE phase of terminal adipogenesis (Figure S8). For KAISO, this is consistent with a recent study demonstrating accelerated cell proliferation in human cells in which KAISO is depleted (Soubry et al., 2010). A role for SMRT in the cell cycle has also been suggested in a recent study by Bhaskara and colleagues (2010) who, based on their findings, even speculated that this may be an ancestral function of this corepressor, which later evolved to mediate cell-cycle-dependent gene expression in a cell-type-specific fashion. The specific involvement of SMRT (NCoRs) in cell-cycle-related processes is rather ambiguous at this point, though, as both enhancing or attenuating effects have been observed (Battaglia et al., 2010). Importantly, it is also unclear whether the proposed mechanism could be cell-type-specific, as it has been shown that some cell lines such as 3T3-L1 require MCE for terminal differentiation, whereas other lines including a human preadipocyte line do not, even though there is general consensus that mitosis checkpoint proteins play an important role (Rosen and MacDougald, 2006). Further investigation in other cell lines or even in vivo will be required to validate the hypothesized role of the SMRT/KAISO complex in terminal differentiation.

A final important observation is the fact that the loss of SMRT results in the upregulation of proadipogenic genes, among which PPAR γ and C/EBP α are the most prominent, although this is not sufficient for SMRT KD cells to spontaneously differentiate. This observation leads to two important questions. First, why does SMRT KD increase the expression of $Ppar\gamma 2$ and $C/ebp\alpha$? One possible mechanism may be a direct release of repression upon SMRT loss as there are two SMRT-binding sites within the *Ppar* γ 2 locus, of which one is also occupied by C/EBP β at D0 and by other early adipogenic factors 4 hr after differentiation induction. In addition, other adipogenic genes such as Ebf1 and Akt1, which are known to activate PPARy (Jimenez et al., 2007; Kim et al., 2010), are also upregulated in SMRT KD cells. Second, why do SMRT KD cells not spontaneously differentiate? As indicated, exposure of SMRT KD cells to the activating PPAR γ ligand rosiglitazone is sufficient to induce terminal adipogenesis, albeit to a lesser extent than cells exposed to complete differentiation medium. This suggests that PPAR γ is not fully transcriptionally active in SMRT KD cells, plausibly because it is counteracted by antiadipogenic processes, of which the upregulation of Wnt5a (next to, for example, Aspn, Trpv6, and

Tnfaip6) in SMRT KD cells could be one. WNT5a is known to phosphorylate SETDB1, which results in the formation of a SETDB1/CDH7 complex that represses PPAR_Y (Takada et al., 2007). Finally, it is worth noting that we also observed the transcriptional and mRNA upregulation of important epithelialmesenchymal transition (EMT) genes such as Twist1 and Snai2 in SMRT KD cells. This may be important given the observation that the induction of EMT endows cells with mesenchymal stem cell properties (Battula et al., 2010), which has been shown to enhance the efficiency of white fat cell differentiation (Isenmann et al., 2009). Together, these findings suggest that SMRT operates at the cross-section of several pro- and antiadipogenic pathways, and that it therefore may have a critical role in controlling the differentiation potential of 3T3-L1 cells by virtue of its ability to integrate cell fate decisions. As such, it may function as a more general gatekeeper of terminal differentiation.

It is clear that future work should now be focused on validating these findings in other cell lines or even in vivo. In addition, it is possible that some of our SMRT-related findings were muted by the presence of the SMRT homolog NCoR1, as we found that the majority of SMRT-bound DNA regions are also enriched for NCoR1 binding. This suggests that SMRT and NCoR1 may be partially redundant and compensate for the absence of one another, as has been proposed earlier (Perissi et al., 2010). It will therefore be of interest in future studies to further dissect the molecular context of the uncovered corepressor interactions as well as their dynamic properties during terminal adipogenesis and possibly to examine the involvement of other corepressors in this process as well.

EXPERIMENTAL PROCEDURES

Cell Culture

3T3-L1 cells were cultured as detailed in Mikkelsen et al. (2010) with the only difference that fetal calf serum was used instead of bovine calf serum (see Supplemental Experimental Procedures for more details).

SMRT, NCoR1, and RNA pol II ChIP-Seq

At the indicated differentiation time points, the cells were fixed using 1% formaldehyde and stored at -80° C. ChIP and Illumina sequencing was performed as explained in Raghav and Deplancke (2012). Data handling and downstream analyses are described in detail in the Supplemental Experimental Procedures.

ChIP for KAISO and H3K9me2

KAISO and H3K9me2 ChIP were performed as described by Reddy et al. (2009) with slight modifications as detailed in the Supplemental Experimental Procedures.

De Novo Motif Identification

The MEME software package was used to identify significant TF motifs in SMRT-bound sequences, which were then compared against known motifs using the TOMTOM motif comparison website (see Supplemental Experimental Procedures for more details).

MITOMI-Based Analysis of KAISO-DNA Interactions

Putative KAISO DNA-binding sites derived from human KAISO and mouse SMRT ChIP-Seq data were prepared for MITOMI analysis as explained in detail in the Supplemental Experimental Procedures. The open-reading frame of mouse KAISO was cloned using Gateway technology and transferred to the pMARE vector as described in Hens et al. (2011) to enable the in vitro expression of KAISO-eGFP fusion. MITOMI experiments were performed as described previously (Maerkl and Quake, 2007).

RNA pol II tags for SMRT peak-associated genes were counted and transformed into the RPKM measure (Mortazavi et al., 2008) to compare the transcriptional activity of genes at different days of differentiation (see Supplemental Experimental Procedures for more details).

Stable Knockdown of SMRT, NCoR1, and KAISO

Lentivirus-mediated shRNA vectors were used to enable stable SMRT, NCoR1, and KAISO protein knockdown in 3T3-L1 cells (see Supplemental Experimental Procedures for more details).

ChIP-qPCR Validation

Random peaks for validation were selected from ChIP-Seq data. Wild-type, SMRT, and KAISO KD stable 3T3-L1 cells were used to ChIP SMRT followed by qPCR for selected genomic regions (for more method details, see Supplemental Experimental Procedures).

RNA Isolation and qPCR for Gene Expression Analysis

Total RNA was isolated using an RNAeasy kit (QIAGEN). RT-qPCR was performed using SuperScript III (Invitrogen), SYBR Green, and the 7900HT Real-Time PCR system (Applied Biosystems). Primers were derived from the GETPrime database (Gubelmann et al., 2011) and are listed in the Supplemental Experimental Procedures.

Immunoprecipitation

SMRT and NCoR1 antibodies were used to pull down KAISO from 3T3-L1 wildtype, shRNA control, KAISO KD, NCoR1 KD, and SMRT KD total cell lysates followed by western blotting to confirm the presence of KAISO (see Supplemental Experimental Procedures for more details).

Cell Cycle Analysis

FACS analysis using propidium iodide was performed to analyze the cell-cycle progression in SMRT KD, KAISO KD, and shRNA control 3T3-L1 cells (see Supplemental Experimental Procedures for more details).

ACCESSION NUMBERS

SMRT, NCoR1, and RNA pol II ChIP-Seq data sets associated with this study are available at ArrayExpress under the accession number E-MTAB-1031.

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures, five tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2012.03.017.

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