

A unique role of cohesin-SA1 in gene regulation and development

Silvia Remeseiro^{1,3}, Ana Cuadrado^{1,3},
Gonzalo Gómez-López², David G Pisano²
and Ana Losada^{1,*}

¹Chromosome Dynamics Group, Molecular Oncology Programme, Spanish National Cancer Research Centre (CNIO), Madrid, Spain and ²Bioinformatics Unit, Structural Biology and Biocomputing Programme, Spanish National Cancer Research Centre (CNIO), Madrid, Spain

Vertebrates have two cohesin complexes that consist of Smc1, Smc3, Rad21/Sccl and either SA1 or SA2, but their functional specificity is unclear. Mouse embryos lacking SA1 show developmental delay and die before birth. Comparison of the genome-wide distribution of cohesin in wild-type and SA1-null cells reveals that SA1 is largely responsible for cohesin accumulation at promoters and at sites bound by the insulator protein CTCF. As a consequence, ablation of SA1 alters transcription of genes involved in biological processes related to Cornelia de Lange syndrome (CdLS), a genetic disorder linked to dysfunction of cohesin. We show that the presence of cohesin-SA1 at the promoter of *myc* and of protocadherin genes positively regulates their expression, a task that cannot be assumed by cohesin-SA2. Lack of SA1 also alters cohesin-binding pattern along some gene clusters and leads to dysregulation of genes within. We hypothesize that impaired cohesin-SA1 function in gene expression underlies the molecular aetiology of CdLS.

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Introduction

In addition to its role in sister chromatid cohesion, essential for accurate chromosome segregation and postreplicative DNA repair (Losada and Hirano, 2005; Peters *et al.*, 2008; Nasmyth and Haering, 2009), cohesin contributes to transcriptional regulation (Rollins *et al.*, 2004; Dorsett *et al.*, 2005; Horsfield *et al.*, 2007; Hallson *et al.*, 2008; Pauli *et al.*, 2008, 2010; Schaaf *et al.*, 2009; Lin *et al.*, 2011), DNA replication (Guillou *et al.*, 2010), V(D)J recombination (Degner *et al.*, 2009) and T-cell receptor rearrangement (Seitan *et al.*, 2011). Chromosome conformation capture (3-C) studies and other experimental evidences suggest that cohesin performs these

functions by promoting the formation of chromatin loops in collaboration with additional factors (Hadjur *et al.*, 2009; Mishiro *et al.*, 2009; Nativio *et al.*, 2009; Hou *et al.*, 2010; Chien *et al.*, 2011; Seitan *et al.*, 2011). One such factor is CTCF, which colocalizes with cohesin at many sites along the human and mouse genomes (Parelho *et al.*, 2008; Rubio *et al.*, 2008; Wendt *et al.*, 2008). Cohesin is also found at non-CTCF-binding sites occupied by tissue-specific transcription factors (TFs), and possibly contributes to set up tissue-specific transcriptional programs (Schmidt *et al.*, 2010). In murine embryonic stem cells, cohesin and the transcriptional coactivator Mediator facilitate DNA looping between the enhancers and promoters of some genes required to maintain pluripotency (Kagey *et al.*, 2010).

The involvement of cohesin in all these different processes that are critical for cell proliferation and differentiation makes it difficult to determine which is most relevant for cohesinopathies, human syndromes caused by mutations in proteins related to cohesin (Bose and Gerton, 2010). The most prevalent cohesinopathy described to date is the Cornelia de Lange syndrome (CdLS), which is caused by heterozygous mutations in the cohesin loader Nipbl (Krantz *et al.*, 2004; Tonkin *et al.*, 2004) or, in a reduced number of cases, in the cohesin subunits Smc1 or Smc3 (Musio *et al.*, 2006; Deardorff *et al.*, 2007). CdLS is characterized by mental retardation, reduced body size, dysmorphic face, upper limb defects and several additional organ abnormalities (Liu and Krantz, 2009). Cells from patients rarely show cohesion defects and instead, microarray studies reveal altered patterns of gene expression (Castronovo *et al.*, 2009; Liu *et al.*, 2009). Nipbl-heterozygous mice exhibit many features of CdLS and display modest but significant transcriptional changes in mouse embryonic fibroblasts (MEFs) and embryonic brains (Kawauchi *et al.*, 2009). Whether the wide range of defects observed in CdLS patients are elicited by expression changes in a few critical genes or by the sum of multiple small alterations is unclear (Muto *et al.*, 2011). Moreover, the exact role of cohesin in CdLS pathogenesis is still far from understood.

Importantly, somatic vertebrate cells have two distinct versions of cohesin that consist of Smc1, Smc3, Rad21/Sccl and either SA1 or SA2 (Losada *et al.*, 2000; Sumara *et al.*, 2000). The two SA proteins show 75% sequence identity along their central region and only differ in short regions at both ends of each protein. Cohesin-SA1 and cohesin-SA2 present similar cell-cycle regulation in terms of loading and dissociation from chromatin (Losada *et al.*, 2000) and are found in all mouse tissues (Remeseiro *et al.*, 2012). However, they are not functionally equivalent. Mice homozygous for a gene-trap allele that abolishes SA1 expression are embryonic lethal (Remeseiro *et al.*, 2012). We have shown that cohesin-SA1 plays a specific role in telomere cohesion that is essential for efficient telomere replication. In contrast, cohesion at centromeres, which is critical for chromosome segregation, relies on cohesin-SA2 (Canudas and Smith, 2009; Solomon

*Corresponding author. Molecular Oncology Programme, Spanish National Cancer Research Centre (CNIO), Melchor Fernández Almagro 3, Madrid 28029, Spain. Tel.: +34 917328000/ext. 3470; Fax: +34 917328033; E-mail: alosada@cnio.es

³These two authors contributed equally to this work

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et al, 2011). Despite their robust centromeric cohesion, chromosome segregation is defective in SA1-deficient cells and often leads to aneuploidy, increasing the incidence of spontaneous tumours in heterozygous animals (Remeseiro *et al*, 2012).

To further understand the functional specificity of cohesin-SA1 and cohesin-SA2, we have compared their genomic distributions. We report, for the first time, the genome-wide binding sites of four different cohesin subunits in both wild-type and SA1-null mouse cells. We show that cohesin-SA1 is enriched at promoters and, more importantly, that it determines the distribution of cohesin. In the absence of SA1, cohesin is significantly less abundant at gene promoters and relocates to genomic positions featured by low cohesin occupancy and absence of CTCF. As a result, gene expression is altered, affecting genes involved in developmental pathways relevant to CdLS.

Results

SA1 is essential for embryonic development

We recently generated a genetically modified mouse model lacking the expression of *Stag1* gene encoding the SA1 cohesin subunit (Remeseiro *et al*, 2012). Viability of SA1-null embryos strongly decreases by E12.5, but some exceptionally survive to E18.5 (see Supplementary Table S1 in Remeseiro *et al*, 2012). Those late embryos present a clear growth delay and general hypoplasia (Figure 1A). Their skin is much thinner, with less hair follicles and a reduced muscle layer compared with their wild-type littermates (Figure 1B, panel I). The developmental delay is also observed in organs such as kidney and liver (Figure 1B, panels II and III, respectively). Interestingly, SA1-null embryos present some features characteristic of CdLS in addition to the reduced body size. These include impairment of lipid metabolism, as illustrated by a dramatically diminished interscapular layer of brown adipose tissue (BAT) (Figure 1B, panel IV), and severe

abnormalities in skeletal and bone development, featured by delayed ossification and defective calcium deposition (Figure 1B, panels V–VII). Both cohesin-SA1 and cohesin-SA2 are ubiquitously expressed in wild-type E17.5 embryos and,

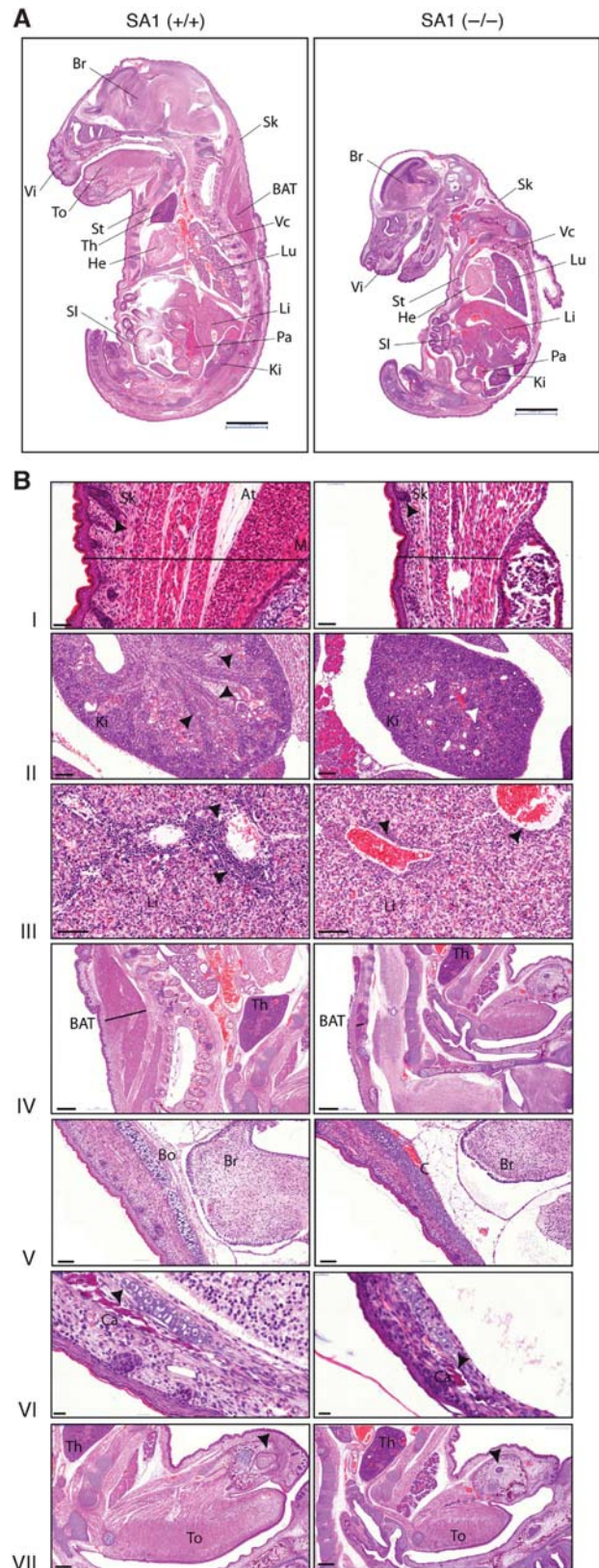


Figure 1 SA1-null embryos present growth delay. Histological analysis of HE-stained tissue sections from E17.5 embryos shows general hypoplasia and developmental delay in SA1-null embryos. (A) SA1-null embryos present a much reduced body size compared with their wild-type littermates. The following organs/tissues are indicated: BAT, brown adipose tissue; Br, brain; He, heart; Ki, kidney; Li, liver; Lu, lung; Pa, pancreas; Si, small intestine; Sk, skin; St, sternum; Th, thymus; To, tongue; Vc, vertebral column; Vi, vibrissa. Scale bars, 2 mm. (B) Detailed sections of different tissues showing either developmental delay or CdLS features. (I) Thinner skin (black line) in SA1-null embryos with less hair follicles (arrowheads), reduced adipose tissue (At) and thinner muscle layer (M). Scale bars, 50 μ m. (II) Kidney sections showing collecting ducts already formed in wild-type (black arrowheads) and not radially arranged in SA1-null embryos. In the latter, glomeruli are dispersed all over as primitive glomeruli at earlier stages (white arrowheads). Scale bars, 100 μ m. (III) Decreased number of haematopoietic precursors in livers from SA1-null embryos compared with wild-type (arrowheads). Scale bars, 100 μ m. (IV) Thickness of the interscapular layer of BAT is dramatically diminished in SA1-null embryos (black line). Scale bars, 500 μ m. (V) Delayed intramembranous ossification in the cranium in the absence of SA1. Notice the presence of cartilage (C) in SA1-null embryos instead of bone (Bo) already formed in the wild-type. Scale bars, 100 μ m. (VI) Defective bone calcification (Ca) in the cranium from SA1-null embryos. Scale bars, 20 μ m. (VII) Delay in dentition in SA1-null embryos. Arrowheads in both sections indicate tooth buds. Scale bars, 500 μ m.

importantly, SA2 expression is not altered in SA1-null embryonic tissues, including those described above (Supplementary Figure S1A). Protein and mRNA levels of other cohesin subunits remain invariable in the absence of SA1 (shown for brain in Supplementary Figure S1B and C). Moreover, the amount of total cohesin bound to chromatin does not change in MEFs lacking SA1 (Figure 4G in Remeseiro *et al*, 2012). Thus, it is unlikely that the inability of cohesin-SA2 to compensate the cohesin-SA1 deficiency has merely a quantitative basis. Instead, cohesin-SA1 must perform specific function(s) in embryonic development that cannot be assumed by cohesin-SA2.

Genome-wide distribution of cohesin-SA1 and cohesin-SA2

In order to assess if a differential distribution of the two cohesin complexes contributes to their functional specificity, we performed chromatin immunoprecipitation followed by massive parallel DNA sequencing (ChIP-seq) in primary MEFs (E12.5) using SA1, SA2, SMC1 and SMC3-specific antibodies (Supplementary Figure S2A and B). Genome-wide analysis of the sequenced tags using MACS peak detection algorithm (see Materials and methods) defined

25 737 binding sites for SA1, 7741 for SA2, 23 994 for SMC1 and 15 546 for SMC3, with a cutoff P -value of 10^{-5} and $FDR < 0.1$ (Figure 2A and B; ChIP-seq data summary in Supplementary Tables S1 and S2). A subset of these regions was further validated by quantitative PCR analysis (ChIP-qPCR; Supplementary Figure S2C, D and E). As expected for proteins forming a complex, a large overlap was observed between the binding sites obtained with SMC1 and SMC3 antibodies, as well as between those seen with SMC1 and either SA1 or SA2 antibodies (Figure 2C). Lack of a more complete overlap is likely attributable to slight differences in the accessibility of the antibodies to the complex depending on its chromatin environment. Since cohesin has been reported to colocalize with the insulator protein CTCF, we compared all our cohesin data sets with a data set of CTCF-binding sites from a murine cell line available online (ENCODE/Stanford/Yale data set). Most positions detected with any given cohesin antibody correspond to a CTCF-binding site, with the SA2 data set showing the lowest overlap (63%; Supplementary Figure S3A). Thus, our ChIP-seq data, obtained with four different antibodies, provide an accurate map of cohesin-binding sites along the mouse genome.

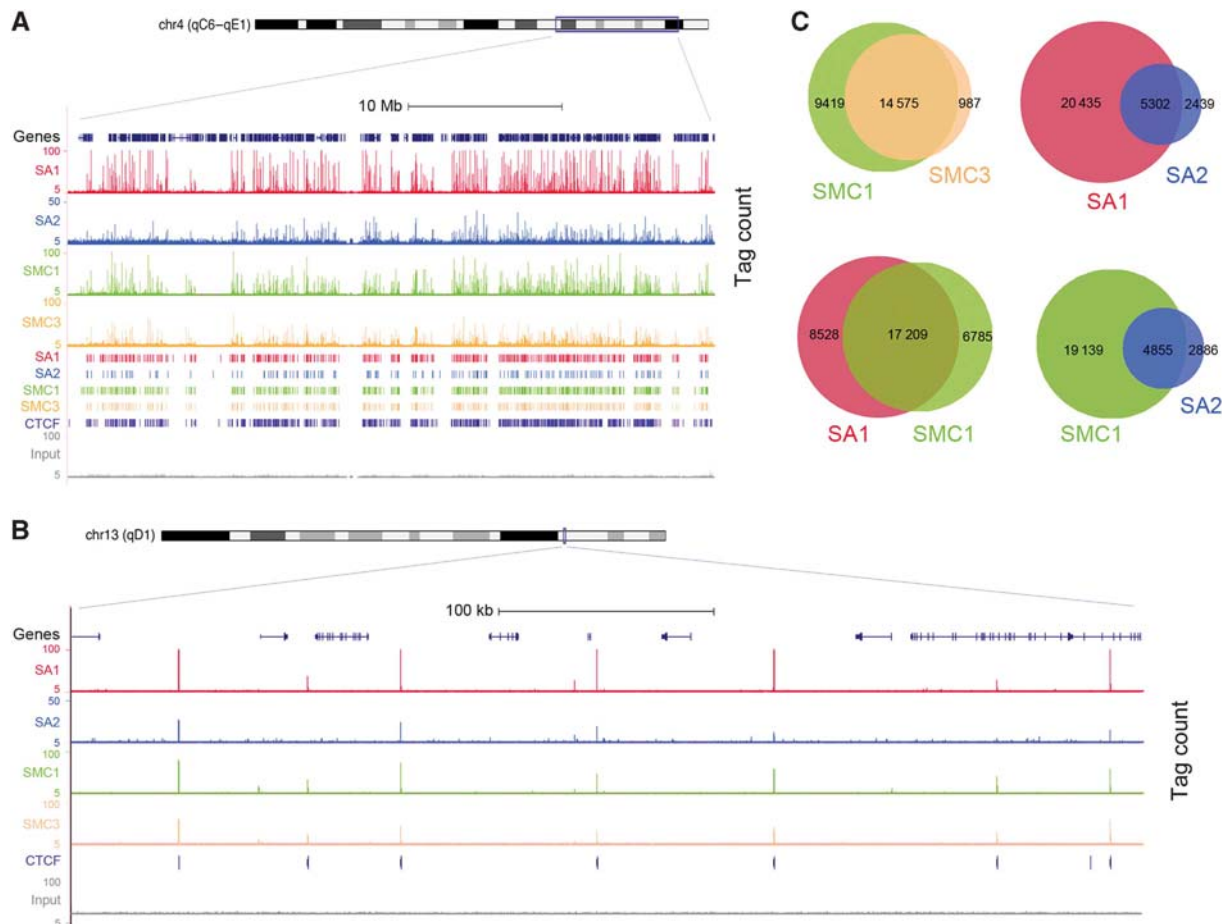


Figure 2 Genome-wide distribution of cohesin-SA1 and cohesin-SA2 in the mouse genome. (A) Representative genomic distribution of SA1, SA2, SMC1 and SMC3 in a region of mouse chromosome 4. Tag counts (upper) and called peak tracks (lower) are shown. A track with CTCF-binding sites (from ENCODE/Stanford/Yale data set) and the input are also depicted. Higher magnification of a region of chromosome 13 is shown in (B). (C) Venn diagrams showing the overlapping between the binding sites identified in wild-type MEFs by ChIP-seq with the indicated antibodies.

Cohesin distribution and enrichment at promoters depend on SA1

Cohesin-SA1 and cohesin-SA2 binding sites are distributed among gene-associated and intergenic regions to a similar extent in wild-type MEFs (Figure 3A, left and middle pie charts). Importantly, cohesin-SA1 is highly enriched at regions 1-kilobase (kb) upstream transcription start sites (TSS) considering that they represent only 0.62% of the mouse genome (Figure 3B, left). The enrichment is significantly less noticeable for cohesin-SA2. This difference is even more remarkable if we compare enrichment at TSS of cohesin-binding sites that are exclusive for SA1 or SA2 (Figure 3B, right). In addition, the frequency of binding at TSS is higher for cohesin-SA1 than for cohesin-SA2 (Figure 3C, left). We next asked whether the absence of SA1 affects cohesin distribution. Indeed, ChIP-seq analysis of SA1-null MEFs with the SA2 antibody resulted in 6349 peaks (P -value of 10^{-5} , $FDR < 0.1$) from which only 45% overlap with the SA2 peaks found in wild-type MEFs (Supplementary Figure S3B; Supplementary Tables S1 and S2). The new positions occupied by SA2 correspond to binding sites preferentially located at intergenic regions (Figure 3A, pie chart on the right). The frequency of SA2 binding at TSS is further reduced in SA1-null cells, which suggests that SA2 is unable to replace SA1 particularly at these genomic positions (Figure 3C, right). Interestingly, we observed a gradual increase in frequency of binding at TSS as the cohesin-bound regions lengthen only in the case of cohesin-SA1, but not for cohesin-SA2 in either wild-type or SA1-null cells (Figure 3D). This indicates that genomic regions containing the highest density of cohesin-SA1 molecules are mainly gene promoters.

To further characterize the redistribution of cohesin-SA2 in the absence of SA1, we performed ChIP-seq with SMC1 and SMC3 antibodies in SA1-null MEFs. The number of cohesin positions detected with these antibodies in the SA1-null MEFs (46 997 and 34 800, respectively) is higher than the number detected with the SA2 antibody, most likely due to a more limited sensibility of the latter (Supplementary Tables S1 and S2). The fact that the number of peaks doubles in both cases with respect to wild-type MEFs suggests a major redistribution of cohesin in the absence of SA1 (Supplementary Figure S3C). Of all SMC1-binding sites identified in SA1-null MEFs, 45% correspond to cohesin-SA1 positions in wild-type cells (group 'a' in Figure 3E). The remaining 55% (group 'b') show significantly lower peak intensity than those in group 'a', and the same is true for the SMC3-binding sites in SA1-null MEFs (groups 'c' and 'd' in Figure 3E; examples are shown in Figure 3F, left). Binding to some of these regions with lower cohesin occupancy was validated by ChIP-qPCR (Supplementary Figure S2C, lower panels). These positions have an additional feature. Whereas most sites bound by SMC1 and SMC3 in wild-type MEFs are also CTCF sites (71 and 82% respectively, see charts in Figure 3F, right), the overlap decreases to 46 and 58% in SA1-null cells, mostly due to the modest overlapping of the new positions with CTCF (groups 'b' and 'd', 19 and 27%, respectively). These results indicate that in the absence of SA1, cohesin spreads to CTCF-negative positions in which the complex shows significantly weaker enrichment compared with the positions defined in the wild-type cells. Taken all together, we conclude that cohesin accumulation at promoters and CTCF sites depends

largely on the presence of SA1. This points to a unique role of cohesin-SA1 in transcriptional regulation.

Transcriptome changes in SA1-null cells affect CdLS-related functions

To obtain a global view of expression changes in the absence of SA1, we analysed the transcriptomes of SA1-null and wild-type MEFs using microarrays (Supplementary Table S3). An enrichment analysis of Gene Ontology (GO) terms revealed profound functional differences between both expression profiles. Transcriptional changes in the SA1-null cells led to upregulation of GO-defined processes related to phagocytosis, endocytosis and apoptosis. In contrast, processes associated with abnormalities observed in CdLS patients, such as limb and skeletal system morphogenesis, heart and lung development and lipid metabolism, were downregulated (Figure 4A; Supplementary Table S4). Moreover, we found a significant overlap (GO Comparative Analysis, $FDR < 0.05$) between the transcriptional changes linked to the loss of cohesin-SA1 and those reported for Nipbl-heterozygous MEFs (Kawauchi *et al*, 2009) (Figure 4B; Supplementary Table S5). A stringent statistical analysis ($FDR < 0.15$) identified 55 differentially expressed genes (DEGs), many of them significantly upregulated in the SA1-null MEFs (Supplementary Table S6). Validation of microarray data was performed by qPCR for a subset of 15 genes (Supplementary Figure S4). To determine if this transcriptional regulation is SA1 specific, we knocked down either SA1 or SA2 in MEFs by siRNA. Transient downregulation of SA1 significantly reduced the expression of 11 out of 14 genes selected from those found to be regulated by SA1 in the microarray analysis (Figure 4C). In contrast, reduction of SA2 expression only affected two of the tested genes, supporting the specificity of cohesin-SA1 in this regulation. Since SA1 is present in the vicinity of the TSS in only six of the DEGs (Supplementary Table S6), it is likely that cohesin-SA1 affects gene expression by additional mechanisms that do not require its presence at promoters. According to recent literature, cohesin promotes the proper topological organization of gene expression domains or gene clusters. Consistent with this role for cohesin-SA1, we observed that 18 out of 55 DEGs are located in close proximity of one another (< 800 kb; Figure 4D). Finally, cohesin may indirectly affect the expression of a gene by regulating a factor required for its transcription (Horsfield *et al*, 2007). As described in the next sections, we have found evidence of the importance of cohesin-SA1 in these different modes of regulation of gene expression by cohesin.

Cohesin-SA1 positively regulates c-myc expression

Myc is a major modulator of cell proliferation, growth and differentiation whose transcription is regulated by cohesin in different organisms (Stedman *et al*, 2008; Rhodes *et al*, 2010). Mutant mice expressing reduced levels of Myc have decreased body mass owing to multiorgan hypoplasia (Trumpp *et al*, 2001). Our ChIP-seq data show that the c-myc gene constitutes one of the most prominent SA1-binding regions in the mouse genome (Figure 5A). Quantitative qPCR analyses in MEFs validate this result and show that SMC1 levels in this region are severely reduced in the SA1-null cells (Supplementary Figure S2D). Expression of myc target genes is significantly altered in these cells ($FDR = 0.06$; Supplementary Figure S5), supporting the rele-

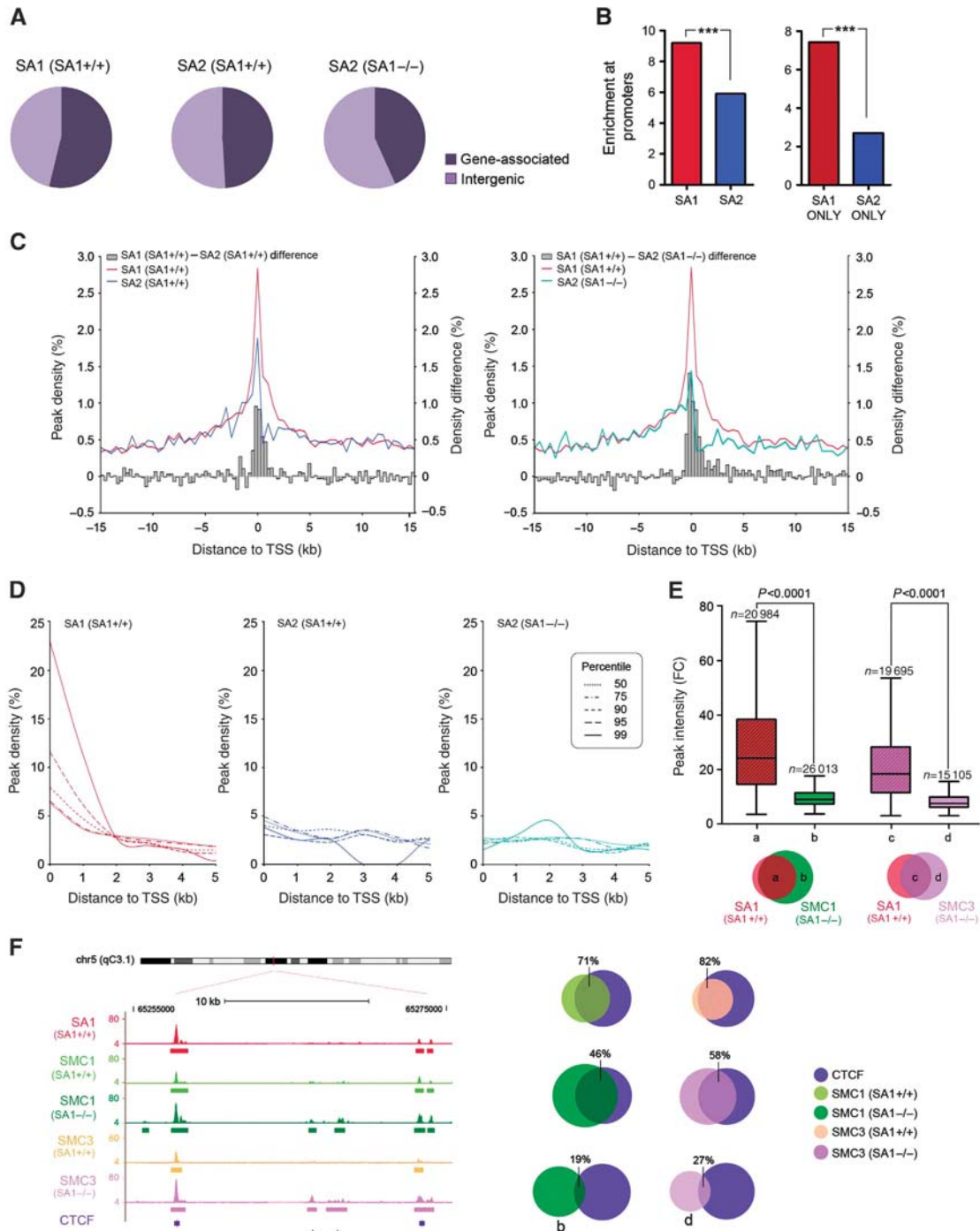


Figure 3 Cohesin distribution depends on SA1. **(A)** Distribution of SA1 and SA2 in SA1 +/+ (wild-type) cells and of SA2 in SA1 -/- cells represented as percentage of peaks detected at gene-associated regions (including 1 kb upstream TSS, Gene Body and 1 kb downstream TTS) and intergenic regions. Notice that SA2 relocates towards intergenic regions in the absence of SA1. **(B)** Left: The percentage of SA1 and SA2 binding sites located in regions 1 kb upstream TSS in wild-type cells was normalized against the frequency of these regions in the genome and displayed as fold enrichment. Right: Same analysis performed for positions that are exclusive for SA1 or SA2 (shadowed bars). *** $P < 0.0001$. **(C)** Cohesin distribution around TSS (± 15 kb) defined as peak density (%). The histogram represents the difference in peak density between SA1 and SA2. **(D)** Frequency of binding at TSS in percentiles of peak length distribution (P50, P75, P90, P95 and P99). **(E)** Peak intensity (measured as fold change) for SMC1 and SMC3 peaks identified in SA1-null MEFs that overlap (groups 'a' and 'c') or not (groups 'b' and 'd') with SA1 peaks, as indicated in the Venn diagrams below the graph. Notice that cohesin positions in groups 'a' and 'c' present significantly higher occupancy (medians = 24.4 and 18.5, respectively) than those in groups 'b' and 'd' (medians = 9.2 and 7.6, respectively). See Supplementary Figure S3B for the number of peaks in each group. **(F)** Left: Representative image showing the redistribution of SMC1 and SMC3 in SA1-null cells towards low occupancy sites (arrowheads). Right: Venn diagrams showing the overlapping between CTCF-binding sites and the cohesin-binding sites indicated, including those defined as groups 'b' and 'd' in **(E)**. More detailed information regarding the peak number within each group is shown in Supplementary Figure S3A and B.

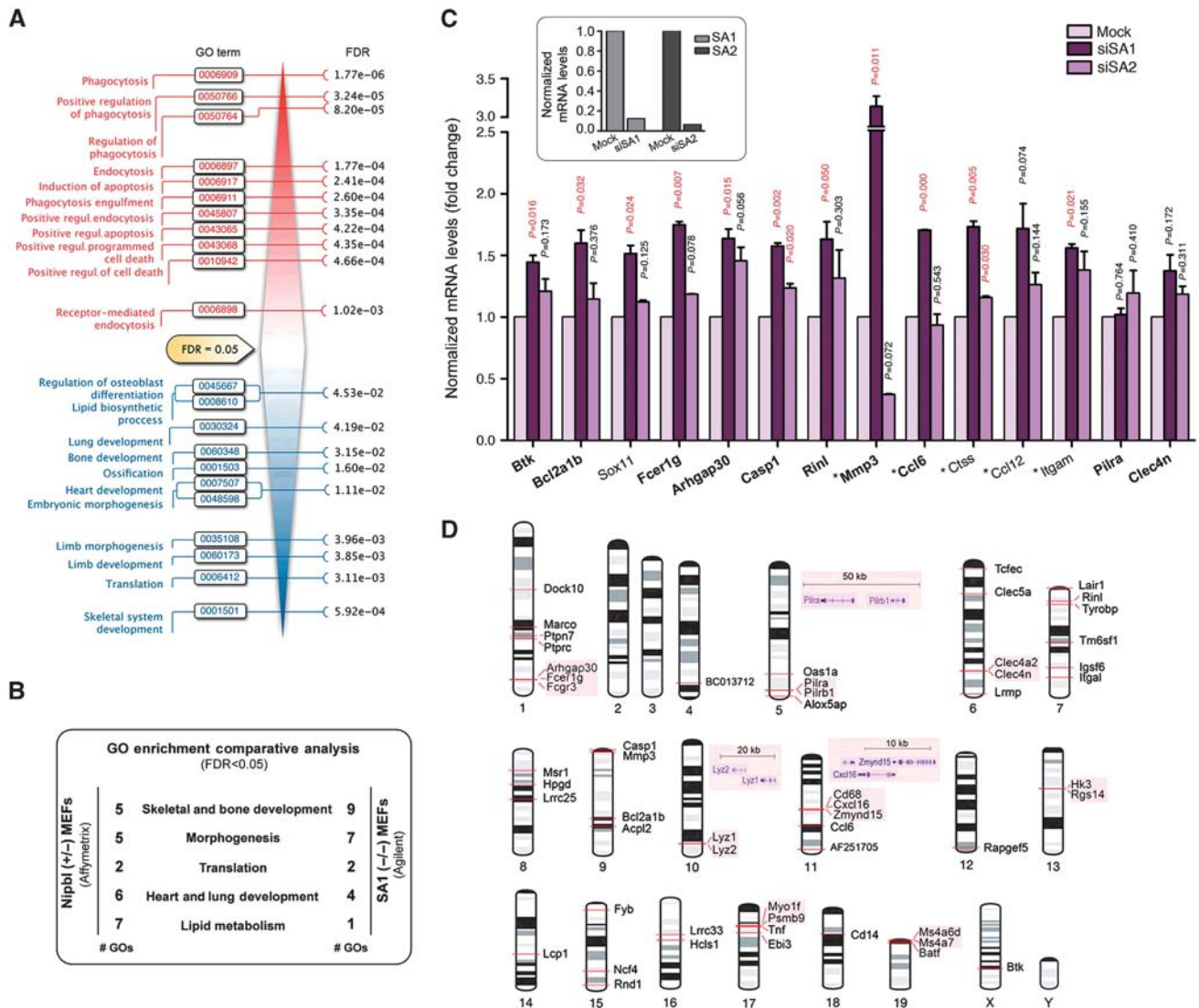


Figure 4 Gene expression changes in the absence of SA1. **(A)** GO analysis (FDR < 0.05) reveals biological processes upregulated (in red) and downregulated (in blue) in SA1-null MEFs compared with wild-type. FDRs for each of the enriched GO terms are indicated. **(B)** GO Comparative Analysis (FDR < 0.05) between transcriptomes from SA1-null and Nipbl-heterozygous MEFs. Common GO terms were grouped in five big biological processes: skeletal and bone development, morphogenesis, translation, heart and lung development and lipid metabolism. The number of GO terms belonging to each group is shown (# GOs). **(C)** Expression of a set of SA1-regulated genes that includes 10 DEGs (shown in bold) and additional genes (Sox11 and skin-related genes, marked with an asterisk) was measured in wild-type MEFs after transfection with SA1 or SA2 siRNAs (results come from triplicate qPCR reactions from two independent experiments). The upper inset shows the efficiency of siRNAs. **(D)** Chromosomal location of the 55 DEGs (FDR < 0.15). Notice that 18 of them are located in close proximity (shadowed).

vance of cohesin-SA1 in positively regulating c-myc. More importantly, ChIP-qPCR performed in brain tissue obtained from wild-type and SA1-null E17.5 embryos indicates that also *in vivo* SA2 does not efficiently replace SA1 (Figure 5B). Myc mRNA and protein levels are reduced in brains from SA1-null embryos, as shown by qPCR and immunostaining (Figure 5C and D, respectively). It is likely that decreased cell proliferation rates due to transcriptional downregulation of c-myc contributes to the lethality of SA1-null embryos. We asked whether other TFs might be regulated by cohesin-SA1. Indeed, gene set analysis revealed that among the transcriptionally altered genes, there is a statistically significant enrichment in genes with binding sites for Pax2 and MafB (Figure 5E, top), two TFs involved in differentiation and development (Cordes and Barsh, 1994; Mansouri *et al*,

1996) whose encoding genes contain cohesin-SA1 at their promoters. Consistently, two of the DEGs are MafB downstream targets (Figure 5E, bottom) and MafB itself is upregulated in SA1-null MEFs (Supplementary Table S3). Therefore, part of the expression changes associated to cohesin-SA1 loss can be secondary to the regulation of genes encoding TFs.

Cohesin-SA1 regulates the expression of gene clusters involved in skin functions

Among DEGs not having cohesin at TSS there are three genes involved in skin development and function (Ccl6, Cxcl16 and Mmp3). Since SA1-null embryos show defects in skin formation (Figure 1B, panel I), we decided to take a closer look at expression changes caused by the absence of SA1 in genes with skin-related functions including immunity, proliferation,

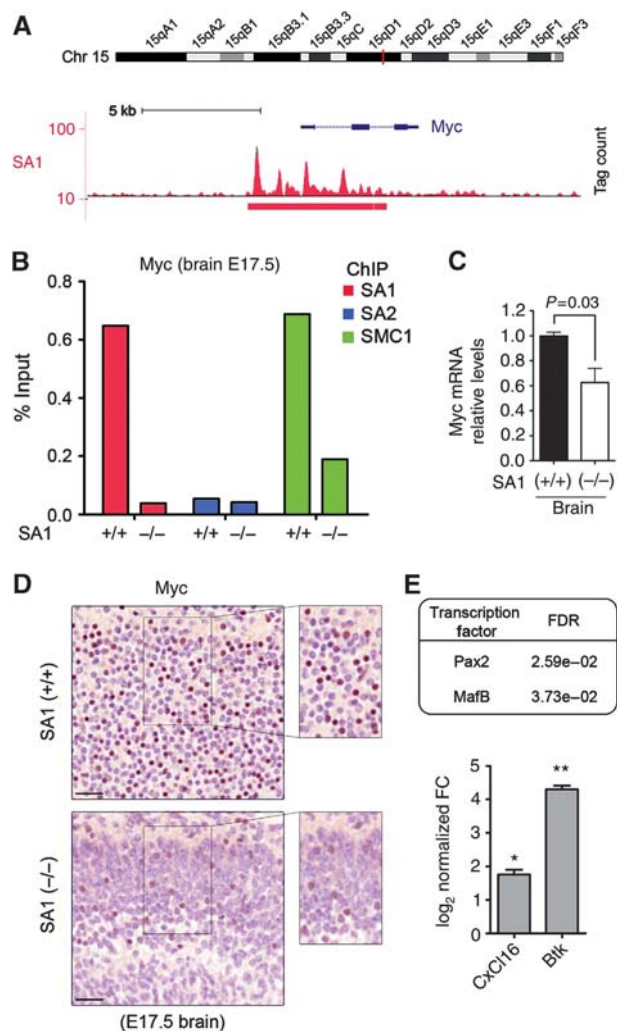


Figure 5 Cohesin-SA1 regulates myc expression. (A) SA1-binding region at myc gene (5350 bp) is the widest in the mouse genome (the median is 531 bp). (B) Validation by ChIP-qPCR of SA1, SA2 and SMC1 binding at myc promoter in wild-type ($n = 12$) and SA1-null ($n = 9$) E17.5 brains. (C) Myc mRNA levels are significantly reduced in SA1-null brains from E17.5 embryos. Three embryonic brains per genotype were used. (D) Immunohistochemistry on E17.5 brains showing reduced Myc protein levels in the cortex of SA1-null embryos. Notice the different cortical structure between wild-type and SA1-null brains. Scale bars, 40 μ m. (E) Table showing two TFs whose target genes are dysregulated in SA1-null cells. Expression levels of two of those target genes (Cxcl16 and Btk) were estimated from three independent qPCR reactions of two clones per genotype. Values are represented as log₂ of fold change (FC) versus wild-type. ** $P < 0.01$, * $P < 0.05$.

skin architecture and motility. Gene Set Enrichment Analysis (GSEA) revealed a highly significant enrichment in those genes in SA1-null MEFs ($FDR < 10^{-3}$; Supplementary Figure S6A). Only 15 out of 88 (17%) skin-related genes whose transcription changes have SA1-binding sites up to 5 kb from their TSS (Supplementary Table S7). Strikingly, 63 of the genes (72%) are located in clusters (labelled in blue in Figure 6A), such as the Ccl and Cxcl clusters involved in skin immune function or the keratin clusters involved in skin structure. Analysis of mRNA levels further confirmed the SA1-specific transcriptional changes for a subset of genes implicated in the immune response (Figure 6B; see also genes labelled with an asterisk in Figure 4C). As shown by ChIP-seq for the keratin cluster in chromosome 11 and for the Cxcl

cluster in chromosome 5, cohesin distribution within the cluster is considerably altered in SA1-null cells, as judged by the appearance of multiple ‘new’ sites with low cohesin occupancy (Figure 6C; Supplementary Figure S6B). At the same time, there is much less cohesin at SA1-binding sites, as exemplified by the reduced binding of SMC1 and SMC3 to a site located in the vicinity of Krt15/19 genes in SA1-null cells (indicated with an arrowhead in Figure 6C; qPCR data in Supplementary Figure S2C, upper panels). We therefore hypothesize that cohesin-SA1 plays an architectural role in the organization of these gene clusters that is essential for regulation of their gene expression.

Cohesin-SA1 regulates the expression of Protocadherins in the brain

A detailed analysis of the transcription data revealed the downregulation of many members of the protocadherin (Pcdh) gene family in SA1-null cells. Most, but not all, Pcdh genes are present in three consecutive clusters (Pcdh a, b and g) at mouse chromosome 18. The expression of the Pcdh genes in these clusters is regulated by multiple promoters and alternative *cis* splicing (Yagi, 2008). Our ChIP-seq data identified SA1-binding sites located precisely at most of the multiple TSS of the clustered Pcdh genes (Figure 7A) and also at non-clustered Pcdh genes (e.g., Pcdh7; validation shown in Supplementary Figure S2E). *In vivo*, ChIP-qPCR performed in the brains from wild-type and SA1-null E17.5 embryos showed on the one hand, the prevalence of SA1 over SA2 at the TSS of several of these genes, and on the other, that SA2 does not efficiently replace SA1 in the SA1-null brains, since SMC1 occupancy in these regions is much reduced (Figure 7B). Given that protocadherins are essential for central nervous system development (Morishita and Yagi, 2007), the contribution of cohesin-SA1 to their transcriptional regulation may be particularly critical in the brain. Indeed, we observed a highly significant downregulation of 7 out of 8 Pcdh genes in the brains of E17.5 SA1-null embryos compared with wild-type (Figure 7C). We had chosen these genes because they had also been found downregulated in the brains of Nipbl-heterozygous mice, leading the authors to propose that reduced levels of protocadherins in the brain might contribute to the mental retardation observed in CdLS patients (Kawauchi *et al*, 2009). Importantly, we here show that regulation of Pcdh gene expression in the brain relies on the presence of cohesin-SA1 at their TSS.

Discussion

Vertebrates have two distinct cohesin complexes that contain either SA1 or SA2 (Losada *et al*, 2000; Sumara *et al*, 2000), but most studies assume that both function in a similar way and/or that cohesin-SA2 is more relevant because it is more abundant in somatic cells (Xiao *et al*, 2011). However, ablation of SA1 results in embryonic lethality in mice and late SA1-null mouse embryos present a clear developmental delay (Remeseiro *et al*, 2012; this study). Thus, cohesin-SA1 and cohesin-SA2 are not functionally equivalent. On one hand, we have shown that cohesin-SA1 is responsible for telomere cohesion whereas cohesin-SA2 is critical for centromere cohesion. On the other hand, we report here that cohesin-SA1 has an important role in gene regulation that cannot be assumed by cohesin-SA2.

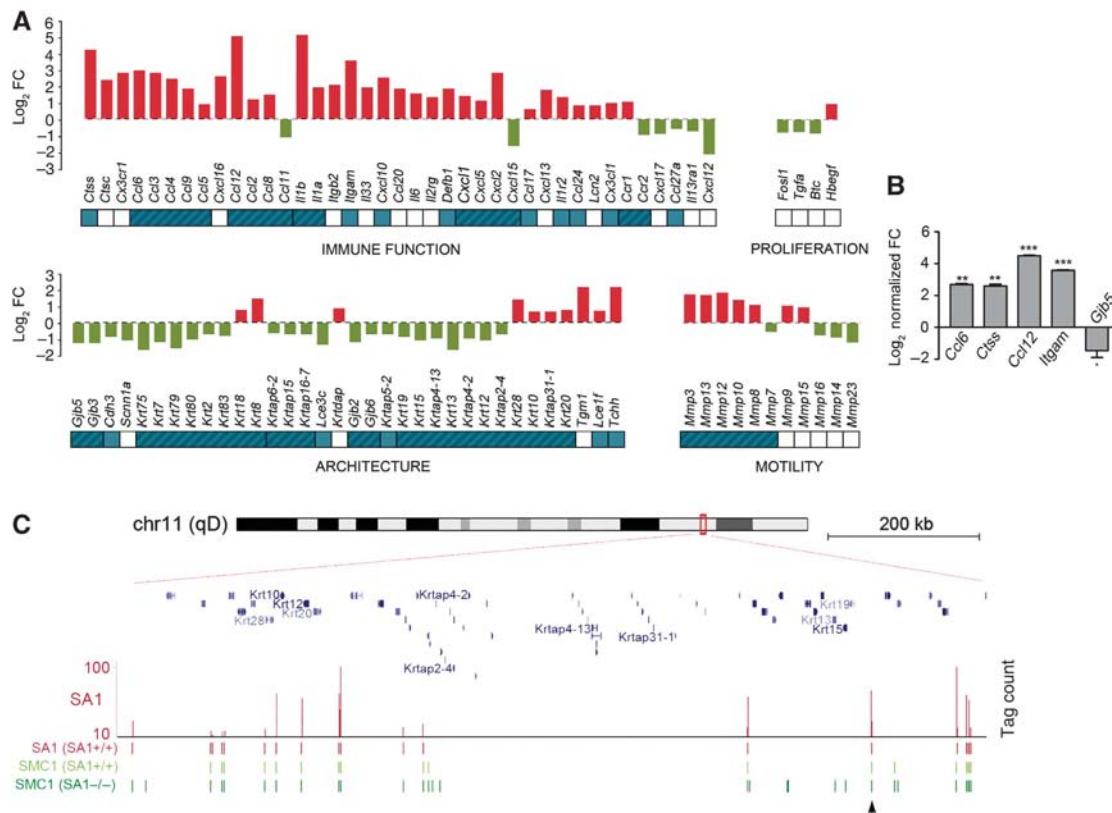


Figure 6 SA1 regulates clustered genes involved in skin development. (A) Transcriptional changes detected in genes involved in skin development and function in SA1-null cells. Bars represent the log₂ FC in SA1-null compared with wild-type cells obtained from microarray analysis. Genes in clusters are depicted in blue and genes belonging to the same cluster are grouped (blue shadow). (B) Validation by RT-qPCR of transcriptional changes in some of the genes shown in (A) (from three independent qPCR reactions of two clones per genotype). ****P* < 0.001, ***P* < 0.01, **P* < 0.05. (C) Detail of SA1 (SA1+/+), SMC1 (SA1+/+) and SMC1 (SA1-/-) binding sites at the Keratin cluster located on chromosome 11. Arrowhead points to a cohesin-SA1-binding site that is validated by ChIP-qPCR in Supplementary Figure S2C.

Differences between SA1 and SA2

We have analysed the genome-wide distributions of SA1 and SA2 in MEFs and found that cohesin-SA1 has a greater propensity for localizing at gene promoters and gene bodies, while cohesin-SA2 prefers intergenic regions. Moreover, in the absence of SA1, cohesin-SA2 (the only cohesin present) redistributes to intergenic regions and fails to accumulate at promoters and other sites nearby genes that could be important for transcriptional regulation, such as sites also bound by CTCF (see model in Figure 8, left). The molecular mechanisms by which cohesin-SA1 regulates gene expression are likely related to the ability of the complex to bring together two DNA sequences not only *in trans* (the sister chromatids), but also *in cis*, thereby facilitating DNA looping (Hadjur *et al*, 2009; Mishiro *et al*, 2009; Nativio *et al*, 2009; Hou *et al*, 2010; Chien *et al*, 2011). These loops may dictate gene expression by promoting or preventing communication between enhancers and promoters in different configurations. One would require the presence of cohesin at the promoter (model in Figure 8, upper right) whereas in other cases, cohesin would be located away from the promoter and would mediate the organization of a gene cluster (Figure 8, lower right). The transcriptional changes observed in SA1-null cells and tissues indicate that cohesin-SA2 cannot fulfil the function of cohesin-SA1. We envision that SA1 and SA2 must confer distinct properties to cohesin. The mechanism by which cohesin associates with chromatin, namely by topological embrace, allows cohesin sliding along DNA (Lengronne

et al, 2004; Ocampo-Hafalla and Uhlmann, 2011). Cohesin-SA1 could be less prone to sliding than cohesin-SA2 or, in other words, more prone to occupy a fixed genomic position. Cohesion, probably the most important function of cohesin-SA2, does not require localization of cohesin at precise sites, whereas regulation of transcription does. The molecular mechanisms underlying the potential differences in mobility between cohesin-SA1 and cohesin-SA2 remain to be elucidated. One possibility is that the interaction of SA1 and SA2 with Wapl and Pds5, two cohesion factors that modulate the association of cohesin with chromatin throughout the cell cycle, has distinct characteristics (Losada *et al*, 2005; Gandhi *et al*, 2006; Gause *et al*, 2010; Nishiyama *et al*, 2010). Another is the preferential interaction of SA1 with proteins present at defined sites such as CTCF, Mediator or Polycomb (Misulovin *et al*, 2008; Parelho *et al*, 2008; Rubio *et al*, 2008; Wendt *et al*, 2008; Nativio *et al*, 2009; Kagey *et al*, 2010; Strubbe *et al*, 2011; Xiao *et al*, 2011). The identification of proteins that specifically interact with either SA1 or SA2 is one goal of our future work.

Cohesin-SA1 and CdLS

The role of cohesin in transcriptional regulation has become particularly relevant for human health after identification of mutations in the cohesin loader Nipbl and in cohesin subunits as a major cause of CdLS and the subsequent finding that cells from CdLS patients do not show overt cohesion defects (Liu and Krantz, 2009; Dorsett, 2011). A mouse

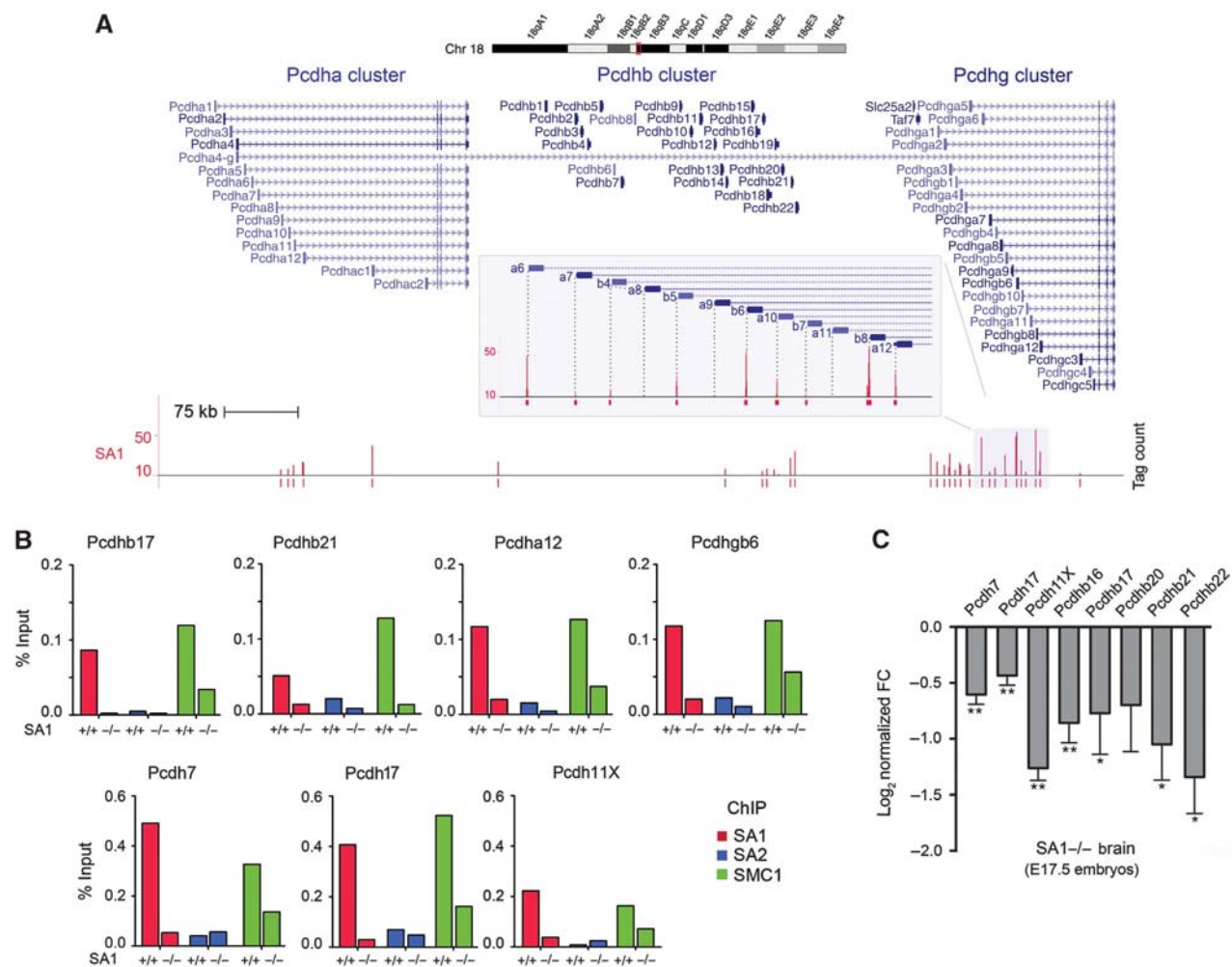


Figure 7 SA1 regulates the expression of protocadherins in mouse brain. (A) Detail of SA1-binding sites at Pcdh clusters located in chromosome 18. Notice the position of SA1 at multiple TSS. (B) SA1, SA2 and SMC1 binding at the TSS of four clustered and three non-clustered Pcdh genes was validated *in vivo* in wild-type ($n = 12$) and SA1-null ($n = 9$) E17.5 brains. (C) Significant downregulation of Pcdh genes in the brains from E17.5 SA1-null embryos (three embryos per genotype and three independent qPCR reactions per condition). Values are represented as \log_2 of FC versus wild-type. ** $P < 0.01$, * $P < 0.05$, Pcdhb20 P -value = 0.13.

heterozygous for a gene-trap mutation in Nipbl recapitulates some of the pathologies observed in CdLS patients including small size, craniofacial anomalies, heart defects, impaired hearing, delayed bone maturation, reduced body fat and behavioural disturbances (Kawauchi *et al*, 2009). Mouse lacking either Pds5A or Pds5B also exhibit CdLS-like features (Zhang *et al*, 2007, 2009). SA1-heterozygous mice do not show apparent CdLS phenotypes, but SA1-null embryos that survive to late stages of embryogenesis (E17.5–18.5) have clear developmental delay, impaired lipid accumulation and delayed ossification, features that resemble CdLS. Limb or heart defects have not been clearly observed in the limited number of embryos examined. In mice, reduction of Nipbl mRNA levels to 70% is sufficient to elicit CdLS phenotypes without causing apparent cohesion defects (Kawauchi *et al*, 2009). In *Drosophila*, reduction of Nipped-B expression to 30% of wild-type levels reduces the stable binding of cohesin SA subunit by the same percentage (Gause *et al*, 2010). How the decrease in Nipbl levels affects loading of cohesin-SA1 and cohesin-SA2 on chromatin in mammalian cells remains to be addressed. One scenario is that it affects more acutely the loading of cohesin-SA1 either because it is less abundant

(Losada *et al*, 2000; Holzmann *et al*, 2010; Remeseiro *et al*, 2012) or because being more frequently found within genes than cohesin-SA2, cohesin-SA1 may be released from chromatin to allow passage of the transcriptional machinery and thus needs to be constantly reloaded by Nipbl. Alternatively, reduction of Nipbl levels may decrease the amount of both cohesins on chromatin but while this barely affects cohesion, it affects gene transcription (Schaaf *et al*, 2009; Heidinger-Pauli *et al*, 2010). Although we cannot rule out the possibility that cohesin-SA2 affects also gene expression, comparative microarray analysis of human glioblastoma cells expressing or not SA2 shows no evidence for such a role (Solomon *et al*, 2011), consistent with our results in MEFs after downregulation of SA2 by siRNA.

In summary, we have found that cohesin-SA1 plays a unique role in transcriptional regulation that is essential for embryonic development and could underlie the aetiology of CdLS. Although the analysis of SA1-null embryonic fibroblasts combining transcriptional profiling and genome-wide distribution data by ChIP-seq is an important first step, we are aware of the necessity of employing a similar strategy in specific tissues or developmental/differentiation stages in

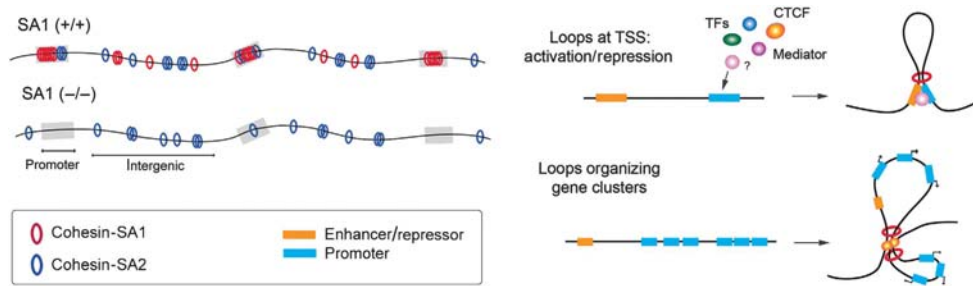


Figure 8 A model for the differential distribution of cohesin-SA1 and cohesin-SA2 and its implications in transcription. Left: Different dynamics and localization of cohesin-SA1 and cohesin-SA2. Cohesin-SA1 is enriched at gene promoters to a much larger extent than cohesin-SA2. In SA1-null cells, cohesin-SA2 fails to accumulate at gene promoters and relocates to intergenic positions. Right: Proposed mechanisms for cohesin-SA1 in regulation of gene expression. Cohesin-SA1 present at gene promoters (upper panel) or in the proximity of genes organized in clusters (lower panel) is required for the formation of loops that arrange the chromatin for gene transcription. The insulator protein CTCF as well as different TFs are likely involved in cohesin-SA1 recruitment to specific genomic positions.

order to understand the contribution of cohesin-SA1 to the establishment of their transcriptomes.

Materials and methods

Identification of SA1, SA2, SMC1 and SMC3 binding sites by ChIP-sequencing

ChIP was performed in SA1-null and wild-type MEFs with custom-made rabbit polyclonal antibodies against SA1, SA2 (described in Remeseiro *et al*, 2012), SMC1 and SMC3 (raised against the C-terminal peptides CEMAKDFVEDDTTHG and CDLTQYPDANPNP-NEQ, respectively), as described (Cuadrado *et al*, 2010) with some modifications. Cells were cross-linked by addition of 1% formaldehyde for 10 min at room temperature, harvested and frozen in liquid nitrogen. Cells were then resuspended in lysis buffer (2×10^7 cells/ml) and sonicated on Covaris system (shearing time 30 min, 20% duty cycle, intensity 10, 200 cycles per burst and 30 s per cycle) in a volume of 2 ml. From 4 to 15 ng of immunoprecipitated chromatin (as quantitated by fluorometry) were electrophoresed on agarose gel and independent sample-specific fractions of 100–200 bp were taken. These samples were processed into sequencing libraries and analysed according to Illumina's 'ChIP-Sequencing Sample Prep Guide' (part #11257047 Rev. A), with the exception that gel extraction was replaced by Agencourt AMPure XP (Beckman Coulter) bead purification. Adapter-ligated library was completed by limited-cycle PCR with Illumina PE primers (14 cycles). DNA libraries were applied to an Illumina flow cell for cluster generation and sequenced on the Illumina Genome Analyzer IIx (GAIIx). Image analysis was performed with Illumina Real Time Analysis software (RTA1.8). Sequence alignment to the reference genome (NCBI/m37/mm9, April 2007) was made with Illumina's ELANDv2 algorithm on its 'eland_extended' mode from within CASAVA-1.7 package, using default settings. Only reads with a unique alignment in the reference genome were used for the peak detection, which was performed using MACS v1.4 setting a FDR < 0.1 and a *P*-value cutoff of 10^{-5} (Zhang *et al*, 2008). All comparisons were done using the input tracks as control, and each one of the data sets as treatment, using the following naming convention: SA1 (SA1+/+), SA2 (SA1+/+), SMC1 (SA1+/+), SMC3 (SA1+/+), SA1 (SA1-/-), SA2 (SA1-/-), SMC1 (SA1-/-) and SMC3 (SA1-/-). In the case of SA1 (SA1+/+) data set, the 176 peaks obtained from SA1 ChIP in SA1-/- cells were cleared. CTCF peaks were obtained from the UCSC Genome Browser track 'MEL CTCF D Pk', part of the ENCODE/Stanford/Yale data set of TF-binding sites (TFBS) in mouse. Genomic interval overlaps and signal distributions were obtained using BEDTools v2.12 (Quinlan and Hall, 2010), PeakAnalyzer v1.3 (Salmon-Divon *et al*, 2010) and custom UNIX shell scripting. All the statistical tests and correlations were calculated using R functions (<http://cran.r-project.org>).

ChIP-qPCR in embryonic brain

The brains from E17.5 embryos were extracted and minced in cold PBS with protease inhibitors cocktail (Roche #11873580001). Small

tissue pieces (<1 mm) were then cross-linked for 20 min RT in fixing solution (1% formaldehyde, 50 mM HEPES-KOH, 100 mM NaCl, 1 mM EDTA and 0.5 mM EGTA). Cross-linking was stopped by adding 1/20 volume of 2.5 M Glycine for 5 min at RT. After two washes in PBS, tissues were resuspended in lysis buffer and processed for further ChIP analysis as described in the above section.

Validation of ChIP-seq results

ChIP-seq results were validated by ChIP-qPCR on immunoprecipitated chromatin from two clones per genotype. Chromosome coordinates of the validated peaks and the corresponding primers are listed in Supplementary Table S8. The relative amount of each amplified fragment was normalized with respect to the amplification obtained from input DNA and represented as percentage of 1 μ g of input DNA.

Gene expression analysis

Total RNA from three wild-type and three SA1-null MEF clones was analysed by two-colour hybridization on Whole Mouse Genome DNA microarrays (G4122F; Agilent), and images were quantified with Agilent Feature Extraction Software (v. 10.1.1). DEGs between SA1-null and wild-type MEFs were obtained by limma (Smyth GK; Bioconductor project; <http://www.bioconductor.org>). FDR adjustment was employed to account for multiple testing. Raw data from gene expression microarray experiments in Nipbl +/- MEFs were kindly provided by the authors (Kawauchi *et al*, 2009). In this case, Affymetrix Murine 430A 2.0 were normalized using Robust Multi-array Average (RMA) algorithm available in Bioconductor's affy package and limma package was used to obtain DEGs.

Functional analysis for GO terms

GO analyses at Biological Process, Cellular Component and Molecular Function were performed using FatiScan tool available at Babelomics suite (<http://www.babelomics.org>). To this end, genes were ranked based on limma's moderated *t* statistic and GO enrichment was evaluated by segmentation test. GO terms showing FDR < 0.05 were considered statistically significant.

Gene set analysis of Myc targets and skin-related genes

GSEA (Subramanian *et al*, 2005) was employed to evaluate the enrichment of custom gene sets in our microarrays experiments. Myc targets were obtained from literature (Chen *et al*, 2008; Kim *et al*, 2008, 2010; Sridharan *et al*, 2009; Smith *et al*, 2010) whereas skin gene set was built from Nagarajan *et al* (2010) and references therein. GSEA was run using gene expression values ranked by limma moderated *t* statistic. After Kolmogorov-Smirnoff testing, those gene sets showing FDR < 0.1, were considered enriched between SA1-null and wild-type MEFs.

Enrichment analysis for target genes of TFs

GSEA for Jaspar TFBS was done using Fatiscan tool available at Babelomics platform (<http://www.babelomics.org>). Genes were

ranked by limma moderated *t* statistic. TFBS showing FDR <0.05 were considered enriched between SA1-null and wild-type MEFs.

mRNA isolation and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated from MEFs using RNeasy Kit (Qiagen) and cDNA was synthesized with SuperScript™ II reverse transcriptase (Invitrogen) using random hexamer primers. An Applied Biosystems 7900HT Fast qRT-PCR was used to determine mRNA levels. GAPDH was used for normalization. Primers used for mRNA amplification are described in Supplementary Table S9.

RNA interference, immunoprecipitation and immunoblotting

Interference of SA1 and SA2 was performed with siGENOME SMARTpool siRNAs from Dharmacon (M-041989 and M-057033, respectively) at a final concentration of 100 nM and using DharmaFECT transfection reagent 1 in the case of C2C12 cells and the Neon transfection system (Invitrogen) in the case of MEFs. ChIP with SA1- and SA2-specific antibodies was performed 72–96 h after transfection. Immunoprecipitation was carried out with Nuclear Complex Co-IP Kit (Active Motif, 54001) from cell extracts according to the manufacturer's instructions, with SA1, SA2, SMC1 and SMC3 specific antibodies. Whole-cell extracts were prepared by lysing and sonicating a cell pellet in SDS-PAGE loading buffer and equal amounts of protein were run in 7.5% Bis/Tris gels followed by western blotting.

Histology and immunohistochemistry

E17.5 embryos were fixed in 10% buffered formalin (Sigma) and embedded in paraffin using standard procedures. In all, 3 μm sections were stained with haematoxylin and eosin (HE) and subjected to histopathological analysis. Anti-myc (Santa Cruz, sc-764), anti-SA1 and anti-SA2 were used for immunohistochemical analysis of 3 μm sections. Positive cells were visualized using 3,3'-diaminobenzidine tetrahydrochloride plus (DAB+) as a chromogen, and counterstaining was performed with haematoxylin.

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Statistical analysis

GraphPad Prism 5 software was used to calculate two-tailed χ^2 test (with Yates' correction) in Figure 3B, Mann-Whitney *U*-test in Figure 3E and two-tailed Student's *t*-test in Figures 4C, 5C, 5E, 6B and 7C, Supplementary Figures S1C and S4. Limma package was used for GO, GSEA and gene expression analysis to obtain DEGs.

Data access

Microarray and ChIP-sequencing data from this study have been submitted to GEO database and have been approved with the following Series reference: GSE32320 (GSE32234 and GSE32319 SubSeries correspond to microarray and ChIP-seq data, respectively).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: AL designed and supervised the study. SR and AC designed, performed, analysed and interpreted all the experiments. DGP and GGL performed and interpreted the ChIP-seq and microarray data analysis, respectively. AL, SR and AC wrote the manuscript with ideas and comments from GGL and DGP.

Conflict of interest

The authors declare that they have no conflict of interest.

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