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Published on: 02 Apr 2021 - bioRxiv (Cold Spring Harbor Laboratory)

Topics: Cellular differentiation, SOX2, Cranial neural crest, Homeobox protein NANOG and Neural crest

Inability to switch from ARID1A-BAF to ARID1B-BAF impairs exit from pluripotency and commitment towards neural crest differentiation in ARID1B-related neurodevelopmental disorders

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Abstract

The BAF complex modulates genome-wide chromatin accessibility. Specific BAF configurations have been shown to have functional consequences, and subunit switches are essential for cell differentiation. *ARID1B* and its paralog *ARID1A* encode for mutually exclusive BAF subunits. *De novo ARID1B* haploinsufficient mutations cause a neurodevelopmental disorder spectrum, including Coffin-Siris syndrome, which is characterized by neurological and craniofacial features. Here, we reprogrammed *ARID1B*$^{+/-}$ Coffin-Siris patient-derived skin fibroblasts into iPSCs, and investigated cranial neural crest cell (CNCC) differentiation. We discovered a novel BAF configuration (ARID1B-BAF), which includes ARID1B, SMARCA4, and eight additional subunits. This novel version of BAF acts as a gate-keeper which ensures exit from pluripotency and commitment towards neural crest differentiation, by attenuating pluripotency enhancers of the SOX2 network. At the iPSC stage, these enhancers are maintained in active state by an ARID1A-containing BAF. At the onset of differentiation, cells transition from ARID1A-BAF to ARID1B-BAF, eliciting attenuation of SOX2 enhancers and pluripotency exit. Coffin-Siris patient cells fail to perform the ARID1A/ARID1B switch, and maintain ARID1A-BAF at pluripotency enhancers throughout CNCC differentiation. This correlates with aberrant SOX2 binding at pluripotency enhancers, and failure to reposition SOX2 at developmental enhancers. SOX2 dysregulation promotes upregulation of the *NANOG* network, impairing CNCC differentiation. ARID1B-BAF directly modulates *NANOG* expression upon differentiation cues. Intriguingly, the cells with the most prominent molecular phenotype in multiple experimental assays are derived from a patient with a more severe clinical impairment.

These findings suggest a direct connection between *ARID1B* mutations, CNCC differentiation, and a pathogenic mechanism for Coffin-Siris syndrome.

Keywords: BAF, ARID1B, Coffin-Siris, pluripotency enhancers, NANOG, SOX2, neural crest, SALL4

Introduction

Cell fate commitment is a complex process that requires timely regulation of developmental genes. This phenomenon is mediated by the concerted activity of transcription factors and chromatin regulators that modulate the interaction between *cis*-regulatory elements (enhancers, promoters) and RNA Polymerase II to elicit gene expression. In this framework, a key role is played by the Brg1/Brm associated factor (BAF) chromatin-remodeling complex. BAF leverages ATP to modulate nucleosome positioning and chromatin accessibility genome-wide [1]. Different configurations of BAF, with context specific functions, have been described, and switches between subunits have been reported to be linked to specific developmental stages[2,3]. All the known BAF configurations require the presence of a subunit containing an AT-rich DNA binding domain (ARID). Namely, in the BAF complex this function is carried out by two mutually exclusive subunits: ARID1A and ARID1B[4-6]. Previous studies in mouse embryonic stem cells (mESCs) have identified a mESC-specific configuration of BAF which regulates pluripotency and self-renewal of the embryonic stem cells (esBAF)[4-6]. Importantly, the esBAF exclusively incorporates ARID1A and not ARID1B. One of these studies also identified a non-canonical version of BAF (gBAF) not containing any ARID subunit and also involved in pluripotency maintenance of mouse embryonic stem cells[4].

De novo haploinsufficient mutations in the *ARID1B* gene cause a spectrum of neurodevelopmental disorders, ranging from Coffin Siris syndrome to non-syndromic intellectual disability[7-12]. Coffin-Siris syndrome is associated with intellectual disability, specific craniofacial features, growth impairment, feeding difficulties and congenital anomalies such as heart and kidney defects[13]. Although other BAF components may also be mutated in this syndrome, the very large majority of mutations (~75%) are in *ARID1B* [11,14,15]. In addition to Coffin-Siris, genome-wide sequencing in unselected cohorts of patients with intellectual disability (ID) shows that *ARID1B* is always in the top-5 of causative genes, explaining about 1% of all ID cases[9,16]. Studies in several mouse models were able to recapitulate the neurological phenotypes typical of the *ARID1B*-associated syndromes[17-20]. Nonetheless, the molecular function of ARID1B in cell fate commitment during human development is still poorly understood.

An important feature of *ARID1B* haploinsufficient individuals is represented not only by severe craniofacial abnormalities, but also by defects of the cardiac and digestive systems, often associated with ineffective migration of the cardiac and enteric neural crest[12]. Further, *ARID1B* is one of the most commonly mutated genes in neuroblastoma, a pediatric tumor of neural crest origin[21]. For all these reasons, the neural crest differentiation represents one of the most suitable models to study the consequences of *ARID1B* mutations in human development.

To investigate the molecular consequences of *ARID1B* haploinsufficient mutations in neural crest differentiation and craniofacial development, we reprogrammed skin fibroblasts of two unrelated *ARID1B*$^{+/-}$ Coffin-Siris patients into induced Pluripotent Stem Cells (iPSCs). Then we used these patient-derived iPSCs to specifically model Cranial Neural Crest Cell (CNCC) differentiation.

Thanks to this approach, we report the discovery of a novel BAF configuration, containing ARID1B, SMARCA4 and eight additional subunits (ARID1B-BAF). In line with the evidence that the esBAF and the gBAF do not contain ARID1B[4-6], we demonstrate that *ARID1B* mutations do not affect self-renewal and pluripotency of human iPSCs. At this stage, the pluripotency is positively regulated by binding of an ARID1A-containing BAF to pluripotency-associated enhancers of the SOX2 and NANOG networks. On the other hand, we show that ARID1B-BAF plays an important role in lineage specification and exit from pluripotency. In fact, ARID1B-BAF is only transiently active during early stages of CNCC differentiation, where it replaces ARID1A-BAF at the SOX2/NANOG enhancers and elicits their repression. Intriguingly, ARID1B-BAF interacts with SALL4 (Spalt Like Transcription Factor 4), which is a multi-zinc-finger transcription factor essential for lineage commitment in early mammalian development, during which it targets sites with the same binding motifs also recognized by SOX2, OCT4 and NANOG[22-25]. This transcription factor can act as both activator and repressor and is dispensable for the maintenance of the

stem cell pluripotency networks, but it ensures that aberrant gene expression programs are not activated during lineage commitment[23].

Importantly, we demonstrate that the *ARID1B*$^{+/-}$ Coffin-Siris cells are unable to switch from ARID1A-BAF to ARID1B-BAF at the onset of the CNCC differentiation, and instead maintain ARID1A-BAF at the pluripotency enhancers throughout the differentiation process. This leads to defective exit from pluripotency and impaired cranial neural crest differentiation. These findings provide evidence for a direct connection between *ARID1B* mutations and a pathogenic mechanism for ARID1B-associated neurodevelopmental syndromes.

Results

Coffin-Siris patient-derived iPSCs are pluripotent and proliferate normally

To investigate the function of ARID1B in craniofacial development, we obtained skin fibroblasts from two unrelated *ARID1B*$^{+/-}$ Coffin-Siris Syndrome patients (hereafter Patient-19 and Patient-26; Fig. 1a), one male and one female, both carrying previously identified *de novo ARID1B* mutations. In detail, Patient-19 presented a nonsense mutation (c.3223C>T;p.Arg1075*; Fig. 1b), while Patient-26 had a frameshift mutation (c.2598del;Tyr867Thrfs*47; Fig. 1b) [10,14]. In both cases, a premature STOP codon was generated (Fig. 1b).

The fibroblasts were reprogrammed into iPSCs by the LUMC hiPSC Hotel (Leiden University). The patient-derived iPSCs exhibit regular morphology (Fig. 1c) and express the pluripotency genes, as shown by both immunofluorescence (Fig. 1d; Supplementary Fig. S1a,b) and RT-qPCR (Fig. 1e). Further, the patient-derived iPSCs grow at the same rate as an *ARID1B*$^{+/+}$ control line (Control line-1; Fig. 1f).

Importantly, the aberrant STOP codon introduced by the mutations is located either upstream (Patient-26) or inside (Patient-19) the AT-Rich Interactive Domain (ARID) (Fig. 1b), which is required for ARID1B's interaction with chromatin[26]. Moreover, in both patients, the new STOP codon is localized upstream of the Nuclear Localization Signal (NLS, Fig. 1b), suggesting that the gene product arising from the mutated allele would not be able to reach the nucleus and the chromatin even in the unlikely case that the transcript escaped non-sense mediated mRNA decay[27]. To test this, we performed cellular fractionation in patient and control iPSCs and conducted an ARID1B western blot on the chromatin fraction with an antibody raised against a peptide in the N-terminus of ARID1B, hence upstream of the mutated regions (sc-32762). Consistent with our hypothesis, the immunoblot on the chromatin fraction shows significantly lower ARID1B protein level in the two patients relative to an *ARID1B*$^{+/+}$ iPSC line (Control line-1; Supplementary Figure S1c). No ARID1B signal was detected in any of the lines in the cytoplasmic fraction (Supplementary Fig S1c).

In summary, *ARID1B* haploinsufficient iPSCs are pluripotent, do not exhibit growth defects, but display significantly less chromatin-bound ARID1B.

The CNCC differentiation is impaired in Coffin-Siris patient-derived iPSCs

We took advantage of a published CNCC differentiation protocol[28], and using Control line-1 we obtained fully differentiated Cranial Neural Crest Cells in 14 days (Fig. 2a,b). A time-course western blot conducted during the CNCC differentiation of Control line-1 revealed that ARID1B protein is highly expressed only during the first week of differentiation, peaking between days 5 and 7, after which is markedly downregulated (Fig. 2c).

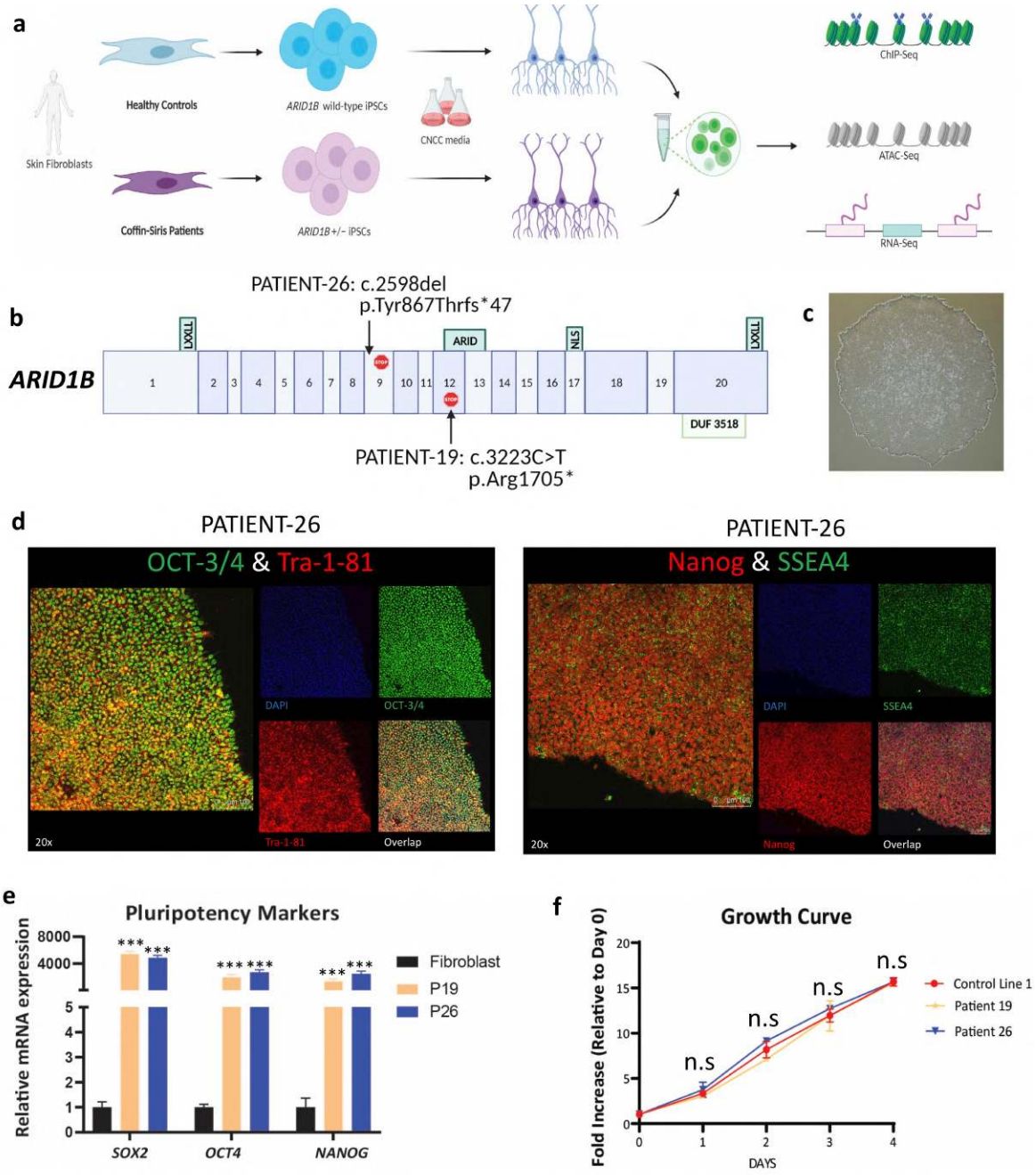


Figure 1 – iPSCs derived from Coffin-Siris patients are pluripotent and proliferate normally. **(a)** Study system: iPSCs were derived from skin fibroblasts of two unrelated Coffin-Siris patients. The iPSCs were used in this study to generate Cranial Neural Crest Cells (CNCCs) and perform genomic experiments to investigate the effect of *ARID1B* mutations. **(b)** Graphical illustration of the *ARID1B* haploinsufficient mutations affecting the two studied patients. The numbers in the gene model refer to *ARID1B's* isoform NM_020732.3. **(c)** Colony of iPSCs derived from Patient-19 showing typical iPSC morphology. **(d, e)** Immunofluorescence and rt-qPCR quantifying the expression of the key pluripotency markers in iPSCs derived from Patient-26. **(f)** Growth curve comparing an *ARID1B*-wt Control iPSC Line with the two patient lines. The patient cells do not exhibit growth impairment.

Next, we induced the differentiation in the two Coffin-Siris lines and the Control line-1 together, and used flow cytometry to measure multiple pluripotency (SSEA-4, TRA-1-60-R) and CNCC (CD10, CD99) surface markers. The cells were sampled at day-zero (iPSC stage), day-5 (mid-point), and day-14 (end of differentiation). Notably, the differentiation was impaired in both patient lines. In fact, in both lines we identified a sizable cell population still double-positive for the pluripotency surface markers even after 14 days (Fig. 2d). This pluripotent population comprised 4.36% and 19.5% of the cells in the two patient lines, respectively (Fig. 2d). In line with this, relative to the control line, a large fraction of the patient cells showed significantly lower expression of the CNCC surface markers even after 14 days of differentiation relative to the control line (Fig. 2e). Together, our data suggest that while *ARID1B* is dispensable for pluripotency, haploinsufficiency of this gene is enough to severely impair differentiation into CNCCs.

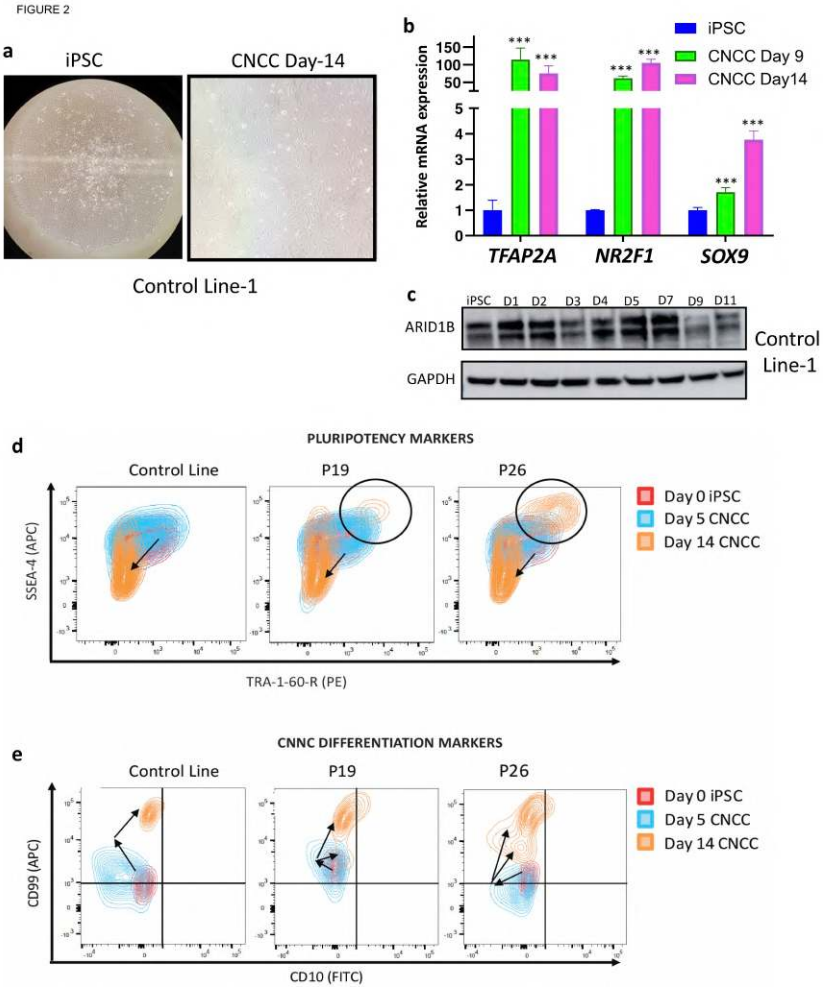


Figure 2 – CNCC differentiation is impaired in the patient cells. (a, b) CNCC differentiation was optimized using an *ARID1B*-wt Control Line. After 14 days, the cells exhibited the classic CNCC morphology and expressed the CNCC markers. **(c)** Time-course immunoblot conducted using Control Line-1 during CNCC differentiation shows that ARID1B is active in the first 7 days of the differentiation, with a peak of activity between day-5 and day-7. The ARID1B protein level strongly decreases after day-7. **(d, e)** Flowcytometry quantifying expression of surface markers for pluripotency and CNCC differentiation in control line-1 and in the two patient lines. A large cell population is still pluripotent in both patients after 14 days (d). The patient lines also show reduced expression of CNCC surface markers after 14 days of differentiation relative to an *ARID1B*-wt Control Line at the same time point (e).

Chromatin accessibility is dysregulated in the differentiating patient cells

We used Next-Generation Sequencing to investigate why *ARID1B* haploinsufficient Coffin-Siris iPSCs did not successfully differentiate into CNCCs. Given that ARID1B protein levels in control cells reach a peak between days 5 and 7, we selected day-5 as a time-point to collect the genomic data. The experiments were conducted with two biological replicates per condition (two control lines, two patient lines). For each condition, a male and a female were included to avoid sex-specific confounding effects. Technical replicates were also performed for each biological replicate. To avoid batch effects, all the biological replicates and conditions were processed together.

Since ARID1B is a component of the BAF chromatin-remodeling complex, we profiled chromatin accessibility with ATAC-seq. Overall, we detected 29,758 ATAC-seq peaks replicated across all replicates and all conditions (patients and controls; FDR <0.05; Fig. 3a). Conversely, 5,540 peaks were specific to the patients (i.e. replicated in all the patient replicates and not detected in any of the controls; hereafter patient-specific ATAC-seq regions; Fig. 3a,b; Supplementary File S1). Finally, only 578 peaks were specific to the controls (hereafter control-specific ATAC-seq regions; Fig. 3a,c; Supplementary File S1).

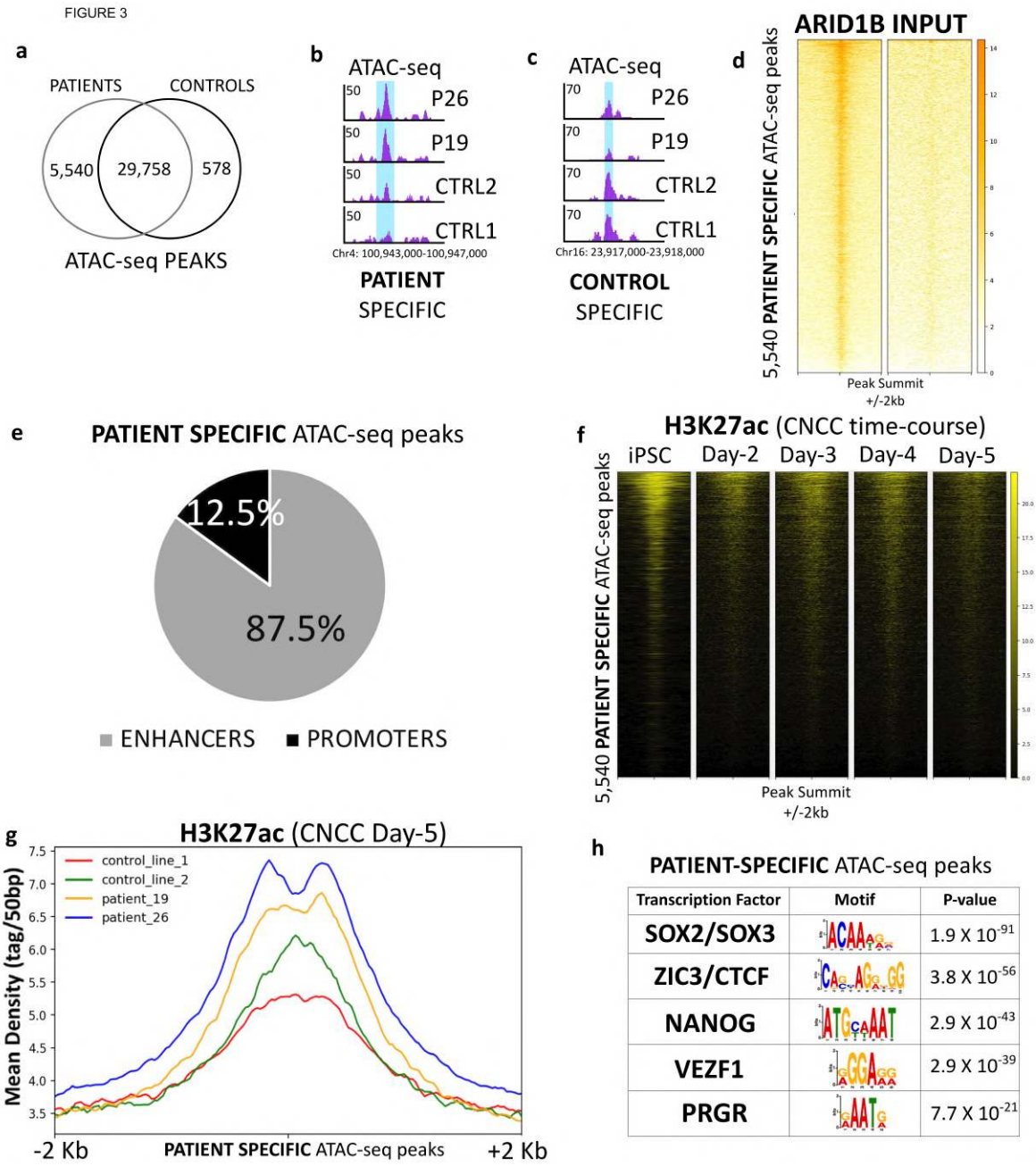
We therefore focused on the 5,540 patient-specific ATAC-seq regions because they represented 91% (5,540/6,118) of all regions with differential chromatin accessibility between patients and controls. ARID1B ChIP-seq performed in the Control Line-1 at the day-5 shows ARID1B binding in all of these regions (Fig. 3d). This suggests that the chromatin accessibility in the 5,540 patient-specific ATAC-seq regions may be directly regulated by ARID1B-BAF. In line with this, ARID1B ChIP-seq performed on all four lines at day-5 of differentiation confirmed that the 5,540 patient-specific ATAC-seq regions are similarly bound by ARID1B in both control lines, while the binding is almost entirely lost in both patient lines (Supplementary Fig. S2a).

The ARID1B-BAF attenuates thousands of enhancers at the onset of CNCC differentiation

To determine the genomic nature of these regions, we associated a gene to each region based on the distance from the nearest Transcription Start Site (TSS). Overall, 87.5% of the ARID1B ChIP-seq peaks were located >10 Kb from the nearest TSS and may represent putative enhancers, while the remaining 12.5% are likely promoters (Fig. 3e). ChIP-seq time-course for H3K27ac in Control Line-1 revealed that many of these regulatory regions are enriched for H3K27ac in iPSCs, while their regulatory activity progressively decreases upon differentiation cues, and by day-5 very little H3K27ac signal is detectable (Fig. 3f). Notably, the gradual decrease in H3K27ac mirrors the steady increase in ARID1B expression detected during the early stages of CNCC differentiation (Fig. 2c). Consistently, the differentiating CNCCs of both patients have significantly higher levels of H3K27ac in these regions relative to the two control lines at the day-5 (Wilcoxon's Rank Sum Test $p < 2.2 \times 10^{-16}$ in all the patient vs control pairwise comparisons; Fig. 3g).

Based on the high H3K27ac signal that the 5,540 patient-specific regions display at the iPSC stage (Fig. 3f), we surmised that these sites could represent cis-regulatory elements important for pluripotency. In line with this hypothesis, DNA-motif analysis on the 5,540 regions revealed that they are enriched for the binding sites of multiple pluripotency factors, including SOX2 and NANOG (Fig. 3h; Supplementary File S2).

Next, we wanted to ensure that the molecular phenotypes observed so far were directly caused by the *ARID1B* mutations, and not by co-occurring mutations in other genes coincidentally shared by both (unrelated) patients. Hence, we employed shRNAs to knock-down ARID1B in the Control Line-1. We were able to obtain a partial knock-down of ARID1B at the iPSC stage (shRNA-1; Supplementary Fig. 2b), which represented a suitable model for *ARID1B* haploinsufficiency. We differentiated the ARID1B-KD iPSCs towards CNCC and again collected the cells at the day-5, and performed ATAC-seq and ChIP-seq for H3K27ac. Notably, both sequencing experiments perfectly recapitulated what we previously observed in the patient lines.



Transcription Factor	Motif	P-value
SOX2/SOX3		1.9×10^{-91}
ZIC3/CTCF		3.8×10^{-56}
NANOG		2.9×10^{-43}
VEZF1		2.9×10^{-39}
PRGR		7.7×10^{-21}

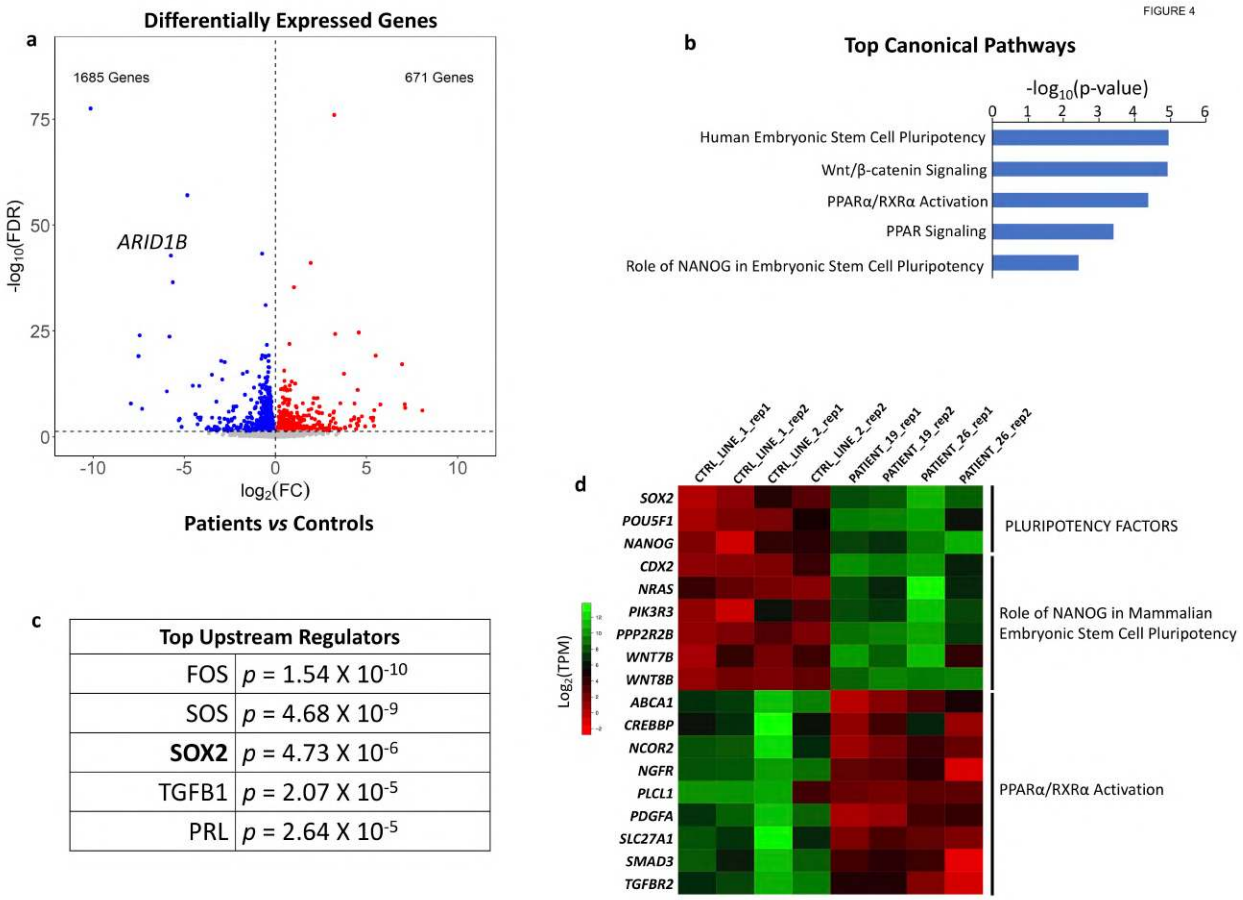
Figure 3 – **Chromatin remodeling at pluripotency enhancers is dysregulated in the patient cells**. **(a)** At CNCC day-5, 29,578 ATAC-seq peaks are shared between patient and control lines. 5,540 peaks are specific of the patients. 578 peaks are specific of the control lines. **(b)** UCSC Genome Browser example of a PATIENT-SPECIFIC ATAC-seq peak. **(c)** UCSC Genome Browser example of a CONTROL-SPECIFIC ATAC-seq peak. **(d)** ARID1B ChIP-seq heatmaps (Control Line-1; CNCC Day-5) show ARID1B binding at nearly all of the PATIENT-SPECIFIC ATAC-seq peaks. The input (collected at CNCC Day-5) was used as a control **(e)** 87.5% of the PATIENT-SPECIFIC ATAC-seq peaks are located > 1kb from a TSS. **(f)** Heatmaps of H3K27ac ChIP-seq time-course at the 5,540 PATIENT-SPECIFIC ATAC-seq peaks (Control Line-1). **(g)** H3K27ac ChIP-seq average profiles centered on the PATIENT-SPECIFIC ATAC-seq regions (CNCC Day-5). **(h)** Motif analysis at the PATIENT-SPECIFIC ATAC-seq regions revealed enrichment for the binding motif of multiple pluripotency factors.

Namely, upon ARID1B-KD, we detected significantly increased chromatin accessibility and H3K27ac signal in the 5,540 patients-specific regions relative to the same iPSC line transduced with a control shRNA (Wilcoxon's Rank Sum Test $p < 2.2 \times 10^{-16}$; Supplementary Fig. S2c,d).
Together, these data indicate that ARID1B-BAF modulates the chromatin accessibility of a specific set of ~4,900 pluripotency enhancers and ~600 promoters that are highly active at the iPSC stage, moderately active at the CNCC differentiation onset, and inactive by day-5 (Fig. 3f). We find that the attenuation of these cis-regulatory elements is impaired in the *ARID1B* haploinsufficient cells, which likely hampers the entire differentiation process towards CNCCs.

"Pluripotency" and "Exit from Pluripotency" genes are dysregulated in the differentiating patient cells

Impaired attenuation of ~4,900 pluripotency-relevant enhancers and ~600 promoters could have a profound effect on gene expression levels. Indeed, RNA-seq conducted on the four lines at CNCC Day-5 identified 2,356 differentially expressed genes, 1,685 of which were downregulated, and 671 upregulated (FDR <5%; Fig. 4a). As expected, *ARID1B* was one of the top downregulated genes in patient CNCCs at the day-5 (Fig. 4a). In stark contrast, only 54 genes were identified as differentially expressed when we performed RNA-seq at the iPSC stage (FDR <5%). This suggests that ARID1B has an important function upon lineage commitment, again mirroring the progressive increase in the ARID1B protein level observed during early differentiation (Fig. 2c). These findings are consistent with previous studies that demonstrated that the mESC BAF complexes (esBAF, gBAF) do not include ARID1B[4-6].
Notably, 598/2,356 (25.4%) of the genes differentially expressed at CNCC day-5 also represented the nearest gene to one of the 5,540 pluripotency enhancers and promoters aberrantly active in the Coffin-Siris patient cells at the same time point (Fisher's Exact Test $p < 0.0001$; Supplementary File S3). These results suggest that over a quarter of the differentially expressed genes are under the direct control of ARID1B-BAF throughout modulation of the chromatin accessibility at the associated enhancers and promoters. As expected, when we compared these 598 genes against the entire set of differentially expressed genes, we found that the 598 genes exhibit enrichment for genes upregulated in patients (Fisher's Exact Test $p < 0.0001$), likely determined by increased activity in the associated enhancers and promoters.
Ingenuity Pathway Analysis on the 598 genes identified five of the top canonical pathways as associated with either pluripotency or exit from pluripotency (Fig. 4b; Supplementary File S4). Wnt-β catenin signaling pathway was also found as enriched. An association between ARID1B and this specific pathway was suggested in recent studies[29,30].
In accordance with the ATAC-seq data, SOX2 was detected among the top upstream regulators (Fig. 4c), and three of the most important pluripotency factors, and specifically *NANOG, SOX2* and *POU5F1* (OCT4), were found as still highly expressed in the patient lines at the day-5 (Fig. 4d).
Both the "*Role in NANOG in Mammalian Embryonic Stem Cell Pluripotency"* pathway and the "*PPARα/RXRα Activation"* pathway were enriched in the 598 genes (Fig. 4b). Namely, the genes belonging to the former pathway were all upregulated, while the genes belonging to the latter were downregulated (Fig. 4d). These two pathways caught our attention because they are thought to antagonize each other. More specifically, NANOG blocks the differentiation of pluripotent cells and establishes the pluripotent state during somatic cell reprogramming. On the other hand, the PPARα/RXRα pathway is activated at the onset of differentiation to promote exit from pluripotency[31]. The activation of PPARα/RXRα elicits the repression of the *NANOG* network to allow efficient exit from the undifferentiated stage[31-33]. Consistent with this, PPARα-inhibitors have been employed to enhance and improve iPSC reprogramming[31].
Taken together, our RNA-seq data suggest that the differentiating *ARID1B*$^{+/-}$ patient lines exhibit a persistent upregulation of multiple pluripotency factors and associated gene networks, along with

downregulation of genes responsible for exit from pluripotency, which impairs the differentiation program to CNCC.



Top Upstream Regulators	
FOS	p = 1.54 X 10^{-10}
SOS	p = 4.68 X 10^{-9}
SOX2	p = 4.73 X 10^{-6}
TGFB1	p = 2.07 X 10^{-5}
PRL	p = 2.64 X 10^{-5}

Figure 4 – "**Pluripotency" and "Exit from Pluripotency" genes are dysregulated in differentiating patient CNCCs.** **(a)** RNA-seq volcano plot shows the differentially expressed genes between patient and control lines at CNCC Day-5. *ARID1B* is one of the top downregulated genes. **(b)** Top canonical pathways (IPA analysis) enriched in the set of 598 differentially expressed genes that also represent the closest gene to a PATIENT-SPECIFIC ATAC-seq peak. **(c)** Top upstream regulators (IPA analysis) enriched in the same set of 598 genes used for panel b. **(d)** RNA-seq heatmap displaying expression patterns at CNCC Day-5 for pluripotency genes, for genes of the NANOG network, and for genes associated to exit from pluripotency (PPARα/RXRα activation pathway).

Aberrant SOX2 and NANOG activity in the *ARID1B* haploinsufficient patient cells

Our experiments indicate that the *ARID1B* haploinsufficient cells fail to attenuate thousands of pluripotency enhancers and promoters enriched for SOX2 and NANOG binding sites (Fig. 3a–h). Further, at day-5 of CNCC differentiation, the expression of *SOX2* and *NANOG* is significantly higher in the patient derived cells than in the controls, and the gene regulatory networks associated with these pluripotency factors are also upregulated (Fig. 4b–d).

Given these results, we set out to investigate the binding profile of SOX2 and NANOG in patient and control lines by ChIP-seq at CNCC day-5. Our spike-in normalized SOX2 ChIP-seq revealed that 3,284/5,540 (59.7%) patient-specific ATAC-seq peaks are characterized by significantly higher SOX2 binding in patients relative to the control lines. In line with this, the chromatin at these regions is accessible in the patient lines but not in the control lines (Fig. 5b). SOX2 is a pioneer factor that is able to bind condensed nucleosomes to open the chromatin for the binding of other factors[34]. As demonstrated by previous studies in mouse in embryonic stem cells SOX2 and other pluripotency pioneer factors (e.g. OCT4) require the BAF complex to perform their pioneer activity[6,34,35]. Our findings indicate that in control conditions ARID1B-BAF complex likely antagonizes the cooperation between other BAF configurations and SOX2, counter-acting the pioneer activity of the latter as soon as the cell differentiation is induced. Further, we identified an additional set of 497 SOX2 peaks specific of the patient lines, which did not exhibit changes in chromatin accessibility. Moreover, we also identified 1,146 SOX2 peaks exclusive of the control lines (Supplementary File S5). Importantly, these control-specific SOX2 peaks were located in proximity to genes associated with neural crest differentiation, including *TFAP2A*, *PAX6*, *PAX7*, *WNT4*, *ENO1*, *C8B*, and *SERBP1* among others. These findings are consistent with two recent studies which suggested that SOX2-chromatin interactions are rewired upon differentiation cues[36,37]. Such rewiring appears impaired in *ARID1B*-haploinsufficient cells, which aberrantly maintain SOX2 at the pluripotency-associated enhancers, and at the same time fail to reposition this transcription factor at the developmental enhancers.

Next, we profiled NANOG at day-5 of differentiation. For this transcription factor, the spike-in normalized ChIP-seq revealed 4,538 peaks unique to the patients (Supplementary File S6). However, in this case, only 219 (4.8%) of the patient-specific NANOG peaks overlapped a patient-specific ATAC-seq peak. We thus interrogated our ATAC-seq data to determine the state of chromatin accessibility at the 4,538 patient-specific NANOG peaks, and overall found no significant changes in accessibility in these regions between the patients and the control lines (Fig. 5c). Notably, nearly a quarter of the patient-specific NANOG peaks were found in regions of repressed chromatin (Fig. 5c,e), consistent with recent studies which suggested that NANOG can bind repressed chromatin like other pioneer pluripotency factors[38,39].

Despite no changes in chromatin accessibility, the NANOG ChIP-seq signal at the 4,538 patient-specific NANOG peaks was significantly higher in the patient than in the control lines (Wilcoxon's Rank Sum Test $p < 2.2 \times 10^{-16}$ in all the patient vs control pairwise comparisons; Fig. 5d,e). We hypothesized that the increased NANOG binding detected in the patients' cells (Fig. 5d) could reflect increased *NANOG* expression (Fig. 4d). In fact, several elegant studies in embryonic stem cells have demonstrated that the downregulation of *NANOG* gene expression marks the transition from naïve to primed state[40-42]. Importantly, it has been shown that *NANOG* expression is modulated by SOX2, which binds a cis-regulatory element in the promoter region of *NANOG*[43,44]. Thus, we examined this cis-regulatory element in detail. As expected, at day-5 of CNCC differentiation, the chromatin accessibility at the promoter-proximal element is significantly higher in the two patient lines than in the two controls (Student's T-Test p=0.0065; Fig. 5f). Accordingly, increased chromatin accessibility correlates with increased SOX2 binding on the cis-regulatory element (Fig. 5f), perhaps explaining the higher *NANOG* gene expression reported in patients at CNCC day-5. Lastly, our shRNA experiments also replicated these findings, demonstrating that the knock-down of ARID1B in the Control line-1 line correlates with a sizeable increase in accessibility at the *NANOG* cis-regulatory element (Fig. 5g), thus suggesting that ARID1B-BAF directly modulates *NANOG* expression dosage at the onset of differentiation.

In sum, the *ARID1B* haploinsufficient lines exhibit persistent activity of two key pluripotency factors (SOX2, NANOG) in the early stages of CNCC differentiation. The aberrant activity of SOX2 and NANOG leads to impaired lineage commitment and inefficient CNCC differentiation.

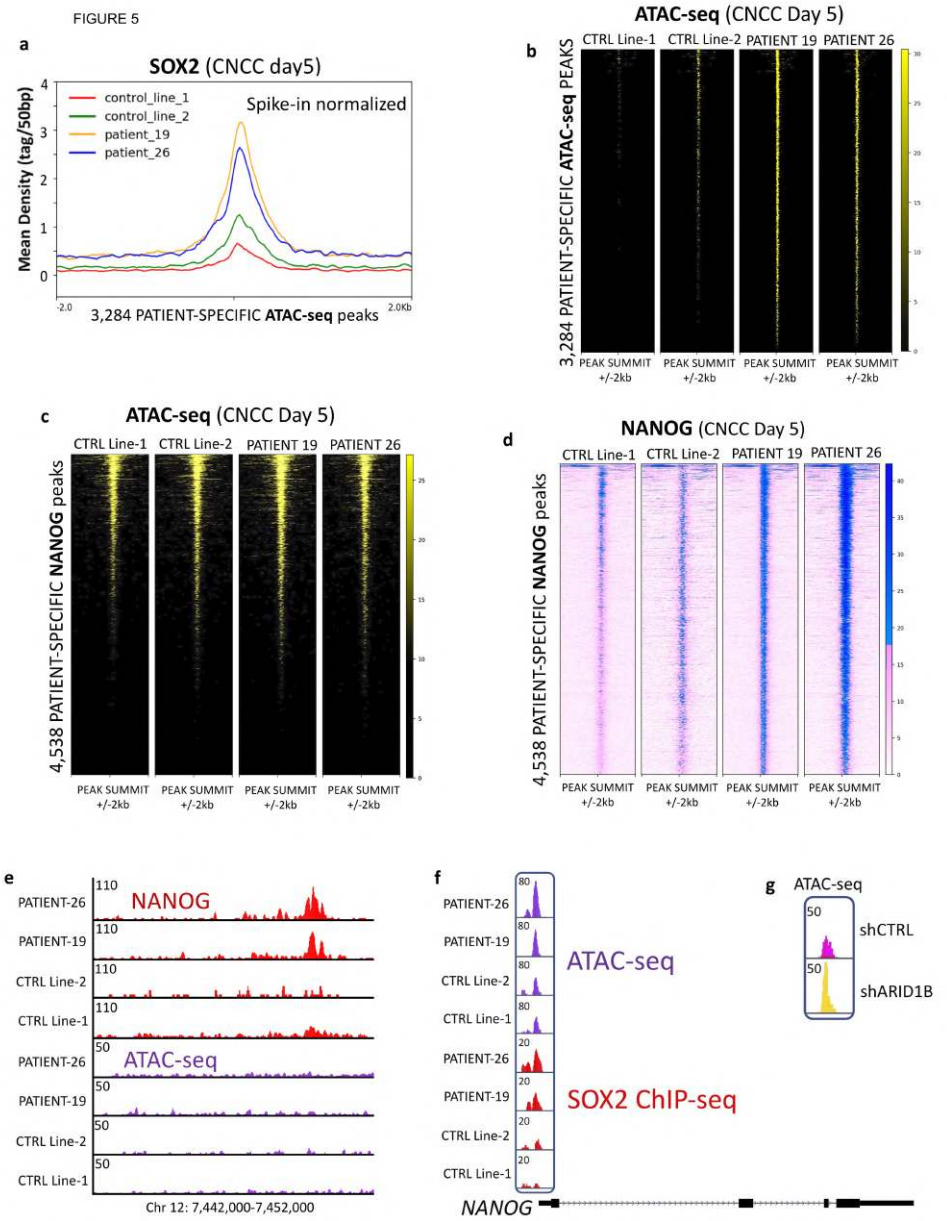


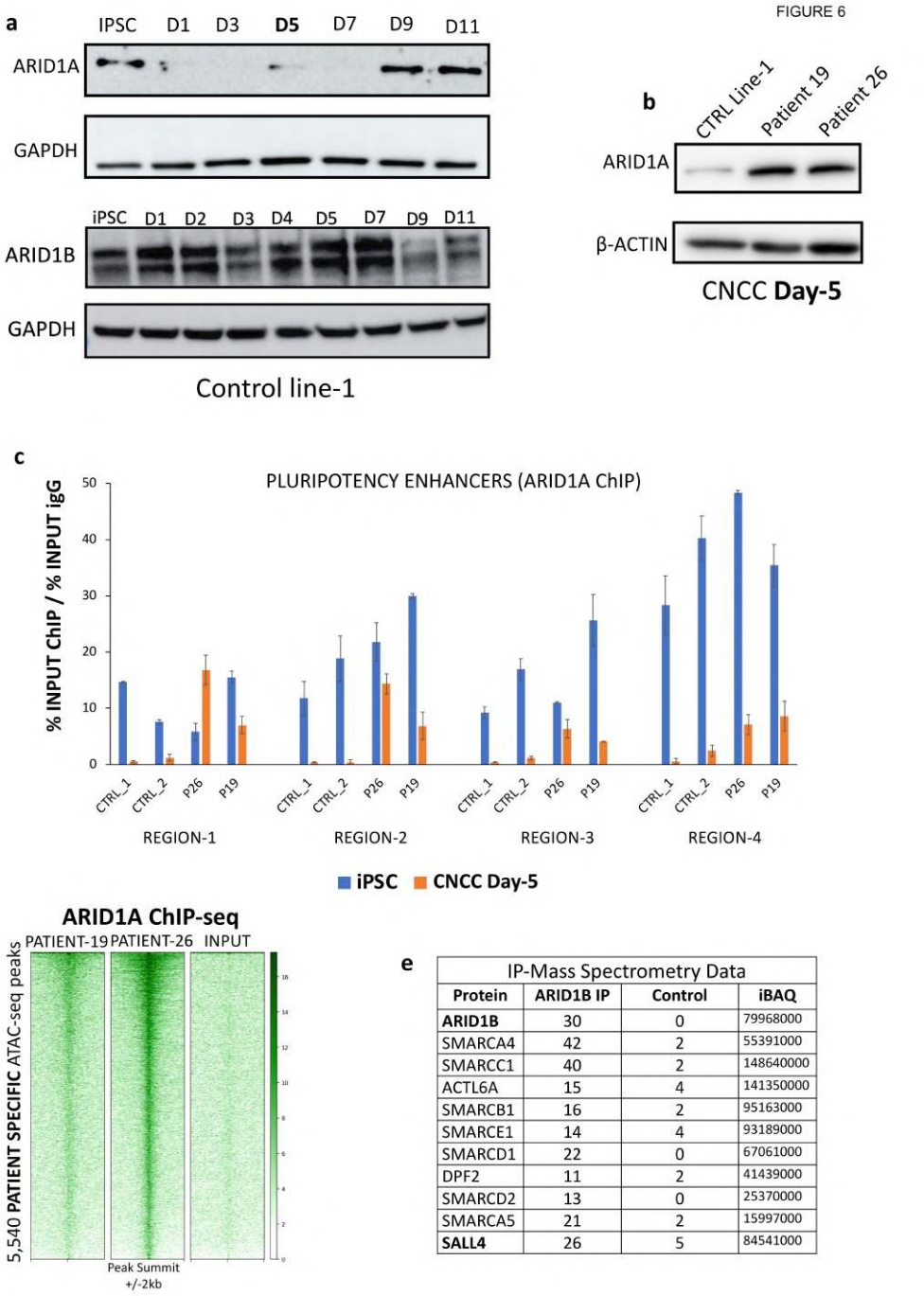
Figure 5 – Aberrant SOX2 and NANOG activity in the patient cells at CNCC Day-5. **(a)** SOX2 ChIP-seq average profile for 3,284 patient-specific ATAC-seq peaks showing patient-specific SOX2 signal (spike-in normalized; CNCC Day-5). **(b)** ATAC-seq heatmaps at the 3,284 peaks shown in Fig. 5a reveal that these regions display increased chromatin accessibility in the patients relative to the two control lines. **(c)** ATAC-seq heatmaps at 4,538 patient-specific NANOG peaks display no changes in accessibility between patient and control lines. **(d)** NANOG ChIP-seq heatmaps at 4,538 patient-specific NANOG peaks (spike-in normalized; CNCC Day-5). **(e)** Example of patient-specific NANOG peak in a region with no chromatin accessibility (CNCC Day-5). **(f)** At CNCC Day-5, a cis-regulatory element in the promoter region of *NANOG* is more accessible in the patients than in the control lines. The same element also displays higher SOX2 binding in the patients than in the controls. **(g)** Knock-down of ARID1B from Control Line-1 also elicits an increase in chromatin accessibility at the cis-regulatory element in the promoter region of *NANOG* (CNCC Day-5).

A switch from ARID1A-BAF to ARID1B-BAF is necessary for exit from pluripotency

We wanted to elucidate how the BAF complex compensates for *ARID1B* haploinsufficiency. As mentioned earlier, ARID1A and ARID1B represent the only two subunits of the BAF harboring an ARID domain, which is leveraged by the complex to interact with the chromatin[26]. A third ARID subunit (ARID2) is exclusive of a different configuration of the complex (pBAF). *ARID2* mutations have shown to cause a neurodevelopmental disorder that does not fully fit the Coffin-Siris syndrome phenotype, although there is some overlap[45]. Compensation mechanisms between ARID1A and ARID1B were recently demonstrated in ovarian cancer[46]. We hence hypothesized that the *ARID1B* haploinsufficient patient cells may compensate for partial loss of ARID1B with ARID1A. To test this, we first assessed ARID1A protein levels in *ARID1B*-wt cells during the course of CNCC differentiation and found that ARID1A exhibits a pattern of activity complementary to ARID1B (Fig. 6a). In agreement with the specific composition of the esBAF, which requires ARID1A[5,6], the human iPSCs show high ARID1A protein level and relatively low ARID1B (Fig. 6a). On the other hand, on day-1 of CNCC differentiation ARID1B is immediately upregulated while the ARID1A protein is completely repressed and is no longer detectable (Fig. 6a). ARID1B remains the only active ARID1 subunit between day-1 and day-7 (Fig. 6a). Finally, after day-7, ARID1B is abruptly downregulated while high ARID1A protein level is repristinated (Fig. 6a). Together, these data suggest that in *ARID1B*-wt conditions the differentiating CNCCs perform multiple switches between ARID1A and ARID1B, the most critical likely taking place as soon as the differentiation is induced. We previously identified the time-frame between days 5 and 7 as the peak of ARID1B expression during CNCC differentiation (Figs. 2c and 6a). In this time-frame ARID1A is not active in wild-type conditions (Fig. 6a). Nonetheless, the differentiating cells from both patients present high ARID1A protein levels at this time point, as opposed to almost no detectable protein in the Control line (Fig. 6b). This confirms that patient cells compensate for the partial loss of ARID1B by maintaining high ARID1A levels throughout the differentiation process. A recent study conducted on liver cells demonstrated that ARID1A-containing BAF and ARID1B-containing BAF may have antagonistic function in the transcriptional regulation of specific genes, with ARID1B acting prevalently as a repressor of enhancer elements, as opposed to the ARID1A, which mostly behaves as an activator[47]. Hence, we hypothesized that the aberrantly high ARID1A protein levels detected in the patient-derived cells during CNCC differentiation might underlie the long-lasting activity of the pluripotency enhancers. Consistently, ARID1A-ChIP followed by qPCR revealed that the pluripotency enhancers are bound by ARID1A at the iPSC stage in both the control and the patient lines at comparable levels (Fig. 6c; *p-values* in Supplementary File S7). On the other hand, at the day-5 ARID1A binding is completely lost in the control lines – which have meanwhile gained ARID1B at the same sites (Fig. 3d) – while it is maintained in both patient lines (Fig. 6c; *p-values* in Supplementary File S7).
To confirm genome-wide that all the 5,540 pluripotency enhancers are bound by ARID1A in the patients, we performed ARID1A ChIP-seq in the patient lines at day-5 of CNCC differentiation. In agreement with the ChIP-qPCR data, the ChIP-seq demonstrated that all the 5,540 pluripotency elements are bound by ARID1A in both patients at CNCC Day-5 (Fig. 6d). The persistent binding of the ARID1A-BAF at the pluripotency enhancers could perhaps explain why the patient cells do not efficiently repress these regions at the onset of the neural crest differentiation.

The ARID1B-BAF complex exclusively incorporates SMARCA4 as ATPase subunit

Finally, we designed a set of experiments to shed light on the composition of the ARID1B-BAF at the day-5 of CNCC differentiation. Thus, we performed immunoprecipitation of endogenous ARID1B followed by mass-spectrometry (IP-MS) in Control Line-1. With this approach, ARID1B coeluted with a total of 9 additional BAF subunits (Fig. 6d). In mammals, the BAF complexes can incorporate two widely interchangeable and mutually exclusive ATPase subunits (i.e. SMARCA2, and SMARCA4). Remarkably, SMARCA4 was the only ATPase subunit identified as coeluting with ARID1B in our IP-MS, while zero peptides of SMARCA2 were detected (Fig. 6e).



e

IP-Mass Spectrometry Data

Protein	ARID1B IP	Control	iBAQ
ARID1B	30	0	79968000
SMARCA4	42	2	55391000
SMARCC1	40	2	148640000
ACTL6A	15	4	141350000
SMARCB1	16	2	95163000
SMARCE1	14	4	93189000
SMARCD1	22	0	67061000
DPF2	11	2	41439000
SMARCD2	13	0	25370000
SMARCA5	21	2	15997000
SALL4	26	5	84541000

Figure 6 – A switch between ARID1A-BAF and ARID1B-BAF is required for a successful exit from pluripotency. (a) Time-course immunoblot conducted using Control Line-1 during CNCC differentiation shows that ARID1A is active at the iPSC stage, and abruptly downregulated at day-1 of differentiation. ARID1A protein level is upregulated again after day-7, mirroring ARID1B's downregulation at the same time point (ARID1B blot duplicated here from Fig. 2c for convenience). **(b)** ARID1A immunoblot: both patient lines display aberrantly high ARID1A's protein level at CNCC Day-5. **(c)** ChIP-qPCR of ARID1A at select pluripotency enhancers: at the iPSC stage the enhancers are bound by ARID1A comparably in both patient and control lines. At CNCC Day-5, the binding is completely lost in the patients while it is at least partially maintained in the patient lines. Coordinates and p-values (T-Test) in Supplementary File S7. **(d)**

ChIP-seq for ARID1A in the two patient lines at CNCC Day-5. Heatmaps are centered on the 5,540 pluripotency enhancers. Input collected at CNCC Day-5 was used as a control. **(e)** Table displaying all the BAF subunits (plus SALL4) that coeluted with ARID1B in the IP-MS performed at Day-5 of CNCC5 differentiation in Control Line-1. Values represent unique peptide numbers. iBAQ values are shown in last column.

This suggests that ARID1B-BAF selectively incorporates only SMARCA4 as a catalytic subunit, while it does not tolerate the incorporation of SMARCA2.

In summary, the IP-MS allowed us to characterize a novel configuration of the BAF complex (ARID1B-BAF), which is active during early stages of cranial neural crest differentiation and includes ARID1B, SMARCA4, and eight additional BAF subunits (Fig. 6e). Intriguingly, the transcription factor SALL4 also coeluted with ARID1B, suggesting a possible interaction with the complex, which was further supported by co-IP (Supplementary Fig. 3). Like ARID1B, SALL4 is also dispensable for the maintenance of the pluripotency networks, while it is essential for lineage commitment in early mammalian development, during which it targets sites with binding motifs also recognized by SOX2, OCT4 and NANOG[22-25]. SALL4 was previously shown to interact with the NuRD repressive complex[23], while interactions with BAF have been largely unexplored. It was recently demonstrated that this transcription factor has affinity for AT-rich regions[48], thus providing further support to the ARID1B-SALL4 interaction. *SALL4* mutations are also associated with developmental syndromes, including Okihiro syndrome, Holt-Oram syndrome, and Townes-Brocks Syndrome[49]. Notably, the *SALL4* gene is downregulated in the Coffin-Siris patients at CNCC day-5 but not in undifferentiated iPSCs, suggesting a possible feedback mechanism between *ARID1B* and *SALL4* during lineage commitment. Future studies will be necessary to support the speculation that SALL4 serves as an intermediator for ARID1B-BAF recruitment at the pluripotency enhancers.

Discussion

ARID1B is a member of the evolutionarily conserved SWI/SNF (BAF) chromatin remodeler[26,50]. *De novo* haploinsufficient mutations in the *ARID1B* gene cause severe neurodevelopmental disorders which affect both physical and cognitive development.

In this study, we investigated the Coffin-Siris-associated *ARID1B* mutations in the context of craniofacial development and report the discovery of a novel function of the BAF complex: attenuation of the gene expression program associated with pluripotency maintenance upon differentiation cues. We found that this repressive function is performed at pluripotency enhancers and promoters by a specific and novel BAF complex configuration (ARID1B-BAF), which is composed of 10 subunits, with the enzymatic activity seemingly carried out exclusively by SMARCA4.

As a consequence of the *ARID1B* mutations, the Coffin-Siris patient cells fail to repress the pluripotency elements. This elicits aberrant SOX2 activity genome-wide, which in turn leads to the upregulation of multiple pluripotency genes, including *NANOG* and its associated gene network, and to the downregulation of the genes responsible for coordinating the exit from pluripotency (PPARα/RXRα pathway). We demonstrate that these pluripotency enhancers are normally maintained in an active state by ARID1A-BAF at the iPSC stage, and subsequently repressed by the ARID1B-BAF at the onset of cranial neural crest differentiation.

A switch between ARID1A-BAF and ARID1B-BAF upon differentiation cues is hence necessary for commitment towards the neural crest lineage.

Other studies have previously suggested that switches between SWI/SNF subunits play important roles in cell fate determination. For example, a switch between the two catalytic subunits SMARCA4 and SMARCA2 mediates the activation of human IFNγ-activated genes[51]. Similarly, a gain of the subunit BAF53a in the neuron-specific BAF (nBAF) is required to control cell cycle exit in the developing neurons[2,3].

With our study, we discovered a novel switch between BAF subunits (ARID1A/ARID1B), critical for the exit from pluripotency. Importantly, a balance between pro-self-renewal and pro-differentiation signals is pivotal for the determination of stem cell fate[52]. We demonstrate that such balance is lost in Coffin-Siris patients, whose cells are unable to perform the ARID1A/ARID1B switch at the pluripotency enhancers at the onset of differentiation. This switch is essential to successfully complete the cranial neural crest differentiation.

Pluripotency is orchestrated by a transcription factor network that needs to be extinguished in an orderly manner to enable lineage commitment and differentiation[52-54]. We find that ARID1B-BAF plays an essential role in this process, by means of a repressive activity at the pluripotency enhancers of the SOX2 and NANOG networks. Similarly, an association between SOX3 and the SMARCA2 ATPase subunit of BAF was recently suggested in a study of neural development in the Nicolaides-Baraitser syndrome[55]. It is worth noting that Coffin-Siris and Nicolaides-Baraitser syndromes share many physical and neurological phenotypes[55-57].

The BAF complex is predominantly considered as a transcriptional activator, which balances out the Polycomb Repressor Complexes (PRC1, PRC2) in the modulation of gene expression[7,58]. Nonetheless, repressing activity for BAF was also reported. For instance, a study conducted on hepatocellular carcinoma cell lines uncovered that ARID1A-containing BAF activates and represses roughly equal numbers of genes, while ARID1B-containing BAF was found to primarily repress enhancer activity[47]. Our experiments corroborate these findings, supporting an enhancer-repressor function for ARID1B-BAF. We demonstrate that the repressive activity of ARID1B-BAF is specific to a set of ~4,900 enhancers and ~600 promoters, enriched for the SOX2 and NANOG binding sites. In *ARID1B*-wt conditions, these cis-regulatory elements are highly active at the iPSC stage, moderately active in the first four days of neural crest differentiation, and finally repressed by the day-5, a time point in which we reported the peak of ARID1B protein expression. The patient cells exhibit aberrant chromatin accessibility at these cis-regulatory elements for many days after the onset of differentiation, enforcing a long-lasting pluripotency signature which persists even after two weeks of differentiation.

Patient-26 derived cells display the most extreme cellular and molecular phenotype, with ~20% of the cells remaining pluripotent at the day-14 of differentiation, likely as a consequence of higher *SOX2* and *NANOG* expression and activity. The cells derived from this patient also show the highest levels of ARID1A binding at these enhancers at the day-5 of CNCC differentiation. Although it is difficult to formally compare disease severity since there are no accepted severity scales for Coffin-Siris syndrome, it is worth noting that Patient-26 presents clinically more severe than Patient-19. For example, Patient-26 was not able to speak at 7 years, whilst Patient-19 started speaking at 4 years. Additionally, the Patient-26 is affected by pyloric stenosis, a congenital anomaly in the digestive system thought to be associated with impaired migration of the enteric neural crest. We consider it unlikely that the difference is caused solely by the mutations in *ARID1B*, since both patients show comparable reduction in ARID1B protein levels. We speculate that additional genetic factors may concur with *ARID1B* haploinsufficiency to determine the clinical severity of the syndrome. Nonetheless, these lines of evidence potentially establish a direct correlation between reduced attenuation of pluripotency enhancers, inefficient exit from pluripotency, impaired cell differentiation, and disease severity (Fig. 7). However, additional experiments with a larger set of patient-derived cell lines would be required to support this model. Furthermore, it would also be important to investigate other differentiation lineages to elucidate whether the ARID1A/ARID1B switch is limited to the neural crest differentiation or if instead it represents a more widespread mechanism utilized by stem cells to exit the pluripotent state and undergo lineage commitment.

Finally, further investigations will be necessary to elucidate the mechanism(s) responsible for the repressive activity of ARID1B-BAF. Recent studies have demonstrated that the function of BAF (including ARID1A-BAF) as transcriptional activator is mediated by the AP-1 transcription factors[55,59,60]. On the other hand, little is known of potential co-factors mediating the repressive function of ARID1B-BAF. The

hypothesis that SALL4 might be mediating such repressive activity is fascinating and it opens up new research directions.

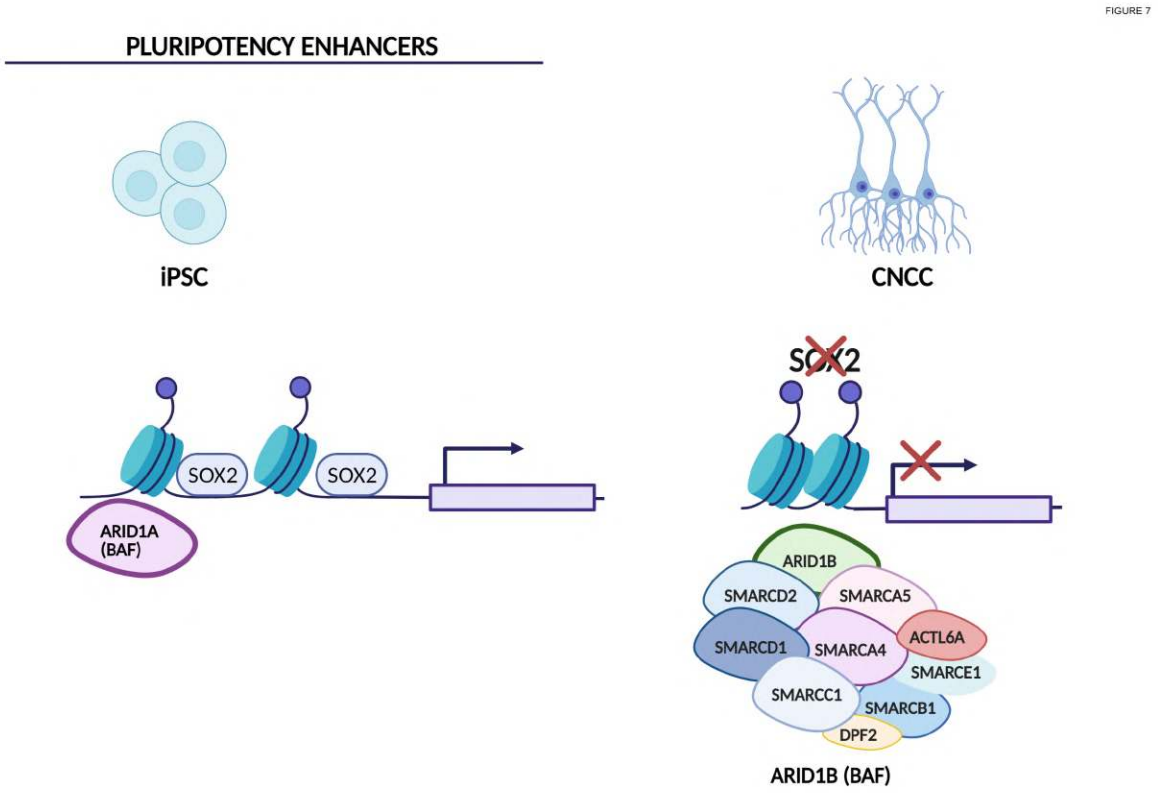


Figure 7 – A novel role for the ARID1B-BAF in the regulation of the exit from pluripotency. In iPSCs, the pluripotency enhancers are maintained in active state by the ARID1A-BAF. When the neural crest differentiation is induced, ARID1B-BAF replaces ARID1A-BAF at these enhancers, eliciting their attenuation. The *ARID1B* haploinsufficient cells fail to perform the ARID1A/ARID1B switch, and maintain ARID1A-BAF at the pluripotency enhancers throughout the differentiation. Consequently, these enhancers remain aberrantly active and bound by SOX2 and NANOG for several days along the differentiation process. As a consequence, the gene network responsible for the exit from pluripotency is not efficiently activated and the CNCC differentiation is impaired.

Acknowledgements

The authors are deeply grateful to the patients and their families, who donated the samples for this research. The authors thank the physicians and technicians who collected and cultured the skin samples. This work was funded by the G. Harold and Leila Y. Mathers Foundation (MT), and the Gisela Thier Fellowship (GWES). The authors thank the Genomic Facilities at Thomas Jefferson University and at the Wistar Institute for the sequencing effort. The authors are grateful to Kelly Vonk (Leiden University) for the help with experimental procedures in the iPSC generation. An invaluable support was provided to the authors by the Stem Cell and Regenerative Neuroscience Center at Thomas Jefferson University, and in particular by Dr. Elizabeth Kropf. Lastly, MT and GWES are thankful to Dr. Samantha Vergano (Children's Hospital of the King Daughters) for making this collaboration and this work possible.

Author contributions: MT, GWES and LP designed the project. GWES recruited the patients and obtained the skin fibroblasts. HMMM and CF reprogrammed the patient fibroblasts into iPSCs and assessed their quality. LD performed initial iPSC characterization experiments. LP performed most of the experiments. PP, ATC, CAO, and SAW contributed to specific experiments (flowcytometry, immunoblots, mass-spectrometry). BC provided intellectual contribution and financial support to PP. MT, LP and SD analyzed the data. MT, GWES, and LP wrote the manuscript, which was read and approved by all the authors.

Data availability: The original genome-wide data generate for this paper are deposited in the GEO database (accession number GSE169654).

MATERIALS AND METHODS

Human iPSC culture

Control iPSC lines were obtained from the iPSC Core of the University of Pennsylvania (Control line-1: SV20 line, male, age 43) and from the Coriell Institute for Medical Research (Camden, NJ. Control line-2: GM23716, female, age 16).

Skin fibroblasts from the two pediatric Coffin-Siris patients (one teenager one young adult) were obtained by the team of Dr. Gijs Santen at Leiden University. Patient 19 is a female, while Patient 26 is a male. The fibroblasts were reprogrammed into iPSCs with the polycistronic lentiviral vector LV.RRL.PPT.SF.hOKSM.idTomato.-preFRT by LUMC human iPSC Hotel as described elsewhere[61,62].

Multiple clones per line were derived. For each clone, pluripotency was assessed by immunofluorescence microscopy using antibodies against NANOG, OCT3/4, SSEA4 and Tra-1-81 under maintenance conditions and antibodies against (TUBB3, AFP and CD31) after spontaneous differentiation into the 3 germ layers as described elsewhere[61]. Clones with proper pluripotent characteristics were selected for downstream usage. Karyotyping by G binding was assessed for all the four lines by the Leiden University Medical Center and Short Tandem Repeat (STR) profiling was performed by the Leiden University Medical Center and and then replicated by the Stem Cell and Regenerative Neuroscience Center at Thomas Jefferson University. The iPSC lines were expanded in feeder-free, serum-free mTeSR™1 medium (STEMCELL Technologies). Cells were passaged ~1:10 at 80% confluency using ReLeSR (STEMCELL Technologies) and small cell clusters (50–200 cells) were subsequently plated on tissue culture dishes coated overnight with Geltrex™ LDEV-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix (Fisher-Scientific).

CNCC Differentiation

The iPSC lines were differentiated into CNCC as previously described[28]. Briefly, iPSCs were treated with CNCC Derivation media: 1:1 Neurobasal medium/D-MEM F-12 medium (Invitrogen), 0.5× B-27 supplement with Vitamin A (50× stock, Invitrogen), 0.5× N-2 supplement (100× stock, Invitrogen), 20 ng/ml bFGF (Biolegend), 20 ng/ml EGF (Sigma-Aldrich), 5 µg/ml bovine insulin (Sigma-Aldrich) and 1× Glutamax-I supplement (100× stock, Invitrogen). Medium (3ml) was changed every day. Three days after the appearance of the migratory CNCC, cells were detached using accutase and placed into geltrex-coated plates. The early migratory CNCCs were then transitioned to CNCC early maintenance media: 1:1 Neurobasal medium/D-MEM F-12 medium (Invitrogen), 0.5× B-27 supplement with Vitamin A (50× stock, Invitrogen), 0.5× N-2 supplement (100× stock, Invitrogen), 20 ng/ml bFGF (Biolegend), 20 ng/ml EGF (Sigma-Aldrich), 1 mg/ml bovine serum albumin, serum replacement grade (Gemini Bio-Products # 700-104P) and 1× Glutamax-I supplement (100× stock, Invitrogen).

ARID1B Knock-down

To make concentrated lentivirus, HEK293T cells were transfected with a pLenti plasmid in which we cloned an shRNA for *ARID1B* (GPP Web Portal: TRCN0000107361). iPSCs were lentivirally transduced by incubating the cells with concentrated virus overnight at 37 C. The next morning the media was changed, and 2 mg/ml puromycin (InvivoGen) were added 24h after infection. After 72 hours, the iPSCs that survived the selection were then differentiated in CNCC using the above described protocol, and collected at Day-5 for the genomic experiments. The cells were kept under puromycin selection for the entire duration of the differentiation. The knock-down efficiency was quantified via western blot.

Flow cytometry analysis of surface markers

To obtain a single cell suspension for flow cytometry analysis, control and patient cells were treated with Accutase for 5 minutes. Cells were then washed with cold PBS-2% FBS and live cells were counted. 1×10^6 cells/condition were resuspended in 100 µL PBS-2% FBS and stained. For pluripotency evaluation, 4 µl of the respective antibodies were used: APC anti-human SSEA-4 antibody (Biolegend, #330417) and PE anti-human TRA-1-60-R antibody (Biolegend, #330609). For analysis of differentiation, 2 µl of the respective antibodies were used: FITC anti-human CD10 (Miltenyi Biotec, #130-124-262) and APC anti-human CD99 (Miltenyi Biotec, #130-121-096). Cells were incubated for 15 min on ice and protected from light, before transferring them into FACS tubes containing additional 300 µL PBS-2% FBS. Flow cytometry data were acquired using a BD LSR II flow cytometer and analyzed with FlowJo Software version 10.7.

Western Blot

For total lysate, cells were harvested and washed three times in 1X PBS and lysed in RIPA buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 500uM DTT) with proteases inhibitors. Twenty µg of whole cell lysate were loaded in Novex WedgeWell 4-20% Tris-Glycine Gel (Invitrogen) and separated through gel electrophoresis (SDS-PAGE) Tris-Glycine-SDS buffer (Invitrogen). The proteins were then transferred to ImmunBlot PVDF membranes (ThermoFisher) for antibody probing. Membranes were incubated with 10% BSA in TBST for 30 minutes at room temperature (RT), then incubated for variable times with the suitable antibodies diluted in 5% BSA in 1X TBST, washed with TBST and incubated with a dilution of 1:10000 of secondary antibody for one hour at RT. The antibody was then visualized using Super Signal West Dura Extended Duration Substrat (ThermoFisher) and imaged with Amersham Imager 680.

Cell fractionation

5×10^6cells/condition were collected and suspended in E1 buffer (50mM HEPES-KOH, 140mM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100, 1mM DTT, 1X Proteinase Inhibitor) followed by a centrifugation step of 1100 g at 4°C for 2min. The cytoplasmic fraction was collected in a fresh tube. Cells were washed two more times with E1 buffer. Pellet was subsequently suspended in E2 buffer (10mM Tris-HCl, 200mM NaCl, 1mM EDTA, 0.5mM EGTA, 1X Proteinase Inhibitor) followed by a centrifugation step of 1100 g at 4°C for 2 min. Nuclear fraction was collected in a fresh tube. Cells were washed two more times with E2 buffer. After the third wash, pellet was suspended in E3 buffer (500mM Tris-HCl, 500mM NaCl, 1X Proteinase Inhibitor) and sonicated for 15 sec (5 sec ON/ 5 sec OFF). Cytoplasmic, nuclear and chromatin fraction were centrifuge at 16000 g for 10min at 4°C.

Antibodies

ARID1B ChIP-Seq: Abcam ab57461. ARID1B western blot: Santa-Cruz sc-32762 and Abcam ab57461. ARID1A ChIP-Seq: GeneTex GTX129433. ARID1A western blot: Cell Signaling Technologies 12354S. Beta-Actin western blot: Cell Signaling Technologies 8457P. SOX2 ChIP-Seq: Active Motif 39843. NANOG ChIP-Seq: R&D Systems AF1997. H3K27ac ChIP-Seq: Abcam ab4729. GAPDH western blot: Cell Signaling Technologies 5174T. CD10 Flow Cytometry: Miltenyi Biotech 130-124-262. CD99 Flow Cytometry: Miltenyi

Biotech 130-121-086. SSEA4 Flow Cytometry: Biolegend 330417. TRA-1-60-R Flow Cytometry: Biolegend 330609. IgG ChIP-qPCR: Cell Signaling Technologies 2729S. Cell Signaling HRP-conjugated anti-rabbit (7074S) and anti-mouse (7076S) were used as secondary antibodies in western blot. Spike-in Antibody: Active Motif 61686. Spike-in Chromatin: Active Motif 53083.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Cells were lysed in Tri-reagent and RNA was extracted using the Direct-zol RNA MiniPrep kit (Zymo research). 600ng of template RNA was retrotranscribed into cDNA using RevertAid first strand cDNA synthesis kit (Thermo Scientific) according to manufacturer directions. 15ng of cDNA were used for each real-time quantitative PCR reaction with 0.1 µM of each primer, 10 µL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) in a final volume of 20 µl, using QuantStudio 3 Real-Time PCR System (Applied Biosystem). Thermal cycling parameters were set as following: 3 minutes at 95°C, followed by 40 cycles of 10 s at 95°C, 20 s at 63°C followed by 30 s at 72°C. Each sample was run in triplicate. 18S rRNA was used as normalizer. Primer sequences are reported in Supplementary Table S1.

ChIP-Seq and ChiP-qPCR

Samples from different conditions were processed together to prevent batch effects.

For SOX2, NANOG and H3K27ac, for each replicate, 10 million cells were cross-linked with 1% formaldehyde for 5 min at room temperature, quenched with 125mM glycine, harvested and washed twice with 1× PBS. The pellet was resuspended in ChIP lysis buffer (150 mM NaCl, 1% Triton X-100, 0,7% SDS, 500 µM DTT, 10 mM Tris-HCl, 5 mM EDTA) and chromatin was sheared to an average length of 200–500 bp, using a Covaris S220 Ultrasonicator. The chromatin lysate was diluted with SDS-free ChIP lysis buffer. For ChIP-seq, 10 µg of antibody (3 µg for H3K27ac) was added to 5 µg of sonicated chromatin along with Dynabeads Protein A magnetic beads (Invitrogen) and incubated at 4 °C overnight. For SOX2 and NANOG ChIP-seq, 10 ng of spike-in Drosophila chromatin (Active Motif) was added to each sample with 2 µg spike-in antibody (Active Motif). On day 2, beads were washed twice with each of the following buffers: Mixed Micelle Buffer (150 mM NaCl, 1% Triton X-100, 0.2% SDS, 20 mM Tris-HCl, 5 mM EDTA, 65% sucrose), Buffer 500 (500 mM NaCl, 1% Triton X-100, 0.1% Na deoxycholate, 25 mM HEPES, 10 mM Tris-HCl, 1 mM EDTA), LiCl/detergent wash (250 mM LiCl, 0.5% Na deoxycholate, 0.5% NP-40, 10 mM Tris-HCl, 1 mM EDTA) and a final wash was performed with 1× TE. Finally, beads were resuspended in 1× TE containing 1% SDS and incubated at 65 °C for 10 min to elute immunocomplexes. Elution was repeated twice, and the samples were further incubated overnight at 65 °C to reverse cross-linking, along with the untreated input (5% of the starting material). On day 3, after treatment with 0.5 mg/ml Proteinase K for 1h at 65 °C, DNA was purified with Zymo ChIP DNA Clear Concentrator kit and quantified with QUBIT.

For ARID1A and ARID1B ChIP-Seq, 10 million cells were cross-linked with EGS (150 mM) for 30min at room temperature followed by a second cross-link with 1% formaldehyde for 15 min at room temperature. The formaldehyde was quenched with by adding glycine (0.125M) for 10 min at room temperature. Cells were washed twice with 1× PBS. Pellet was resuspended in buffer LB1 (50 mM Hepes-KOH, 140 mM NaCl, 1 mM EDTA, 10% Glycerol, 0.5% NP-40, 0.255 Triton X-100), incubated 10 min at 4 °C followed by a centrifugation step of 600g for 5 min at 4 °C. Pellet was suspended in buffer LB2 (10 mM Tris-HCl, 20 mM NaCl, 1 mM EDTA, 0.5 mM EGTA) incubated 10 min at 4 °C followed by a centrifugation step of 600g for 5 min at 4 °C. Cells were then resuspended in buffer LB3 (10 mM Tris-HCl, 200 mM NaCl, 1mM EDTA, 0.5 mM EGTA, 0.1% Na-DOC, 0.5% N-laurosylsarcosine) incubated 10 min at 4 °C followed by a centrifugation step of 600g for 5min at 4 °C. Pellet was suspended in LB3 and chromatin was sheared to an average length of 200–500 bp, using a Covaris S220 Ultrasonicator. For each sample, 15 ug of sonicated chromatin was incubated at 4 °C overnight along with Dynabeads Protein G conjugated with 10ug of antibody. On day 2, beads were washed once with each of the following buffers: WB1 (50 mM Tris-HCl, 150 mM NaCl, 0.15 SDS, 0.1% Na-DOC, 1% Triton X-100, 1 mM EDTA), WB2 (50 mM Tris-HCl, 500 mM NaCl, 0.15 SDS,

0.1% Na-DOC, 1% Triton X-100, 1 mM EDTA), WB3 (10 mM Tris-HCl, 250 mM LiCL, 0.55 NP-40. 0.55 Na-DOC, 1 mM EDTA), TE Buffer (10 mM Tris-HCl, 1mM EDTA). Finally, beads were resuspended in EB (10 mM tris-HCl, 0.55 SDS, 300 mM NaCl, 5mM EDTA) and incubated at 65 °C for 30 min to elute immunocomplexes. Elution was repeated twice, and the samples were further incubated overnight at 65 °C to reverse cross-linking, along with the untreated input (5% of the starting material). On day 3, after treatment with 0.5 mg/ml Proteinase K for 1h at 65 °C.

For all ChIP-seq experiments, barcoded libraries were made with NEB ULTRA II DNA Library Prep Kit for Illumina, and sequenced on Illumina NextSeq 500, producing 75bp SE reads.

For ChIP-qPCR, on day 1 the sonicated lysate was aliquot into single immunoprecipitations of 2.5×10^6 cells each. A specific antibody or a total rabbit IgG control was added to the lysate along with Protein A magnetic beads (Invitrogen) and incubated at 4 °C overnight. On day3, ChIP eluates and input were assayed by real-time quantitative PCR in a 20 µl reaction with the following: 0.4 µM of each primer, 10 µl of PowerUp SYBR Green (Applied Biosystems), and 5 µl of template DNA (corresponding to 1/40 of the elution material) using the fast program on QuantStudio qPCR machine (Applied Biosystems). Thermal cycling parameters were: 20sec at 95 °C, followed by 40 cycles of 1sec at 95°C, 20sec at 60°C.

ChIP-seq Analyses

After removing the adapters, the sequences were aligned to the reference hg19, using Burrows Wheeler Alignment tool (BWA), with the MEM algorithm[63] . Aligned reads were filtered based on mapping quality (MAPQ > 10) to restrict our analysis to higher quality and likely uniquely mapped reads, and PCR duplicates were removed. We called peaks for each individual using MACS2[64] (H3K27ac) or Homer[65], at 5% FDR, with default parameters.

RNA-Seq

Cells were lysed in Tri-reagent (Zymo research) and total RNA was extracted using Quick-RNA Miniprep kit (Zymo research) according to the manufacturer's instructions. RNA was further quantified using DeNovix DS-11 Spectrophotometer while the RNA integrity was checked on Bioanalyzer 2100 (Agilent). Only samples with RIN value above 8.0 were used for transcriptome analysis. RNA libraries were prepared using 1 µg of total RNA input using NEBNext® Poly(A) mRNA Magnetic Isolation Module, NEBNext® UltraTM II Directional RNA Library Prep Kit for Illumina® and NEBNext® UltraTM II DNA Library Prep Kit for Illumina® according to the manufacturer's instructions (New England Biolabs).

RNA-Seq Analyses

Reads were aligned to hg19 using STAR v2.5[66], in 2-pass mode with the following parameters: --quantMode TranscriptomeSAM --outFilterMultimapNmax 10 - -outFilterMismatchNmax 10 --outFilterMismatchNoverLmax 0.3 --alignIntronMin 21 -- alignIntronMax 0 --alignMatesGapMax 0 --alignSJoverhangMin 5 --runThreadN 12 -- twopassMode Basic --twopass1readsN 60000000 --sjdbOverhang 100. We filtered bam files based on alignment quality (q = 10) using Samtools v0.1.19[63]. We used the latest annotations obtained from Ensembl to build reference indexes for the STAR alignment. Kallisto[67] was used to count reads mapping to each gene. RSEM[68] was instead used to obtain FPKM (Fragments Per Kilobase of exon per Million fragments mapped). We analyzed differential gene expression levels with DESeq2[69], with the following model: design = ~condition, where condition indicates either CTRL or Patients.

ATAC-Seq

For ATAC-Seq experiments, 50,000 cells per condition were processed as described in the original ATAC-seq protocol paper[70]. ATAC-seq data were processed with the same pipeline described for ChIP-seq, with one modification: all mapped reads were offset by +4 bp for the forward-strand and -5 bp for the reverse-

strand. After peak calling (MACS2), peaks replicated in all 4 lines (hereafter consensus peaks) were used for downstream analyses.

Nuclear extract, IP and LC-MS/MS

After collection, cells were washed twice with ice cold PBS before resuspension in co-IP buffer (20mM Tris pH 7.9, 100mM NaCl, 0.1% NP-40, 0.5mM DTT, protease inhibitors), and rotated for 5 minutes at 4°C. After spinning down at 2000rpm for 10 minutes, the nuclear pellet was resuspended in buffer C (20mM Tris pH 8.0, 1.5mM MgCl2, 0.42M NaCl, 25% glycerol, 0.2mM EDTA, 0.5mM DTT, protease inhibitors), dounce homogenized (with B pestle), and incubated at 4°C for 30 minutes. The extract was centrifuged at 12,000rpm for 30 minutes, and the supernatant was kept as nuclear extract. The nuclear extract was dialyzed overnight in BC80 (20mM Tris pH 8.0, 80mM KCl, 0.2mM EDTA, 10% glycerol, 1mM B-mercaptoethanol, 0.2mM phenylmethylsulfonyl fluoride (PMSF)), cleared, and stored at -80°C. For the IP, 1.5mg of nuclear extract was incubated for 3 hours at 4°C with 6µg ARID1B antibody and 50µL of Dynabeads Protein A, and the control IP was performed with 0.75mg of nuclear extract and 25µL of Dynabeads Protein A. Beads were washed three times with co-IP buffer, followed by a final wash with 0.05% NP-40 in PBS. Elution was performed by agitation in 0.1M glycine pH 3.0 for one minute, and 1M Tris base pH 11.0 was added to neutralize the pH of the eluate. Eluates were prepared for SDS-PAGE and run on a Novex WedgeWell 10% Tris-Glycine Gel (Invitrogen) with Tris-Glycine-SDS buffer (Bio-Rad), at 110V for 10 minutes. The gel was stained with Colloidal Blue staining kit (Invitrogen), and further processed at the proteomics facility at the Wistar Institute. Briefly, the gel lanes were excised, reduced with TCEP, alkylated with iodoacetamide, and digested with trypsin. Tryptic digests were analyzed using LC-MS/MS (a standard 90 minute LC gradient on the Thermo Q Exactive HF mass spectrometer). MS/MS spectra were searched with full tryptic specificity against the UniProt human database (10/02/2020) using MaxQuant 1.6.17.0, and also searched for the common protein N-terminal acetylation, Asn deamidation, and Met oxidation. Protein and peptide false discovery rate was set at 1%.

Statistical and genomic analyses

All statistical analyses were performed using R v3.3.1. BEDtools v2.27.1[71] was used for genomic analyses. Pathway analysis was performed with Ingenuity Pathway Analysis Suite (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis). Motif analyses were performed using the Meme-Suite[72], and specifically with the Meme-ChIP application. Fasta files of the regions of interest were produced using BEDTools v2.27.1. Shuffled input sequences were used as background. E-values < 0.001 were used as threshold for significance[72].

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