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RAPID COMMUNICATION

BAF45D regulates spinal cord neural stem/progenitor cell fate through the SMAD-PAX6 axis

Mutations in BRG1-associated factor 45D (BAF45D, also known as double PHD fingers 2 (DPF2)) are linked to Coffin-Siris syndrome (CSS).[1] However, the underlying molecular mechanisms remain ill-defined. Here, we identified that wild-type but not CSS-associated BAF45D mutants increased the expression of PAX6,[2] a neural stem cell marker, and phosphorylated SMAD3 (p-SMAD3) in spinal cord neural stem cells (NSCs) derived from H9 human embryonic stem cells. Both BAF45D and SMAD3 are required for the induction of p-SMAD3 and PAX6 together with STAT3 and SMAD7 by retinoic acid (RA). In the presence of RA, BAF45D knockdown decreased the expression of genes that regulate stem cell pluripotency. Moreover, the same genes in the spinal cord NSCs had more binding sites with BAF45D than those in H9 cells. Furthermore, Gene Ontology (GO) assay results indicated that BAF45D targets more pathways responsible for stem cell maintenance in NSCs. Finally, BAF45D is associated with BRG1, p-SMAD3, and PAX6. Together, these findings suggest a role for BAF45D in modulating PAX6 expression through SMAD signaling, highlighting the importance of BAF45D in neural stem/progenitor cell fate commitment.

Human NSCs are a promising cell source for disease modeling and cell transplantation therapy. We induced H9 cells (Fig. S1A, D) into spinal cord NSCs that expressed PAX6, NESTIN, and SOX1 (Fig. S1). HOXC9, a spinal cord marker, was also detected in NSCs (Fig. S1N, P). The spontaneous differentiation of NSCs was also examined (Fig. S1Q–V). To explore the role of BAF45D in spinal cord NSCs, lentivirus-mediated infection of wild-type BAF45D, BAF45D mutant carrying a tryptophan (W) substitution of cysteine (C) at position 330 (C330W), and BAF45D mutant carrying a histidine (H) substitution of arginine (R) at position 350 (R350H) was performed. The results showed that BAF45D increased PAX6 and p-SMAD3 expression in a PHD2-dependent manner (Fig. 1A, B). Using the P19 mouse embryonal carcinoma stem cell line, which expresses no human antigen (Fig. S2), we also discovered that forced expression of FLAG-tagged BAF45D increased PAX6 and p-SMAD3 expression in a PHD2-dependent manner (Fig. 1C–E), connecting PAX6 levels with the phenotypes of CSS-related gene mutations.

Next, we performed RA-induced neural differentiation of P19 cells in the presence of negative control (NC), *Gapdh*, and *Baf45d* siRNAs (Fig. S3). We found that BAF45D was required for the expression of PAX6 and p-SMAD3 but not total SMAD3 (Fig. S3E–H). Moreover, consistently with the morphological changes in P19 cells (Fig. S3I), the quantitative real-time PCR results (Fig. S3J) showed that *Pax6* mRNA levels were significantly decreased by *Baf45d* siRNA, while the mRNA levels of *Tuj1* and *Gfap* were not decreased, indicating a role of BAF45D in neural stem/progenitor cell fate specification. To screen related genes involved in PAX6 induction, an RNA-seq assay was performed using P19 cells that were treated with NC siRNA and *Baf45d* siRNA, followed by RA induction (Fig. S4A). Even though RA can increase BAF45D, BAF45D knockdown was proven by the decreased FPKM values (Fig. S4B). Differential expression gene analysis revealed that 169 genes were increased and 225 genes were decreased (Fig. S4C) among 13509 genes (Fig. S4D). In particular, BAF45D knockdown significantly decreased the expression of some genes related to neural function (Fig. S4E and Table S1).

Through an RNA-seq assay, we found that some genes that regulate stem cell pluripotency were significantly decreased upon knockdown of BAF45D (Fig. 1F; Fig. S4 and Table S2), implying that BAF45D may play a role in maintaining stem cell fate. To investigate the function of this transcription factor in spinal cord NSCs, we performed a CUT&Tag assay (Fig. S5A–E). BAF45D-bound genes were associated with neural signaling and were increased significantly in spinal cord NSCs compared to H9 cells (Fig. S5F). Next, more BAF45D-binding sites of the gene set in the NSCs regulating stem cell pluripotency, which had a high score, were found

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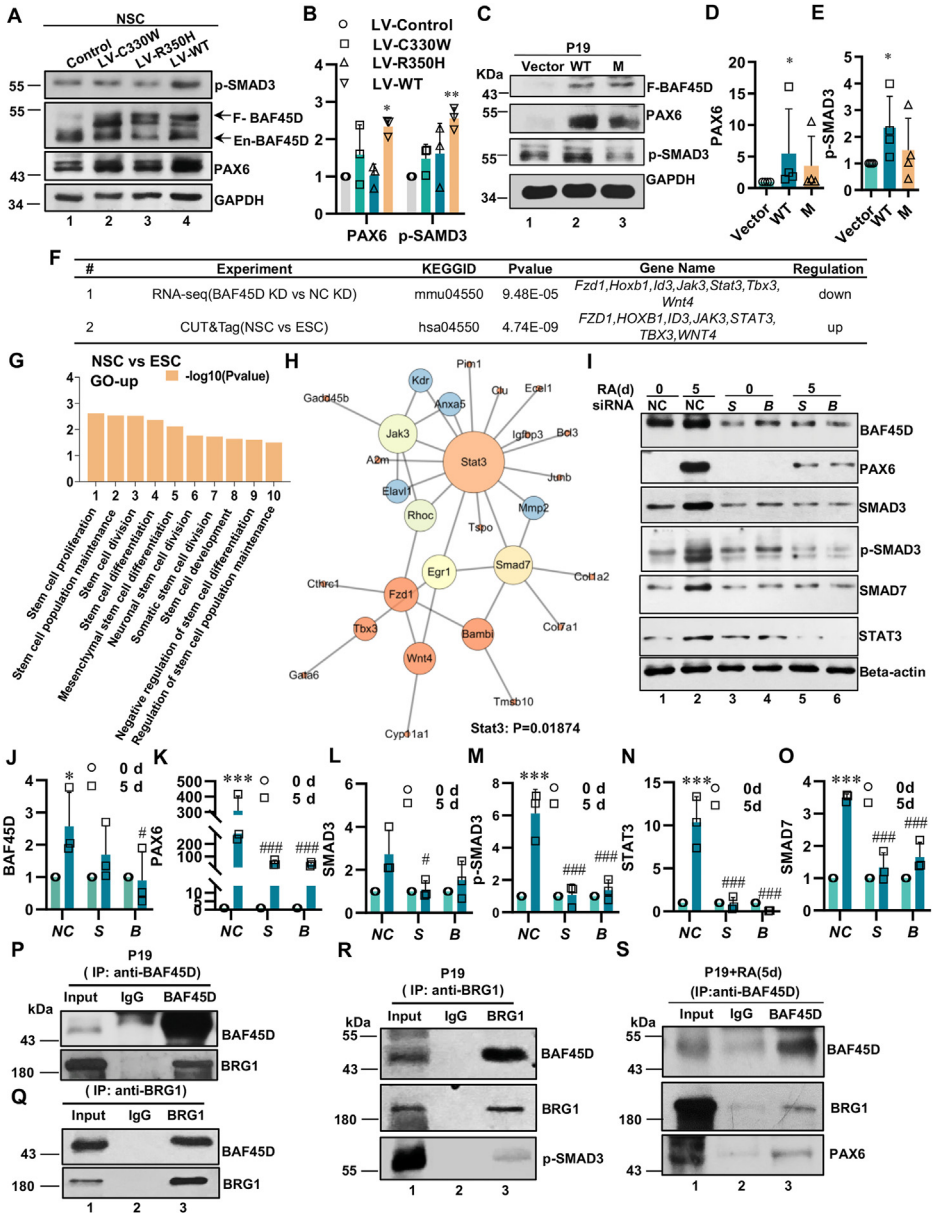

Figure 1 BAF45D contributes to spinal cord neural stem/progenitor cell fate commitment by regulating PAX6 expression through SMAD signaling. **(A, B)** Spinal cord neural stem cells (NSCs) were infected with control lentiviruses (Control) and lentiviruses expressing wild-type BAF45D (LV-WT), C330W (LV-C330W), and R350H mutant BAF45D (LV-R350H). The infected NSCs were harvested on the 3rd day of infection and subjected to immunoblotting (IB) assays using the indicated antibodies (A). Statistical analysis of the IB data was performed (B). **(C—E)** P19 cells were transfected with pFLAG-vector (Vector), wild-type FLAG-BAF45D (WT), and mutant FLAG-BAF45D (M) plasmids for 72 h and subjected to IB assay for the indicated proteins (C). Statistical analysis of the relative expression levels of PAX6 and p-SMAD3 in the transfected P19 cells (D and E). $^{*}P < 0.05$, by one-way ANOVA. The data are from four replicates. WT, wild type. M, PHD2 mutant. **(F)** Regulation of similar gene sets related to signaling pathways influencing pluripotency was analyzed by RNA-seq assay in retinoic acid (RA)-treated P19 cells and analyzed by CUT&Tag assay upon BAF45D knockdown in human spinal cord NSCs. **(G)** Gene Ontology (GO) assay for the significantly increased BAF45D binding sites in the NSCs. **(H)** The typical protein—protein interaction (PPI) network (analyzed by Cytoscape) of the key downregulated factors related to the SMAD signaling pathway by BAF45D knockdown is shown. The *p* values of the significantly downregulated Stat3 are shown. The node sizes have a positive correlation with the number of associated edges. The node colors are related to the clustering coefficient. **(I)** P19 cells were transfected with NC, Smad3 #1327 (S), and BAF45D (B) siRNAs, followed by RA induction for 5 days. Then, the P19-derived cells were subjected to an IB assay to determine the expression of the indicated proteins. **(J—O)** Statistical analysis of the expression levels of BAF45D (J), PAX6 (K), SMAD3 (L), p-SMAD3 (M), STAT3 (N), and SMAD7 (O) before (0 d) and after (5 d) RA induction. The data are from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (*versus* 0 d). $\#P < 0.05$, $\#\#P < 0.01$ and $\#\#\#P < 0.001$ (*versus* NC group), by one-way ANOVA. **(P, Q)** Mutual interaction between BAF45D and BRG1 in normal P19 cells. Immunoprecipitation assays were performed using anti-BAF45D (P) and anti-BRG1 (Q) antibodies in P19 cells. Then, an IB assay was performed using the indicated antibodies. **(R, S)** Immunoprecipitation assays were performed using the anti-BRG1 antibody in undifferentiated P19 (R) cells and the anti-BAF45D antibody in differentiated P19 (S) cells, followed by an IB assay using the indicated antibodies.

than in the H9 cells (Fig. 1F; Fig. S6A). In NSCs, *SOX2*, a gene responsible for NSC maintenance, and *SMAD7*, a gene responsible for the negative regulation of SMAD signaling, exhibited more binding sites with BAF45D (Fig. S6B, C). A recent report indicated that loss of BAF53a resulted in decreased chromatin accessibility at *SOX2* binding sites, which failed to maintain the proliferation of neural stem/progenitor cells.[3] Here, more BAF45D-binding sites of both *SMAD7* and *SOX2* were identified in NSCs than in H9 cells, indicating that BAF45D may target both genes to control neural stem/progenitor cell fate. To our surprise, the CUT&Tag GO analysis results (Table S3) confirmed that the BAF45D binding genes related to stem cell maintenance in the NSCs were ranked higher than those in the H9 cells (Fig. 1G; Fig. S6D, E), suggesting that BAF45D may play a crucial role in maintaining neural stem cell fate. Moreover, STAT3, a protein negatively associated with SMAD signaling, was identified as one of the core factors of the typical protein—protein interaction (PPI) network that was composed of the key decreased factors by BAF45D knockdown (Fig. 1H and Table S4), suggesting that SMAD signaling may be involved in RA-induced PAX6 expression.

To determine whether SMAD3 is required for the induction of PAX6, STAT3, and SMAD7, we screened three *Smad3* siRNAs (#787, #1158, and #1327) (Fig. S7A, B) and found that siRNA #1327 was more efficient than the other two siRNAs (Fig. S7C—H). Next, to further determine the role of BAF45D and SMAD3 in STAT3 and SMAD7 expression, P19 cells transfected with NC, #1327 *Smad3* (*S*) and *Baf45d* (*B*) siRNAs were treated with RA for 5 days. The results indicated that RA induced the expression of PAX6, p-SMAD3, STAT3, and SMAD7 in the NC group (Fig. 1I). Moreover, compared to the NC group, both the BAF45D and SMAD3 knockdown groups had decreased expression of PAX6, p-SMAD3, STAT3, and SMAD7 (Fig. 1I—O). Our results support a recent report that SMAD3 depletion inhibits PAX6 during the differentiation of limbal stem/progenitor cells into epithelial cells,[4] highlighting that PAX6 levels may be stabilized by positive and negative autoregulation *in vivo*. Finally, interactions among BAF45D, BRG1, p-SMAD3, and PAX6 were revealed by immunoprecipitation assays (Fig. 1P—S). Interestingly, CSS is a special disorder that has hair, nail, and central nervous system phenotypes. A recent paper showed that more or fewer hair follicles and thicker or thinner epidermis were related to the expression patterns of the *PAX6* gene.[5] Based on these findings and our data, we cannot exclude the possibility that the *PAX6* gene may also be related to the nonneural phenotypes of CSS, which warrants rigorous study in the near future.

Author contributions

Xueying Chen and Xiujuan Hu: data curation, formal analysis, validation, investigation, visualization, methodology, and writing. Jing Jiang: data curation, formal analysis, validation, investigation, and visualization. Jie Tao: formal analysis and methodology. Lihua Liu: resources and project administration. Shengyun Fang: resources and review. Yuxian Shen, resources, methodology, and supervision. Qingsong Hu: methodology and supervision. Chao Liu: conceptualization, data curation, formal analysis, validation, investigation, visualization, methodology, supervision, administration, funding acquisition, and writing.

Conflict of interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.05.007.

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