ORIGINAL ARTICLE

CHD6 regulates the topological arrangement of the CFTR locus

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Abstract

The control of transcription is regulated through the well-coordinated spatial and temporal interactions between distal genomic regulatory elements required for specialized cell-type and developmental gene expression programs. With recent findings *CFTR* has served as a model to understand the principles that govern genome-wide and topological organization of distal intra-chromosomal contacts as it relates to transcriptional control. This is due to the extensive characterization of the DNase hypersensitivity sites, modification of chromatin, transcription factor binding sites and the arrangement of these sites in *CFTR* consistent with the restrictive expression in epithelial cell types. Here, we identified CHD6 from a screen among several chromatin-remodeling proteins as a putative epigenetic modulator of *CFTR* expression. Moreover, our findings of CTCF interactions with CHD6 are consistent with the role described previously for CTCF in *CFTR* regulation. Our results now reveal that the CHD6 protein lies within the infrastructure of multiple transcriptional complexes, such as the FACT, PBAF, PAF1C, Mediator, SMC/Cohesion and MLL complexes. This model underlies the fundamental role CHD6 facilitates by tethering cis-acting regulatory elements of *CFTR* in proximity to these multi-subunit transcriptional protein complexes. Finally, we indicate that CHD6 structurally coordinates a three-dimensional structure between intragenic elements of *CFTR* bound by several cell-type specific transcription factors, such as CDX2, SOX18, HNF4 α and HNF1 α . Therefore, our results reveal new insights into the epigenetic regulation of *CFTR* expression, whereas the manipulation of *CFTR* gene topology could be considered for treating specific indications of cystic fibrosis and/or pancreatitis.

Introduction

Gene expression depends on the ability of distal genomic elements, some regarded as well-defined gene enhancers bound by transcription factors, to access the core transcriptional machinery to faithfully execute a well-coordinated transcriptional program (1). In this regard, *CFTR* has been considered as a model gene to determine the dynamics and mechanisms of genome-wide DNA topology that determine the fate of gene transcription (2,3). This is due to the relatively extensive characterization of the DNase I hypersensitivity sites (HSs) (4), modification of chromatin and transcription factor binding elements that occur *in vivo* (5–7) among specific studies that map nucleosome position (8). These results are coupled with the restrictive expression of *CFTR* in epithelial cell types (2). Moreover, *CFTR* is composed of multiple HSs consistent with its expression pattern in the cell types examined and directly corresponding with the extent of histone acetylation/deacetylation as a measurement of chromatin access (5,9,10). Regulation of *CFTR* topology has been characterized through interaction of both proximal and remote HSs as landmarks by chromosome conformation capture (3C) analysis to determine the frequency of the interactions corresponding with the cell types examined (11). The transcription factor CTCF bound to DNA in chromatin has remained a consistent mark that identifies the putative position of distal elements linked with basal transcriptional elements or promoters consistent with establishing boundaries in chromatin (12). More recently, cohesin subunits that actuate chromatin folding and selective conformation are considered a basis through which distal genomic regulatory elements are functionally looped or tethered toward the promoter of many genes (13) that include *CFTR* (14). Several regulatory elements within *CFTR* could function within a hub that forms according to cell-type and tissue-specific manners to organize chromatin required for *CFTR* transcription. One such regulatory element resides within the first intron of *CFTR*, whereby, several transcription factors, CDX2, TCF4 and HNF1 α (5,15) are bound tightly and directly reflect the level of *CFTR* transcription.

Members of the chromodomain helicase DNA (CHD)-binding family of enzymes belong to the SNF2 superfamily of ATP-dependent chromatin remodelers (16). Genetic, biochemical and structural studies demonstrate that CHD proteins are important regulators of transcription and play critical roles during developmental processes (17). CHD6 has been shown to directly interact with RNA polymerase II (RNAPII) (18), however, confirmation of CHD6 modulating RNAPII activity remains unclear. Human variation of CHD6 is evident in a spectrum of genetic diseases (19,20), but a direct link to any disease process remains uncertain.

Here, we describe the role of CHD6 in coordinating chromatin structure necessary to accommodate *CFTR* expression. We find that CHD6 cooperates extensively with multiple transcriptional complexes as an organized hub for expression of *CFTR*. Moreover, resolution of the immune-specific CHD6 complex reveals subunits that incorporate distinct complexes that participate in mechanisms for chromatin structure and gene transcription. These studies advance the role of CHD6 in regulating the chromatin topology of *CFTR* as an exemplary genetic model for this mechanism. Enforcing favorable chromatin topology to override the stringencies placed on *CFTR* expression where genetic lesions alter expression may model an approach to modulate *CFTR* expression where specific indications of cystic fibrosis and/or pancreatitis rely on more expression of *CFTR*.

Results

CHD6 is a novel chromatin remodeler of *CFTR*

To identify novel regulators of chromatin architecture for expression of the *CFTR* locus, we performed a short hairpin RNA (shRNA) library screen using a panel of custom-modified MISON[®] shRNAs (Sigma) designed for targeting the expression of 89 chromatin-modifying or remodeling proteins derived from The RNAi Consortium (21). Following infection and selection in the pancreatic adenocarcinoma cell line CF-PAC, we measured expression of *CFTR* by multiplexed quantitative polymerase chain reaction (qPCR) through independent positions of the transcript by qPCR to statistically affirm the abundance of the entire *CFTR* transcript. The change of *CFTR* transcript abundance was normalized against levels of *HPRT* and *GAPDH* mRNA. The screen revealed several positive regulators such as CHD6, CHD8, the components of the SWI-SNF complex Smarcc1/BAF155 and BRG1 and the FACT complex subunits SUPT16H and SSRP1, while CHD4 appeared to be a negative transcriptional regulator of *CFTR* (Fig. 1A). Given the strong modulation of the relative steady-state values of *CFTR*, we considered CHD6 as an interesting candidate to study epigenetic mechanisms for regulation of *CFTR* expression. To verify that CHD6 was bona fide regulator of *CFTR* expression, CF-PAC cells were infected with lentivirus encoding shRNA directed against CHD4, CHD6, and CTCF as a control or with scrambled shRNA-expressing lentiviruses. Loss of CHD4 or CHD6 proteins resulted in an increase or decrease, respectively, of *CFTR* mRNA levels (Fig. 1B). Consistent with a role of CTCF in positively regulating *CFTR* expression, *CFTR* transcript levels were significantly reduced upon CTCF silencing (Fig. 1B). To validate this, we analyzed the effect of CHD6 on *CFTR* expression in another cell type relevant to *CFTR* expression, the intestinal carcinoma cell line Caco2. CHD6 shRNA-infected cells showed a mild (less than 2-fold) but highly reproducible *CFTR* repression (Fig. 1C).

CHD6 is associated with multiple chromatin-modifying and chromatin-remodeling complexes

We showed that CHD6 regulates *CFTR* expression. To gain insight into molecular partners associated with CHD6 we achieved large-scale immunoaffinity chromatography studies in the human embryonic carcinoma cell line NTera-2 using immunoaffinity chromatography and mass spectrophotometry (MS) methods to identify protein composition of CHD6 interacting proteins. The CHD6-containing immune complex was then resolved by a silver-stained sodium dodecyl sulfate-polyacrylamide (4–12%) gradient gel and molecular weight determination was resolved by fractionation with a Superose 6[™] gel filtration column, and the indicated fractions were analyzed by western blot of CHD6 as shown (Fig. 2A–C). Protein identification performed by multidimensional protein identification technology (MudPIT) to establish distributed normalized spectral abundance factors (dNASF) determined the peptide composition and coverage of the CHD6 interactome using procedures previously described (23). Fractionation of the CHD6 complex identified key elements of chromatin and transcriptional regulatory proteins. Individual protein interactions were validated by immunoprecipitation (Fig. 2C). Among the CHD6-binding proteins, we detected CHD8, the FACT subunits SUPT16H and SSRP1; the chromatin organizer CTCF; the components of the cohesin complex SMC3 and MCM3AP; the acetyltransferase TAF9/TAFII31 of the transcription factor IID (TFIID) complex; the components of the MLL1/MLL complex involved in histone methylation and demethylation

Figure 1. Targeting chromatin-modifying proteins by a viral shRNA library identifies CHD6 as a candidate chromatin remodeler of CFTR. (A) Representative heatmap generated from shRNA-mediated loss of the respective chromatin-remodeling proteins in the CF-PAC pancreatic adenocarcinoma cell line following three independent trials indicated on the left. Relative levels of CFTR were evaluated by qPCR and compared and normalized with the control shRNA following shRNA-mediated loss of each of the chromatin-remodeling proteins shown. (B) Direct quantitative PCR of CFTR mRNA corresponding to loss of CHD4, CHD6 and CTCF in CF-PAC cells by lentiviral shRNA. Results are mean $\pm$ SEM of three independent experiments. *Significant difference compared with the control group, $P \leq 0.003$ for CHD6 and $P \leq 0.008$ for CTCF. Corresponding immunoblots indicate the relative protein levels of the targeted proteins by shRNAs. (C) Depletion of CHD6 by introducing lentiviral-transduced shRNA in Caco2 cells results in loss of CFTR expression relative to the shRNA control. Results are mean $\pm$ SEM of five independent experiments. *Significant difference compared with the control group, $P \leq 0.0001$.

ASH2L, WDR5 and UTX/KDM6B; the bromodomain-containing protein BRD7, a novel BAF-specific SWI/SNF subunit and the component of the transcriptional PAF1 complex (PAF1C). See

further details in Supplementary material, Table S1. Relative abundance of proteins is indicated by dNASF, revealing a network whose components have roles in nucleosome remodeling,

Figure 2. Immunoaffinity purification of the CHD6 complex. (A) Silver stain visualization of protein eluted by immunoprecipitation of nuclear enriched CHD6 from NTera2 embryonic carcinoma cells. Slices of the PAGE gel were subjected to tandem MS/MS for protein analysis and identification. (B) Immunoblot detecting CHD6 from fractions following gel filtration on Sepharose GL of the immunocomplex of nuclear CHD6 shown in (A). (C) Immunoblotting was performed to detect individual protein subunits identified in (A). (D) CHD6-associated proteins with statistically significant dNSAF values ($P < 0.05$) were identified in three MudPIT analyses in the graph shown. An integrated and modified STRING network (22) of associated proteins identified through the immunoaffinity CHD6 complex was generated and presented above the graph. In total, 148 proteins were identified as being strongly enriched with high confidence ($P \leq 0.05$), some of which are organized within additional multi-subunit complexes highlighted in the light gray background. Spheres shown represent only the high confidence ($P < 0.05$) CHD6-associated proteins identified in two of the three MudPIT analyses performed. A summary of CHD6-associated proteins that associate with other transcription factors is presented (Supplementary material, Table S1).

chromosome organization, RNAPII-mediated transcription and histone modification (Fig. 2D). In all, our data suggest that CHD6 fits within a hierarchical core involving several multi-subunit transcriptional complexes.

CHD6 is recruited specifically to the CFTR locus together with other transcription factors

Because, we, and others, previously demonstrated that a strong regulatory element function involves the first intron of CFTR (2,5,24), we next tested whether CHD6 was bound within this region. For that purpose, chromatin immunoprecipitation (ChIP) assays were performed in CF-PAC and Caco2 cells transduced with CHD6 shRNA or a shRNA control, and several sites along the CFTR locus were analyzed. ChIP analysis demonstrated strongest enrichment of CHD6 at position III (intron 1) and a notable enrichment at positions II (promoter), IV (intron 11) and I (5'). Loss of CHD6 results in loss of enrichment in both CF-PAC and Caco2 cells (Fig. 3A). Given the interaction between CHD6 and the architectural protein CTCF and the previous description of CTCF bound within CFTR (14,25), we assessed whether CHD6 was required for enrichment of CTCF at specific sites in CFTR. Our results reveal the requirement of CHD6 for maintaining the integrity for CTCF recruitment. We (5) and others (6,26) have previously shown that factors such as hepatocyte nuclear factor 1 α (HNF1 α), caudal homeobox protein 2 (CDX2) and T-cell factor 4 (TCF4) among others can bind directly to the intronic element within intron 1 and are necessary for enhancer activity. To provide further insight on the interaction between CHD6 and the intronic element within intron 1, we performed similar

ChIP experiments with specific antibodies against several transcription factors. Specific occupation by CHD6 and CTCF in position III (intron 1) correlates with the presence of SUPT16H, TAF9, HNF4 α , SOX18, PTF1 α , HNF1 α and CDX2 (Fig. 3B and Supplementary material, Fig. S1), which diminishes upon CHD6 silencing.

In order to further characterize the interaction between CHD6 and CDX2, we performed immunoprecipitation studies, where nuclear extracts of Caco2 cells were immunoprecipitated with CHD1 and CHD6 antibodies and immunoblotted against CDX2. Data indicate the association between CDX2 with CHD6, but not CHD1 (Fig. 3C). Furthermore, reciprocally testing interactions with antibodies against CDX2, Suv39H1 and RNAPII we confirm the interaction between CHD6, CDX2 and RNAPII [S2p] (phosphorylated serine 2). To determine if these two factors occupy CFTR locus simultaneously, we precipitated Caco2 chromatin first with CHD6 antibody and, after extensive washing, again with CDX2, RNA PolII and Suv39H1 antibodies. In the case of CDX2 and RNAPII, sequential immunoprecipitation (Re-ChIP) revealed amplification of the correct element within intron 1 of CFTR (site III) (Fig. 3C, right bottom panel). These results indicate that CHD6 and CDX2 occupy the same chromatin within a regulatory element of the first intron.

Moreover, it was determined by ChIP-seq that global distribution of CDX2 and TCF4 genomic binding are directly coupled and strongly overlap with HNF1 α and HNF4 α sites in Caco2 cells (27). We then tested whether interactions between CDX2 and HNF1 α , TCF4 and CHD6 occur within the local chromatin environment. Immunoprecipitation studies using a purified chromatin-associated fraction showed that this interaction is indeed taking place in a more biological context (Supplementary material,

Figure 3. CHD6 and CTCF binding to the *CFTR* locus. **(A)** ChIP analysis of CHD6 and CTCF binding at six sites along the *CFTR* locus (positions I to VI) in Caco2 and CF-PAC cells transduced with CHD6 shRNA or a shRNA negative control. Percent enrichment is shown relative to input (% input) DNA determined by qPCR. IgG controls are shown for position III in both cell lines. Sites I–VI correspond to their location from 5' to 3' direction in the locus and primer sequences are listed in Supplementary material, Table S2. Results are mean $\pm$ SEM of triplicates and are representative of at least five independent ChIP experiments. **(B)** ChIP-qPCR experiments measuring relative enrichment of SUPT16H, TAF9, HNF4 α , SOX18, PTF1A, HNF1 α and CDX2 at the *CFTR* first intron. Percent relative enrichment (% input) is calculated over the input. Results are mean $\pm$ SEM of triplicates and are representative of minimum of five independent experiments. *Significant difference compared with the control group, * $P \leq 0.02$, ** $P \leq 0.002$, *** $P \leq 0.0002$. See also Supplementary material, Figure S1. **(C)** Immunoprecipitation of CHD1 and CHD6 in Caco2 nuclear extracts. Immunoprecipitation followed by immunoblotting of CDX2 is shown (left panel). Immunoprecipitation of CDX2, Suv39H1 and the S2p specific RNAPII in Caco2 nuclear lysates followed by immunoblotting to detect CHD6 is shown (right top panel). A small percentage of the input used prior to immunoprecipitation experiments was used to detect constitutive β -actin protein. Sequential ChIP (Re-ChIP) experiments on *CFTR* first intron was conducted. CHD6 immunoprecipitates were subjected to a second immunoprecipitation with the antibodies against CDX2, RNA PolII and Suv39H1 (right bottom panel). See also Supplementary material, Figure S2.

Figure 4. CHD6 is required for long-range interactions within the *CFTR* locus. (A) 3C analysis showing relative interaction frequencies between the promoter and other sites along the locus in Caco2 cells transduced with control and CHD6 shRNA lentiviruses. The x-axis represents the position relative to the TSS and the y-axis represents the interaction frequency relative to the interaction frequency between two *HindIII* fragments within the ubiquitously expressed *ERCC3* gene. Results are mean $\pm$ SEM of triplicates and are representative of at least two independent experiments. See also Supplementary material, Figure S3. (B) Schematic overview of the CHD6 3C-ChIP assay in Caco2 cells. (C) PCR fragments amplified with primers for the first intron (B + C) and for the region between 5' and first intron (A + C). Templates in each panel include 3C input (cross-linked chromatin *HindIII*-digested and ligated), α -CTCF 3C and α -CHD6 3C (chromatin that was immunoprecipitated with CTCF and CHD6 antibodies prior to ligation) and IgG 3C (chromatin immunoprecipitated with IgG as a negative control). Correspondent qPCR-3C data are shown in the right panel. Results are mean $\pm$ SEM of triplicates and are representative of at least three independent experiments. Restriction sites are shown in the schematic representation of the *CFTR* locus and primer sequences are listed in Supplementary material, Table S2. (D) Simplified model of the CHD6 and CTCF mediated interactions in the human *CFTR* locus.

Fig. S2) that provides strong evidence for CHD6, CDX2 and TCF4 as positive regulators of CFTR.

Chromatin architecture of CFTR requires CHD6 and CHD8

For the most part intrinsic control of epithelial cell-type expression of CFTR requires tethering distinct regulatory elements with the CFTR TSS. Long-range looping interactions between the CFTR promoter and specific DHS sites have been shown in Caco-2 cells among other cell types relevant to CF pathology by quantitative chromosome conformation capture (q3C) (28). To test the hypothesis that CHD6 mediates long-range chromosomal interactions within the CFTR locus as a component of coordinated control of CFTR expression, we performed q3C with CHD6 loss by shRNA in Caco-2 cells. Formaldehyde cross-linked nuclei from control and CHD6 knock-down cells were subjected to *Hind*III digestion and subsequent q3C analysis. We used a reverse primer located in the *Hind*III fragment that contains the CFTR TSS and paired it with multiple forward primers located close to *Hind*III fragments, some of them within relevant DHS sites, across the CFTR locus (Supplementary material, Table S2). We determined the digestion efficiency for future accurate quantification. Using a dilution series of digested and re-ligated control library template generated with three minimally overlapping BAC clones, each forward primer was shown to function with the reverse primer with a >90% efficiency. Ligation products were then quantified by quantitative PCR to analyze the ligation efficiency (or 'interaction frequency'), which was normalized using the interaction frequency measured between two *Hind*III fragments within the ubiquitously expressed ERCC3 locus. In accordance with what others have previously described, in control Caco-2 cells we detect interactions between the promoter and *Hind*III fragments located in intron 1, intron 11 and region 3' to the gene. However, the interaction frequencies observed in CHD6 knock-down cells were significantly reduced compared with those in control cells, specifically the interaction peaks in intron 1 and intron 11, confirming the importance of CHD6 protein in the looping of active CFTR. Furthermore, loss of CHD8 also leads to loss of looping interactions (Supplementary material, Fig. S3), suggesting a similar role for the other member of the family in organizing chromatin architecture.

Next we performed ChIP-3C as previously described (29,30) to identify long-range chromatin looping of the CFTR locus mediated specifically by CHD6. We digested cross-linked chromatin isolated from Caco2 cells with *Hind*III and immunoprecipitated with CHD6 and CTCF-specific antibodies. We then ligated this DNA and amplified it by PCR using the combination of primers shown in Figure 4B. We found that both CHD6 and CTCF antibodies can readily immunoprecipitate 3C products determining the interaction between the promoter and intron 1 (B + C) and more modestly between the 5' region and intron 1 (A + C) (Fig. 4C). The method we chose uses ChIP to pull down specific protein-bound chromatin interactions from such 3C chromatin fragments, and one would also co-precipitate the non-specific chromatin fragments that are attached to the specific interaction complexes. As such, it is likely that the use of the standard 3C protocol with the addition of ChIP just as it is would lead to high levels of false positives. To address this issue, one particular ChIP-3C protocol uses urea ultracentrifugation purification of ChIP complexes to reduce the amount of non-enriched noise from the ChIP. Also, to ascertain whether intra-chromosomal collision captured by ChIP-3C is valid we used numerous replicates and cloning verified by Sanger sequencing to be assured of the recovery of the precise fragments following ligation of *Hind*III

fragments. Therefore, these results provide strong statistical confidence that CHD6 is present within the corresponding chromatin loops. A model based on our data is shown in Figure 4D suggests that CHD6 functions in combination with CTCF and other interacting and associated nuclear factors to coordinate the topology of the CFTR locus.

Discussion

Here, we show through a modified lentiviral shRNA screen of chromatin-modifying and remodeling proteins that CHD6 demonstrated strong capacity for modulating CFTR transcription *in vivo* in the CF-PAC cell line (Fig. 1). Within the scope of immunoaffinity purification and quantitative tandem MS analysis, we resolved and validated the composition of the 2.8 MDa nuclear CHD6 complex that is consistent with the assembly of various multi-subunit complexes involving transcription, chromosome structure and RNA processing among others (Fig. 2). Existing studies showed that CTCF functionally interacts with the SNF2-like chromodomain CHD8 (31). We find that, like CHD8, the subfamily paralogue CHD6 interacts with CTCF (Fig. 2). Our studies reveal that CHD6 plays a role in organizing the highly ordered structure of chromatin surrounding CFTR in cooperation with CTCF and CHD8. While much attention was initially placed on characterizing transcription factors associated with CFTR transcription initiation (32), our understanding has greatly evolved in how multiple distal sites cooperate through both general and cell-type transcription factors to contribute both temporally and spatially to the arrangement in chromatin that dictate transcription programs for CFTR (6). In light of this concept, we now provide the role of CHD6 to function as an integrative hub for cell-type transcription factors CDX2, TCF4, HNF1 α and HNF4 α as our studies begin to provide a view for mechanisms directing the long-range association between regulatory regions of CFTR directed through the chromo-helicase DNA-binding domain 6 (CHD6) protein.

The above data provide evidence that strongly support a role of CHD6 in chromatin loop formation as an underlying mechanism of CFTR transcriptional control. In summary, our studies now illuminate the direct role of CHD6, CHD8 and CTCF in combination with a number of transcription factors in coordinating the genomic topology of CFTR. Such new insights may provide novel epigenetic approaches to modify CFTR expression where modulation of chromatin topology surrounding CFTR will have potential in addressing specific clinical indications linked to defects in CFTR expression.

Materials and Methods

Additional methods are described in the Supplementary Material.

shRNA screen of chromatin-remodeling proteins

We used a shRNA screen with a custom-modified MISSION[®] LentiPlex[®] pooled shRNA library (Sigma) designed to target ~89 chromatin regulatory proteins in CF-PAC1 cells according to instructions by the supplier (Sigma). More detailed explanation of the lentiviral shRNA screen is provided in Supplementary Materials, Material and Methods.

Quantitative tandem MS and MudPIT

Immunoaffinity purification of CHD6 complex and MudPIT analysis were performed essentially as described previously (23)

with changes noted here. Human embryonal carcinoma Ntera2 cells were harvested, and nuclear extracts were prepared using Dounce homogenization. CHD6 complexes were immunoprecipitated using anti-CHD6 antibodies and precipitated with trichloroacetic acid and subjected to MudPIT analysis similar to what was described previously (23). Analysis of tandem MS and MudPIT data is provided in Supplementary Material, Materials and Methods.

q3C

3C was performed as described previously (33,34) with minor modifications described in the Supplementary Material, Materials and Methods.

ChIP-3C

ChIP-3C was performed as described previously (30) with modifications described in the Supplementary Material, Materials and Methods.

Supplementary Material

Supplementary Material is available at HMG online.

Author Contributions

A.S., S.L. and M.J.W. conceived the study. A.S., T.P., S.L., N.B., F.A. and M.J.W. carried out the experiments. A.S., T.P., S.L., F.A., F.Z., W.Z. and M.J.W. analyzed data. A.V., N.S.L., N.B., F.J.S. and J.A.W. provided technical and conceptual advice. M.J.W. supervised the experiments. A.S. and M.J.W. wrote the manuscript. All authors participated in editing the manuscript.

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Conflict of Interest statement. None declared.

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References

- Belmont, A.S. (2014) Large-scale chromatin organization: the good, the surprising, and the still perplexing. *Curr. Opin. Cell Biol.*, **26**, 69–78.
- Ott, C.J., Blackledge, N.P., Kerschner, J.L., Leir, S.H., Crawford, G.E., Cotton, C.U. and Harris, A. (2009) Intronic enhancers coordinate epithelial-specific looping of the active CFTR locus. *Proc. Natl. Acad. Sci. USA*, **106**, 19934–19939.
- Gheldof, N., Smith, E.M., Tabuchi, T.M., Koch, C.M., Dunham, I., Stamatiouyannopoulos, J.A. and Dekker, J. (2010) Cell-type-specific long-range looping interactions identify distant regulatory elements of the CFTR gene. *Nucleic Acids Res.*, **38**, 4325–4336.
- Nuthall, H.N., Moulin, D.S., Huxley, C. and Harris, A. (1999) Analysis of DNase-I-hypersensitive sites at the 3' end of the cystic fibrosis transmembrane conductance regulator gene (CFTR). *Biochem. J.*, **341**(Pt 3), 601–611.
- Paul, T., Li, S., Khurana, S., Leleiko, N.S. and Walsh, M.J. (2007) The epigenetic signature of CFTR expression is co-ordinated via chromatin acetylation through a complex intronic element. *Biochem. J.*, **408**, 317–326.
- Gillen, A.E. and Harris, A. (2012) Transcriptional regulation of CFTR gene expression. *Front. Biosci.*, **4**, 587–592.
- Kerschner, J.L. and Harris, A. (2012) Transcriptional networks driving enhancer function in the CFTR gene. *Biochem. J.*, **446**, 203–212.
- Yigit, E., Bischof, J.M., Zhang, Z., Ott, C.J., Kerschner, J.L., Leir, S.H., Buitrago-Delgado, E., Zhang, Q., Wang, J.P., Widom, J. et al. (2013) Nucleosome mapping across the CFTR locus identifies novel regulatory factors. *Nucleic Acids Res.*, **41**, 2857–2868.
- Phylactides, M., Rowntree, R., Nuthall, H., Ussery, D., Wheeler, A. and Harris, A. (2002) Evaluation of potential regulatory elements identified as DNase I hypersensitive sites in the CFTR gene. *Eur. J. Biochem.*, **269**, 553–559.
- Li, S., Moy, L., Pittman, N., Shue, G., Auferio, B., Neufeld, E.J., Leleiko, N.S. and Walsh, M.J. (1999) Transcriptional repression of the cystic fibrosis transmembrane conductance regulator gene, mediated by CCAAT displacement protein/cut homolog, is associated with histone deacetylation. *J. Biol. Chem.*, **274**, 7803–7815.
- Lewandowska, M.A., Costa, F.F., Bischof, J.M., Williams, S.H., Soares, M.B. and Harris, A. (2010) Multiple mechanisms influence regulation of the cystic fibrosis transmembrane conductance regulator gene promoter. *Am. J. Respir. Cell Mol. Biol.*, **43**, 334–341.
- Wallace, J.A. and Felsenfeld, G. (2007) We gather together: insulators and genome organization. *Curr. Opin. Genet. Dev.*, **17**, 400–407.
- Kagey, M.H., Newman, J.J., Bilodeau, S., Zhan, Y., Orlando, D. A., van Berkum, N.L., Ebmeier, C.C., Goossens, J., Rahl, P.B., Levine, S.S. et al. (2010) Mediator and cohesin connect gene expression and chromatin architecture. *Nature*, **467**, 430–435.
- Gosalia, N., Neems, D., Kerschner, J.L., Kosak, S.T. and Harris, A. (2014) Architectural proteins CTCF and cohesin have distinct roles in modulating the higher order structure and expression of the CFTR locus. *Nucleic Acids Res.*, **42**, 9612–9622.
- Mouchel, N., Henstra, S.A., McCarthy, V.A., Williams, S.H., Phylactides, M. and Harris, A. (2004) HNF1alpha is involved in tissue-specific regulation of CFTR gene expression. *Biochem. J.*, **378**, 909–918.
- Marfella, C.G. and Imbalzano, A.N. (2007) The Chd family of chromatin remodelers. *Mutat. Res.*, **618**, 30–40.
- Hall, J.A. and Georgel, P.T. (2007) CHD proteins: a diverse family with strong ties. *Biochem. Cell. Biol.*, **85**, 463–476.
- Lutz, T., Stoger, R. and Nieto, A. (2006) CHD6 is a DNA-dependent ATPase and localizes at nuclear sites of mRNA synthesis. *FEBS Lett.*, **580**, 5851–5857.
- Kalscheuer, V.M., Feenstra, I., Van Ravenswaaij-Arts, C.M., Smeets, D.F., Menzel, C., Ullmann, R., Musante, L. and Ropers, H.H. (2008) Disruption of the TCF4 gene in a girl with mental retardation but without the classical Pitt-Hopkins syndrome. *Am. J. Med. Genet. A.*, **146A**, 2053–2059.
- Yamada, K., Fukushi, D., Ono, T., Kondo, Y., Kimura, R., Nomura, N., Kosaki, K.J., Yamada, Y., Mizuno, S. and Wakamatsu, N. (2010) Characterization of a de novo balanced t(4;20)

- (q33;q12) translocation in a patient with mental retardation. *Am. J. Med. Genet. A.*, **152A**, 3057–3067.
21. Ketela, T., Heisler, L.E., Brown, K.R., Ammar, R., Kasimer, D., Surendra, A., Ericson, E., Blakely, K., Karamboulas, D., Smith, A.M. et al. (2011) A comprehensive platform for highly multiplexed mammalian functional genetic screens. *BMC Genomics*, **12**, 213.
 22. Armean, I.M., Lilley, K.S. and Trotter, M.W. (2013) Popular computational methods to assess multiprotein complexes derived from label-free affinity purification and mass spectrometry (AP-MS) experiments. *Mol. Cell. Proteomics*, **12**, 1–13.
 23. Banck, M.S., Li, S., Nishio, H., Wang, C., Beutler, A.S. and Walsh, M.J. (2009) The ZNF217 oncogene is a candidate organizer of repressive histone modifiers. *Epigenetics*, **4**, 100–106.
 24. Rowntree, R.K., Vassaux, G., McDowell, T.L., Howe, S., McGuigan, A., Phylactides, M., Huxley, C. and Harris, A. (2001) An element in intron 1 of the CFTR gene augments intestinal expression in vivo. *Hum. Mol. Genet.*, **10**, 1455–1464.
 25. Li, Y., Huang, W., Niu, L., Umbach, D.M., Covo, S. and Li, L. (2013) Characterization of constitutive CTCF/cohesin loci: a possible role in establishing topological domains in mammalian genomes. *BMC Genomics*, **14**, 553.
 26. McCarthy, V.A., Ott, C.J., Phylactides, M. and Harris, A. (2009) Interaction of intestinal and pancreatic transcription factors in the regulation of CFTR gene expression. *Biochim. Biophys. Acta*, **1789**, 709–718.
 27. Verzi, M.P., Hatzis, P., Sulahian, R., Philips, J., Schuijers, J., Shin, H., Freed, E., Lynch, J.P., Dang, D.T., Brown, M. et al. (2010) TCF4 and CDX2, major transcription factors for intestinal function, converge on the same cis-regulatory regions. *Proc. Natl. Acad. Sci. USA*, **107**, 15157–15162.
 28. Ott, C.J., Suszko, M., Blackledge, N.P., Wright, J.E., Crawford, G. E. and Harris, A. (2009) A complex intronic enhancer regulates expression of the CFTR gene by direct interaction with the promoter. *J. Cell. Mol. Med.*, **13**, 680–692.
 29. Fullwood, M.J. and Ruan, Y. (2009) ChIP-based methods for the identification of long-range chromatin interactions. *J. Cell. Biochem.*, **107**, 30–39.
 30. Cai, S., Lee, C.C. and Kohwi-Shigematsu, T. (2006) SATB1 packages densely looped, transcriptionally active chromatin for coordinated expression of cytokine genes. *Nat. Genet.*, **38**, 1278–1288.
 31. Ishihara, K., Oshimura, M. and Nakao, M. (2006) CTCF-dependent chromatin insulator is linked to epigenetic remodeling. *Mol. Cell*, **23**, 733–742.
 32. Pittman, N., Shue, G., LeLeiko, N.S. and Walsh, M.J. (1995) Transcription of cystic fibrosis transmembrane conductance regulator requires a CCAAT-like element for both basal and cAMP-mediated regulation. *J. Biol. Chem.*, **270**, 28848–28857.
 33. Hagege, H., Klous, P., Braem, C., Splinter, E., Dekker, J., Cathala, G., de Laat, W. and Forne, T. (2007) Quantitative analysis of chromosome conformation capture assays (3C-qPCR). *Nat. Protoc.*, **2**, 1722–1733.
 34. Miele, A., Gheldof, N., Tabuchi, T.M., Dostie, J. and Dekker, J. (2006) Mapping chromatin interactions by chromosome conformation capture. *Curr. Protoc. Mol. Biol.*, Chapter 21, Unit 21.11.