Characterizing Intracellular Localization and Chromatin Remodeling Role of CHD6 in Human Fetal Development and in Glioblastoma

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Abstract— **CHD6, a member of the CHD family of DNA-binding, chromatin-modifying proteins, has been shown to be upregulated in glioblastoma. Using immunofluorescence the intracellular region to which CHD6 localizes can be determined. Through the use of chromatin Immunoprecipitation (ChIP), candidate genomic loci enriched for CHD6 can be determined. Immunofluorescence and ChIP results for CHD6 using a current protocol are presented. Notably, immunofluorescence of fetal germinal matrix and adult subventricular zone showed principally cytoplasmic localization of CHD6, contrary to expectation. CHD6 ChIP showed partial enrichment in certain genes of possible significance to glioblastoma in fetal tissue relative to the positive control. A higher sample size is needed to test and evaluate the significance of these findings. The results presented here reveal new insights into the changes in the epigenetic landscape contributing to glioblastoma and the manipulation of CHD6 expression could be considered for treating specific incidents of glioblastoma.**

I. INTRODUCTION

The set of proteins that constitute epigenetic regulators include so-called 'writers,' 'editors' and 'readers' of DNA methylation or histone post-translational modifications[5]. Whereas 'writers' are responsible for *de novo* modifications to DNA or DNA-bound histone proteins and 'editors' are responsible for chemically altering or reverting the modification placed by 'writers', 'readers' modulate and mediate interaction between proteins and protein complexes, including the transcriptional machinery[5]. Dysfunction or deregulation of epigenetic 'writers,' 'editors' or 'readers,' is widely implicated in stem cell activation and tumorigenesis[5].

Among the epigenetic 'readers' is a subset of chromodomain-containing proteins, the Chromodomain Helicase DNA-binding, or CHD, family of chromatin modifiers[1,4,5,8,12]. The CHD proteins are distinguished from other chromodomain-containing chromatin readers by their tandem chromodomains[1,4,5,8], which have been shown to interact with methylated lysine residues correlated with either repressed transcription or active transcription (H3K27me3 and H3K4me3)[1,4,11,12]. Within the larger family of CHD proteins, are three subfamilies distinguished by their varying functional or conserved domains. Class I CHD proteins contain CHD1 and CHD2. The Class I CHD proteins are the best characterized in terms of function and structure[,4,8,12]. Chromatin Immunoprecipitation (ChIP) experiments, which utilize antibodies to selectively precipitate proteins bound to specific genomic regions, have been conducted to elucidate the DNA regions enriched for CHD1[12]. Class II CHD proteins are distinguished by their dual PHD fingers, or plant homeodomains, and are composed of CHD3, CHD4 and CHD5[1,4,8,12]. Class III CHD proteins are composed of CHD6, CHD7, CHD8 and CHD9[1,4,8,12]. They are distinguished from the other two classes by their SANT (Switching-defective protein 3, Adaptor 2, Nuclear-receptor corepressor, Transcription Factor III B) and BRK (Brahma and Kismet) domains, which lie close to the proteins' C-terminus[1,4,12]. The precise function of the BRK domain is unknown[4]; however, it has been shown to interact with the transcriptional repressor CTCF[1,8], potentially coordinating chromatin loops[7].

Of considerable interest in the third CHD class is the poorly characterized nucleosome-remodeling protein CHD6. Though its precise mechanism of action has not been established[8,12], CHD6 has been reported to be upregulated in glioblastoma[3,10]. As a result, insight into the intracellular localization of CHD6 as well as its enrichment in genetic loci of interest to glioblastoma, such as *Olig2*[2], *EGFR (Epidermal Growth Factor Receptor)*[2] and *GFAP (Glial Fibrillary Acidic Protein)*[2] using immunofluorescence and ChIP would provide a more holistic understanding of CHD6 expression and the epigenetic landscape of glioblastoma pathology.

Immunofluorescence and ChIP experiments were performed to characterize the intracellular localization and chromatin-remodeling role of CHD6. Notably, immunofluorescence of fetal germinal matrix and adult subventricular zone showed principally cytoplasmic localization of CHD6, contrary to expectation. Through the use of ChIP, candidate genomic loci enriched for CHD6 were elucidated. CHD6 ChIP showed partial enrichment at the promoter of *EGFR*, one gene of possible significance to glioblastoma[2], in fetal and glioblastoma tissues, relative to a presumed positive control locus where CHD6 has been previously demonstrate to bind[8]. However, higher sample size is needed to test and evaluate the significance of these findings.

II. METHODS

A. Immunofluorescence

Paraffinized tissue sections (4 micrometers) previously fixed in 10% formalin were deparaffinized in xylene. Sections were rehydrated gradually through washes in graded alcohols: wash in 100% ethanol twice for 10 minutes each, then 95%, 75%, 50% ethanol for 5 minutes each then deionized water for 5 minutes. Slides were boiled in citrate-based buffer (pH=6) for 5 minutes then cooled on bench top for 30 minutes. Specimen

was blocked in blocking buffer for 60 minutes using 0.25% TritonX and 1% normal donkey serum. Cells were incubated with anti-GFAP (rat) and CHD6 (rabbit) primary antibodies overnight at a concentration of 1:100. Sections were washed 3 times in PBS. Sections were then incubated for 1 hour with fluorochrome-conjugated secondary antibodies. Secondary antibodies for Rat (pink fluorescence) and rabbit (green fluorescence) were used. Following three more PBS washes, specimens were incubated with DAPI nuclear stain (blue fluorescence) for 5 minutes. Sections were examined using confocal microscopy.

B. ChIP and qPCR Analysis

DNA binding of CHD6 was assessed via chromatin immunoprecipitation (ChIP) assays. Frozen post-mortem brain tissue or neurosurgical de-identified specimens (adult cortex n=3, fetal germinal matrix n=3, glioblastoma n=3) were minced manually using razor blades in a Petri dish. Minced tissue was suspended in 1400uL 1% formaldehyde fixative and rotated for 10 minutes at room temperature. Formaldehyde fixation was quenched with 93.3uL 2M glycine (125mM final concentration) and rotated for 5 minutes at room temperature. Chromatin was sheared to 150-600bp[9] and subjected to immunoprecipitation with antibodies against CHD6 or IgG (rabbit)[2,9] as negative control (12-370, Millipore, 5 mg) after pre-incubation with magnetic Dynabeads from rabbits (11203D, Invitrogen). Ten percent of the lysate was saved as "input" for normalization analysis. The chromatin-associated DNA was purified using the QIAquick PCR Purification Kit (28104, Qiagen, Valencia, CA) and quantified by qPCR (Stratagene Mx3000P) using primers designed within the *EGFR* promoter (EGFRprom), *ZIC1*, a reputed gene enriched for CHD6, *Olig2*, and *B2M*, a housekeeping gene (Appendix 1). The data was analyzed as percent input recovery relative to *ZIC1*, the presumed positive control. Melting curves were analyzed to ensure specificity of the primers. The efficiency for all primers was calculated, deemed appropriate, and accounted for in the analysis[2].

III. RESULTS

A. CHD6 Localizes to the Cytoplasm in Fetal Germinal Matrix and Adult Subventricular Zone

Immunofluorescence staining of fetal germinal matrix showed principally strong cytoplasmic localization of CHD6 (Figure 2). There was high cell abundance in the fetal tissue compared to the adult tissue, consistent with the findings by Samuelsen et.al[6]. CHD6 staining appeared comparatively stronger in the fetal germinal matrix than in adult subventricular zone. GFAP, stained in pink, was clearly expressed in the radial glia projecting out of the germinal matrix. Notably, CHD6's principally cytoplasmic localization seemingly conflicts with its reputed function as a DNA-binding protein and its hypothesized nuclear localization[12]. However, strong cytoplasmic staining could have obscured nuclear staining, and ChIP studies indicate the presence of locus-specific nuclear CHD6 presence. Further experimentation on CHD6 is required to determine whether it is natively found in the nucleus or if it translocates to DNA in response to some stimulus, as is seen in other transcription factors[14].

Intriguingly, there is remarkable CHD6 signal seen in the ependymal layer of the subventricular zone (Figure 1), which has not been previously demonstrated. Previous ChIP experiments have shown enrichment of CHD6 in CF-PAC, a pancreatic adenocarcinoma cell line, and Caco2, a colon cancer cell line[8]. Interestingly, pancreatic ductal cells (from which pancreatic adenocarcinoma arises), enterocytes, and ependymal cells have simple columnar structure[17,18]. Enterocytes and ependymal cells also have a similar brush border on their apical surface[17]. Whether these commonalities are related to CHD6 expression remains to be verified.

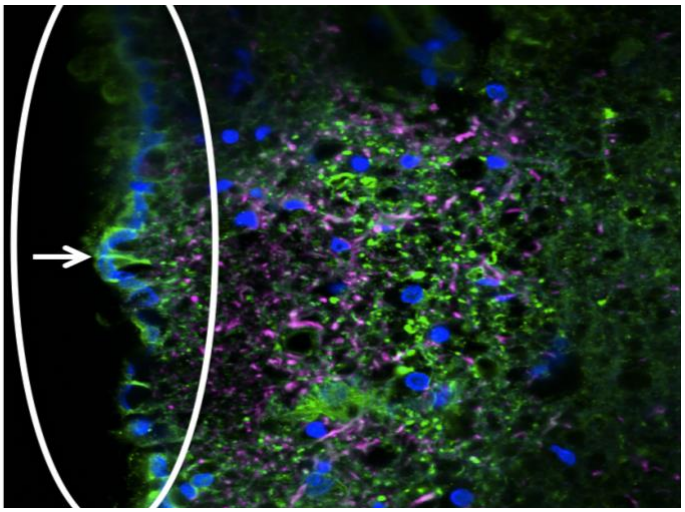

Figure 1
Adult subventricular zone under the confocal microscope. Note cytoplasmic localization of CHD6 and robust CHD6 signal in the ependymal layer, as indicated by the white arrow. GFAP fluoresces pink, CHD6 is green and DAPI is blue.

B. CHD6 may be enriched at the EGFR Promoter in Fetal Tissue and Glioblastoma

ChIP was performed on adult Cortex (n=3), fetal germinal matrix (n=3) and Glioblastoma (n=3) tissue types to assess for *in vivo* binding of CHD6 at genomic loci of potential interest in fetal development and gliomagenesis. The enrichment of CHD6 in each of the genomic loci of interest was calculated as percent of input recovery, and it was normalized to percent input enrichment of CHD6 at ZIC1, the positive control locus (Figure 4). The expectation was to see enrichment of CHD6 at gene loci, which are dysfunctionally upregulated in

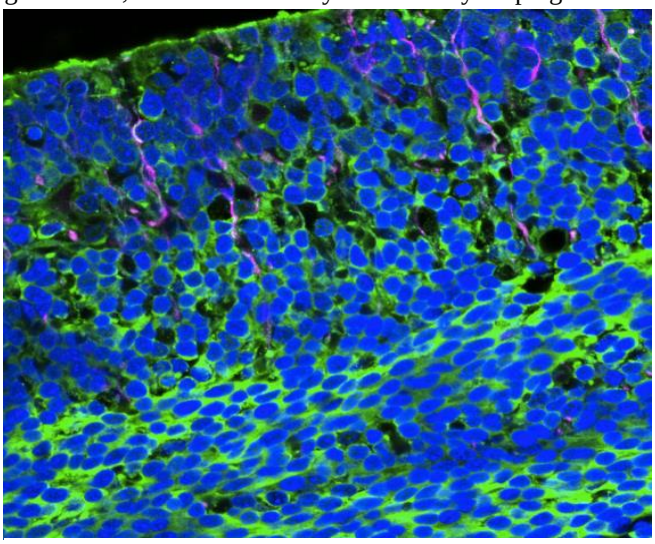

Figure 2
Note the principally cytoplasmic localization of CHD6 and strong CHD6 signal. Fetal germinal matrix under the confocal microscope. GFAP fluoresces pink, CHD6 is green and DAPI is blue.

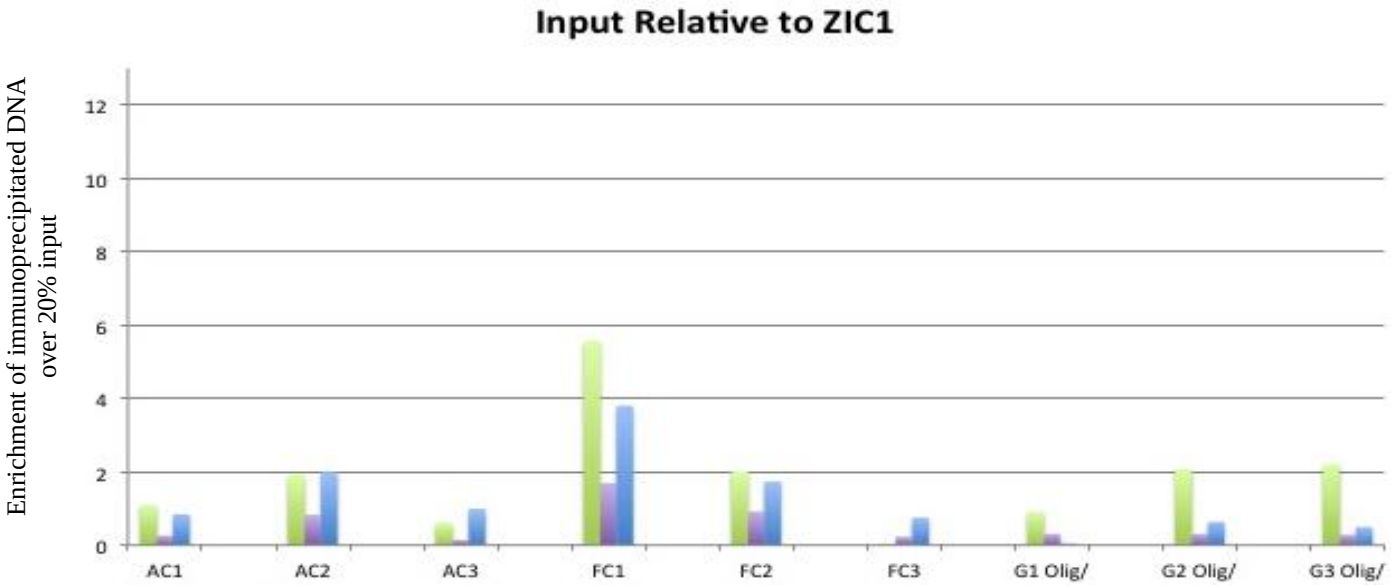

Figure 3
Enrichment of CHD6 binding is represented as percent input, relative to *ZIC1*, the positive control locus. Loci assessed include, EGFR promoter (green), B2M (purple), and *Olig2* (blue).

glioblastoma, such as at *Olig2* and *EGFR*, since the chromodomains of CHD6 bind methylated lysine residues associated with active transcription[11]. This preliminary data reveal greatest CHD6 enrichment at the *EGFR* promoter in fetal and glioblastoma samples, and at the *Olig2* promoter in fetal samples, relative to *ZIC1*. Unfortunately, significance cannot be established in this data since the qPCR amplification of IgG samples yielded more than one amplicon product.

IV. Discussion

The immunofluorescence staining of both fetal germinal matrix and adult subventricular zone showed principally cytoplasmic localization of CHD6. This contradicts the hypothesis that CHD6 is strictly relegated to DNA-binding in the nucleus. One possible explanation for these results is that most CHD6 could natively reside in the cytoplasm but translocate to the nucleus in response to binding of a cofactor. Lathrop et. al. report that CHD6 precipitates in complex with the transcription factor Nrf2, which is trafficked to the nucleus in response to cellular oxidative stress[13,14]. Analysis of the primary amino acid sequence of CHD6 reveals several potential nuclear localization signals (NLS)[19]. One possible mechanism for CHD6's counterintuitive intracellular localization could be that CHD6's NLS sequences are obscured by protein folding but are subsequently revealed following binding of a cofactor. Upon the emergence of the NLS sequences, importin, the protein responsible for protein import into the nucleus, could shuttle CHD6 to the genetic loci where CHD6 exerts its action as a chromatin remodeler.

Both the immunofluorescence and ChIP experiments relied on primary antibodies effective at recognizing CHD6. Prior to the immunofluorescence and ChIP, no experiments validating the antibody and proving its efficacy in precipitating CHD6 were performed. Furthermore, although no published literature has elaborated on the finding that CHD6 is enriched at *ZIC1*, *ZIC1* was used as the positive control locus in the ChIP experiment. Sancho et. al., independently reported that *ZIC1* would act as an effective positive control. In designing primers for *ZIC1*, the UCSC Genome Browser was consulted for candidate loci that could be enriched for CHD6. Candidate loci were determined using enrichment of transcription factor CTCF as proxy for CHD6 since Sancho et. al. reported CTCF acting in complex with CHD6[8]. To date, there has been no brain tissue ChIP-seq data for CHD6 upon which to base *ZIC1* as a positive control more accurately. Operating under the conjecture that CHD6 is enriched at *ZIC1*, based on the assertion by Sancho *et. al.* is an assumption in this experiment. Another limitation of the data is the lack of a negative control. Since CHD6 is a chromatin remodeler that colocalizes with RNA Polymerase II[8], an effective negative control would be loci of constitutively heterochromatic regions of DNA, such as telomeres or centromeres. Since these loci are constitutively heterochromatic, they are not transcribed and therefore enrichment of RNA Polymerase II and CHD6 would be unlikely in those regions. The qPCR amplification of the IgG samples yielding more than one amplicon could suggest the presence of primer dimers on the melting curve due to very little DNA pulled down in the immunoprecipitation. As a result, the experiment must be repeated with higher sample size in order to establish significance and make a strong assertion based on the ChIP data.

Future experiments will seek to titrate parameters for and effectuate a ChIP protocol using MNase, as opposed to sonication, for chromatin fragmentation. More ChIP experiments on adult cortical, fetal and glioblastoma tissue isolated from postmortem brain are needed to confirm or deny the results presented here. Also, immunofluorescence on epileptic brain specimens might shed further light on the question of CHD6's translocation to the nucleus. Epileptic seizures are characterized by failure to reuptake glutamate released into the synapse, resulting in prolonged and uncontrolled neuronal depolarization[15]. This behavior is highly taxing on neurons and often results in a state of cellular

oxidative stress called excitotoxicity[16]. Examining such oxidatively stressed tissue through immunofluorescence could enhance the understanding of CHD6's intra cellular localization.

Going forward, experiments should seek to elucidate the coordination between CHD6 and CTCF[8] in the formation of loops within chromatin[7]. These loops signify the topological landscape of chromatin and are as impactful to transcription as the molecular epigenetic landscape of histones and DNA[7]. To further the body of knowledge around chromatin loops and a potential role for CHD6 in catalyzing their formation, co-immunoprecipitation (co-IP) of CHD6 should be performed. If CHD6 precipitates in complex with CTCF and cohesin, it can be determined whether CHD6 has a correlative role in coordinating chromatin loops.

The above data provide evidence that suggests a role for CHD6 in binding chromatin at actively transcribed loci, such as the *EGFR* promoter or *Olig2* in fetal cortical and glioblastoma tissue. The results presented here reveal new insights into the changes in the epigenetic landscape contributing to glioblastoma. Manipulation of CHD6 expression could be considered for treating specific indications of glioblastoma by decreasing transcription of inappropriately expressed genes such as *Olig2* and *EGFR*; however, further research is needed to fully understand CHD6's role in gliomagenesis.

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