From X-inactivation to neurodevelopment: CHD8-transcription factors (TFs) competitive binding at regulatory regions of CHD8 target genes can contribute to correct neuronal differentiation

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A B S T R A C T

The chromodomain helicase DNA-binding protein 8 (CHD8) is a chromatin remodeler whose mutation is associated, with high penetrance, with autism. Individuals with *CHD8* mutations share common symptoms such as autistic behaviour, cognitive impairment, schizophrenia comorbidity, and phenotypic features such as macrocephaly and facial defects. *Chd8*-deficient mouse models recapitulate most of the phenotypes seen in the brain and other organs of humans. It is known that CHD8 regulates - directly and indirectly - neuronal, autism spectrum disorder (ASDs)-associated genes and long non-coding RNAs (lncRNAs) genes, which, in turn, regulate fundamental aspects of neuronal differentiation and brain development and function. A major characteristic of CHD8 regulation of gene expression is its non-linear and dosage-sensitive nature. *CHD8* mutations appear to affect males predominantly, although the reasons for this observed sex bias remain- unknown. We have recently reported that CHD8 directly regulates X chromosome inactivation (XCI) through the transcriptional control of the Xist long non-coding RNA (lncRNA), the master regulator of mammalian XCI. We identified a role for CHD8 in regulating accessibility at the Xist promoter through competitive binding with transcription factors (TFs) at Xist regulatory regions. We speculate here that CHD8 might also regulate accessibility at neuronal/ASD targets through a similar competitive binding mechanism during neurogenesis and brain development. However, whilst such a model can reconcile the phenotypic differences observed in *Chd8* knock-down (KD) vs knock-out (KO) mouse models, explaining the observed CHD8 non-linear dosage-dependent activity, it cannot on its own explain the observed disease sex bias.

Autism is generally acknowledged to have a higher prevalence in males compared to females. This understanding is based on epidemiological studies, including those involving twins, as well as robust clinical observations that consistently reveal a greater ratio of autism diagnoses in males than in females. The specific male-to-female ratio in autism varies, but it is commonly reported to range from around 3:1 (Loomes et al., 2017) to approximately 2:1 (Zwaigenbaum et al., 2012). Importantly, these ratios represent a general pattern and should not be interpreted as an absolute fact that females are less likely to be on the autism spectrum (see below).

The chromodomain helicase DNA-binding protein 8 (CHD8) is an ATP dependent chromatin remodeler whose mutation is associated, with high penetrance, with autism (Bernier et al., 2014), showing specific

sex-biased phenotypes (Weissberg and Elliott, 2021). CHD8 is a gene that encodes a protein involved in chromatin remodeling, which is the process of altering the structure and accessibility of DNA in the cell nucleus. CHD8 belongs to the CHD family of proteins, which are ATP-dependent chromatin remodelers. The CHD8 protein consists of several functional domains, including two chromodomains, an SNF2-like helicase/ATPase domain, and a DNA-binding domain. These domains enable CHD8 to interact with DNA and other proteins, allowing it to regulate gene expression and chromatin organization (Weissberg and Elliott, 2021). The CHD8 protein is known to play a crucial role in multiple biological processes, particularly during embryonic development and brain development. It has been found to be highly expressed in the developing brain, particularly in neural progenitor cells, which are

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responsible for generating different types of neurons. By interacting with other chromatin remodelers such as CHD7 (Batsukh et al., 2010) and transcription factors (Sood et al., 2020), CHD8 can modulate the accessibility of genes, influence the binding of RNA pol II/transcription factors, and potentially impact the transcriptional elongation process. Recently it has been shown that *Chd8* mutation affects H3K36 methylation levels that in turn affect alternative splicing and, therefore, finely tuning gene expression (Kerschbamer et al., 2022). All this evidence highlights the multifaceted multirole role of CHD8 in orchestrating gene expression and chromatin dynamics.

Patients carrying a heterozygous mutation in the *CHD8* gene show autistic behaviour, gastrointestinal symptoms and specific facial and skeletal features such as increased occipitofrontal circumference (OFC), and macrocephaly (Bernier et al., 2014). Mutations in this gene have also been associated with schizophrenia (McCarthy et al., 2014). *Chd8* haploinsufficiency in some mouse models have revealed an autistic-like phenotype, including repetitive/stereotyped behaviour, increased anxiety (Katayama et al., 2016), and altered social behaviour (Katayama et al., 2016; Suetterlin et al., 2018). Increased brain size has been reported in all haploinsufficiency models so far (Bernier et al., 2014; Katayama et al., 2016; Suetterlin et al., 2018; Sugathan et al., 2014). Altered neuronal connectivity (Suetterlin et al., 2018), and impairment of gastrointestinal motility (Bernier et al., 2014) have also been reported with however considerable phenotypic variability between different *Chd8* haploinsufficiency mouse models (Suetterlin et al., 2018). In mouse models and mouse/human cells, *Chd8* haploinsufficiency has also been associated with a generalised dysregulation of gene expression (Katayama et al., 2016), through direct and indirect effects of *Chd8* depletion on global gene expression (Sugathan et al., 2014; Cotney et al., 2015), a phenotype compatible equally with the altered neurogenesis and increased brain size (Bernier et al., 2014; Sugathan et al., 2014) including zebrafish models.

In studies conducted on *Chd8* knockout (KO) or hypomorphic models, sex-biased phenotypes have been reported in both males and females, which appear to be associated with the specific type of mutation. Research using CHD8 mouse models has revealed differences in behavioral and physiological characteristics between male and female mice carrying CHD8 mutations. These differences encompass variations in social behavior, repetitive behaviors, cognitive abilities, and brain structure. For instance, a study published in 2018 investigated the behavior of male and female mice with Chd8 mutations and found that male mice with *Chd8* mutations exhibited more severe social deficits and repetitive behavior compared to female mice with the same mutation (Jung et al., 2018). Cherepanov et al. also showed sex-specific differences between male and female *Chd8* heterozygous mouse model, in particular, they reported that both male and female *Chd8* heterozygous mice exhibited anxiogenic behavior, while only females showed depressive symptoms. Locomotion, social avoidance, and sociability were normal in heterozygous mice compared to wild-type (WT) mice (Cherepanov et al., 2021). However, their CHD8+/DSL males had impaired social preference, while females showed an increased response to novelty (Cherepanov et al., 2021). Noticeably, in this mouse model, oxytocin injection partially improved these behavioural phenotypes. Another study from the Basson lab demonstrated changes in neuronal connectivity in CHD8 mutant mice, indicating effects on brain development and function with little sex-specific differences (Suetterlin et al., 2018). Overall, these findings suggest that CHD8 mutations may impact males and females differently in terms of their behavioral and neurodevelopmental outcomes. The specific effects of CHD8 mutations on males and females warrant further investigation to gain a more comprehensive understanding of the sex-specific differences associated with CHD8-related phenotypes.

Chd8 Knock-Out (KO) animals show embryonic lethality at embryonic day E7.5/8 albeit in the absence of sex differences (Nishiyama et al., 2004) with a lack of proliferation apparent from embryonic day E5.5, from which day on the cells start degenerating (Nishiyama et al.,

2004). Such observations are consistent with the reported repressive role for CHD8 in p53-mediated transcription (Nishiyama et al., 2004) via H1 recruitment at target gene regulatory regions (negative regulation of gene expression) (Nishiyama et al., 2009). In contrast, *CHD8* downregulation has been shown to reduce the expression of neuronal and ASD genes (Sugathan et al., 2014; Cotney et al., 2015) regulating cortical neurogenesis through the activation of the *Wnt* signalling and cell cycle genes (Durak et al., 2016) (positive regulation of gene expression), with significant differences between different experimental models (Suetterlin et al., 2018; Durak et al., 2016; Hurley et al., 2021). Interestingly, CHD8 has also been reported to regulate long non-coding RNAs (lncRNAs) expression (Wilkinson et al., 2015). Collectively, this data indicates that CHD8 might act as both an activator and a repressor of gene transcription through positive and negative remodeling of chromatin at specific target genes.

Recently the Basson lab, has uncovered a non-linear, dosage-dependent role for *Chd8* in the regulation of brain size and gene expression in the developing cortex (Hurley et al., 2021). Interestingly, *Chd8* haploinsufficiency or reducing CHD8 protein levels to 35% of wildtype (WT), referred to as mild hypomorphs, is associated with brain hyperplasia, while reducing CHD8 levels to 10-15% of WT levels (severe hypomorphs) or deleting *Chd8* from early neural progenitors resulted in reduced brain size and increased apoptosis in the embryonic neocortex (Hurley et al., 2021). Transcriptional analysis revealed the deregulation of ~2000 genes in the *CHD8* hypomorphs, including neuronal genes and genes implicated in cell cycle progression, as previously reported (Durak et al., 2016). In the Chd8 severe hypomorph and null animal brains, the authors reported greater deregulation at a transcriptional level (Hurley et al., 2021), including deregulation of the p53 pathways, consistent with early embryonic phenotypes in complete null animals (Nishiyama et al., 2009).

Recent work from the Avner lab (Cerase et al. (2021)) reported a similar non-linear effect of *Chd8* mild and severe/full CHD8 depletion on global transcriptional cell output and on the transcription of Xist RNA (the master regulator of XCI (Boeren and Gribnau, 2021; Cerase and Tartaglia, 2020)) during neuronal differentiation (Pintacuda and Cerase, 2015). The authors showed that CHD8 is mainly binding transcriptionally permissive chromatin regions, decorated by the H3K4me3 mark, working as an activator/facilitator of transcription. They went on to show that a mild *CHD8* KD leads to modest gene deregulation in ES-derived neuronal progenitors (Cerase et al., 2021). Conversely, severe *CHD8* KD and *CHD8* null cells showed a greater degree of gene deregulation with hundreds to thousands of genes being deregulated (including neuronal/ASD genes) (Cerase et al., 2021), potentially through direct and indirect/adaptive transcriptional response, as previously reported (Sugathan et al., 2014; Cotney et al., 2015). While a mild *CHD8* KD leads to a decrease of Xist RNA expression during differentiation, in severe KD and full *Chd8* null cells, the authors observed a substantial increase of Xist transcription and accessibility at Xist regulatory regions (Cerase et al., 2021). Applying a classical model by which CHD8 actively remodels the chromatin at target genes regulatory regions through influencing the spacing of the nucleosome array (positive gene regulation) or regulating H1 binding (negative gene regulation), or cooperative binding with pluripotency factors (Sood et al., 2020), cannot fully explain the non-linear effect of CHD8 depletion on gene expression seen in these two studies (Hurley et al., 2021; Cerase et al., 2021). Cerase et al. (2021), has suggested alternatively that the discrepancies between mild and severe *CHD8* KD can be explained by competitive TF binding at *Chd8* target genes. The authors have shown that while in the WT situation, CHD8 is an activator of Xist gene, in the absence of CHD8, the binding of certain transcription factors may be enhanced (Fig. 1). In particular, they showed increased binding of YY1, a strong Xist activator (Makhlouf et al., 2014), at Xist regulatory regions in the absence of the CHD8 protein (Cerase et al., 2021). Indeed, a consensus analysis of CHD8 ChIP-seq peaks, revealed a shared binding site between CHD8 and the YY1 proteins at ~75% of identified CHD8
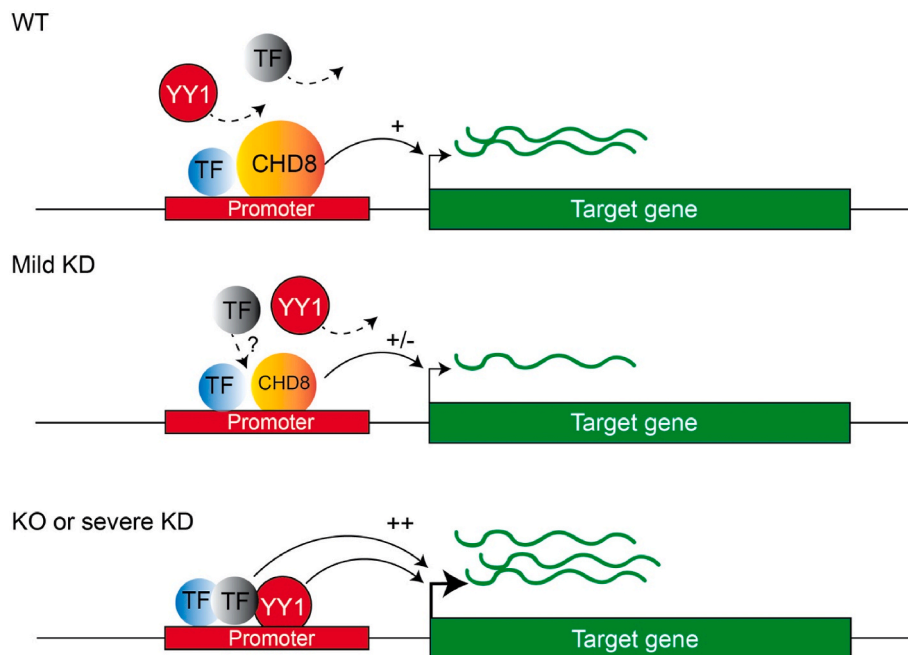
Fig. 1. *CHD8 regulates target gene expression in a non-linear fashion through competition with YY1 and other transcription factors.* In the wild-type (WT) situation, CHD8 binds target genes through recognition of H3K4me2-3 by its chromodomains and through interaction with histones and accessory proteins, to activate target gene expression (top panel). In mild knock-down (KD) conditions, a <50% reduction of CHD8 reduces the expression of target genes (middle panel). Residual CHD8 binding does not allow YY1 and/or other TFs to efficiently access target gene regulatory regions. In *CHD8* knock-out (KO) or severe KD conditions, in the absence of *CHD8*, YY1 is now allowed to bind to target genes and deregulate gene target expression. Note: competitive binding with TFs other than YY1 is also possible and likely to cause gene deregulation (up/down-regulation) through direct and indirect effects (not shown in the picture, see main text for more information). A representative scenario has only been depicted for clarity and simplicity. Cooperative binding is also likely to play a role in the regulation of CHD8 gene targets (Sood et al., 2020).

peaks (Cerase et al., 2021). Noticeably, of the ~2484 differentially regulated genes in the *Chd8* knockout (neuronal progenitors), 1203 promoters are bound by CHD8 and of those at least 391 also by YY1 (Cerase et al., 2021). Competition and/or the involvement of other TFs and/or other chromatin remodelers (Cerase et al., 2021) remains to be fully excluded.

Such a model appears to better explain the non-linear dosage-dependent effect reported in the various *CHD8* depletion models (Hurley et al., 2021; Cerase et al., 2021) as compared to classic genetic models and could be extended to aspects of gene regulation, such as the regulation of alternative splicing (Kerschbamer et al., 2022), other than the classical positive (Sugathan et al., 2014; Cotney et al., 2015) and negative chromatin remodeling (Nishiyama et al., 2009) initially associated with CHD8 activities or cooperative binding (Sood et al., 2020). This working model can obviously be applied beyond the field of XCI and CHD8 regulation to the wider interpretation of non-linear gene regulation in Mendelian disease phenotypes. As XCI is not on the whole affected in *CHD8* mutants, while the proposed model may help in the interpretation of data from different genetic systems, it cannot, by itself alone, explain the male-biased phenotypes observed in *CHD8* animal models.

Can the model suggested by Cerase and Avner explain sex-differences and sex bias? As in their study XCI is not majorly affected (perhaps slightly enhanced), it is possible that abnormal silencing of genes that otherwise escape XCI can contribute to some extent to the observed sex bias in mouse models. As this data was generated in differentiating ESCs (Pintacuda and Cerase, 2015), it's not possible to simply translate these results to neurons and neuronal development. Furthermore, these data were only obtained in female lines and the equivalent experiments in the exact experimental conditions in males is currently not available.

The reasons of the observed sex bias in autism in humans are not known. It is plausible that autism presents itself differently in females or that diagnostic criteria may exhibit a bias toward identifying autism

traits in males. In fact, when considering ASD symptoms as a continuous spectrum that encompasses mild/very-mild phenotypes, contrasting males-to-female ratios have been reported, indicating that females can carry a greater etiological burden than males (Robinson et al., 2013). This finding strongly supports the theory of a female protective effect (FPE). The presence of this effect may be attributed to the X chromosome, which contains an abundance of genes that regulate brain development and function (Pallier et al., 2022) and the random inactivation of X chromosomes in females (XCI) (Boeren and Gribnau, 2021). As a result, the mosaic expression of paternal versus maternal X chromosomes could contribute to the observed FPE. Ongoing research aims to enhance our understanding of the underlying factors contributing to the gender imbalance in autism diagnosis and to investigate the potential for underdiagnosis or misdiagnosis of autism in females.

Declaration of Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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