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REVIEW
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Transcription Pause and Escape in Neurodevelopmental Disorders

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Transcription pause-release is an important, highly regulated step in the control of gene expression. Modulated by various factors, it enables signal integration and fine-tuning of transcriptional responses. Mutations in regulators of pause-release have been identified in a range of neurodevelopmental disorders that have several common features affecting multiple organ systems. This review summarizes current knowledge on this novel subclass of disorders, including an overview of clinical features, mechanistic details, and insight into the relevant neurodevelopmental processes.

Keywords: transcriptional pausing, RNApol2, neurodevelopmental disorders, Cornelia de Lange Syndrome, intellectual disability

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INTRODUCTION

Gene transcription is a highly regulated process that ultimately determines cellular identity and response to external stimuli. Transcription often occurs in bursts (Wan et al., 2021) and is primarily regulated at the level of burst initiation and promoter proximal RNApol2 (RNA polymerase 2) pausing (Bartman et al., 2019). Pausing takes place following RNApol2 gene entry and recruitment of the general transcription factor TFIIH to the preinitiation complex, which results in melting of the DNA template and rapid progression of RNApol2 to the pause site, 20–120 nucleotides downstream of the TSS (transcription start site). Release from the paused state requires the action of the P-TEFb (Positive Transcription Elongation Factor b) complex, whose kinase module phosphorylates RNApol2 and associated pausing factors to enable entry into productive elongation. Transcription of virtually all genes was shown to be dependent on P-TEFb activity (Jonkers et al., 2014) and significant accumulation of paused RNApol2 was observed at a fraction of these (Day et al., 2016).

Establishment and in particular release from pausing is a highly regulated process involving multiple factors that often also act in other phases of the transcription cycle. Central to pause-release is P-TEFb recruitment to paused RNApol2 as part of a complex with BRD4 (bromodomain containing protein 4) or the SEC (super elongation complex), assisted by Mediator and the PAF1 (polymerase-associated factor 1) complex (Lu et al., 2016). Moreover, whilst BRD4 is not absolutely required for CDK9 recruitment, it is necessary for the assembly of a productive elongation complex (Winter et al., 2017).

Interestingly, pathogenic variants in multiple transcriptional pausing regulators have been identified in neurodevelopmental disorders (NDDs), including CdLS (Cornelia de Lange Syndrome) and CdLS-like disorders (**Figure 1**). Some of these regulators play a direct role in P-TEFb recruitment (e.g., SEC, BRD4) while others are linked to transcriptional pausing through physical interaction (i.e., NIPBL) or by experimental evidence from cellular systems (e.g., Mediator and PAF1c). Gain-of-function mutations in *AFF4*, encoding a subunit of the SEC, result in the CdLS-related CHOPS (OMIM# 616368) (Cognitive development and coarse facies, Heart defects, Obesity, Pulmonary involvement, and Short stature and skeletal dysplasia) syndrome (Izumi et al., 2015). Heterozygous loss-of-function (LoF) mutations in *BRD4* similarly result in a CdLS-like

syndrome (Olley et al., 2018) and NIPBL, whose disruption is the most frequent genetic cause of CdLS, was linked to transcriptional pausing *via* its interaction with the Integrator complex (van den Berg et al., 2017). Mutations in subunits of the Mediator and PAF1 complex have been identified in several intellectual disability (ID) syndromes and haploinsufficiency of SETD5, one of the most frequent genetic causes of idiopathic ID, was recently linked to pausing defects (Deliu et al., 2018). These observations causally link transcriptional pausing defects to NDDs well beyond CdLS.

In this review we will present current insights into the mechanism and gene regulatory implications of RNApol2 pausing and discuss in detail the involvement of specific pausing regulators in the aetiology of several NDDs.

MECHANISTIC OVERVIEW OF PROMOTER PROXIMAL PAUSING

Establishment and Maintenance of RNApol2 Pausing

Transcription initiation involves establishment of the preinitiation complex (PIC), comprised of general transcription factors (GTFs) and RNApol2. TFIIH, the last GTF to be recruited to the PIC, is required both for initiation and establishment of promoter proximal pausing (reviewed in Chen et al., 2018). Its helicase activity mediates melting of the DNA template to enable open complex formation, while its CDK7 kinase module phosphorylates Ser7 and, notably, Ser5 residues in the RNApol2 CTD (C-terminal domain), resulting in escape from the PIC and progression to the pause site.

RNApol2 pausing is stabilized and shielded from premature termination by the association of DSIF (DRB-sensitivity inducing factor) and NELF (negative elongation factor). Structural studies on the paused elongation complex have shown that NELF stabilizes the paused state by limiting RNApol2 intramolecular mobility and nucleotide triphosphate (NTP) active site entry and by interfering with binding to elongation factors, including TFIIS (Vos et al., 2018b). Diversified roles in the establishment, maintenance, and release of paused RNApol2 have been described for the PAF1 and Integrator complexes, which will be discussed in more detail in the next section.

Release From Pausing

Release from pausing requires the kinase activity of the P-TEFb complex, consisting of CDK9 and (in most cases) Cyclin T1. CDK9 activity is regulated in a multistep process. First, activation of CDK9 requires phosphorylation of a conserved threonine residue (Thr286) in its T-loop region, primarily catalyzed by TFIIH-subunit CDK7 (Larochelle et al., 2012). Most of the active, nuclear P-TEFb complexes are subsequently sequestered in an inhibitory complex comprised of the 7SK small nuclear (sn) RNA, capping enzyme MePCE, LARP7 and HEXIM1/2 proteins (Barboric et al., 2005; Egloff et al., 2006). Release from the 7SK snRNP (small nuclear ribonucleoprotein) complex is regulated by various enzymatic activities catalyzing post-translational modifications (e.g., HEXIM ubiquitination and Cyclin T acetylation) or modifying the 7SK snRNA structure [reviewed in Bacon and D'Orso (2019)]. Active P-TEFb can then be brought to paused RNApol2 by sequence specific TFs (transcription factors) or as part of a complex with BRD4 or the SEC.

In vitro, RNApol2 escape from the paused state requires P-TEFb, PAF1c (PAF1 complex) and the elongation factor SPT6 (Vos et al., 2018a). Active CDK9 phosphorylates NELF, DSIF, and Ser2 residues in the RNApol2 CTD. Phosphorylated NELF dissociates from the paused elongation complex, while phosphorylated DSIF turns into a positive elongation factor that remains associated with elongating RNApol2. Recent studies based on acute depletion of DSIF-subunit SPT5 indeed confirm that this factor plays an essential role in both maintenance of pausing and elongation processivity (Aoi et al., 2021; Hu et al., 2021).

Role of Pausing in Gene Regulation

RNApol2 pausing was first described for the *Drosophila Melanogaster hsp70* heat shock gene (Gilmour and Lis, 1986; Rougvie and Lis, 1988), where it was thought to enable rapid transcriptional responses to changes in the environment. Genome-wide studies have since shown that it is a widespread phenomenon at all transcribed genes, both in *Drosophila* and mammalian cells (Zeitlinger et al., 2007; Jonkers et al., 2014).

Several roles for RNApol2 pausing in gene regulation have been proposed, as reviewed in Adelman and Lis (2012). Pausing could provide a time window for the association of capping enzymes and elongation factors, ensuring subsequent optimally productive elongation. It was also observed that genes with a high pausing index intrinsically favor nucleosome occupancy over the transcription start site (TSS), suggesting that pausing may contribute to maintenance of a nucleosome free region that enables rapid transcription re-initiation (Gilchrist et al., 2010). Furthermore, by providing an additional level at which gene expression can be controlled, RNApol2 pausing allows for fine-tuning of transcriptional responses through integration of multiple signaling events (Adelman and Lis, 2012). Indeed, computational modeling and specific experimental perturbations showed that biological stimuli impinge on burst initiation and pause release to affect transcriptional output (Bartman et al., 2019). Two independent studies have further linked those two processes by showing that pause duration directly influences transcription initiation rate (Gressel et al., 2017; Shao and Zeitlinger, 2017). Finally, in developing *Drosophila* embryos, strong promoter-proximal pausing contributes to rapid acquisition of transcriptional synchrony required for coordinated responses in tissue development (Lagha et al., 2013).

Estimates on the average length of pause duration vary depending on the chosen model system and methodology. Several studies using inhibitors of transcription initiation or pause-release (i.e., triptolide or flavopiridol) followed by RNApol2 tracking over time determined median pause durations of between 5 and 20 min in *Drosophila* and mouse cells (Henriques et al., 2013; Jonkers et al., 2014; Shao and Zeitlinger, 2017). By introducing a CDK9 analog sensitive mutation, Gressel et al. (2017) could very rapidly and specifically inhibit CDK9 and track its effect on RNApol2 dynamics. Median pause durations

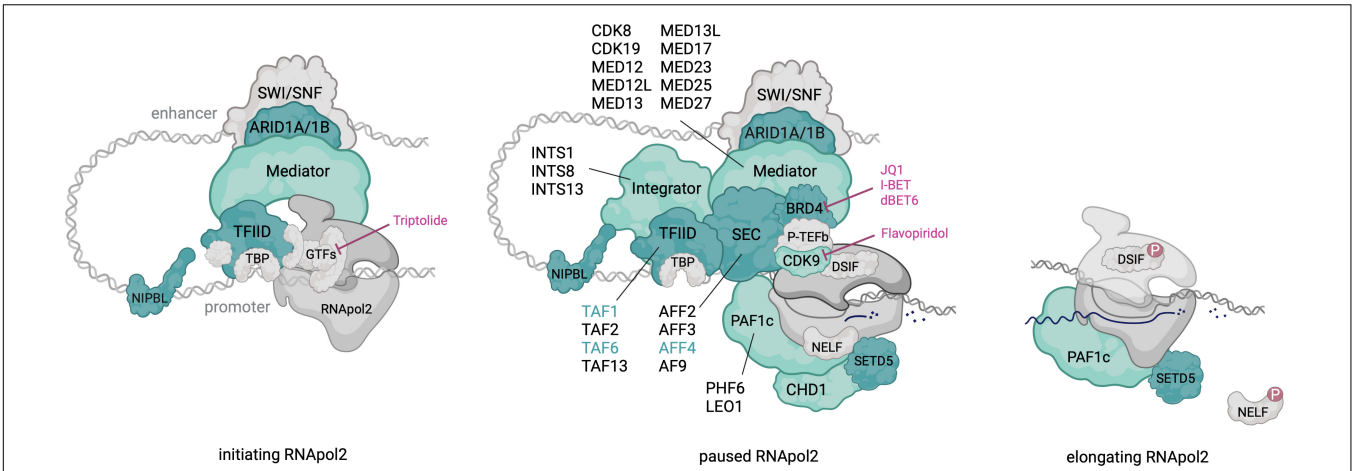


FIGURE 1 | An overview of RNApol2 in initiating, paused and elongating state with transcriptional pausing regulators that contribute to NDDs colored in green. For multi-subunit complexes, subunits with pathogenic NDD variants are outlined. Dark green shading and font highlights factors in which pathogenic variants result in a CdLS-like phenotype. Discussed therapeutics and their targets in the paused RNApol2 complex are indicated. Created with BioRender.com.

measured within the range of 1–2 min. Even shorter pause durations of less than 1 min were detected by fluorescent recovery after photobleaching (FRAP) on GFP-tagged RNApol2 acting in endogenous gene transcription (Steurer et al., 2018) or on an engineered gene array (Darzacq et al., 2007). Interestingly, both these studies also found that a large fraction (∼90%) of paused RNApol2 fails to enter productive elongation and prematurely terminates. These data are important to keep in mind when interpreting downstream effects of pausing deregulation on the steady-state transcriptome.

TFIID

The TFIID complex consists of TATA box-binding protein TBP and TBP-associated factors (TAFs). It is mostly known for its function as a general transcription factor (GTF) during transcription initiation, where it recognizes several core promoter motifs (e.g., the TATA-box) and, upon promoter binding, recruits RNApol2. Together with other GTFs and RNApol2, the preinitiation complex (PIC) is formed [as reviewed by Roeder (1996)].

However, *in vitro* assays indicate that the presence of TBP is sufficient for PIC formation (Fant et al., 2020), and structural analysis of TFIID shows that it also resides downstream of promoter elements at the pausing site (Cianfrocco et al., 2013; Nogales et al., 2017). Moreover, DNA elements that bind TFIID are enriched at pausing sites in *Drosophila* (Hendrix et al., 2008; Lee et al., 2008; Shao et al., 2019). In line with these results, multiple TAFs have been found to interact with various components of the SEC, including AF9, EAF1 and CDK9 (Biswas et al., 2011; Yadav et al., 2019). *TAF6* knockdown leads to reduced TFIID stability and a loss of interaction with AF9, cyclinT1 and CDK9, resulting in reduced recruitment of TFIID, SEC and RNApol2 to target genes (Yadav et al., 2019). Furthermore, *TAF1* and *TAF2* knockdown causes a widespread increase in transcription at protein coding genes, and a decrease of promoter-proximal pausing (Fant et al., 2020), thus leading to the hypothesis that TFIID may function directly in the regulation of transcriptional pausing.

Being an important transcriptional regulator, TFIID defects are associated with numerous diseases, including cancer (Oh et al., 2017; Xu et al., 2018) and neurodegenerative disease (Makino et al., 2007; Herzfeld et al., 2013; Aneichyk et al., 2018). Several of the TFIID proteins are also associated with neurodevelopmental disorders. The X-linked gene *TAF1* encodes for the largest subunit of TFIID and its mutation is also most frequently described to cause neurodevelopmental delay (Stenson et al., 2014; Niranjan et al., 2015; Hu et al., 2016; Gudmundsson et al., 2019; Kahrizi et al., 2019; Okamoto et al., 2020). Two large studies have described a total of 41 individuals that present with global developmental delay, ID, microcephaly, short stature, characteristic facial dysmorphologies and generalized hypotonia (O'Rawe et al., 2015; Cheng et al., 2019). Two of these individuals were initially diagnosed with CdLS (see below) due to their craniofacial features, growth failure, ID and specific limb malformations. Although there is a large overlap between patient phenotypes, it is hard to distinguish a specific facial gestalt, which could be due to the widespread distribution of *TAF1* missense variants, covering all TAF1 domains (Cheng et al., 2019).

Notably, all disease-causing mutations discovered thus far were either hemizygous or homozygous missense variants, or gene duplications, indicating that TAF1 loss-of-function is potentially lethal. Indeed, *taf1* knockout in zebrafish caused embryonic lethality with deregulated genes enriched for those involved in neurodevelopmental processes (Gudmundsson et al., 2019). In mice and human several ubiquitously expressed TAF1 isoforms have been described, while neuronal tissue expresses an isoform that includes a 6-nucleotide long microexon (Makino et al., 2007). Microexon inclusion is temporally regulated and the resulting neuronal isoform N-TAF1 is predominantly

expressed in postmitotic neurons (Capponi et al., 2020). It was postulated by the authors that such cell-type specific splicing events could contribute to tissue-specific disease phenotypes of ubiquitously expressed genes. Whether and how the reported missense variants affect transcriptional pause-release remains to be investigated.

Similar to TAF1, a CdLS phenotype was also discovered in a patient carrying a homozygous missense mutation in *TAF6*, resulting in the first autosomal recessive form of CdLS (Yuan et al., 2015). A second homozygous missense variant causing global developmental delay and syndromic ID was discovered by two independent studies (Alazami et al., 2015; Yuan et al., 2015). Both mutations caused a reduction in interaction of TAF6 with other TFIID subunits (Yuan et al., 2015). A total of ten patients have been identified with four genotypic TAF2 variants, in all cases comprising homozygous missense mutations (Najmabadi et al., 2011; Halevy et al., 2012; Hellman-Aharony et al., 2013; Thevenon et al., 2016; Lesieur-Sebellin et al., 2021). Patients present with global developmental delay, moderate to severe ID, microcephaly and abnormalities in the corpus callosum (reviewed by Lesieur-Sebellin et al., 2021). Furthermore, a single study has identified four patients with *TAF13* mutations from two unrelated families (Tawamie et al., 2017). Similar to *TAF2* and *TAF6* mutation, the disease phenotype is caused by homozygous missense variants. Patients present with developmental delay, mild ID and microcephaly; however, they do not show any dysmorphic facial features. Biochemical and transcriptome analysis on these TAF13 variants indicate a reduced heterodimerization with TAF11, and deregulation of a large set of genes (Tawamie et al., 2017).

Lastly, multiple cases of 6q subtelomeric deletions, characterized by developmental delay, intellectual disability, microcephaly, seizures and dysmorphic features, were linked to the loss of TBP (Eash et al., 2005; Rooms et al., 2006). However, $Tbp^{+/-}$ mice do not show significant behavioral abnormalities indicative of cognitive impairment compared to WT mice, while $Tbp^{-/-}$ mice show very early embryonic lethality (Martianov et al., 2002; Rooms et al., 2006). Nevertheless, discovery of a patient with mild ID, difficulty walking and abnormal movement related to a homozygous deletion resulting in a frameshift in *TBP,* does further indicate a role for TBP in neural development (Monies et al., 2017).

Although not all of TFIID subunits have (yet) been associated with neurodevelopmental disorders, some have been further studied for their impact in neuronal development. *TAF4* is highly expressed in cortical neural stem cells *in vitro*, where it is believed to regulate neuronal differentiation together with intracellular signaling factor RanBPM (Brunkhorst et al., 2005). *Taf4a* knockout mice die at E9.5 and show severe growth retardation, and obvious patterning and morphogenesis defects (Langer et al., 2016). Moreover, $Taf4a^{-/-}$ ESCs are unable to differentiate into glutamatergic neurons *in vitro* due to impaired PIC formation at differentiation genes (Langer et al., 2016). *Taf9b* is upregulated during neuronal differentiation of mouse ES cells and *Taf9b* knockout causes downregulation of neuronal genes such as *Tubb3*, both *in vitro* and *in vivo* (Herrera et al., 2014).

In summary, the high number of TFIID components found associated with neuronal defects upon mutation or loss indicates that misregulation of TFIID broadly impacts neuronal differentiation. Moreover, the CdLS diagnosis for mutations in TAFs implicated in the regulation of pausing warrants further investigation into this link, as will be discussed below.

CDK9

CDK9 is widely expressed in all human tissues (De Luca et al., 1997) and plays a role in several diseases, including HIV infection and multiple cancers (Egloff, 2021). In total six patients have been described that carry variants in *CDK9* resulting in CHARGE (coloboma, heart defects, atresia choanae, growth retardation, genital abnormalities and ear abnormalities)-like syndrome (OMIM#214800) (Shaheen et al., 2016; Maddirevula et al., 2019; Nishina et al., 2021). Five of these patients carry homozygous non-synonymous variant p.Arg225Cys and in one patient compound heterozygous missense variants (i.e., p.Ala288Thr and p.Arg303Cys) were detected.

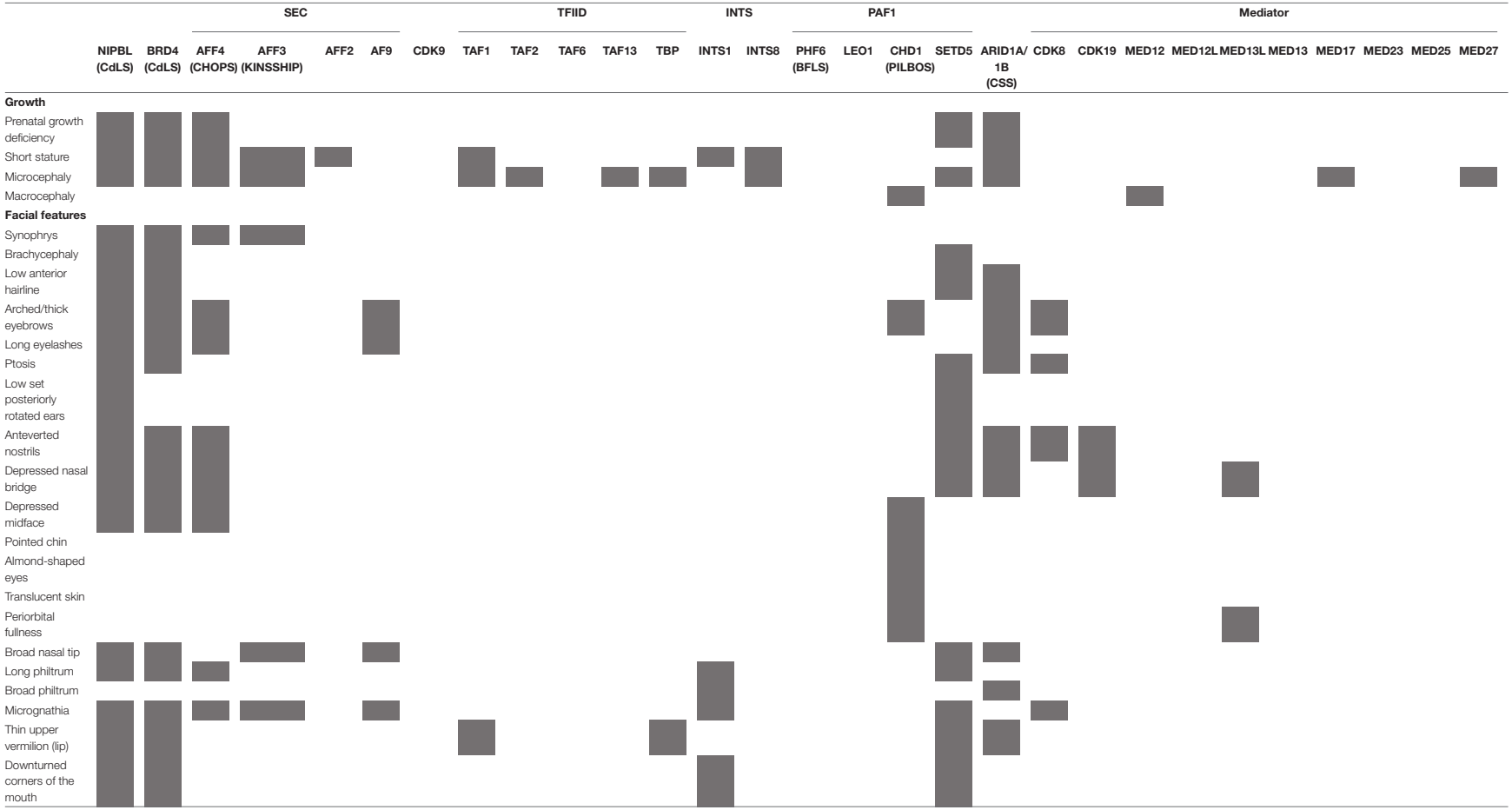
CHARGE syndrome was initially identified in patients with mutations in the chromodomain helicase DNA-binding protein CHD7 and it frequently features intellectual disability and global developmental delay (Vissers et al., 2004). Similarly, the reported CDK9 variants were associated with global developmental delay (5 cases), intellectual disability (2 cases), microcephaly (2 cases), cerebral (3 cases) and cerebellar (3 cases) atrophy, epileptic seizures (3 cases) and myelination defects (1 case) (see also **Table 1** for an overview of clinical symptoms). The three affected amino acids are highly conserved amongst vertebrates and locate in the catalytic kinase domain of CDK9. Patient-specific recombinant CDK9 variants showed reduced kinase activity *in vitro*, suggesting that loss of function of CDK9 causes the phenotype (Nishina et al., 2021). To what extent this decrease in enzymatic activity affects RNApol2 pause release remains to be determined.

The developmental role of CDK9 has been studied in several model organisms. In zebrafish embryos, CDK9 inhibition with flavopiridol or depletion with morpholinos resulted in increased apoptosis and an underdeveloped forebrain and midbrain (Matrone et al., 2016). In mice, homozygous loss of Cdk9 is lethal whereas heterozygous loss causes abnormal morphology of the heart, skin and epididymis (Munoz-Fuentes et al., 2018). P-TEFb was found be required for retinoic acid (RA)-induced neuronal differentiation of neuroblastoma cells (De Falco et al., 2005; Ghosh et al., 2018). Which neurodevelopmental pathways are affected by the reported CDK9 missense variants should be topic of further investigation.

BRD4

Several factors involved in the recruitment of P-TEFb to paused RNApol2 have been implicated in neurodevelopmental disorders with a CdLS-like phenotype, including BRD4. BRD4 is part of the

TABLE 1 | Overview of clinical symptoms.



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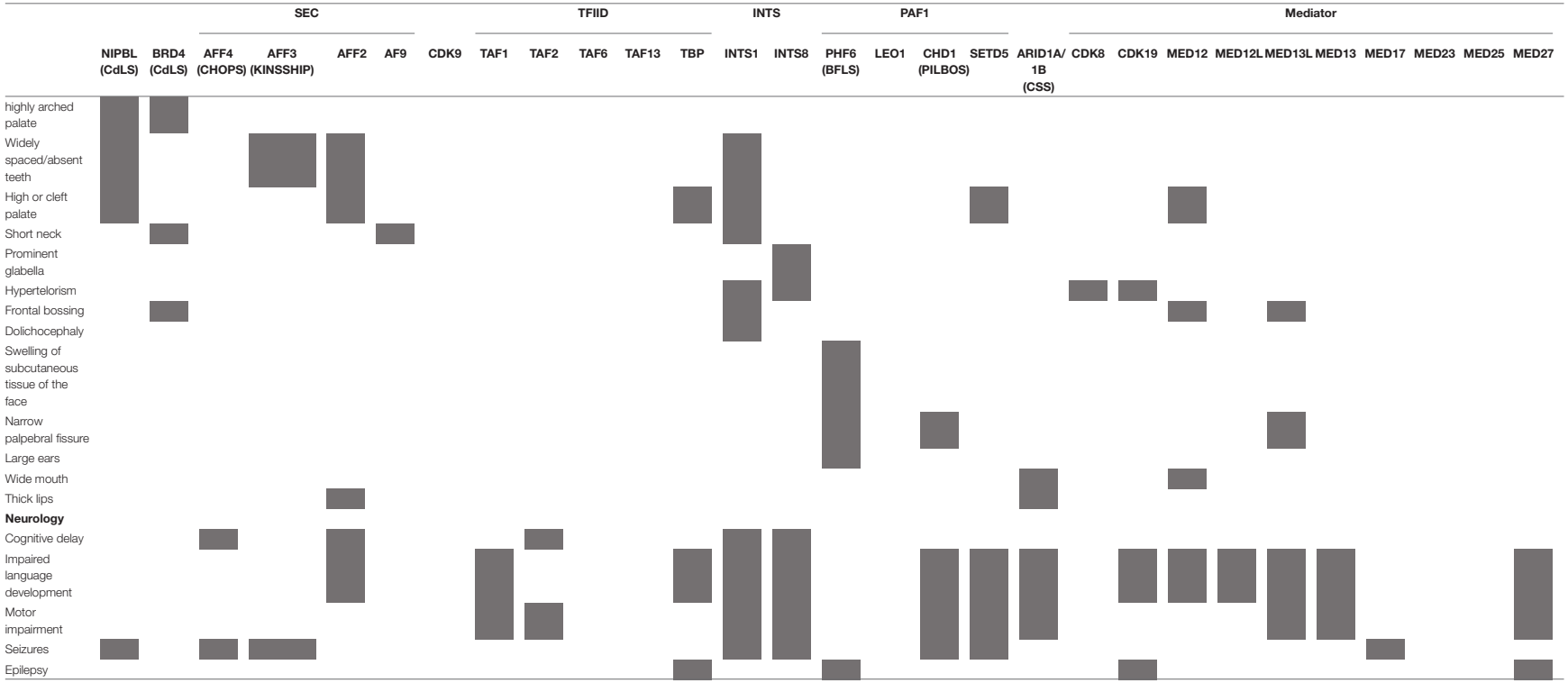

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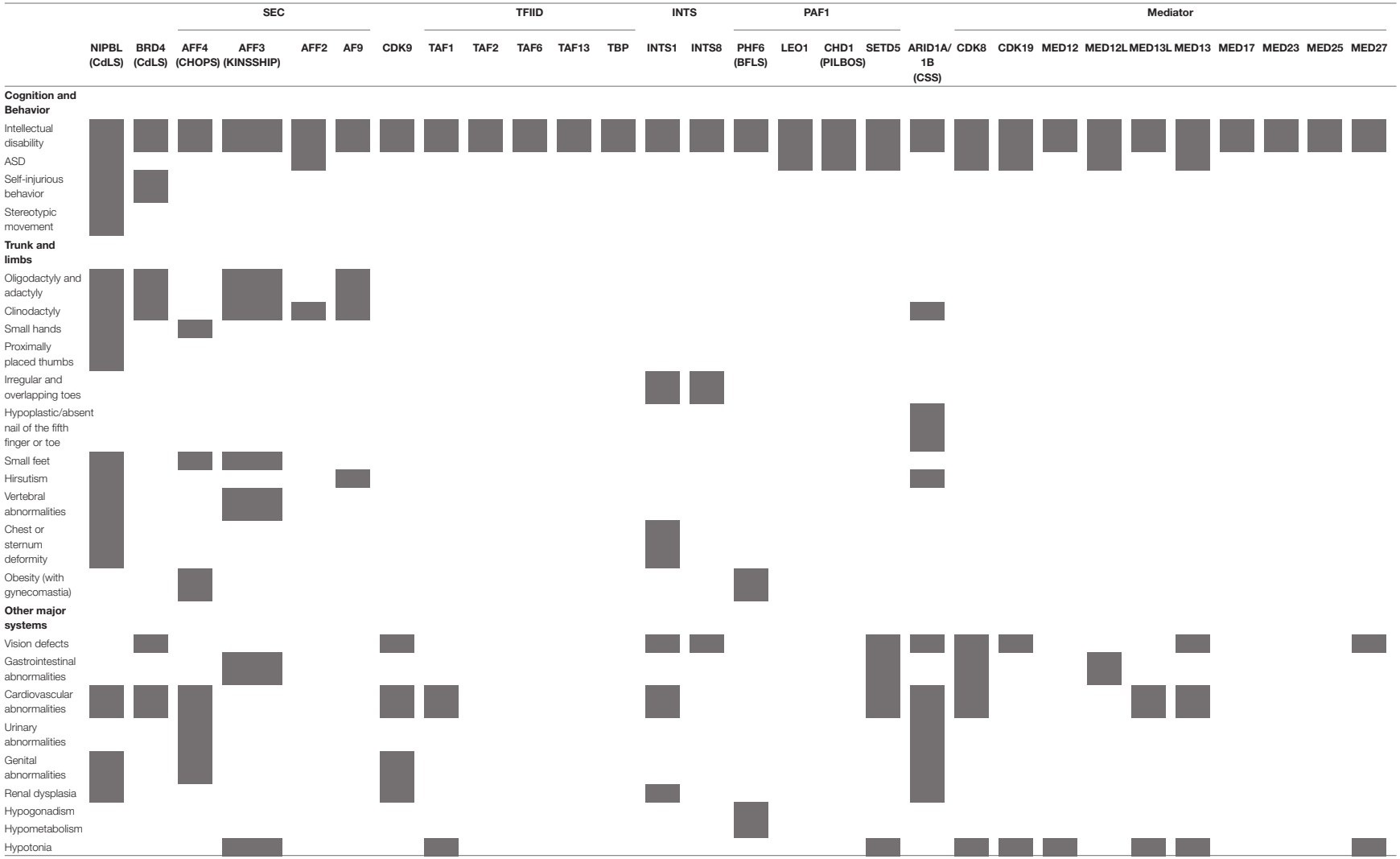

bromodomain and extra-terminal domain (BET) family, together with BRD2, BRD3 and the testis specific BRDT (Shi and Vakoc, 2014). The bromodomains of these proteins can bind acetylated lysines at histones and transcription factors, mediating their recruitment to active chromatin (Dey et al., 2003; Wu et al., 2013; Shi and Vakoc, 2014).

The BET inhibitors such as JQ1 and I-BET result in chromatin dissociation of BRD4 and subsequent deregulation of global gene expression (Filippakopoulos et al., 2010; Dawson et al., 2011; Xu and Vakoc, 2017). Rapid BET protein degradation by dBET6 resulted in accumulation of paused RNApol2 and a severe loss of Ser2-phosphorylated, elongating RNApol2 (Winter et al., 2017), an effect linked to BRD4 and not BRD2 or BRD3 as shown by degron-based depletion studies (Arnold et al., 2021; Zheng et al., 2021).

BRD4 contains a unique interaction domain for P-TEFb (Bisgrove et al., 2007) and interacts with other important pausing factors such as the Mediator complex (Jiang et al., 1998; Jang et al., 2005; Wu and Chiang, 2007), the PAF1 complex, and DSIF (Yu et al., 2015; Arnold et al., 2021). A systematic analysis in HeLa cells has shown that BRD4 recruits P-TEFb specifically to DSIF-subunit SPT5 (Lu et al., 2016). However, BET protein degradation or targeted BRD4 depletion did not impact chromatin association of P-TEFb (Winter et al., 2017; Muhar et al., 2018). This observation supports the hypothesis that BRD4 functions as an allosteric activator of P-TEFb, allowing it to work efficiently once in proximity to the paused complex (Schroder et al., 2012; Itzen et al., 2014).

Other roles of BRD4 in the regulation of transcription have also been described. For example, BRD4 can facilitate elongation independently of P-TEFb (Kanno et al., 2014). The ET domain of BRD4 interacts with several factors to drive transcription activation (Rahman et al., 2011) and BRD4 can function as an atypical kinase to phosphorylate Serine 2 in the CTD of RNApol2 *in vitro* (Devaiah et al., 2012). BRD4 specific and pan-BET protein degradation, resulting in widespread transcription continuation downstream of the termination zone, suggested a role for BRD4 in 3′-processing and transcription termination (Arnold et al., 2021). Lastly, BRD4 levels are particularly high at super-enhancers (SEs) where, together with MED1, it is described as a component of liquid–liquid phase separated transcriptional condensates (Boija et al., 2018; Sabari et al., 2018).

Heterozygous, multigenic deletions in chromosome 19, encompassing *BRD4*, have been linked to intellectual disability in multiple probands (Jensen et al., 2009; Bonaglia et al., 2010; van der Aa et al., 2010; Gallant et al., 2011; Jelsig et al., 2012; Olley et al., 2018; Alesi et al., 2019). Moreover, recent studies identified four patients with intragenic mutations in BRD4, resulting in a CdLS-like phenotype characterized by intellectual disability, microcephaly, developmental delay, and many of the CdLS facial features (Olley et al., 2018; Rentas et al., 2020). Mutations included two non-sense variants and two missense variants in the second bromodomain of BRD4, leading to impaired chromatin-association. In mice, heterozygous loss of Brd4 leads to early postnatal mortality, severe prenatal growth failure, reduced body fat, and abnormalities of the craniofacial skeleton (Houzelstein et al., 2002). These features are also commonly found in CdLS, suggesting BRD4 haploinsufficiency and, by extension, deregulated transcriptional pausing as likely cause of the CdLS-like phenotype.

Other functional effects of BRD4 haploinsufficiency besides transcriptional imbalance have also been proposed as alternative causes of the CdLS-like phenotype. Impaired regulation of DNA repair but not transcription was found in BRD4 mutated mESCs and in CdLS patient lymphoblastoid cells (LCLs) (Olley et al., 2018). Similarly, CdLS cells show increased DNA damage sensitivity (Vrouwe et al., 2007). Proper DNA repair is imperative for neural development (Frank et al., 2000; Gao et al., 2000) and mutation of proteins involved in DNA damage repair are often associated with neurodevelopmental defects [reviewed in Lee et al. (2016)], suggesting that defective DNA repair may also contribute to the CdLS-like phenotype of BRD4 heterozygous LoF patients.

Several studies have addressed BRD4 function in the central nervous system. In the adult mouse brain, *Brd4* is predominantly expressed in neurons, where it regulates immediate early gene (IEG) expression (Korb et al., 2015). Rapid induction of IEGs in response to neuronal activity relies on the presence of promoter proximal paused RNApol2 (Saha et al., 2011). IEGs are essential for consolidation of synaptic modification and memory function (Frey et al., 1989, 1996; Nguyen et al., 1994; Messaoudi et al., 2002). Consequently, BET protein inhibition with JQ1 reduced expression of synaptic proteins and resulted in long-term memory deficits (Korb et al., 2015). Together, this suggests a critical role for BRD4 in transcription regulation and neuronal activation during memory formation.

Dysregulation of BRD4 has been causally linked to Rett Syndrome (Xiang et al., 2020) and fragile X Syndrome (FXS) (Korb et al., 2017), two of the most prevalent neurodevelopmental disorders. FXS, caused by loss of the translation repressor FMRP (fragile X mental retardation protein) is characterized by intellectual disability, behavioral deficits, and autism spectrum disorder (ASD). *Brd4* transcripts were identified as direct targets of FMRP, resulting in elevated Brd4 protein levels in *Fmr1* knockout mice. Treatment of these FXS modeling mice with JQ1 reversed aberrant neuronal spine density and gene expression, as well as atypical social and repetitive behavior. Similar beneficial effects of JQ1 were observed in human and mouse models of Rett syndrome (RTT), caused by loss of function of the X-linked gene encoding MeCP2 (methyl-CpG binding protein 2). Increased chromatin binding of BRD4 in *in vitro* differentiated human RTT interneurons, and in MGE (medial ganglionic eminence) and cortex mimicking RTT organoids, caused extensive transcriptional dysregulation that was reverted upon exposure to JQ1. Importantly, JQ1 treatment of RTT modeling $MeCP2^{-/Y}$ mouse pups improved short term survival and slowed down phenotypic progression.

Collectively, these studies highlight the importance of BRD4 dosage during neurodevelopment. They also underscore the feasibility of postnatal phenotypic reversal of some aspects of NDDs and suggest that at least part of the pathology results from aberrant gene regulation in fully differentiated postmitotic neurons. Rebalancing of transcription pause regulation and

elongation can thus be used as a therapeutic strategy to ameliorate symptoms related to NDDs.

NIPBL

NIPBL (Nipped-B-like) encodes for the protein delangin, the human homolog of fly Nipped-B protein and fungal sister chromatid cohesion protein 2 (SCC2), which together with SCC4 forms a complex that is necessary for cohesin loading onto chromosomes. Recent studies physically and functionally linked NIPBL to the regulation of transcriptional pausing (van den Berg et al., 2017; Olley et al., 2018; Luna-Pelaez et al., 2019). Here, the diverse roles of NIPBL in gene and chromatin architecture regulation will be considered in the context of neural development.

Gene variants in cohesin core components and regulatory proteins are identified as the cause of CdLS (OMIM# 122470, 300590, 300882, 610759, and 614701), a dominant and genetically heterogeneous neurodevelopmental disorder with physical, cognitive and behavioral characteristics (Kline et al., 2018). CdLS prevalence is estimated to be around 1:10,000 – 1:30,000 live births (Kline et al., 2007). Characteristic features include craniofacial anomalies, intellectual disability, psychomotor delay, pre- and postnatal growth retardation, upper limb malformations, hirsutism, and affected gastrointestinal and visceral organ systems (overview of clinical symptoms in **Table 1**).

Heterozygous LoF or missense variants in NIPBL are identified in approximately 70% of cases whereas variants in SMC1A, SMC3, RAD21, and HDAC8 account for another 5% of (non-)classic cases with overlapping and often milder phenotypes (Liu and Baynam, 2010; Ansari et al., 2014). Heterozygous NIPBL LoF variants are localized throughout the coding sequence and associate with more severe phenotypes, while milder missense variants locate predominantly to important functional domains at the interface with DNA, MAU2, RAD21, SMC1, and SMC3 (Mannini et al., 2013; Shi et al., 2020). Compensatory expression from the intact *NIPBL* allele is frequently observed and a reduction of ∼15% in expression is enough to observe a clinical phenotype (Deardorff et al., 1993). Furthermore, somatic mosaicism for *NIPBL* mutations is reported in 10–23% of 'classic CdLS' diagnosed patients (Huisman et al., 2013; Braunholz et al., 2015; Latorre-Pellicer et al., 2021).

Consistent with the function of NIPBL as cohesin loading factor, CdLS patient-derived NIPBL$^{+/-}$ lymphoblastoid cells (LCLs), *Nipbl*$^{+/-}$ mouse embryonic fibroblasts (MEFs), and fetal liver cells exhibit reduced global or local cohesin binding and defective 3D genome organization (Liu and Krantz, 2009; Chien et al., 2011; Newkirk et al., 2017). Formation of such chromatin loops by loop extrusion relies on an active holoenzyme consisting of cohesin and NIPBL-MAU2 (Davidson et al., 2019; Kim et al., 2019).

Although reduced Nipbl levels in a CdLS mouse model did not affect bulk cohesin loading, deregulated genes showed reduced cohesin binding (Remeseiro et al., 2013). In the absence of overt chromosome segregation defects (Castronovo et al., 2009), deregulated gene expression likely underlies neuronal dysfunction in CdLS (Castronovo et al., 2009; Kawauchi et al., 2009; Remeseiro et al., 2013). In addition, NIPBL recruits cohesin to sites of double-strand breaks (DSBs) for DNA repair under control of MDC1, RNF168 and HP1γ (Oka et al., 2011).

A direct link to gene regulation was established for Nipped-B, the fly homolog of delangin, which was found to regulate Notch signaling and other developmental pathways by facilitating enhancer-promoter communication (Krantz et al., 2004; Tonkin et al., 2004). In mammalian cells, Zuin et al. (2014) subsequently showed that NIPBL binds to promoters of active genes to regulate their expression, independent of cohesin. In addition, there are many transcription factors amongst the NIPBL target genes that are differentially expressed in CdLS. These findings indicate that NIPBL influences transcription in several ways; by loading cohesin complexes that regulate genes *via* chromatin insulation and chromosomal long-range interactions, directly by binding at gene promoters and indirectly through regulation of TF expression.

NIPBL was linked to the regulation of transcriptional pausing *via* its interaction with the Integrator complex in mouse neural progenitor cells (van den Berg et al., 2017). NIPBL genomic binding was enriched at promoters containing paused RNApol2 and important for the regulation of neuronal migration genes (e.g., *Sema3a, Nrp1, Plxnd1*, and *Gabbr2*) and consequently for normal cortical neuron migration *in vivo*. Defects in neuronal migration and subsequent aberrant neuronal positioning disrupt neural circuit formation and have been causally linked to intellectual disability and seizures, both features of CdLS (Liu and Krantz, 2009).

Evidence for a role of NIPBL and cohesin in transcriptional pausing has also been found in *Drosophila*, where promoter-proximal cohesin binding correlated with a significantly higher pausing index that was similarly affected by Nipped-B or Rad21 depletion (Fay et al., 2011; Schaaf et al., 2013). Taken together, these findings implicate transcriptional pausing defects in the aetiology of CdLS, a hypothesis further supported by the causal linkage of variants in bona-fide pausing regulators BRD4, AFF4 and Integrator complex to CdLS-like disorders, as discussed in other sections of this review.

To what extent NIPBL function in gene regulation can be uncoupled from cohesin function remains unclear. Widespread NIPBL binding in the absence of cohesin was detected at active promoters and enhancers in mouse embryonic fibroblasts, neural progenitor cells, and lymphoblastoid cells (Zuin et al., 2014; Busslinger et al., 2017; van den Berg et al., 2017). However, the recently solved cryo-EM structure of the fission yeast and human cohesin-NIPBL-DNA complex suggests that the NIPBL-MAU2 loading complex forms an integral part of DNA-bound cohesin (Higashi et al., 2020; Shi et al., 2020). In addition, *in vitro* reconstitution assays show that NIPBL-MAU2 is required for cohesin-mediated loop extrusion (Davidson et al., 2019; Kim et al., 2019).

In support of a role for the NIPBL-MAU2-cohesin holoenzyme in gene regulation, Weiss et al. (2021) recently reported significant overlap in deregulated genes between

patient-derived, NIPBL haploinsufficient cortical neurons and mouse postmitotic neurons acutely depleted of RAD21. Deregulated genes were enriched for neuronal functions related to signaling processes, synaptic transmission, learning and behavior. Disrupted 3D genome organization and transcriptional control in these post-mitotic cortical mouse neurons furthermore emphasized that cohesin is continuously required for neuronal gene expression. Further research is required to answer the question whether NIPBL variants cause deregulated gene expression in CdLS directly, *via* dysregulated pausing, *via* reduced cohesin function, or both.

SUPER ELONGATION COMPLEX

The super elongation complex (SEC) comprises various elongation factors and incorporates active P-TEFb. The AF4/FRM2 family (AFF) forms the scaffold; the canonical SEC contains either AFF1 or AFF4, whereas SEC-like complex 2 and 3 contain AFF2 or AFF3, respectively. These scaffolding proteins interact with 11—19 lysine-rich leukemia (ELL) 1, ELL2 or EEL3, the ELL-associated factor EAF1 or EAF2 and eleven-nineteen leukemia (ENL) or AF9 (He et al., 2010; Lin et al., 2010, 2011; Smith et al., 2011).

Initially, many of the SEC components were identified as translocation partner of the mixed lineage leukemia (MLL) gene (Thirman et al., 1994; Lin et al., 2010). Upon MLL-fusion, SEC is recruited to MLL target genes where it promotes transcription elongation (Mueller et al., 2009; Lin et al., 2010; Yokoyama et al., 2010). This role can be directly linked to its association with P-TEFb. In addition, SEC interacts with important pausing factors such as Mediator (Takahashi et al., 2011; Lens et al., 2017), PAF1c (Kim et al., 2010; He et al., 2011; Wier et al., 2013) and the Integrator complex (Gardini et al., 2014). PAF1c and Mediator recruit SEC to phosphorylate NELF subunits and the RNApol2 CTD, resulting in release of NELF and entry into elongation (Lu et al., 2016). Acute degron-based AFF4 depletion resulted in increased promoter-proximal pausing and decreased RNApol2 in the gene body of heat shock induced genes (Zheng et al., 2021), confirming its role in transcription pause release.

The SEC has been found to regulate expression of many IEGs and developmental control genes involved in neuronal lineage commitment, such as *HOX* genes (Yokoyama et al., 2010; Lin et al., 2011; Luo et al., 2012). Dosing SEC-activity in *Drosophila* neuroblasts is essential to maintain the right balance between self-renewal and differentiation (Liu et al., 2017). Perhaps unsurprisingly, mutations in SEC subunits lead to neurodevelopmental syndromes such as Fragile XE ID, CHOPS and KINSSHIP syndrome (Pramparo et al., 2005; Striano et al., 2005; Izumi et al., 2015; Voisin et al., 2021).

AFF4

AF4/FRM2 family member 4 (AFF4) is essential for SEC stability and proper transcription in metazoans (He et al., 2010; Lin et al., 2010). Initially, three patients with a CdLS-like phenotype (intellectual disability, short stature and craniofacial dysmorphism) were identified that carried missense mutations in *AFF4*. Absence of certain typical CdLS features, including microcephaly, lead to the delineation of a novel syndrome called CHOPS for Cognitive impairment, Coarse facies, Obesity, Pulmonary involvement, Short stature and skeletal dysplasia (OMIM# 616368) (Izumi et al., 2015).

Currently, 12 individuals have been identified with mutations in *AFF4* leading to CHOPS syndrome (Izumi et al., 2015; Raible et al., 2019; Kim et al., 2021). In all cases, a missense mutation was found in the highly conserved ALF homology domain (Bitoun and Davies, 2005), which interacts with the E3 ubiquitin ligase SIAH1 to regulate AFF4 protein stability (Oliver et al., 2004). Increased AFF4 protein stability and chromatin association, resulting in upregulation of transcriptional targets also found upregulated in CdLS, has been proposed as the causative mechanism (Izumi et al., 2015).

AFF3/LAF4

Lymphoid nuclear protein related to AF4 (LAF4), also known as AFF3, is also an MLL fusion partner (Ma and Staudt, 1996; von Bergh et al., 2002). Like the other AFF proteins, AFF3 functions as a scaffolding protein for interaction with AF9 or ENL and P-TEFb to form SEC-like 3 (Bitoun et al., 2007; Luo et al., 2012). In this manner, it mediates transcriptional activity through regulation of transcription pausing of a specific subset of genes, including imprinted genes such as *XIST* (Luo et al., 2016; Wang et al., 2017; Zhang et al., 2019). Aff3 overexpression predominantly leads to gene upregulation in the mouse cortex, indicating a positive role in transcription (Moore et al., 2014). Dysregulation of AFF3 has been associated with various diseases such as rheumatoid arthritis (Stahl et al., 2010) and breast cancer (To et al., 2005).

A role for AFF3 in neural development was implicated after the discovery of a folate sensitive fragile site (FSFS), encompassing a CGG repeat expansion called FRA2A, in the *AFF3* gene promoter (Anneren and Gustavson, 1981; Murthy et al., 1990; Tukun et al., 2000). Hypermethylation of CGG repeats in these FSFS results in silencing of the surrounding locus, which is often associated with intellectual disability (Debacker and Kooy, 2007). FRA2A hypermethylation indeed leads to *AFF3* silencing and is associated with impaired motor and language skills (Metsu et al., 2014).

To date, AFF3 deletions (2) and missense (16) variants have been identified in 18 individuals with developmental delay and intellectual disability (Steichen-Gersdorf et al., 2008; Shimizu et al., 2019; Voisin et al., 2021). Interestingly, all missense variants located to the ALF domain of AFF3, similar to AFF4 missense variants (Raible et al., 2019). However, in contrast to the reported AFF4 variants, AFF3 protein stability was not affected (Voisin et al., 2021). Together with the observed gene deletions, this suggests that AFF3 heterozygous LoF causes the observed phenotype.

Besides developmental delay and intellectual disability, most patients carrying AFF3 variants presented with encephalopathy, skeletal dysplasia, failure to thrive, microcephaly and global brain atrophy (Steichen-Gersdorf et al., 2008; Shimizu et al., 2019; Voisin et al., 2021). Despite similarity to CHOPS, specific characteristics suggested a novel syndrome called KINSSHIP for horseshoe kidney, Nievergelt/Savarirayan type of mesomelic

dysplasia, seizures, hypertrichosis, intellectual disability, and pulmonary involvement (OMIM# 619297) (Voisin et al., 2021). AFF3 missense variants or deletions cause a much more severe phenotype than FRA2A-associated *AFF3* gene silencing. This could be explained by a lack of AFF3 inactivation in the first few weeks after fertilization (Willemsen et al., 2002) or, alternatively, AFF3 silencing could be tissue specific (Voisin et al., 2021).

In mice, *Aff3* is expressed in cortical neurons during the initial steps of differentiation and is downregulated in the postnatal cortex (Britanova et al., 2002). Similarly, in humans, *AFF3* is highly expressed in the fetal brain and diminished in adults (Hiwatari et al., 2003). *Aff3* homo- and heterozygous knockout causes skeletal defect, and homozygous knockouts also show abnormal skull shape, kidney defects, brain malformations and neurological anomalies, similar to features presented in KINSSHIP probands (Voisin et al., 2021). Aff3 depletion in the developing mouse cortex resulted in neuronal migration defects that may explain the developmental delay and ID identified in humans with AFF3 haploinsufficiency (Moore et al., 2014).

AFF2/FMR2

AF4/FRM2 family member 2 (*AFF2*), often referred to as *FMR2*, is an X-linked gene and known to encode a transcription activator (Hillman and Gecz, 2001). In contrast to the other AFF family members, AFF2 is not associated with ALL fusion. In the SEC, it functions as scaffolding protein by binding ENL or AF9 and P-TEFb to form SEC-like 2, which regulates a specific subset of genes (Luo et al., 2012).

Like AFF3, AFF2 also contains an FSFS site, located in the 5'UTR (untranslated region) of exon 1. CGG repeat expansion at this site leads to AFF2 silencing and can result in FRAXE intellectual disability (Gecz et al., 1996; Gu et al., 1996). FRAXE ID can be mild to severe, and include cognitive impairment, delayed language development, autistic behavior, and characteristics such as a long, narrow face, mild facial hypoplasia, a high-arched palate, irregular teeth, hair abnormality, angiomata, clinodactyly, thick lips, and nasal abnormalities (Flynn et al., 1993; Knight et al., 1993; Gecz et al., 1996; Gu et al., 1996).

Intragenic variants and chromosomal disruption of *AFF2* can also cause a similar phenotype (Gedeon et al., 1995; Honda et al., 2007; Sahoo et al., 2011; Stettner et al., 2011), indicating that it is indeed the hemizygous loss of AFF2 that leads to ID. Deletions always encompass the highly conserved ALF domain that is also affected in AFF3 and AFF4 LoF variants, underscoring its functional importance in relation to the ID phenotype. Dysregulation or other missense mutations are also associated with epilepsy (Timms et al., 1997; Moore et al., 1999) and ASD (Mondal et al., 2012).

Aff2 is expressed in the subventricular zone (SVZ) and cortical plate of the mouse cortex (Chakrabarti et al., 1998; Gu and Nelson, 2003; Vogel and Gruss, 2009). *Aff2* knockout mice show impaired learning and memory performance and increased long-term potentiation in the hippocampus (Gu et al., 2002). Furthermore, *AFF2*-null neurons show reduced synaptic activity (Deneault et al., 2018) and silencing of *AFF2* in patients leads to deregulation of IEGs previously implicated in neuronal migration and activation, such as *JUN* and *FOS* (Perez-Cadahia et al., 2011; Bjorkblom et al., 2012; Melko et al., 2013). Dysregulation of *JUN* and *FOS* has been implicated in other ID disorders (e.g., related to Mediator complex mutations) (Hashimoto et al., 2011), suggesting they may be important downstream effector genes.

AF9/MLLT3

AF9, also known as *MLLT3*, is one of the most common fusion partners of the *MLL* gene, and is often associated with leukemia (Strissel et al., 2000). Upon interaction with histone methyltransferase Dot1L, AF9 acts as an epigenetic modifier at specific genes to cause both activation and repression (Zhang et al., 2006; Bitoun et al., 2007; Buttner et al., 2010). A general role in transcription pause regulation was found for AF9, and its homolog ENL, through its contribution to the SEC (like) complexes (Lin et al., 2010; He et al., 2011). Here, the specific YEATS domains of AF9 and ENL interact with the PAF complex, resulting in recruitment of SEC to paused RNApol2 (He et al., 2011). Upon loss of AF9 and ENL, P-TEFb recruitment to NELF-A and the RNApol2 CTD is disrupted, inhibiting its phosphorylation and thereby pause release (Lu et al., 2016).

In two patients, *de novo* translocations of chromosome 4 and 9, *t*(4;9) were identified to cause a disruption of the *AF9* gene, resulting in neurodevelopmental delay with intellectual disability, growth delay, seizures and ataxia (Pramparo et al., 2005; Striano et al., 2005). Long-read sequencing in a patient with intellectual disability and facial dysmorphism rendered AF9 heterozygous LoF as likely causative gene (Hiatt et al., 2021).

Af9 knockout mice display perinatal lethality (Collins et al., 2002). *Af9* is expressed in various brain regions, including the cortex, the hippocampus, cerebellar cortex and at the midbrain/hindbrain boundary (Vogel and Gruss, 2009). In the developing cortex, *Af9* expression prevents premature differentiation of TBR2-positive intermediate progenitor cells (IPCs) in the subventricular zone (SVZ) (Buttner et al., 2010). In human ESCs, AF9 interacts with the 5-methylcytosine dioxygenase TET2 to activate neural targets and support neural commitment (Qiao et al., 2015).

Taken together, these studies indicate that various SEC components have an important role in neural development, albeit not exclusively in the context of the SEC. Comparing patient phenotypes and commonly deregulated pathways should reveal the contribution of pausing dysregulation to the neurological symptoms.

INTEGRATOR COMPLEX

The Integrator complex is a $>$ 1 MDa protein complex that is conserved across metazoans and consists of 14 subunits (Baillat et al., 2005; Malovannaya et al., 2011; Chen et al., 2012). It can directly bind the Ser7-phosphorylated RNApol2 CTD and, through catalytic subunit INTS11, mediate endonucleolytic 3′-end cleavage of many nascent RNAs (Egloff et al., 2010; Baillat and Wagner, 2015). Initially described as required for termination of small-nuclear RNA (snRNA) transcription, Integrator is now known to control processing and expression

of other non-polyadenylated RNApol2 transcripts, including enhancer RNAs (eRNAs) (Lai et al., 2015; Elrod et al., 2019), telomerase RNAs (Rubtsova et al., 2019), viral miRNAs (Cazalla et al., 2011), replication-dependent histones, and long non-coding RNAs (Skaar et al., 2015).

A role for Integrator complex in the regulation of protein coding genes was first described in 2014, when two independent groups demonstrated its association with paused RNApol2. Integrator was shown to be required for initiation and pause-release of EGF-responsive IEGs in HeLa cells, where it interacts with NELF and is required for recruitment of the SEC (Gardini et al., 2014; Stadelmayer et al., 2014). Contrary to this stimulatory role in pause-release, Integrator complex has also been described as attenuator of transcription impinging on paused RNApol2. It stimulates premature termination through endonucleolytic cleavage of nascent RNA associated with the pausing complex (Elrod et al., 2019; Tatomer et al., 2019). Indeed, INTS9 or INTS11 depletion mainly resulted in upregulation of Integrator-bound genes both in *Drosophila* and human cells. Furthermore, association with protein phosphatase 2A (PP2A) provides Integrator with an alternative catalytic function to dampen transcriptional output through dephosphorylation of the RNApol2 CTD and DSIF-subunit SPT5 (Huang et al., 2020; Zheng et al., 2020; Vervoort et al., 2021).

Biallelic mutations in Integrator complex Subunits INTS8 have been identified in three siblings that manifest with a rare and recessive neurodevelopmental syndrome (Oegema et al., 2017). Features include severe intellectual disability, seizures, impaired speech development, motor impairment, facial dysmorphism and limb anomalies. Brain MRI scans showed microcephaly and structural brain abnormalities such as cerebellar hypoplasia, reduced volume of the pons and brainstem, and periventricular heterotopia, a cortical neuron migration defect (**Table 1**). The compound heterozygous INTS8 variants encompass a predicted missense mutation (c.893A > G, p.Asp298Gly) leading to an unstable transcript, and a nine-base-pair in-frame deletion leading to the deletion of three amino acids (c.2917_2925del, p.Glu972_Leu974del, or 'ΔEVL') (Oegema et al., 2017). INTS8-ΔEVL showed reduced association with the Integrator complex and RNApol2, leading to instability of other subunits and an overall loss of complex integrity. This resulted in misprocessing of UsnRNA, splicing defects and gene expression changes affecting neuronal differentiation (Oegema et al., 2017). In a separate study, INTS8 was shown to be required for association of PP2A with the Integrator complex (Huang et al., 2020). INTS8 depletion resulted in increased RNApol2 CTD and SPT5 phosphorylation, stimulating pause-release and thereby upregulation of Integrator target genes.

To date, ten patients carrying biallelic *INTS1* mutations have been reported in literature. They include homozygous missense (Krall et al., 2019) or non-sense variants (Oegema et al., 2017), as well as a combination of missense and either frameshift or non-sense variants (Krall et al., 2019; Zhang et al., 2020). All reported patients presented with growth and cognitive delay, severe language impairment, facial dysmorphism and cataracts. Skeletal malformations, in particular of the chest wall, and motor impairment were also frequently noted. Mechanistically it is not clear how INTS1 variants impact on Integrator complex function, although its potential role as scaffolding subunit could affect the function of the entire complex. In addition, subunit cross-regulation has been reported in zebrafish models, where ints1 depletion had a negative impact on expression of other Integrator subunits (Krall et al., 2019).

Depletion of Ints1 and Ints11 from neural progenitors in the developing mouse brain resulted in neuronal migration defects, linked to aberrant semaphorin signaling (van den Berg et al., 2017). Similar defects were observed upon disruption of NIPBL, a novel interactor of the Integrator complex and prominent causal factor in CdLS. Interestingly, multiple overlapping clinical features between CdLS and INTS mutations have been reported in literature, which are summarized in **Table 1**. Besides growth and cognitive delays, common facial abnormalities (e.g., micrognathia, downturned corners of the mouth, widely spaced teeth), pectus deformity and renal malformations were frequently reported (Kline et al., 2018; Krall et al., 2019). In line with the data from mouse neural progenitor cells, this indeed suggests dysregulation of common gene regulatory pathways as underlying cause of the observed clinical features.

ARID1A/ARID1B

AT-rich interactive domain-containing protein 1A (ARID1A) and 1B (ARID1B) are one of the main, mutually exclusive subunits of the switch/sucrose non-fermentable (SWI/SNF)-like brahma-associated factor (BAF) complex, a multiprotein ATP-dependent chromatin remodeling complex composed of conserved core- and variant subunits (Raab et al., 2015; Mashtalir et al., 2018). SWI/SNF complexes play important roles in epigenetic regulation of gene expression, lineage specification, and maintenance of stem cell pluripotency (Euskirchen et al., 2011; Raab et al., 2015). ARID1A-containing complexes are particularly involved in tumor suppression, and ARID1A is the most frequently mutated chromatin regulator across all human cancers (Lawrence et al., 2014). In particular, ovarian clear cell carcinoma (OCCC) carries the highest prevalence of ARID1A mutations (∼57%) (Jones et al., 2010).

Mutations in *ARID1A* and *ARID1B* are also an important cause of Coffin-Siris Syndrome (CSS; OMIM 135900), a rare autosomal-dominant neurodevelopmental syndrome (Tsurusaki et al., 2012). CSS is characterized by intellectual disability, growth deficiency, microcephaly, coarse facial features and hypoplastic or absent nail of the fifth finger or toe (Coffin and Siris, 1970). Approximately 60% of affected individuals carry a germline mutation in one of six SWI/SNF subunit genes (SMARCB1, SMARCA4, SMARCA2, SMARCE1, ARID1A, and ARID1B) or a small set of additional genes (Vergano et al., 1993; Santen et al., 2012, 2013; Tsurusaki et al., 2012, 2014; Wieczorek et al., 2013). ARID mutations are mostly truncating, LoF mutations or whole-gene deletions, suggesting that haploinsufficiency is the likely cause of the observed neurodevelopmental phenotype (Bögershausen and Wollnik, 2018). Mostly heterozygous LoF mutations in ARID1A have been identified in around 5% of classic CSS cases (Vergano et al., 1993; Tsurusaki et al., 2012),

whilst four CSS-like patients with *ARID1A* microduplications have been described (Bidart et al., 2017). *ARID1B* mutations have been found in up to 62% of (often milder) CSS cases and also explain a significant fraction (0,4–1,0%) of idiopathic ID cases that are often accompanied by speech impairment and agenesis of the corpus callosum hallmarks (Santen et al., 2012; Tsurusaki et al., 2012; Wieczorek et al., 2013; Grozeva et al., 2015).

Several studies have addressed the contribution of ARID1A and ARID1B in SWI/SNF-mediated gene regulation. These concluded that ARID1A-containing BAF acts as both transcriptional activator and repressor, whereas ARID1B-BAF mainly functions as repressor of enhancer activity (Raab et al., 2015). In addition, ARID1A and ARID1B were required for maintenance of global chromatin accessibility (Kelso et al., 2017) and, in the case of ARID1A, for genome compartmentalization (Wu et al., 2019). Interestingly, Trizzino et al. (2018) recently provided evidence for a role of ARID1A and ARID1B in transcription pause release. Knockdown of ARID1A reduced RNApol2 pausing on active genes and globally diminished Ser5-phosphorylation of the RNApol2 CTD. The pausing defect could be rescued by upregulation of ARID1B, suggesting that both ARID1A and ARID1B control transcription *via* RNApol2 pausing and that dysregulated pausing likely mediates effects of ARID1A loss in cancers and possibly also neurodevelopmental disorders.

Several studies have addressed the role of Arid1a and Arid1b in the developing mouse brain. Cortex-specific homozygous deletion of *Arid1a* resulted in reduced cortical thickness linked to inhibition of IPC proliferation and decreased production of deep layer neurons (Liu et al., 2021). In contrast, Arid1b deletion mainly affected ventral forebrain progenitors, suggesting differential requirement for Arid1a and Arid1b in distinct cellular compartments (Moffat et al., 2021). Indeed, CSS-mimicking *Arid1b*-heterozygous mice mainly showed defects in interneuron development (Jung et al., 2017). Reduced proliferation and increased apoptosis in progenitors of the lateral and medial ganglionic eminences (LGE and MGE) resulted in an overall decrease in $GABA^+$ and $Parvalbumin^+$ interneurons in the cortex. These developmental defects resulted in CSS-reminiscent behavioral abnormalities, including impaired cognitive function and social interaction, and increased anxiety-like and repetitive behavior (Celen et al., 2017; Jung et al., 2017). Interestingly, heterozygous Arid1b loss caused a strong reduction in Ser5-CTD phosphorylated RNApol2 at target gene promoters (Jung et al., 2017), warranting further investigation into the role of transcriptional pausing misregulation in CSS.

PAF1 COMPLEX

The Polymerase-Associated Factor 1 complex (PAF1c) is a multifunctional and highly conserved protein complex that regulates all stages of the RNA transcription cycle [recently reviewed in Francette et al. (2021)]. PAF1c was discovered as a novel RNApol2-interacting complex in *Saccharomyces cerevisiae* 25 years ago (Wade et al., 1996), and foundational studies in budding yeast have elucidated the diverse ways *via* which it controls gene expression (Wade et al., 1996; Shi et al., 1997; Mueller and Jaehning, 2002). PAF1c is composed of subunits PAF1, CTR9, CDC73, LEO1, RTF1 and, in human cells, SKI8 (Mueller and Jaehning, 2002). In higher eukaryotes, PAF1c is recruited to promoters and enhancers of active genes, where it directly binds to the CTD and outer surface of RNApol2, as well as to elongation factor SPT4/5 (DSIF) (Yu et al., 2015; Chen et al., 2021).

In recent years, the role of PAF1c in regulation of RNApol2 pause-release has been studied in detail. PAF1c strongly associates with P-TEFb and both factors show interdependent recruitment to target gene promoters (Yu et al., 2015). RNAi-mediated PAF1c depletion, depending on the cell line and specific study, resulted in either increased or decreased RNApol2 pausing (Yu et al., 2015; Chen et al., 2021). In both cases, observed effects were linked to alterations in P-TEFb recruitment. Interestingly, in zebrafish neural crest (NC) progenitors, loss of Paf1c could be compensated by loss of Cdk9, suggesting that at crucial NC genes Paf1c and P-TEFb act antagonistically (Jurynec et al., 2019). These contrasting findings may indicate that PAF1c function is context- and gene-specific. Detailed structural studies of the activated RNApol2 elongation complex showed that PAF1c displaces NELF, suggesting it mainly acts to promote pause-release (Vos et al., 2018a).

Pathogenic variants in genes encoding subunits or interactors of PAF1c have been identified in various neurodevelopmental processes and ID disorders. Most well-described are mutations in the X-linked gene encoding the PHD-Like Zinc Finger Protein 6 (PHF6) that associates with PAF1c and causes the ID disorder Börjeson–Forssman–Lehmann syndrome (BFLS; OMIM 301900) (Lower et al., 2002; Zhang et al., 2013). Besides ID, BFLS is characterized by epilepsy, hypometabolism, hypogonadism, obesity with gynecomastia, swollen subcutaneous facial tissues, narrow palpebral fissure, and large ears (**Table 1**) (Lower et al., 2002; Jahani-Asl et al., 2016). Eight different mutations, including two truncating non-sense variants and 6 different missense variants, were identified in seven familial and two sporadic cases of BFLS.

Phf6 is highly expressed in embryonic and early postnatal stages of mouse brain development (Lower et al., 2002). RNAi-mediated Phf6 depletion *via in utero* electroporation of the embryonic mouse brain was shown to profoundly impair neuronal migration *in vivo*, leading to formation of white matter heterotopias that displayed neuronal hyperexcitability (Zhang et al., 2013). Paf1 depletion phenocopied this migration phenotype, suggesting important PHF6-PAF1c co-operation in transcription regulation of neurodevelopment.

PAF1c core subunit LEO1 was identified as candidate neurodevelopmental disease gene in a large meta-analysis study that combined ID and ASD patient *de novo* mutations with CNV morbidity data (Coe et al., 2019). This finding was confirmed in a targeted sequencing study 1 year later, where LEO1 mutations were linked to intellectual disability and autistic behavior (Wang et al., 2020). Paternally inherited deletions in the LEO1 promoter linked to increased LEO1 expression were also associated with ASD (Brandler et al., 2018), suggesting that PAF1c subunit imbalance may contribute to the neurodevelopmental phenotype.

To date, PAF1c subunit CTR9 has not been causally linked to NDDs in humans, despite a clear neurological phenotype in *Drosophila*. In this model organism, embryonic or early larval lethality of Ctr9 mutants could be partially rescued by re-expression of Ctr9 in the nervous system and mutant embryos contained increased numbers of neuroblasts and dividing progeny (Bahrampour and Thor, 2016). Moreover, a role for Ctr9 in controlling terminal neuronal differentiation was proposed, as evidenced by downregulation of several neuropeptides.

Of note, besides PHF6, several additional PAF1c interactors have been implicated in pausing regulation and NDD, most prominently SETD5 (Osipovich et al., 2016) and CHD1 (Lee et al., 2017). The ATP-dependent chromatin remodeller CHD1 is recruited to actively transcribed genes by PAF1c (Lee et al., 2017), where it enables RNApol2 promoter escape by removing the nucleosome barrier (Skene et al., 2014). Heterozygous *CHD1* missense variants have been identified as the cause of developmental delay, autism, speech apraxia and facial dysmorphic features in Pilarowski–Bjornsson syndrome (OMIM #602118) (Pilarowski et al., 2018). SETD5 harbors H3K36 methyltransferase activity and is important for PAF1c recruitment to common target genes (Sessa et al., 2019; Li et al., 2021b). Setd5 depletion from hematopoietic stem cells resulted in decreased pausing indices, with a concomitant increase in elongating RNApol2 and upregulated target gene expression (Li et al., 2021b). *SETD5* heterozygous LoF variants are a leading cause of idiopathic ID and ASD (Kuechler et al., 2015; Deliu et al., 2018; Powis et al., 2018) and have also been linked to a CdLS-like phenotype (Parenti et al., 2017). Setd5 haploinsufficient mice show cognitive impairment and behavioral abnormalities linked to increased progenitor proliferation and a loss of synaptic contacts (Deliu et al., 2018; Sessa et al., 2019; Nakagawa et al., 2020).

MEDIATOR COMPLEX

The Mediator complex interacts with different TFs and is implicated in almost every aspect of transcription regulation [reviewed by Jeronimo and Robert (2017)], chromatin architecture [reviewed by Andre et al. (2021)], and DNA repair (Soutourina and Werner, 2014). Due to its many functions, Mediator is often associated with cancer and developmental disease (Schiano et al., 2014; Yin and Wang, 2014). Mediator is composed of 30 subunits, organized in four parts: head, middle, tail and kinase. Each of these modules contains a specific set of Mediator subunits, and the specific composition of Mediator varies. The head and middle part interact with RNApol2 and general TFs at promoter sites, while the tail interacts with sequence specific TFs at enhancer sites (Tsai et al., 2014, 2017; Robinson et al., 2016). It is therefore hypothesized that Mediator, together with cohesin, facilitates chromatin looping to allow proximity of enhancers and promoters and enable PIC assembly (Kagey et al., 2010; Soutourina, 2018). In addition, Mediator, together with TFs, BRD4 and RNApol2, enables liquid-phase separation to form condensates of transcriptional machinery at super enhancers (Cho et al., 2018; Sabari et al., 2018).

Mediator was also found to regulated pausing in various ways. *In vitro*, Mediator overcomes the inhibitory activity of Gdown1 (Hu et al., 2006; Jishage et al., 2012), a stabilizer of promoter-proximal pausing, suggesting that Mediator might alleviate Gdown1-mediated blocking of paused RNApol2 (Cheng et al., 2012). Moreover, loss of MED14, which forms the interaction point between head and middle modules of Mediator, results in loss of promoter proximal RNApol2 (Jaeger et al., 2020). Mediator also regulates transcription pausing through interaction with pausing factors. Various Mediator subunits are found to directly interact with SEC, BRD4 and DSIF. Metazoan specific MED26 interacts with the EAF subunit in SEC, allowing recruitment of active P-TEFb to the pausing complex (Lu et al., 2016). Consequently, depletion of MED26 interrupts SEC recruitment, RNApol2 CTD phosphorylation and expression of *c-MYC* and *HSP70* genes (Takahashi et al., 2011). Furthermore, MED1 and MED23 recruit active P-TEFb associated with BRD4 to paused RNApol2 (Lu et al., 2016). In line with these findings, *Med23* knockout mESCs showed decreased P-TEFb binding to selected genes (Wang et al., 2013).

The kinase module associates with Mediator in a reversible manner and is best described out of all Mediator modules. It comprises of CDK8, CCNC, MED12, and MED13. In vertebrates, paralogs of CDK8, MED12, and MED13 are CDK19, MED12L, and MED13L, respectively. Although the exact function of these paralogs is unknown, they incorporate into the Mediator complex in a mutually exclusive manner (Daniels et al., 2013). CDK8 depletion leads to reduction of SEC recruitment to the promoter site of serum induced genes, resulting in reduced gene expression (Donner et al., 2010). Similarly, SEC is recruited to hypoxia-inducible genes in a CDK8 dependent manner (Galbraith et al., 2013), suggesting a role for Mediator's kinase module in transcription pause regulation.

Kinase Module

Variants in all proteins of the kinase module, except for CCNC, have been implicated in neurodevelopmental delay, indicating an important role for this module in neuronal development. A total of fourteen patients have been described with 10 different *de novo* heterozygous missense mutations in CDK8 (Calpena et al., 2019; Uehara et al., 2020). These patients suffer from hypotonia, ID and variable facial dysmorphisms, as well as agenesis of the corpus callosum. For CDK19, 15 patients were identified that presented with a similar phenotype (Mukhopadhyay et al., 2010; Chung et al., 2020; Zarate et al., 2021), suggesting that both kinases perform similar, non-redundant functions. Besides one case of CDK19 haploinsufficiency, all disease-causing missense variants for CDK8 and CDK19 are localized in the kinase domain (Chung et al., 2020; Zarate et al., 2021), indicating that loss of kinase activity contributes to the neurological defects that involve reduced dendritic branching and altered dendrite morphology (Mukhopadhyay et al., 2010).

From all kinase module subunits, variants in the X-linked gene *MED12* have most frequently been identified in NDDs. Hemizygous variants in males have been described to cause Opitz–Kaveggia syndrome (OMIM# 305450), Ohdo syndrome (OMIM# 300895), and Lujan–Fryns syndrome (OMIM# 309520)

(Risheg et al., 2007; Schwartz et al., 2007; Vulto-van Silfhout et al., 2013). These three syndromes are very similar, as they all encompass ID, macrocephaly, hypotonia, abnormalities in the corpus callosum and typical facial features. However, most patients with MED12 variants, including a total of 25 females (Li et al., 2021a; Polla et al., 2021), show syndromic or non-syndromic ID without a specific disease phenotype [reviewed in Plassche and Brouwer (2021) and Srivastava and Kulshreshtha (2021)]. Non-sense, missense, and splice-site variants localize to all protein domains, resulting in a large spectrum of phenotypes with varying severities of ID and developmental delay. Recently, seven individuals were identified with mutations in MED12L, a MED12 paralog (Nizon et al., 2019). These encompass a wide variety of heterozygous mutations, such as duplication, deletion or single-nucleotide variants. Patients present with ID, developmental delay, speech impairment, and sometimes abnormalities in the corpus callosum.

Although the exact mechanism by which MED12 causes this neuronal phenotype remains to be elucidated, several relevant pathways have been described. For example, MED12 interacts with G9a and REST to regulate neuronal gene expression (Ding et al., 2008, 2009). Moreover, MED12 interacts with Gli3 to activate SHH target genes (Zhou et al., 2006) and some of the reported MED12 missense variants were unable SHH target gene expression in *Med12* null mice (Zhou et al., 2012). Importantly, several MED12 variants affected expression of IEGs such as *JUN*, *FOS* and *EGR1*, which is controlled at the level of pause-release (Donnio et al., 2017).

MED13 and in particular MED13L variants have been linked to various neurodevelopmental aberrations. MED13 was first described as candidate ID gene in a patient with short stature and mild dysmorphisms caused by an 800 kb deletion (Boutry-Kryza et al., 2012). MED13 variants were subsequently described in patients with ASD (Iossifov et al., 2014; Rk et al., 2017) and, recently, missense or truncating variants were identified in thirteen patients presenting with developmental delay, ID, and speech disorders (Snijders Blok et al., 2018). MED13L patients show developmental delay and ID and many of the phenotypes also observed for MED13 and MED12 variants, such as ASD and hypotonia (Asadollahi et al., 2013; Adegbola et al., 2015; Cafiero et al., 2015; van Haelst et al., 2015; Torring et al., 2019; Plassche and Brouwer, 2021). Most frequently, this is caused by a heterozygous loss-of-function of MED13L, and the most severe phenotypes seem to be caused by missense mutations, indicative of a dominant-negative effect (Smol et al., 2018). However, the exact mechanism and pathways remain unknown. In a recent mouse study, Hamada et al. (2021) showed that MED13L protein is highly expressed in the ventricular zone of the cerebral cortex and is also detectable in the developing hippocampus and cerebellum.

Tail and Head Module

Although proteins of the kinase module are most often described in connection with neuronal development, Mediator components MED17 (head), MED23, MED25, and MED27 (tail) have also been found to cause neurodevelopmental disorders upon mutation. In seven individuals, biallelic mutations of MED23 were identified to cause ID (Hashimoto et al., 2011; Trehan et al., 2015). A homozygous variant found in five of these patients was shown to have altered interaction with enhancer bound TFs, resulting in dysregulation of IEGs *JUN* and *FOS* (Hashimoto et al., 2011).

The first disease causing variant identified in MED25, a homozygous missense mutation in the SH3 recognition domain, was detected in a consanguineous family in which 23 individuals presented with Charcot-Marie-Tooth disease type 2B2, a peripheral axonal neuropathy (Leal et al., 2009). Two other studies identified a total of 14 individuals with moderate to severe ID, who were also affected by two different homozygous *MED25* missense mutations (Basel-Vanagaite et al., 2015; Figueiredo et al., 2015). In one study patients also presented with abnormalities in the eye, palate and corpus callosum (Basel-Vanagaite et al., 2015). The missense mutation found in this study drastically impairs MED25 interaction with other Mediator components.

Interestingly, autosomal recessive variants in MED27 and MED17 result in a very similar disease phenotype, characterized by developmental delay, spasticity, seizures, microcephaly and cerebellar atrophy (Kaufmann et al., 2010; Meng et al., 2021). Metazoan-specific subunit MED27 forms the junction between the head and tail modules of the Mediator complex, where it interacts with MED14 and MED17 (Rengachari et al., 2021). Disrupted interaction between head and tail modules might therefore contribute to the neurodevelopmental abnormalities. Indeed, in zebrafish, Med27 loss of function disrupts dopaminergic amacrine cells and serotonergic neurons resulting in size reduction of head, eye, jaw, and eventually leading to lethality 6 days post fertilization (Durr et al., 2006). In fly and chicken, disruption of Med27 also leads to embryonic lethality (Gokcezade et al., 2014; Li-Kroeger et al., 2018; Tsujino et al., 2019). Together these results clearly indicate an important role for Med27 in embryonic and neuronal development.

Taken together, it can be concluded that many of the Mediator components are already found to be essential for neuronal development and, when mutated, can cause syndromic ID in various forms. Despite phenotypic variety, intellectual disability arises in almost all cases. Interestingly, many NDD-causing mutations converge on the kinase module, which has been implicated in the regulation of transcriptional pause-release (Donner et al., 2010; Galbraith et al., 2013; Poot, 2020). Therefore, it would be of particular interest to further delineate the molecular and developmental defects downstream of these mutations.

CONCLUSION

Transcription pause-release is increasingly being recognized as an important step in the regulation of gene expression. Here we have highlighted several neurodevelopmental disorders that are likely caused by dysregulated transcriptional pausing. To what extent the reported variants affect pause duration in the developing brain and which genes and pathways are most affected should be subject of future studies. Several experimental

approaches can be used to measure pause duration. For example, RNApol2 positional information obtained from relatively small cell populations using CUT&RUN-based methods (Meers et al., 2019) can be used to calculate gene-specific pausing indices. Furthermore, nascent RNA-sequencing techniques such as precision nuclear run-on (PRO) sequencing (Kwak et al., 2013) or mammalian native elongating transcript sequencing (mNET-seq) (Nojima et al., 2015) map active RNApol2 with (near-) nucleotide resolution in a highly quantitative manner. Recent adaptations (Judd et al., 2020) mean that it will become feasible to apply these techniques to small, pure cell populations isolated from developing human brain organoids, thereby potentially enabling the establishment of a direct link between transcriptional pausing and neurodevelopmental defects.

Shared clinical features and diagnosis (notably the frequent occurrence of CdLS-like characteristics) suggest a common underlying mechanism. In this respect it will be interesting to investigate a possible role in the regulation of transcription pause-release for additional factors that, when mutated, result in a CdLS-like appearance (i.e., KMT2A and ANKRD11). Indeed, a link between KMT2A and transcriptional pausing has been suggested in HIV latency reversal, where KMT2A displaces PAF1c and facilitates SEC recruitment (Gao et al., 2020). Following the reverse logic, variants in known pausing regulators [e.g., TRIM28 (Bunch et al., 2014)] should be given special consideration during genetic diagnosis. Furthermore, gene editing techniques and advanced *in vitro* models of human brain development (e.g., brain organoids) now provide an excellent opportunity to uncover the disease-relevant neurodevelopmental pathways typically affected by dysregulated pause-release. We envision that this knowledge can directly translate into improved diagnostics in the clinic, by providing evidence for gene variant causality and through transcriptome-based diagnostics. Finally, as transcription pause-release is a process amenable to drug intervention, it forms a potentially promising target for drug-mediated therapeutic intervention in NDDs.

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All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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