The regulation of glial-specific splicing of Neurexin IV requires HOW and Cdk12 activity

Floriano Rodrigues, Leila Thuma and Christian Klämbt*

SUMMARY

The differentiation of the blood-brain barrier (BBB) is an essential process in the development of a complex nervous system and depends on alternative splicing. In the fly BBB, glial cells establish intensive septate junctions that require the cell-adhesion molecule Neurexin IV. Alternative splicing generates two different Neurexin IV isoforms: Neurexin IV\textsuperscript{exon3}, which is found in cells that form septate junctions, and Neurexin IV\textsuperscript{exon4}, which is found in neurons that form no septate junctions. Here, we show that the formation of the BBB depends on the RNA-binding protein HOW (Held out wings), which triggers glial specific splicing of Neurexin IV\textsuperscript{exon3}. Using a set of splice reporters, we show that one HOW-binding site is needed to include one of the two mutually exclusive exons 3 and 4, whereas binding at the three further motifs is needed to exclude exon 4. The differential splicing is controlled by nuclear access of HOW and can be induced in neurons following expression of nuclear HOW. Using a novel in vivo two-color splicing detector, we then screened for genes required for full HOW activity. This approach identified Cyclin-dependent kinase 12 (Cdk12) and the splicesosomal component Prp40 as major determinants in regulating HOW-dependent splicing of Neurexin IV. Thus, in addition to the control of nuclear localization of HOW, the phosphorylation of the C-terminal domain of the RNA polymerase II by Cdk12 provides an elegant mechanism in regulating timed splicing of newly synthesized mRNA molecules.

KEY WORDS: HOW, Raf, Cdk12, Prp40, Neurexin IV, Drosophila, Glia, Splicing, Blood-brain barrier

INTRODUCTION

The regulation of gene expression is central to all aspects of development. This is particularly obvious when we consider that the number of genes present in the genome of C. elegans, Drosophila or human is surprisingly similar. The complexity of gene expression, however, dramatically increases due to differential splicing (Black, 2003; Keren et al., 2010; Li et al., 2007; Matlin et al., 2005). About 95% of all human pre-mRNAs are subject to alternative splicing, of which 4.3% carry mutually exclusive spliced exons (Koscienly et al., 2009; Pan et al., 2008; Wang et al., 2008). In Drosophila, roughly 88% of all genes are subject to splicing and 60.7% of these genes are subject to alternative splicing (Graveley et al., 2011).

Differential splicing is not only required for neuronal lineages but is also needed for the development of glial cells (Edenfeld et al., 2006; Wu et al., 2002). Drosophila glia arise during embryogenesis through the activity of the master regulatory gene held out wings (how) (Einheber et al., 1997; Rios et al., 1997b). how interacts with one of the isoforms encoded by the hel out wings (how) gene (Edenfeld et al., 2006). Three different HOW isoforms are generated that all share a KH RNA-binding domain and differ in their C-terminal tails. The short isoform, HOW(S), binds Crn in the cytoplasm and is able to shuttle to the nucleus. The long isoform, HOW(L), is only found in the nucleus and cannot bind Crn (Edenfeld et al., 2006; Volk et al., 2008). The
middle form is predicted by FlyBase and is currently not further analyzed. Drosophila how mutants have altered glial differentiation and the HOW protein is able to bind Nrx-IV mRNA (Edenfeld et al., 2006). Mice that are mutant for the how homologue called quaking (Qk – Mouse Genome Informatics) develop severe demyelization phenotypes resembling the Drosophila glial differentiation phenotypes shown by crn and how mutants (Ebersole et al., 1996; Edenfeld et al., 2006; Sidman et al., 1964; Wu et al., 2002; Zhao et al., 2010).

Quaking/HOW proteins are members of the STAR (signal transduction and activation of RNA) family of RNA-binding proteins implicated in the control of pre-mRNA splicing, mRNA stability and mRNA transport (Artzt and Wu, 2010; Edenfeld et al., 2006; Larocque et al., 2002; Vernet and Artzt, 1997; Volk, 2010; Zhao et al., 2010). STAR proteins, which are often regulated by post-translational mechanisms, bind to a short sequence motif in pre-mRNA molecules, which is similar to the recognition site for post-translational mechanisms, bind to a short sequence motif in pre-mRNA molecules, which is similar to the recognition site for splice factor 1 (Artzt and Wu, 2010; Galarneau and Richard, 2005; Wu et al., 2002; Zhao et al., 2010). STAR proteins, which are often regulated by pre-mRNA molecules, which is similar to the recognition site for splice factor 1 (Artzt and Wu, 2010; Galarneau and Richard, 2005; Wu et al., 2002; Zhao et al., 2010). STAR proteins, which are often regulated by pre-mRNA molecules, which is similar to the recognition site for splice factor 1 (Artzt and Wu, 2010; Galarneau and Richard, 2005; Wu et al., 2002; Zhao et al., 2010). STAR proteins, which are often regulated by pre-mRNA molecules, which is similar to the recognition site for splice factor 1 (Artzt and Wu, 2010; Galarneau and Richard, 2005; Wu et al., 2002; Zhao et al., 2010).

In Drosophila, the Nrx-IV gene encodes two equally sized mutually exclusively spliced exons, of which one is flanked by HOW-binding sites. These exons encode two related Discoidin domains and their mutually exclusive splicing results in cell adhesion proteins with altered binding capabilities involved in different aspects of neuron-glia interaction (Edenfeld et al., 2006; Stork et al., 2009). The exon 4-containing Nrx-IV mRNA is expressed mainly in neurons. It generates a protein that interacts with the Ig-domain protein Wrapper, which is expressed by the midline glial cells. By contrast, we show here that the exon 3-containing Nrx-IV mRNA is expressed by cells that form pSJ.

Here, we have analyzed the role of HOW and its influence on differential splicing during glial maturation. We show that HOW is required for the establishment of the BBB and directly affects the splicing of Nrx-IV pre-mRNA. To further dissect the function of HOW, we generated several splicing reporters and assayed the relevance of individual HOW response elements. During mutually exclusive splicing, HOW is required for inclusion of exon3 and, thus, the presence of HOW is associated with the formation of the pleated septate junctions in glial cells. To link the activity of HOW to the general control of glial maturation, we performed a genetic screen and identified Raf kinase, Cyclin-dependent kinase 12 (Cdk12) and Pp40 as important regulators required for full activity of HOW.

MATERIALS AND METHODS

Fly work
All crosses were performed on standard food. The following genotypes were used: Nrx-IVp30 (Baumgartner et al., 1996), how<sup>awv</sup> (Nabel-Rosen et al., 1999), how<sup>Lp06788</sup> (Exelixis), repoGal4(II) (Lee and Jones, 2005), repoGal4(III) (Sepp and Auld, 1999), elav<sup>155</sup>Gal4, elavGal4(III) (Lin et al., 1995), how<sup>248</sup>Gal4 (Brand and Perrimon, 1993), UAS-EGFP<sup>RE7</sup> (B.-Z. Shiloh, Weizmann Institute of Science, Rehovot, Israel), UAS-how<sup>RNAi</sup>, UAS-cry<sup>RNAi</sup>, UAS-Cdk12<sup>RNAi</sup> (VDRC), UAS-hPABP<sup>23ap</sup>, UAS-ra<sup>RNAi</sup>, UAS:lamEGFP, GFP- balancers (Bloomington) and UAS-pp40<sub>IP3</sub><sup>RNAi</sup> (Ni et al., 2011). Germ line transformation was carried out as described previously (Bischof et al., 2007).

Dextran injections
Stage 17 embryos were injected as described previously (Stork et al., 2009).

Tissue fixation and histological analysis
Immunohistochemical methods were performed as described before (Stork et al., 2009). All fluorescent samples were recorded on a LSM 710 confocal microscope (Zeiss). Fluorescent measurements were carried out using ImageJ (http://imagej.nih.gov/ij/index.html).

Antibodies
The following antibodies were used: anti-EGFP (Invitrogen); anti-mCherry (Clontech); anti-repo (DSHB, B. Altenhein, University of Mainz, Germany); anti-dCdk12(1 (A. Greenleaf, Duke University, Durham, NC, USA); and anti-HRP-Cy5 (Molecular Probes). Alexa Fluor dyes 488, 568 and 633 were used (Molecular Probes).

Isolation of RNA
Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer’s instructions. Tissue-specific mRNA was isolated from embryos expressing human poly(A)-binding protein in either glial cells or neurons (Yang et al., 2005).

RT-PCR
Total RNA (1 µg) or 100 ng of tissue-specific mRNA extracts were used for reverse-transcribed PCR using SUPERscript II polymerase with Oligo(dT)12-18 primers (Invitrogen) following the manufacturer’s protocol.

PCR and restrictions
The used primer combinations were partially designed with Primer3 (Untergasser et al., 2007) and standard PCR was performed using Taq DNA polymerase (NEB). Restrictions were carried out according to the manufacturer’s instructions (Roche).

Neurexin IV detection
NrxIVSplice2for::CGCCCTTCAGGACTATTCT
NrxIVSplice7rev::CTTTAAGTCTGACGGCATAG

Reporter cloning
NrxIV_2-5_for::ccacCGCCTTTACtgACTATTCT
NrxIV_2-5_rev::GTAATGCGACGCTAGACTCCAC
mCherry_3.0_Neo_for::catgcatgcatgGAGGAGGAAATGGTGAGCAAGGGC-GGCGAGGAGGAATG
mCherry_3.0_Neo_rev::catgcatgctgGAGGAGGAATGGTGAGCAAGGGCGAGGAGGATACG
EGFP_3.0_xba_for::ctagcatggcatgGAGGAGGAAATGGTGAGCAAGGGCGAGGAGGATACG
EGFP_3.0_sperev::ggaactagtCTAATGTACAGCTCGTCCATGC

RTPCR
Generation of constructs
UAS-HOW(S)-3HA and UAS-HOW(L)-3HA were generated using Gateway cloning in a custom-made pUASTattB rFA 3HA vector. Wild-type DNA spanning Nrx-IV exons 2-5 was amplified with proofreading Phusion polymerase (Finnzymes). An engineered KpnI was included to ensure translation and the fragment was inserted into the expression vector pUASTattB rFA EGFP (R. Stephan, unpublished), containing a C-terminal EGFP-tag resulting in the reporter nrxIV 2.0.

Site-directed mutagenesis was carried out to eliminate a NcoI restriction site located in the intron 5’ of exon 3. For nrxIV 3.0, EGFP was inserted into the XbaI restriction site of exon 3 and mCherry was inserted into the NcoI restriction site of exon 4. All constructs were integrated into the landing sites zh-68E and zh-86F<sup>BP</sup> (Bischof et al., 2007).

Site-directed mutagenesis (SDM)
To generate base pair changes, QuikChange II XL Site-Directed Mutagenesis Kit was used according to the manufacturer’s instructions (Agilent Technologies/Stratagene). The primers for SDM were partially designed using QuikChange Primer Design Program (Agilent Technologies/Stratagene).

Δ3 HRE
Δ3HRE_Abw441l_for::GCAGACGCGAGGTTACTGTCaCagCGCTTTAAG-GATGGCGCAGT

DEVELOPMENT

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how mutants show abnormal splicing of Nrx-IV

In the next step, we characterized the Nrx-IV splicing pattern of how mutant embryos. Total mRNA from stage 16 embryos was reverse transcribed. Subsequently, PCR reactions with primers
spanning the alternatively spliced region were performed (Fig. 2A, arrows). To discriminate between exon 3- and exon 4-containing isoforms, we used endogenous restriction sites (XbaI in exon 3 and NcoI in exon 4) (Fig. 2A). Nrx-IVexon3 containing cDNAs generate 359 and 271 bp fragments, whereas restriction of Nrx-IVexon4-containing cDNAs generates 423 and 207 bp fragments. howstru mutants show a different distribution of the Nrx-IV isoforms compared with the wild-type splicing pattern, having more Nrx-IVexon4 (Fig. 2B). To quantify the levels of the different splicing forms, we measured the intensity of the exon-specific DNA fragments (highlighted by red and green dots in Fig. 2B). In wild type, the exon4/exon3 ratio is 0.66 ± 0.15 s.d.; quantification of DNA bands of five independent experiments), in howstru type, the exon4/exon3 ratio is 0.66 (±0.15 s.d.; quantification of fragments (highlighted by red and green dots in Fig. 2B). In wild forms, we measured the intensity of the exon-specific DNA containing cDNAs generates 423 and 207 bp fragments.

To further dissect glial-specific splicing, we generated a set of reporter constructs encompassing the differentially spliced region of Nrx-IV (Fig. 2D, Fig. 3A). In the construct nrxIV_2.0, exon 5 is partially replaced by a EGFP cassette to monitor expression of the transgene, and exon 2 is engineered to contain an ATG-start codon (Fig. 3A). The expression control is mediated via UAS elements, allowing expression of the reporter specifically in neurons, using elavGal4, or in glial cells, using repoGal4. In embryos, splicing of the nrxIV_2.0 reporter closely mimics that found in the endogenous Nrx-IV gene (compare Fig. 2C,D).

**How positive glia expresses NrxIVexon3**
To circumvent mRNA isolation, we generated the nrxIV_3.0 reporter. We inserted a EGFPstop sequence in exon 3, resulting in a translational stop whenever exon 3 is used. In addition, we added a mCherrystop sequence in exon 4, resulting in a translational stop when exon 4 is used (Fig. 3B). Upon transfection of such a construct into S2 cells, 45% of the cases we noted concomitant generation of exon 3- and exon 4-containing mRNAs. In the remaining cells, either exon 3 splicing or exon 4 splicing is observed (Fig. 3C-F; in 30% of the cells only exon 3 and in 25% of the cells only exon 4 splicing is detected). This indicates that even in S2 cells a stable commitment towards a specific splicing reaction can be made.

The nrxIV_3.0 reporter can also be used to monitor splicing specificity with a single cell resolution in Drosophila embryos (Fig. 3G-N). When expressed in glia using repoGal4, we noted only green fluorescence indicating splicing of the exon 3 (Fig. 3G,H; the CNS is indicated by broken lines). By contrast, following expression in neurons using elavGal4, both nrxIV_3.0 transcripts...
can be detected in the CNS (Fig. 3K-N, red and green). Similar results were obtained when we analyzed splicing in the larval nervous system (supplementary material Fig. S2A-H). In summary, this analysis supports the notion that the BBB-forming glia express only the Nrx-IV-exon3 transcript.

To determine the HOW expression domain, we used the 24BGal4 insertion into the how locus (how24BGal4), which faithfully recapitulates how expression (Brand and Perrimon, 1993; Fyrberg et al., 1997) (Fig. 3, supplementary material Fig. S3). Prominent how expression is noted in some CNS glia, which, based on their position, correspond to the subperineurial glia that constitute the BBB (Fig. 3O-Q). No how expression is noted in neurons. In addition, when we expressed the nrxIV_3.0 reporter using the how24BGal4 driver in embryos, all cells express the green exon 3 cassette, indicating that, in embryos, the presence of HOW is associated with the selection of exon 3 (supplementary material Fig. S2M-P). When we expressed the nrxIV_3.0 reporter in larvae using the how24BGal4 driver, most cells appear green, but we noted a few cells in the nervous system that express only the red exon 4 cassette (supplementary material Fig. S2I-L, arrow).

Nrx-IV splicing requires HOW response elements

We had previously shown that HOW is able to bind the Nrx-IV pre-mRNA (Edenfeld et al., 2006). Four potential HOW-binding sites (HOW response elements, HREs) are evolutionary conserved (Fig. 4). HRE1 is situated 5' of exon 3, and three binding sites, HRE2 to
HRE4 are clustered within 40 bp just 3’ of exon 3. Within the splicing reporter nrxIV_2.0, we mutated all individual HREs, as well as several combinations, and tested the consequences for glial cell-specific splicing (Fig. 5, supplementary material Fig. S4).

In wild-type glia, the nrxIV_2.0wt construct is almost exclusively spliced towards the exon 3-containing mRNA (Fig. 5C,D, green dots). Upon mutation of the three 3’ HREs, an aberrant splicing pattern is noted with mRNA molecules containing both, exon 3 and exon 4 (Fig. 5C, white dotted boxes, supplementary material Fig. S5). The different HREs appear to act in a redundant fashion and stronger effects are noted in the triple mutant (Fig. 5C, sh2+3+4). The deletion of all 3’ HREs (sh3) has an even stronger impact on splicing. The removal of the 5’ HRE increases the number of transcripts lacking both exon 3 and exon 4 (Fig. 5C, red dotted boxes). This phenotype is in particularly prominent in quadruple mutants, where all HREs are mutated (Fig. 5C, sh2+3+4). The deletion of all 3’ HREs (sh3) has an even stronger impact on splicing.

The removal of the 5’ HRE increases the number of transcripts lacking both exon 3 and exon 4 (Fig. 5C, red dotted boxes). This phenotype is in particularly prominent in quadruple mutants, where all HREs are mutated (Fig. 5C, sh2+3+4). The restriction analysis of the PCR products shown in Fig. 5C indicated no shift of the glial splicing pattern (Fig. 5D). In conclusion, these data suggest that binding of HOW at the three 3’ HREs is needed to suppress inclusion of exon 4 and the 5’-binding site is required for general inclusion of one of the two exons in the final mRNA.

Identification of additional factors regulating HOW function
The above data are compatible with the following model underlying HOW function. HOW(L) acts as a constitutive factor promoting the selection of nrxIVexon3 in glia and also functions, when ectopically expressed, in neurons. However, in wild-type glia the formation of septate junctions occurs significantly later, after HOW expression is initiated. Thus, HOW is likely to be regulated post-translationally to instruct Nrx-IVexon3 splicing in glia. As HOW can be phosphorylated (Nir et al., 2012), we tested the influence of several kinases on the splicing activity following an RNAi-based (or expression of dominant-negative isoforms) screening approach (supplementary material Table S1).
We determined the effects of gene silencing on the splicing of the nrxIV_2.0 reporter specifically in larval glial cells. Fifteen kinases were tested and silencing of two of them, Raf and Cdk12, resulted in a clear shift of Nrx-I^peton3 to Nrx-I^peton4 splicing (Fig. 6B, supplementary material Table S1).

Cdk12 protein is a nuclear localized kinase that phosphorylates the C-terminal domain (CTD) of the RNA polymerase II during transcriptional elongation (Bartkowiak et al., 2010) (Fig. 9). The phosphorylated CTD is bound by Prp40, a subunit of the U1 snRNP (Morris and Greenleaf, 2000; Neubauer et al., 1997). Prp40, in turn, has been shown to interact with the HOW-binding protein Cm/Cfl1 (Chung et al., 1999; Edenfeld et al., 2006). Thus, Cdk12 is in a position to facilitate splicing of pre-mRNAs that have bound the HOW protein (Fig. 9).

Provided that phosphorylation of the CTD by Cdk12 favors the recruitment of the U1 snRNP subunit Prp40 and thus the recruitment of Cm/HOW, one would postulate that knockdown of prp40 gene function affects alternative splicing of Nrx-IV. To assay a cell-type specific reaction, we monitored the splicing of the nrxIV_2.0 splice reporter following pan-glial co-expression of prp40RNAi. Wild-type neurons predominantly express exon 4-containing Nrx-IV transcripts, whereas glia express both exon 3- and exon 4-containing Nrx-IV transcripts. Upon silencing of prp40 function, we noted a significant increase in the level of the neuronal splicing form (Fig. 6L), indicating that Prp40 acts in the same pathway as Cdk12 and HOW.

Cdk12 is required for HOW-dependent splicing of Nrx-IV

To test the model that Cdk12 regulates HOW function, we first determined the relationship of the two genes in epistasis experiments. In a gain-of-function approach, we asked whether the ability of HOW(L) to induce glial-specific splicing in larval neurons requires Cdk12 activity. Cdk12 is expressed ubiquitously and localizes to the nucleus (Fig. 3R, supplementary material Fig. S3D) (Bartkowiak et al., 2010). We used the fluorescent-based nrxIV_3.0 reporter, which, in contrast to the nrxIV_2.0 reporter, shows some expression of the Nrx-I^peton3 isoform in neurons. Expression of ectopic HOW(L) in neurons results in late larval lethality. The surviving larvae show reduced brain size and reduced amounts of the neuronal splicing isoform (Fig. 8B-C’). To determine the ratio of transcripts, we determined the quotient of the mean fluorescence intensity of the EGFP and mCherry signals in the brain lobes using ImageJ (Fig. 8B,F). Expression of HOW(L) resulted in a significant shift of the isoform quotient from 0.63 to 1.67, reflecting the decreased expression of exon4 mRNAs (<10^{-2}; Fig. 8C,F). Upon expression of HOW(L) and concomitant silencing of Cdk12, the larval lethality is rescued, demonstrating that HOW(L) acts at least in part through Cdk12. The splicing pattern of the nrxIV_3.0 reporter is now shifted to a ratio of 1.19, suggesting that the presence of Cdk12 is needed for full HOW(L) activation (<10^{-2}; Fig. 8D,F). Knock down of only Cdk12 does not alter the splicing pattern of the nrxIV_3.0 reporter (ratio is 0.67;
Fig. 8E,F). These data indicate that HOW(L) requires Cdk12 activity for its function in promoting the presence of a glial-specific spliced Nrx-IVexon3 mRNA.

DISCUSSION

Differential splicing is a key element in generating the amazing complexity of higher nervous systems. Through relatively few regulatory elements, a single gene can generate several different isoforms with potential distinct cellular functions. In Drosophila, differential splicing is required for the correct glial development. Here, we have dissected the role of the STAR-family member HOW in controlling such a differential splicing event at the Nrx-IV locus, which is pivotal for the generation of the BBB.

Nrx-IV exons 3 and 4 are spliced in a mutually exclusive manner. They share DNA sequence identity of 60% and encode related Discoidin domains, which provide distinct adhesive properties (Stork et al., 2009; Vogel et al., 2006). Within glial cells, expression of Nrx-IVexon3 predominates and participates in the formation of septate junctions. Interestingly, the binding partner of Nrx-IV at the Drosophila septate junctions, Neuroglian, or the Caspr-binding partner at the septate-like junctions in vertebrates,
Neurofascin, are also subject to cell-type specific, differential splicing (Basak et al., 2007; Genova and Fehon, 2003; Hassel et al., 1997; Hortsch et al., 1990; Volkmer et al., 1996). Differential splicing appears to be of more general relevance during the formation of septate junctions. The fly homologue of the membrane-skeleton protein 4.1, Coracle, binds to Nrx-IV and mediates the linkage of the septate junctions to the cytoskeleton (Fehon et al., 1994; Lamb et al., 1998; Ward et al., 1998). Differential splicing of coracle generates at least four different splice variants that encode four distinct proteins (Drysdale and Crosby, 2005). RT-PCR experiments indicate that the Coracle-PB isoform is generated in a HOW-dependent manner (F.R. and C.K., unpublished).

STAR proteins, like HOW, bind sequence motifs in the pre-mRNA of their targets (Galarneau and Richard, 2005; Israeli et al., 2007; Ryder et al., 2004; Ryder and Williamson, 2004). Following site-specific mutation of all HREs, we could show that HRE1 may be needed for general exon definition. The mutation of this sequence motif leads to increased exon skipping of both exon 3 and exon 4, suggesting a crucial role for HRE1 in general splicing, possibly affecting the branch point of this intron (Arning et al., 1996; Berglund et al., 2008; Kramer and Utans, 1991). The HRE2, HRE3 and HRE4 elements influence mutually exclusive splicing. Upon mutation of these motifs, both exons are left in the mRNA more frequently, which suggests their function in exon selection. Such an effect was not observed in neurons (data not shown). Thus, these HREs seem to play a role in exon selection.

The HOW isoforms share an identical KH RNA-binding domain (Volk, 2010; Volk et al., 2008). HOW(S) predominantly localizes to the cytoplasm and HOW(L) is found mostly in the nucleus of glial cells. Here, we showed that nuclear HOW is sufficient for the induction of glial-specific splicing in neurons. Interestingly, both HOW isoforms can partially rescue the how mutant phenotype. HOW(S) appears to have higher rescuing abilities as both transgenes are inserted in the same chromosomal landing site (Bischof et al., 2007), resulting in identical expression levels, we assume that HOW(S) must be efficiently transported into the nucleus to promote Nrx-IV splicing. Because, following overexpression of the HOW(S), most of the protein stays in the cytoplasm, the shuttle mechanism(s) directing HOW(S) into the nucleus must be very tightly regulated. Possibly, HOW(S) has better rescuing abilities than HOW(S), but not HOW(L), can facilitate the nuclear import of the splice factor Crn (Edenfeld et al., 2006).
STAR family proteins are phosphorylated on several residues (Matter et al., 2002; Stoss et al., 2004; Nir et al., 2012). In the past, it has been established that the HOW homolog Sam68 is phosphorylated by MAPK the regulation of which is controlled by Raf (Matter et al., 2002). Indeed, expression of a dominant-negative Raf protein in glia shifted the splicing pattern towards the neuronal form, suggesting a role for receptor tyrosine kinase signaling for glial differentiation as it has been demonstrated at several other instances (Franzdottir et al., 2009; Michailov et al., 2004).

In addition, we noted that silencing of Cdk12 resulted in a shift of the splicing pattern towards the neuronal form. Cdk12 is a broadly expressed serine/threonine kinase that also contains stretches of arginine- and serine-rich sequences (SR domains) known to be present in RNA-processing proteins, which regulate splicing, nuclear export and stability of the mRNA (Chen et al., 2006; Huang and Steitz, 2005). Drosophila Cdk12 is associated with the C-terminal domain (CTD) of the RNA polymerase II (RNAPII) and phosphorelates Ser2 (Bartkowiak et al., 2010) (Fig. 9). The CTD of RNAPII acts as an assembly platform that controls transcription and pre-mRNA processing (Egloff and Murphy, 2008). Phosphorylated CTD in turn is recognized by Prp40 (Morris and Greenleaf, 2000), which belongs to the U1 snRNP. Moreover, a direct interaction between Prp40 and Crocked neck like factor 1 and Greenleaf, 2000), which belongs to the U1 snRNP. Moreover, phosphorylation of CTD by Cdk12 is expressed in the nucleus of almost all cells (Fig. 3R; supplementary material Fig. S3D). To further decipher the role of Cdk12 during splicing, we have used a loss-of-function allele. Homozygous mutant animals are lethal at the beginning of larval development. However, these mutants show no splicing defects, most probably owing to strong maternal contributions (data not shown).

The formation of the BBB implies the maturation of septate junctions only in fully differentiated subperineurial glial cells. Thus, the timing of splicing of pre-mRNAs encoding septate junction proteins is crucial and most likely regulated by two independent signaling cascades. We propose that the mRNA-binding protein HOW integrates these signaling events and is key in determining cellular differentiation.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material

References


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<th>Name</th>
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<td>Ex3 -&gt; Ex4</td>
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<td>downstream of receptor kinase</td>
<td>drk</td>
<td>dsRNA</td>
<td>Ex3</td>
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<td>Son of sevenless</td>
<td>sos</td>
<td>dsRNA</td>
<td>Ex3</td>
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<td>Ras oncogene at 85D</td>
<td>ras85d</td>
<td>dsRNA / dominant negative</td>
<td>Ex3</td>
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<tr>
<td>Downstream of raf1</td>
<td>dsor1</td>
<td>dsRNA</td>
<td>Ex3</td>
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