A feedback loop mediated by degradation of an inhibitor is required to initiate neuronal differentiation

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Neuronal differentiation is regulated by proneural genes that promote neurogenesis and inhibitory mechanisms that maintain progenitors. This raises the question of how the up-regulation of proneural genes required to initiate neurogenesis occurs in the presence of such inhibition. We carried out loss and gain of gene function, an interaction screen for binding partners, and biochemical analyses to uncover the regulation, developmental role, and mechanism of action of a ubiquitination adaptor protein, Btbd6a (BTB domain containing 6a). We find that the proneural gene *neurog1* up-regulates *btbd6a*, which in turn is required for up-regulation of *neurog1*. Btbd6a is an adaptor for the Cul3 ubiquitin ligase complex, and we find that it binds to the transcriptional repressor Plzf (promyelocytic leukemia zinc finger). Btbd6a promotes the relocation of Plzf from nucleus to cytoplasm and targets Plzf for ubiquitination and degradation. *plzfa* is expressed widely in the neural epithelium; when overexpressed, it inhibits neurogenesis, and this inhibition is reversed by *btbd6a*. The antagonism of endogenous *plzfa* by *btbd6a* is required for neurogenesis, since the block in neuronal differentiation caused by *btbd6a* knockdown is alleviated by *plzfa* knockdown. These findings reveal a feedback loop mediated by degradation of an inhibitor that is essential for progenitors to undergo the transition to neuronal differentiation.

[*Keywords*: Neurogenesis; ubiquitination; Plzf; Cul3 adaptor]

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The development of the nervous system requires precise control of the differentiation of neurons from neural progenitor cells. The initial steps of neuronal differentiation are regulated by members of the proneural basic helix–loop–helix (bHLH) transcription factor family (Campuzano and Modolell 1992; Bertrand et al. 2002). Proneural gene expression occurs widely at low levels in neural progenitors that are competent but not committed to undergo neuronal differentiation. Neurogenesis is initiated only in those cells in which proneural gene expression is up-regulated, in turn activating downstream genes required for subsequent steps of neuronal differentiation (Vaessin et al. 1994; Culi and Modolell 1998). In the vertebrate CNS, this cascade is mediated by members of the neurogenin and achaete–scute families of proneural genes and their downstream targets, including the bHLH transcription factors NeuroD and NeuroM.

The vertebrate CNS forms over a period of many days or weeks, during which progenitor cells differentiate to form distinct cell types at different stages. Consequently, it is essential that a pool of progenitors is maintained that is available for subsequent neurogenesis, and that an appropriate amount of neuronal differentiation occurs at any specific stage. The correct balance between the maintenance of progenitors and neuronal differentiation is regulated by a number of transcriptional regulators that inhibit neurogenesis (Bertrand et al. 2002; Ross et al. 2003). In one mechanism, nascent neurons laterally inhibit the differentiation of their neighbors via activation of the Notch receptor (Louvi and Artavanis-Tsakonas 2006). Proneural genes up-regulate the expression of Delta or Serrate/Jagged ligands, which activate Notch in adjacent cells. Notch activation leads to the up-regulation of specific members of the Hes/Her family of transcriptional repressors, which in turn inhibit both the expression and function of proneural genes (Ross et al. 2003; Kageyama et al. 2005). This lateral inhibition of neurogenesis is relieved once the differentiating neuron migrates away from the ventricular zone, such that progenitors can then compete with each other to initiate neuronal differentiation.

Another type of mechanism that limits the amount of neurogenesis involves inhibitors, expressed independently of Notch activation, that repress the transcription or function of proneural genes. This inhibition is mediated by a number of factors that include specific members

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of the Hes/Her family (Geling et al. 2003; Bae et al. 2005) and Id proteins that sequester essential E protein cofactors of proneural proteins and promote Hes1 gene expression (Norton 2000; Bai et al. 2007). In some cases, inhibitors are expressed in specific spatial domains in the neural epithelium, where they serve to block neurogenesis (Bally-Cuif and Hammerschmidt 2003), while others are more widely expressed and may act to limit the activity of proneural transcription factors. An important question is how proneural genes overcome such widespread inhibition to become up-regulated to the threshold required for neuronal differentiation. A number of mechanisms have been found to mediate this up-regulation by direct positive feedback or by inhibition of an inhibitor (Bertrand et al. 2002; Gibert and Simpson 2003; Kageyama et al. 2005). Such mechanisms can ensure that there is a discrete switch to neurogenesis once sufficient proneural activity has been achieved to initiate positive feedback.

In studies to identify functions of Btbd6 (BTB domain containing 6), a putative ubiquitin ligase adaptor protein, we uncovered a role of targeted protein degradation in the initiation of vertebrate neurogenesis. We find that, during primary neurogenesis in zebrafish, *neurogenin1* (*neurog1*) up-regulates expression of *btbd6a*, which in turn is required for the up-regulation of *neurog1* and neurogenesis. Btbd6a acts as a ubiquitination adaptor protein that binds to the transcriptional repressor Plzf (promyelocytic leukemia zinc finger). *plzfa* is expressed widely in the neural epithelium during primary neurogenesis, where it acts to inhibit *neurog1* expression and neuronal differentiation. *Btbd6a* blocks the inhibition of neurogenesis by *plzfa*, decreases the amount of Plzf in the nucleus, and promotes its degradation. These findings reveal a positive feedback loop downstream from a proneural gene, mediated by an adaptor protein that targets the degradation of a widely expressed inhibitor of neurogenesis.

Results

In an in situ hybridization screen of a chick hindbrain cDNA library (Christiansen et al. 2001), we identified a novel BTB domain gene expressed in cells that have a scattered distribution in the developing hindbrain and spinal cord, and are at a higher density in r5 and r6 (data not shown). This pattern of expression correlates with the birth of hindbrain reticular neurons and spinal interneurons that are the first to differentiate during chick development (Sechrist and Bronner-Fraser 1991). As preliminary studies suggested that zebrafish homologs are also expressed in early differentiating neurons, we set out to use this system to analyze its regulation and function.

btbd6 is a member of a conserved gene family

We deduced the sequence of zebrafish homologs of the BTB domain gene by sequencing of cDNA clones and predictions from cDNA and genome sequence databases. This revealed that the encoded polypeptide has an arrangement of motifs characteristic of a small family of genes present in all animal genomes (Stogios et al. 2005): an N-terminal BTB domain, BACK domain, and C-terminal PHR domain (Supplemental Fig. 1A). The BTB domain mediates protein–protein interactions and is present in diverse proteins, including transcription factors and ubiquitination adaptors (Kelly and Daniel 2006; Perez-Torrado et al. 2006). The PHR domain is a motif identified as a tandem repeat in hiw/rpm-1/pam, a RING finger gene initially implicated in synaptic growth (Wan et al. 2000; Zhen et al. 2000), and the BACK domain is present in several proteins in association with PHR or Kelch domains (Stogios and Prive 2004; Stogios et al. 2005). This combination of motifs suggests that BTB–BACK–PHR proteins may act as ubiquitin ligase adaptors that target specific proteins for degradation. In vertebrate genomes, the BTB–BACK–PHR family is comprised of four genes, termed *BTBD1*, *BTBD2*, *BTBD3*, and *BTBD6* (Supplemental Fig. 1B). The gene found in our screen corresponds to *BTBD6*, which, due to genome duplication in fish (Postlethwait et al. 2004), has two zebrafish orthologs that we designate as *btbd6a* and *btbd6b*.

btbd6a is expressed during primary neurogenesis downstream from neurog1

We carried out in situ hybridization studies to determine the developmental expression patterns of the zebrafish *btbd6* orthologs, and found that both are expressed in the developing nervous system: *btbd6a* in the CNS (Fig. 1A–D), and *btbd6b* in cranial ganglia (data not shown). We focused subsequent analysis on *btbd6a*. At the three-somite (3s) stage, expression occurs in three longitudinal columns in the hindbrain and anterior spinal cord (Fig. 1A). Subsequently, expression occurs in a punctate pattern along the length of the developing spinal cord (Fig. 1B,C) and, by 24 h, has become restricted to the posterior spinal cord (Fig. 1D). These aspects of *btbd6a* expression are strongly reminiscent of the pattern of primary neurogenesis in zebrafish. In addition, *btbd6a* expression occurs in a dynamic pattern in specific hindbrain segments and in the midbrain and forebrain (Fig. 1A–D).

To determine when *btbd6a* is expressed during primary neurogenesis, we carried out double in situ hybridizations to compare it with molecular markers of different steps of neuronal differentiation. In regions of primary neurogenesis, we detected *btbd6a* transcripts in cells that express low or high levels of *neurog1* (Fig. 1E–E‴). In comparisons with markers of later steps of differentiation, we found that *btbd6a* transcripts are coexpressed with *isl1*, which marks a subset of primary neurons (Fig. 1F–F‴), and there is a partial overlap with Elav (HuC), which marks all post-mitotic neurons (data not shown). These data suggest that *btbd6a* expression is initiated with, or shortly after, *neurog1* expression; maintained during early steps of neuronal differentiation; and down-regulated during terminal differentiation. The overlap with *neurog1* expression occurs only during primary neurogenesis, as *neurog1* is expressed more widely than *btbd6a* at later stages (data not shown).

The results of our gene expression studies suggest that *btbd6a* may be up-regulated downstream from *neurog1*. To test this, we analyzed the effect of morpholino

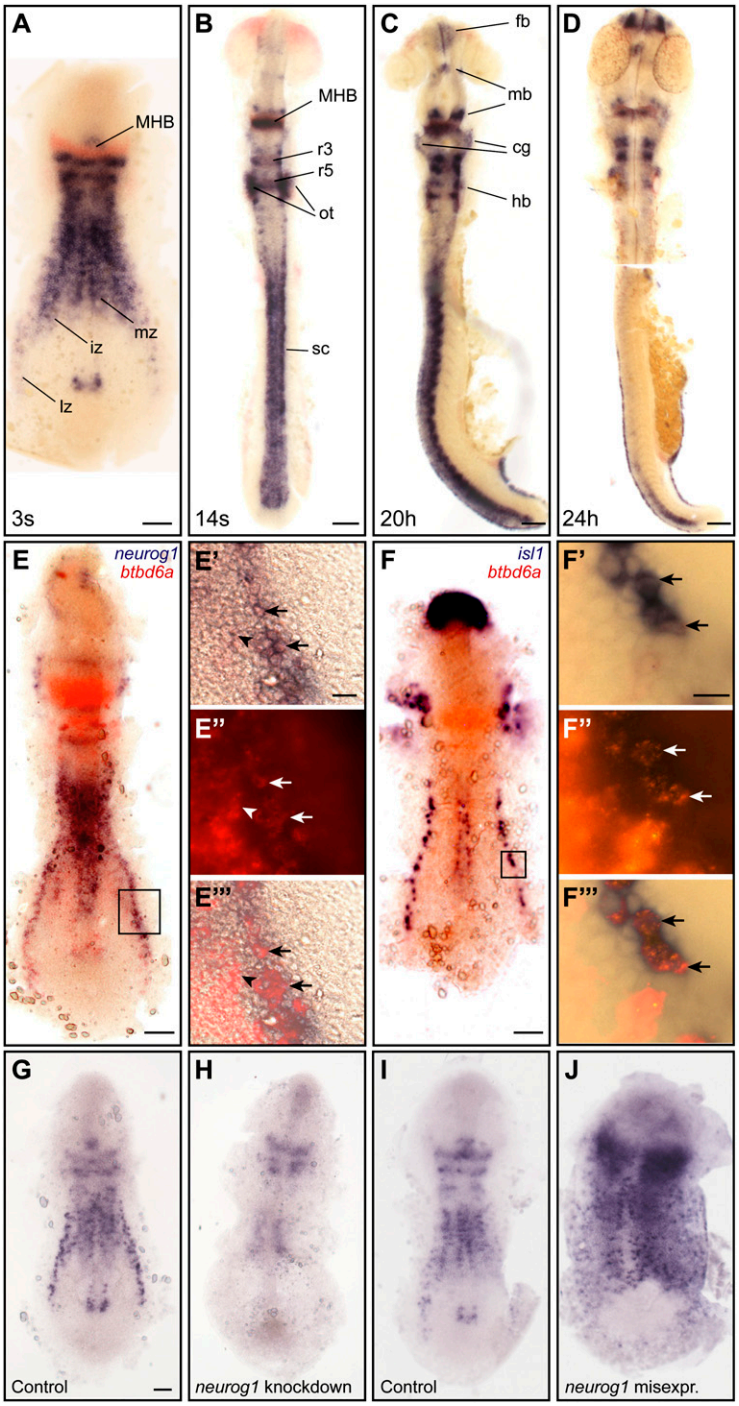

Figure 1. *btbd6a* is expressed during primary neurogenesis downstream from neurog1. (*A–D*) Expression pattern of *btbd6a* as determined by in situ hybridization. At 3s, *btbd6a* transcripts are in rostrocaudal stripes in the posterior neural plate characteristic of the lateral zone (lz), intermediate zone (iz), and medial zone (mz) of primary neurogenesis. At 14s and 20 h, expression occurs widely in the spinal cord (sc) and, by 24 h, has become restricted to the posterior spinal cord. Expression also occurs in a dynamic segmental pattern in the hindbrain (hb), at the mid-hindbrain boundary (MHB), cranial ganglia (cg), midbrain (mb), and forebrain (fb). *pax2a* and *krox20* expression (red signals) mark the mid-hindbrain boundary and r3/r5, respectively. (Ot) Otic placode. (*E,F*) Double in situ hybridizations to compare expression of *btbd6a* (red signal) with *neurog1* and *islet1* (*isl1*), as markers of different stages of neuronal differentiation (blue signals). *E′–E‴* and *F′–F‴* are bright-field, red fluorescence, and superimposed views of the areas indicated in *E* and *F*, respectively. (*E–E‴*) *btbd6a* transcripts are detected in cells with high (arrows) or low (arrowhead) levels of *neurog1* expression, corresponding to differentiating neurons and progenitors, respectively. The weaker signal for *btbd6a* in cells with high *neurog1* expression is due to masking of red fluorescence by strong blue staining. The widespread lower-level expression of *neurog1* in progenitors is not detected, as blue signal development was for a short period to avoid excessive masking. (*F–F‴*) *btbd6a* transcripts are detected in differentiating neurons that express *isl1* (arrows), as well as in other neuronal cell types. (*G,H*) Embryos injected with 1.6 ng of either control (*G*) or *neurog1* (*H*) MO were analyzed at 3s for *btbd6a* expression. Knockdown of *neurog1* leads to a major decrease of *btbd6a* expression in neurogenic zones, except medial neurons, whereas segmental expression is not affected (31 out of 31 embryos). (*I,J*) Fifty picograms of *lacZ* control (*I*) or *neurog1* RNA (*J*) were injected at the one-cell stage, and *btbd6a* expression was analyzed in 3s embryos. Overexpression of Neurog1 induces ectopic *btbd6a* expression (28 out of 28 embryos). All embryos are shown in dorsal views. Bars: E′–E‴,F′–F‴, 10 μm; all other panels, 100 μm.

oligonucleotide (MO)-mediated knockdown of *neurog1* and found that this leads to a major decrease in the expression of *btbd6a* associated with primary neurogenesis (Fig. 1G,H). Medial neurons still express *btbd6a* following *neurog1* knockdown (Fig. 1H), consistent with studies showing that another proneural gene promotes differentiation of these primary motor neurons (Cornell and Eisen 2002). To further analyze the relationship with *neurog1*, we tested the effect of overexpressing *neurog1* and found that this leads to ectopic expression of *btbd6a* (Fig. 1I,J). These results show that *btbd6a* is up-regulated downstream from *neurog1* and, based on the overlap and relative timing of their normal expression, is likely to be a direct or early indirect target of Neurog1 during primary neurogenesis.

Knockdown of btbd6a inhibits neurogenesis

To determine whether *btbd6a* has any role in neurogenesis, we first carried out MO-mediated gene knockdowns. Analysis of sequence databases suggested that

two alternatively spliced *btbd6a* transcripts are expressed in which *btbd6a2* lacks specific N-terminal coding sequences present in *btbd6a1* (Supplemental Figs. 2, 3A). The *btbd6a2* transcript is predicted to encode a shorter protein in which translational initiation occurs at a more C-terminal methionine compared with *btbd6a1*. In RT–PCR assays, we found that *btbd6a1* is expressed throughout early zebrafish development, and *btbd6a2* up-regulated from 9 h when neurogenesis is initiated (Supplemental Fig. 3B). We were not able to determine the expression pattern of the alternative transcripts, as the short isoform-specific probes are not sensitive enough, but these results raised the possibility that they are coexpressed during neurogenesis. We therefore designed MOs predicted to block translation of each transcript, and used these alone or in combination. We found that knockdown of *btbd6a1* or *btbd6a2* alone leads to a mild decrease in the expression of late markers of neurogenesis, but had no detectable effect on *neurog1* expression (Fig. 2A–C,F–H,K–M). In contrast, knockdown of both *btbd6a* transcripts leads to a major decrease in the expression of *neurog1* and downstream markers of neuronal differentiation (Fig. 2D,I,N). Several lines of evidence strongly suggest that this inhibitory effect is specific: Higher amounts of each *btbd6a* MO alone had little effect on *neurog1* expression; control MO had no specific effect on neurogenesis; similar results were obtained after coinjection of *p53* MO (data not shown), which blocks nonspecific effects caused by activation of p53 by some MOs (Robu et al. 2007); and there is a similar effect of dominant-negative forms of Btbd6a (described below). These results reveal that *btbd6a* is essential for the increase in *neurog1* expression required for neurogenesis.

Overexpression of Btbd6a promotes neurogenesis

The findings that *btbd6a* knockdown inhibits *neurog1* expression, yet *btbd6a* is expressed downstream from *neurog1*, suggest that Btbd6a may act in a positive feedback loop required to up-regulate *neurog1*. This predicts that Btbd6a overexpression will promote the expression of *neurog1*. To test this, we microinjected *btbd6a* RNA at the two-cell stage to express it in one-half of zebrafish embryos, together with *lacZ* RNA as a reporter. We found that ectopic expression of Btbd6a leads to a major increase in the expression of *neurog1* and later markers of neuronal differentiation (Fig. 2E,J,O). Notably, the increase occurred within the zones of primary neurogenesis rather than there being a loss of the intervening nonneurogenic zones. These results suggest that *btbd6a* promotes neurogenesis via the up-regulation of *neurog1*. Consistent with this, we found that knockdown of *neurog1* leads to a loss of primary neurogenesis in embryos that overexpress Btbd6a (30 out of 34 embryos) (data not shown).

btbd6a encodes a putative ubiquitin adaptor protein

Some BTB domain proteins act as adaptor proteins that target specific proteins for ubiquitination and degradation by assembling them with the Cullin3 (Cul3) ubiquitin ligase complex (Furukawa et al. 2003; Geyer et al. 2003; Pintard et al. 2003; Xu et al. 2003; Petroski and Deshaies 2005). These proteins bind to Cul3 via a BTB domain and to their target via a motif such as a Kelch or Math domain (Krek 2003). By analogy, BTB–PHR proteins may act as adaptors. To test this, we generated cell lines expressing epitope-tagged full-length or truncated forms of Btbd6a (Fig. 3A) and analyzed whether these can bind to Cul3. We found that full-length Btbd6a coimmunoprecipitates with Cul3, whereas Btbd6a lacking the PHR domain does not, and that there is a lower amount of coimmunoprecipitation of Btbd6a lacking the BTB domain compared with the full-length protein (Fig. 3B). We analyzed the subcellular distribution of these tagged Btbd6a proteins and found that full-length protein is present mainly but not exclusively in the cytoplasm (Fig. 3C,F,I), Btbd6aΔPHR is present in the nucleus (Fig. 3D,G,J), and Btbd6aΔBTB is present at similar levels in the cytoplasm and nucleus (Fig. 3E,H,K). The extent to which full-length or

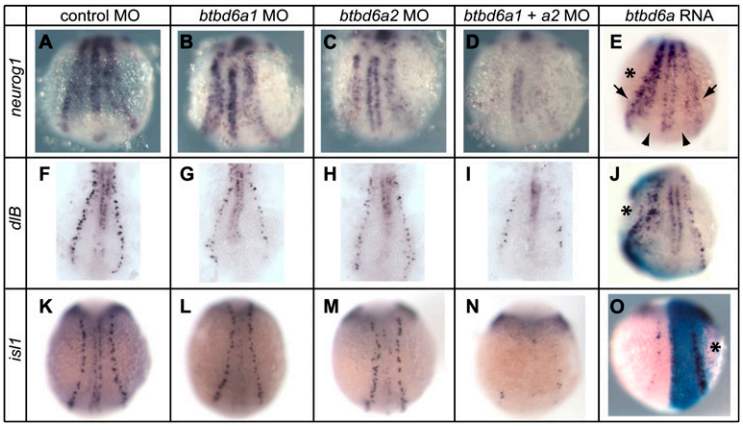

Figure 2. Knockdown or misexpression of *btbd6a* affects primary neurogenesis. Knockdown of *btbd6a1* (3.2 ng of MO) (*A–D,F–I,K–N*) or of *btbd6a2* (0.8 ng of MO) (*A–C,F–H,K–M*) has no detectable effect on *neurog1* expression, and causes a mild decrease in expression of *dlb* and *isl1*. (*D,I,N*) Knockdown of both transcripts (3.2 ng of *btbd6a1* MO + 0.8 ng of *btbd6a2* MO) leads to a major decrease in expression of *neurog1* (34 out of 55 embryos), *dlb* (46 out of 53), and *isl1* (45 out of 86). The decrease in *neurog1* expression is not a nonspecific effect due to the increased total amount of MO, as it was not observed after injection of 6.5 ng of *btbd6a1* MO. Injections were carried out at the one-cell stage, and embryos were analyzed at 3–4s. (*E,J,O*) Overexpression of *btbd6a2* leads to increased expression of *neurog1* (36 out of 42), *dlb* (14 out of 15), and *isl1* (20 out of 22) within neurogenic domains (arrow), while the intervening nonneurogenic zones (arrowhead) are not affected. Thirty-five picograms of *btbd6a2* RNA were coinjected with *lacZ* RNA into one cell at the two-cell stage to achieve expression in one-half (asterisk) of embryos, and were analyzed at 3–5s by staining for LacZ activity followed by in situ hybridization. Overexpression of *lacZ* alone has no effect on neurogenesis.

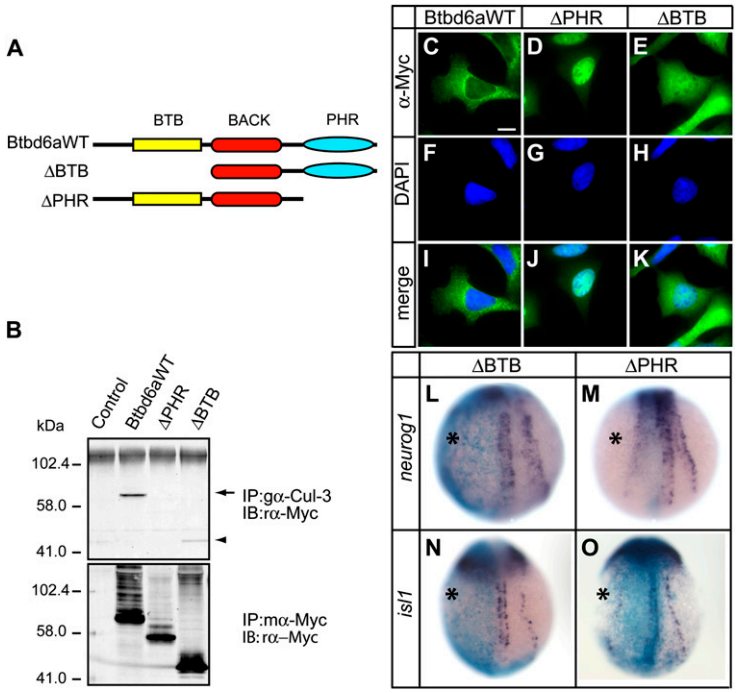

Figure 3. Binding and functional studies of deletion mutants suggest that Btbd6a is a Cul3 adaptor. (*A*) Depiction of full-length (wild type, WT) and truncated (ΔPHR and ΔBTB) Btbd6a constructs used. (*B*) Western blots of immunoprecipitations from HEK293 cell lines with stable expression of myc epitope-tagged Btbd6a1. Full-length Btbd6a (arrow), and, to a lesser extent, Btbd6aΔBTB (arrowhead), coimmunoprecipitates with endogenous Cul3, whereas Btbd6aΔPHR does not. (IP) Immunoprecipitation; (IB) immunoblot; (g) goat; (m) mouse; (r) rabbit; (α) anti. (*C–K*) Detection of the subcellular location of full-length or truncated Btbd6a in HEK293 cell lines. Epitope-tagged Btbd6a1 was detected with anti-myc antibody (green signal, first row) and nuclei with DAPI staining (blue signal, second row); merged views are shown in the third row. Full-length Btbd6a is detected mainly but not exclusively in the cytoplasm (*C,F,I*), Btbd6aΔPHR was detected in the nucleus (*D,G,J*), and Btbd6aΔBTB was detected at similar levels in the nucleus and cytoplasm (*E,H,K*). (*L–O*) Effect of overexpressing truncated Btbd6a on neurogenesis. Three-hundred-fifty picograms of RNA encoding truncated forms of Btbd6a were coinjected with *lacZ* RNA into one cell at the two-cell stage to achieve expression in one-half (asterisk) of zebrafish embryos, which were analyzed at 3–5s. Overexpression of Btbd6aΔBTB (*L,N*) or Btbd6aΔPHR (*M,O*) leads to a decrease in *neurog1* (45 out of 61 and 18 out of 23 embryos, respectively) and *isl1* (35 out of 53 and 41 out of 57, respectively) expression. As a control, expression of Ntl in mesoderm was analyzed and was found to be unaffected by overexpression of either of the truncated Btbd6a proteins (30 out of 30) (data not shown).

truncated Btbd6a is present in the cytoplasm correlates with binding to Cul3, which could reflect that the subcellular distribution of Btbd6a is affected by binding to cytoplasmic Cul3 and/or that the PHR and BTB domains are involved in trafficking between nucleus and cytoplasm.

A prediction of Btbd6a acting as an adaptor protein is that truncated proteins will act in a dominant-negative manner by preventing the assembly of a target protein with the Cul3 complex. To test this, we microinjected RNA at the two-cell stage to express truncated forms of Btbd6a in one-half of zebrafish embryos. We found that expression of Btbd6aΔBTB or Btbd6aΔPHR leads to a major decrease in neurogenesis compared with the nonexpressing half (Fig. 3L–O). These findings support the idea that Btbd6a acts as an adaptor protein.

Btbd6a interacts with Plzf

The results of loss-of-function and gain-of-function experiments, together with evidence that Btbd6a may act as a ubiquitination adaptor, can most easily be explained by Btbd6a promoting the degradation of an inhibitor of neurogenesis. This raises the question of the identity of proteins that bind to Btbd6a. To address this, a yeast two-hybrid screen of a zebrafish embryo cDNA library was carried out using Btbd6a as bait. In addition to obtaining hits for Cul3, this screen identified Plzf as potentially interacting with Btbd6a. Plzf was an interesting candidate, since this BTB domain zinc finger gene acts as a transcriptional repressor, maintains progenitors for myeloid differentiation and spermatogenesis (Zelent et al. 2001; Kotaja and Sassone-Corsi 2004; McConnell and Licht 2007), and is expressed widely in the mouse nervous system (Avantaggiato et al. 1995; Cook et al. 1995). We therefore next addressed the questions of whether Plzf does bind to Btbd6a, and whether there is overlapping expression of these genes consistent with an interaction during nervous system development.

We carried out immunoprecipitation assays to determine whether Plzf binds to Btbd6a and, if so, which domains of Btbd6a are required. We found that full-length Btbd6a and Btbd6aΔPHR bind to Plzf, although the latter coimmunoprecipitates with Plzf to a lesser extent than full-length Btbd6a (Fig. 4A). In contrast, Btbd6aΔBTB failed to coimmunoprecipitate with Plzf, and thus the BTB domain appears to be essential for binding to Plzf but not to Cul3 (Fig. 3B). The finding that distinct domains of Btbd6a are required to bind to Plzf and Cul3 supports the possibility that it acts as an adaptor protein.

To address whether *plzf* is coexpressed with *btbd6a* during nervous system development, we studied the expression of the two zebrafish *plzf* orthologs. We found that, during early stages of primary neurogenesis, *plzfb* is expressed in the forebrain, midbrain, and hindbrain (Fig. 4B), and that *plzfa* expression occurs more widely in the neural epithelium, at highest levels in the forebrain and midbrain, and in a posterior-to-anterior gradient in the caudal neural plate at 3–6s (Fig. 4C,D). The broad expression of *plzfa* overlaps with the expression of *btbd6a* in the zones of primary neurogenesis in the caudal neural plate (Fig. 4E,F). These data suggest that *plzfa* is widely expressed in neural progenitors within which *btbd6a* is upregulated in differentiating neurons, consistent with

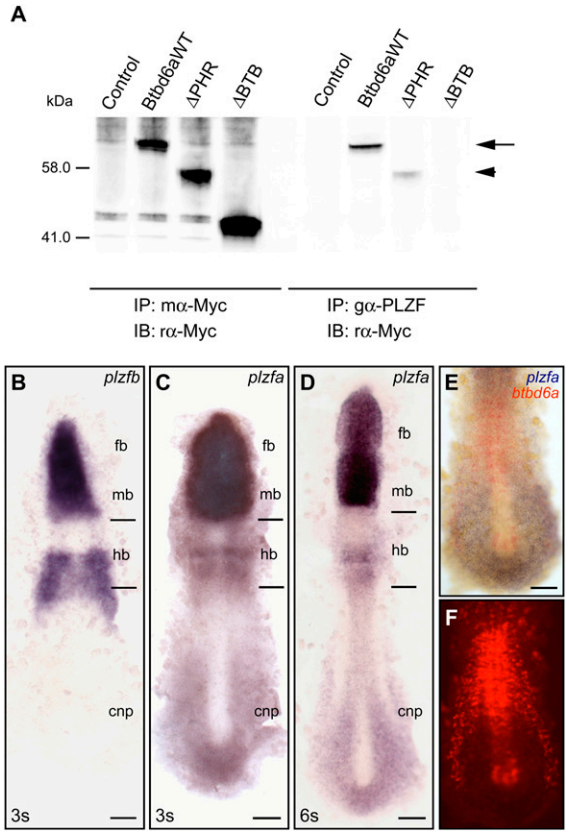

Figure 4. Plzf interacts with Btbd6a and is widely expressed during primary neurogenesis. (*A*) Coimmunoprecipitation assays in cell lines with stable expression of myc-tagged full-length (wild type [WT]) or truncated (ΔPHR and ΔBTB) Btbd6a (see the legend for Fig. 3). The panels show immunoprecipitations of Btbd6a (*left* side), and of PLZF followed by immunodetection of Btbd6a (*right* side). Full-length Btbd6a (arrow), and, to a lesser extent Btbd6aΔPHR (arrowhead), coimmunoprecipitates with PLZF, whereas Btbd6aΔBTB does not. (*B–D*) Expression pattern of *plzfb* (*B*) and *plzfa* (*C,D*). *plzfb* transcripts are detected in the forebrain, midbrain, and hindbrain, whereas *plzfa* expression occurs more widely in the developing CNS. At 3s and 6s, *plzfa* is expressed at high levels in the forebrain and midbrain, and in a posterior-to-anterior gradient in the caudal neural plate (cnp). (*E,F*) Double in situ hybridization to detect *btbd6a* (red signal in *E*; shown in fluorescence in *F*) and *plzfa* (blue signal in *E*) at 4s. In the posterior neural plate, *plzfa* expression overlaps with *btbd6a*, except in the tailbud, where there is a pulse of *neurog1* and *btbd6a* expression prior to primary neurogenesis. Bars: *B–E*, 100 μm.

a potential interaction between Plzfa and Btbd6a during neurogenesis.

Plzfa is an inhibitor of neurogenesis and is antagonized by Btbd6a

The finding that *plzfa* is widely expressed in the neural epithelium raises the possibility that it has a role in the control of neurogenesis. To analyze this, we first carried out gain-of-function experiments by microinjecting *plzfa* RNA to overexpress it in one-half of embryos. We found that overexpression of *plzfa* leads to a decrease in *neurog1* and *isl1* expression compared with the control uninjected side (Fig. 5A,B). These data raise the prospect that the up-regulation of Btbd6a may act to relieve the inhibition of neurogenesis by *plzfa*. We therefore analyzed the effect of coexpression of *btbd6a* on the inhibition of neurogenesis by overexpressed *plzfa*. We found that coexpression of increasing amounts of *btbd6a* with a fixed amount of *plzfa* leads to a progressive increase in neurogenesis from the low level that occurs in the presence of *plzfa* alone (Fig. 5C). At high doses of *btbd6a*, the inhibitory effect of exogenous *plzfa* is more than overcome and there is a greater amount of neurogenesis than in controls.

To test the role of endogenous *plzfa*, we next carried out gene knockdowns with antisense MOs, and found either no change or only a small increase in the amount of neurogenesis (data not shown). That the effect is mild may be explained first by the existence of endogenous mechanisms to degrade Plzf in progenitors selected to differentiate (tested below), and second by Notch-mediated lateral inhibition of neurogenesis. The expression of *plzfa* in progenitors is not altered by blocking Notch activation with the inhibitor DAPT (Supplemental Fig. 4A,B), and overexpression of *plzfa* strongly inhibits neurogenesis after blocking of Notch activity (Supplemental Fig. 4C,D). The inhibition of neurogenesis by Plzfa is thus not a component of, or dependent on, the Notch pathway. We propose that, in progenitors adjacent to differentiating neurons, strong Notch activation is sufficient to inhibit neurogenesis in the absence of *plzfa*. However, in cells that become selected during lateral inhibition to have lower Notch activity, a decrease in Plzfa activity may be required to enable differentiation. This predicts that, if Notch activation is partially blocked such that it is close to the threshold required to inhibit neurogenesis, knockdown of *plzfa* will now lead to an increase in neurogenesis. We therefore titrated DAPT to a concentration that causes a mild increase in neurogenesis. We found that, under these conditions, knockdown of *plzfa* leads to a significant increase in the amount of neurogenesis (Fig. 5D; Supplemental Fig. 4F–I). These findings demonstrate that Notch activity is sufficient for the lateral inhibition of differentiation in the absence of *plzfa*, and suggest that down-regulation of Plzf activity enables the differentiation of progenitors with low Notch activity.

Antagonism of plzfa by btbd6a is required for neurogenesis

The observation that *btbd6a* overexpression leads to a substantial increase in neurogenesis, whereas *plzfa* knockdown does not, suggests that Btbd6a also acts on another target that inhibits neurogenesis. Since such an unidentified target could have a predominant role, this raises the question of whether antagonism of Plzfa by Btbd6a is required for neurogenesis. To test this, we analyzed whether knockdown of *plzfa* rescues the effect of *btbd6a* knockdown. We found that, whereas *btbd6a* knockdown leads to an almost complete block in differentiation, when combined with *plzfa* knockdown, all embryos have normal amounts of neurogenesis (Fig. 5E–G). The antagonism

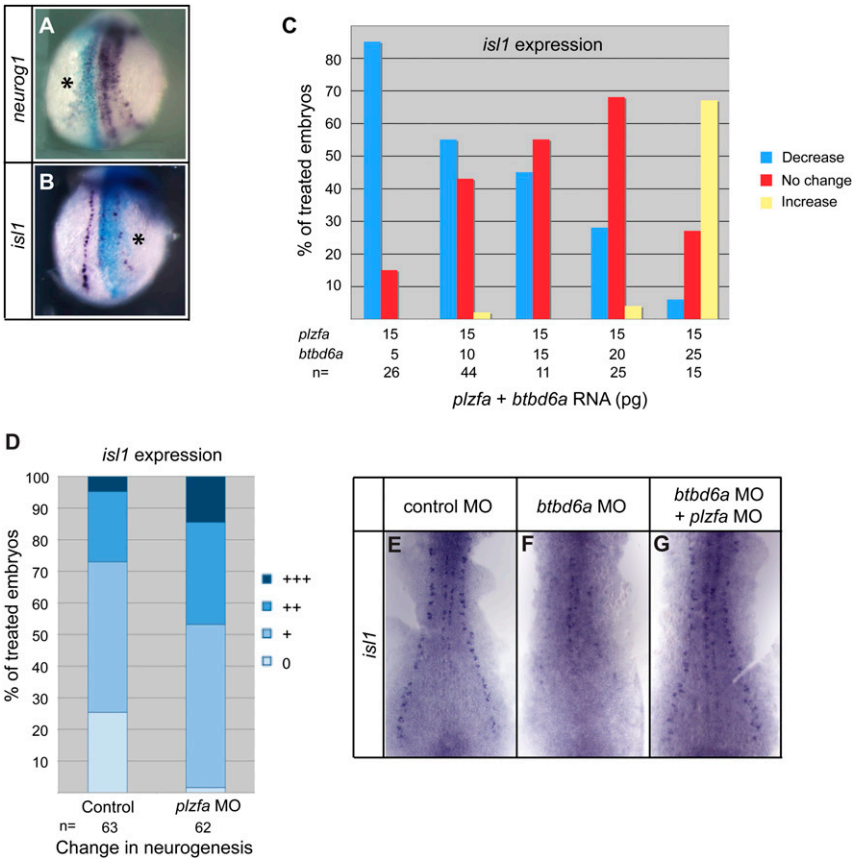

Figure 5. Plzfa is an inhibitor of neurogenesis and is antagonized by Btbd6a. (*A*,*B*) Effect of Plzfa overexpression on neurogenesis. *plzfa* and *lacZ* RNA were coinjected into one cell at the two-cell stage to express them in one-half (asterisk) of embryos, which were analyzed at 4s. Overexpression of *plzfa* leads to a down-regulation of *neurog1* (36 out of 38 embryos) and *isl1* (41 out of 44) expression. As a control, expression of Ntl in mesoderm was analyzed and was found to be unaffected by overexpression of Plzfa (17 out of 17) (data not shown). (*C*) Bar chart of the effect of coinjecting a fixed amount of *plzfa* RNA with increasing amounts of *btbd6a2* RNA, as indicated, with the number of embryos analyzed indicated *below*. The phenotypes were scored as a clear decrease (blue), no change (red), or increase (yellow) in the number of *isl1*-expressing cells at 4s compared with controls. (*D*) Bar chart depicting the effect of MO-mediated knockdown of *plzfa* on neurogenesis when Notch signaling is attenuated. *plzfa* MO or control MO was injected into one- to four-cell embryos in which Notch activation was partially blocked with DAPT. Embryos at the 4s stage were scored for the number of *isl1*-expressing cells, as shown in Supplemental Figure 4F–I: (0) no increase; (+) mild increase; (++) intermediate increase; (+++) high increase. Knockdown of *plzfa* leads to a significant increase in neurogenesis ($P < 0.01$). (*E–G*) *plzfa* knockdown reverses the effect of *btbd6a* knockdown on neurogenesis. Double knockdown of *btbd6a* (as described in the legend for Fig. 2) leads to a major decrease in neurogenesis (32 out of 37 embryos) (*F*) compared with control knockdowns (*E*). (*G*) Following coinjection of 5 ng of *plzfa* MO with *btbd6a* MOs, all embryos have a similar amount of neurogenesis as in control knockdowns (37 out of 37). Bar,100 μm.

of *plzfa* by *btbd6a* is thus essential for the transition of progenitors to neuronal differentiation.

Btbd6a targets Plzf for degradation

Our finding that *btbd6a* antagonizes the inhibition of neurogenesis by *plzfa* can be explained by a model in which Btbd6a decreases the amount of Plzf protein by targeting it for degradation. To test this, we carried out cotransfection experiments in HEK293 cells to express a fixed amount of Plzfa with different amounts of Btbd6a, or as a negative control with Btbd6aΔBTB that does not bind Plzf. We found that increasing amounts of Btbd6a expression lead to a progressive decrease in the steady-state level of Plzf protein (Fig. 6A,C), whereas this decrease does not occur after expression of Btbd6aΔBTB (Fig. 6B,C). To determine whether Btbd6a promotes ubiquitination of Plzf, cells expressing Plzf with or without Btbd6a were incubated in the presence of the proteasome inhibitor MG132, and then immunoprecipitation of Plzf followed by Western blot analysis of ubiquitin was carried out. We found that, in the presence of Btbd6a, there is a major increase in the amount of high-molecular-weight ubiquitinated protein that is immunoprecipitated (Fig. 6D). We therefore conclude that the inhibition of neuronal differentiation by Plzf is relieved by the up-regulation of Btbd6a, and that Btbd6a promotes the ubiquitination and degradation of Plzf protein.

Btbd6a promotes nuclear export of Plzf

By analogy with studies of the Cul3 adaptor Keap1 (McMahon et al. 2003; Cullinan et al. 2004; Sun et al. 2007), it was possible that, in addition to promoting degradation, Btbd6a regulates the nucleocytoplasmic distribution of Plzf protein. To test this, we injected RNA encoding HA-tagged Plzf into one-cell-stage zebrafish embryos, and then in the same embryos injected RNA encoding myc-tagged Btbd6a into one cell at the two- to four-cell stages to achieve mosaic expression. We found that, in cells expressing Btbd6a, there was a major decrease in the amount of Plzf in the nucleus and increase in the cytoplasm compared with cells that do not express exogenous Btbd6a (Fig. 6E–H).

Discussion

The correct balance between the initiation of neurogenesis versus maintenance of neural progenitors is achieved by inhibitory mechanisms that limit the up-regulation of

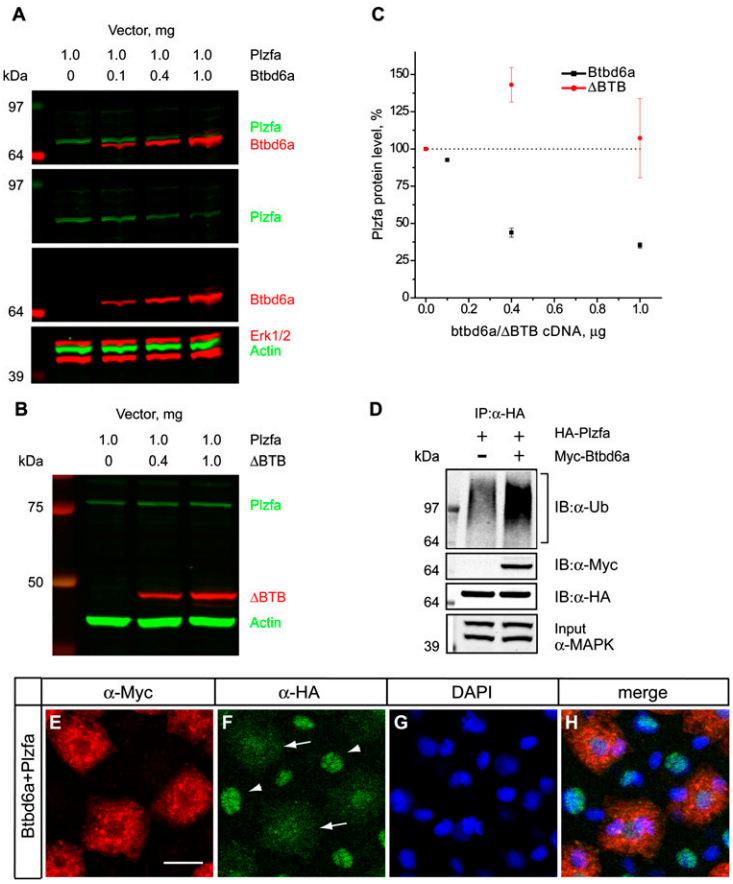

Figure 6. Btbd6a promotes ubiquitination, degradation, and nuclear export of Plzfa. (*A–C*) Effect of Btbd6a on steady-state level of Plzfa protein. A fixed amount of HA-tagged *plzfa* cDNA was cotransfected with increasing amounts of myc-tagged *btbd6a1* (*A*) or *btbd6aΔBTB* (*B*) cDNA, as indicated. Western blotting was carried out to detect Plzf, Btbd6a, and Erk and/or actin as loading controls. There is a progressive decrease in the level of Plzfa protein with increasing amounts of Btbd6a expression (*A*), but no decrease following coexpression with Btbd6aΔBTB, which does not bind Plzfa (*B*). (*C*) The graph is a representative of three independent experiments, with the Plzfa level normalized to actin staining. (*D*) Btbd6a mediates ubiquitination of Plzfa protein. HEK293 cells were transfected with HA-tagged *plzfa* with or without myc-tagged *btbd6a*, and, after 24 h, were treated with proteasome inhibitor MG132 for 4 h. Extracts were immunoprecipitated with anti-HA antibody and immunoblotted with anti-ubiquitin (α-UB), anti-myc, and anti-HA antibodies. MAPK was used as a loading control. (*E–H*) Btbd6a regulates the subcellular localization and amount of nuclear Plzfa protein. Fifteen picograms of RNA encoding HA-tagged Plzfa were injected in one-cell-stage zebrafish embryos, and then 35 pg of RNA encoding Myc-tagged full-length Btbd6a were injected at the four-cell stage to achieve mosaic expression. The subcellular localization of expressed proteins was detected with anti-Myc (*E*) and anti-HA (*F*) antibodies, and nuclei were stained with DAPI (*G*). In cells coexpressing Btbd6a, less Plzfa protein is found in the nucleus and more is found in the cytoplasm (arrows in *F*) compared with cells devoid of exogenous Btbd6a (arrowheads in *F*).

proneural gene expression. We uncovered a novel feedback loop required for primary neurogenesis that is mediated by a ubiquitin adaptor protein, Btbd6a. We show that the proneural gene *neurog1* up-regulates expression of *btbd6a*, that Btbd6a decreases nuclear levels and promotes degradation of the transcriptional repressor Plzf, and that *plzfa* is widely expressed in the neural epithelium and inhibits *neurog1* gene expression. Whereas knockdown of *btbd6a* leads to a major decrease in neurogenesis, its overexpression is sufficient to increase the amount of neurogenesis and to overcome the inhibition of neurogenesis by *plzfa*. The functional antagonism of *plzfa* by *btbd6a* is essential for neuronal differentiation, since *plzfa* knockdown alleviates the block in neurogenesis that occurs following knockdown of *btbd6a*. The up-regulation of *neurog1* gene expression required for primary neurogenesis is thus enabled by positive feedback in which a widely expressed inhibitor is targeted for degradation.

Feedback loops in the initiation of neurogenesis

Previous studies have revealed other feedback loops that promote neurogenesis (Bertrand et al. 2002; Gibert and Simpson 2003; Kageyama et al. 2005). One type of mechanism involves the inhibition of a repressor through binding; for example, in the vertebrate CNS, where proneural genes up-regulate expression of Hes6, which by heterodimerizing with Hes1 prevents it from inhibiting the expression and activity of proneural proteins (Bae et al. 2000; Koyano-Nakagawa et al. 2000). The regulatory logic of the role of Btbd6a and Plzfa is similar to this cascade, except that it involves targeted degradation of an inhibitor rather than formation of an inactive complex. As Btbd6a overexpression leads to a greater increase in neurogenesis than *plzfa* knockdown, Btbd6a may also target the degradation of another inhibitory factor. The widespread expression of an inhibitor of proneural gene expression that is itself inhibited or degraded downstream from proneural genes has two consequences. First, it sets a threshold to ensure that the initiation of differentiation is confined to cells in which sufficient proneural activity has been achieved. Second, once there is enough proneural activity to achieve positive feedback, this will underlie a discrete switch from a progenitor to neuronal differentiation. The degradation of an inhibitor may make such a progression less reversible than mechanisms involving binding and competition of activators and inhibitors.

Although the genes involved are not homologous, the roles of *plzfa* and *btbd6a* in primary neurogenesis are similar to *Tramtrack* and *Phyllopod* in *Drosophila*. *Tramtrack* encodes a BTB zinc finger transcriptional repressor that inhibits specific fates of photoreceptor and sensory organ cells (Xiong and Montell 1993; Guo et al. 1995). This inhibition is relieved by up-regulation of

Phyllopod, which acts as an adaptor to bring Tramtrack to the Sina ubiquitin ligase (Li et al. 2002), thus targeting Tramtrack for degradation (Li et al. 1997; Tang et al. 1997). In the eye, *phyllopod* expression is regulated upstream of proneural genes by activation of Raf and Ras1 (Chang et al. 1995; Dickson et al. 1995). However, in sensory organ progenitor cells, *phyllopod* expression is up-regulated downstream from *achaete–scute* proneural genes (Pi et al. 2004), and thus acts in a feedback loop analogous to that mediated by *btbd6a* in primary neurogenesis.

In contrast to our findings, a recent study has concluded that a *Xenopus laevis* homolog of *btbd6* is required for late steps of neuronal differentiation, since knockdown led to decreased expression of late but not early markers (Bury et al. 2008). Although this difference could be due to species-specific functions, we observed a similar decrease in late and not early neuronal markers following knockdown of either of the alternative *btbd6a* transcripts, whereas knockdown of both blocks the onset of neurogenesis. The knockdown of one transcript in *Xenopus* (Bury et al. 2008) may thus inhibit late but not early differentiation steps due to a partial blocking of *btbd6* function. These observations beg the question of why partial knockdown of Btbd6a has a stronger effect on late than on early markers of differentiation. One potential explanation is that Btbd6a targets the degradation of an inhibitor acting at multiple steps in the transcriptional cascade of neuronal differentiation; consequently, partial blocking of *btbd6a* function would have a cumulative inhibitory effect on late markers.

Relationship with lateral inhibition of neurogenesis

Our findings raise the question of the relationship between *btbd6a*, *plzf*, and the selection process that occurs due to Notch-mediated lateral inhibition (Fig. 7A). During lateral inhibition, high Notch activity in progenitors adjacent to differentiating neurons up-regulates *hes* genes, leading to inhibition of the expression of proneural genes and downstream Notch ligands. In contrast, Notch activity is low in cells that become selected to differentiate, thus alleviating the inhibition of proneural gene expression by Notch-dependent *hes* genes. Our finding that *btbd6a* function is required for primary neurogenesis reveals that the decrease in Notch activity is not sufficient to enable neuronal differentiation. Rather, it is required that Btbd6a is up-regulated in order to promote degradation of Plzf that otherwise would inhibit neurogenesis. Consistent with this, endogenous *plzfa* contributes to the inhibition of neurogenesis under conditions of low Notch activity, and knockdown of *plzfa* alleviates the block in differentiation that occurs following *btbd6a* knockdown. We therefore conclude that the feedback loop mediated by Btbd6a is essential in the progression from the selection of progenitors for differentiation to the initiation of neurogenesis (Fig. 7B).

Roles of PLZF

PLZF was discovered as a cause of specific forms of acute promyelocytic leukemia in which chromosomal translocation generates an abnormal fusion between PLZF and Retinoic Acid Receptor α (RARα) protein (Chen et al. 1993; Zelent et al. 2001). The PLZF-RARα fusion protein acts as a dominant-negative retinoic acid receptor and blocks retinoid signaling, whereas the reciprocal RARα-PLZF fusion interferes with PLZF repressor activity (He et al. 2000; Zelent et al. 2001). The induction of leukemia by these fusion proteins is in part due to *PLZF* being required to inhibit the growth and differentiation of myeloid precursors (Shaknovich et al. 1998; Yeyati et al. 1999; McConnell et al. 2003). Furthermore, *PLZF* is required for maintenance and self-renewal of spermatagonial stem cells (Buaas et al. 2004; Costoya et al. 2004). Our findings suggest that, in parallel with other mechanisms, *plzfa* inhibits the differentiation of progenitor cells during primary neurogenesis. These findings raise the prospect that PLZF contributes to the maintenance of progenitors in diverse cell lineages, perhaps by the direct or indirect repression of genes that promote differentiation.

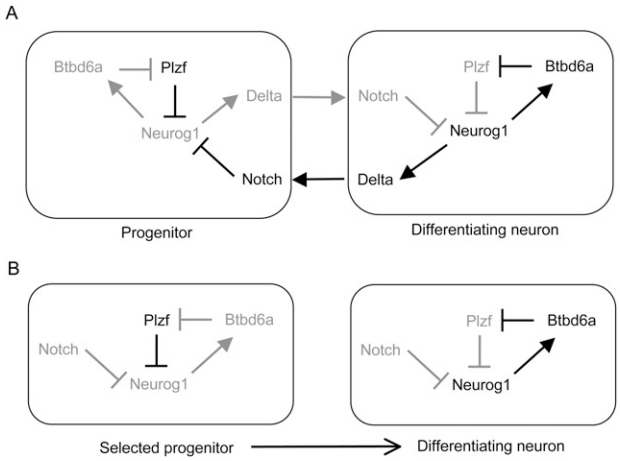

Figure 7. Model of the role of Btbd6a in neurogenesis. Depiction of the regulatory relationships deduced in this study, placed in the context of Notch-mediated lateral inhibition. (*A*) In progenitors, Notch signaling is dominant in the inhibition of differentiation by repressing the up-regulation of *neurog1*. The higher-level expression of *neurog1* required for neuronal differentiation is enabled by the up-regulation of Btbd6a, leading to degradation of Plzf, which inhibits neurogenesis. (*B*) Decreased Notch activation during the selection of cells to differentiate is not sufficient to initiate neurogenesis. The promotion of Plzf degradation by Btbd6a is thus essential for the transition from progenitor selection to the initiation of neuronal differentiation.

Ubiquitination and subcellular localization

Our finding that Btbd6a overexpression leads to decreased nuclear and increased cytoplasmic levels of Plzf protein is consistent with studies showing that ubiquitination of Plzf correlates with a shift in its distribution from nucleus to cytoplasm (Kang et al. 2008). This raises the question of the relationship between subcellular localization and ubiquitination. An attractive model is suggested by studies of the Keap1 adaptor protein that regulates the nucleocytoplasmic location and ubiquitination of the Nrf2 transcription factor. Keap1 affects Nrf2 localization in part by

sequestering it with cytoplasmic Cul3, leading to Nrf2 degradation (McMahon et al. 2003; Cullinan et al. 2004). In addition, binding of Keap1 to Nrf2 in the nucleus promotes export of the complex to the cytoplasm, where binding to Cul3 and degradation then occur (Sun et al. 2007). Similarly, Btbd6a could decrease the amount of Plzf available in the nucleus for transcriptional repression by promoting its nuclear export and/or by sequestering Plzf with Cul3 in the cytoplasm leading to ubiquitination and degradation.

Whereas the BTB domain of a number of adaptor proteins is capable of binding Cul3 (Geyer et al. 2003; Krek 2003; Pintard et al. 2003; Xu et al. 2003; Petroski and Deshaies 2005; Bury et al. 2008), our finding that Btbd6aΔBTB binds Cul3 indicates that the remaining region of Btbd6a, which is comprised of a BACK and PHR domain, is sufficient to mediate the interaction. Similarly, the BTB domain of Keap1 is not required for its interaction with Cul3 (Kobayashi et al. 2004). Furthermore, we find that the BTB domain of Btbd6a is required for binding to Plzf, consistent with it providing the specificity to recruit a substrate to the Cul3 complex. It will therefore be interesting to elucidate at the structural level how different ubiquitination adaptors mediate specific binding to Cul3 and to the proteins targeted for degradation.

Concluding remarks

Inhibitors of cell differentiation are widely used during development to regulate the maintenance of progenitors versus initiation of differentiation. Our finding of a pathway mediating the targeted degradation of an inhibitor of primary neuronal differentiation raises the question of whether analogous mechanisms operate elsewhere in the nervous system and other tissues. It will be interesting to determine whether other ubiquitination adaptors act downstream from transcription factors that regulate the onset of cell differentiation.

Materials and methods

Cloning and sequence analysis

A chick *btbd6* cDNA clone was isolated from a subtracted chick embryo hindbrain cDNA library (Christiansen et al. 2001). Zebrafish homologs were identified through BLAST searches (Altschul et al. 1997) of the NCBI and Ensembl databases, and the full-length cDNA sequences were determined from the following ESTs: *btbd6a1* (IMAGE ID: 4199804), *btbd6a2* (5334339 and 5334701), *btbd6b* (3714223 and 4955953). Full-length zebrafish *plzfa* cDNA was obtained from IMAGE ID: 3815539 and *plzfb* from Ensembl (ENSDARG00000015680), and was confirmed by RT–PCR of RNA from 2s- 13s-stage zebrafish embryos. All ESTs were purchased from RZPD or MRC Geneservice. For phylogenetic tree construction, sequences were aligned by the CLUSTAL W method (Thompson et al. 1994) using Lasergene (DNASTAR).

Generation of constructs

btbd6a and *plzfa* constructs in pCS2 + MT vector were created by PCR from full-length cDNA clones with the following primers: full-length Btbd6a1, CCGGAATTCCGTTCTCATGCCCGCTGC and TACGTAACCCCCCTACTCTCTCTTC; Btbd6a1ΔBTB, CC GGAATTCGGAGGCGCGAAATGCATG and TACGTAACCCC CCTACTCTCTCTTC; Btbd6a1ΔPHR, CCGGAATTCCGTTC TCATGCCCGCTGC and TACGTACACAGCGTCCACGGTA GC; Btbd6a2, CCGGAATTCGATGGCGGCGGAACTGTA and TACGTAGGATCAGTCCAAGTACTCAT; Plzfa, CCGGAATT CTATGGATTTGACTAAAATGGG and TACGTATCAGACGT AGCAGAGGTAG.

HA-tagged Plzfa was generated from the pCS2 + MT/plzfa construct by replacement of the myc tag. The following constructs have been described: *lacZ* (Xu et al. 1999), *neurog1* (Blader et al. 1997).

RT–PCR

The manufacturer's protocols were used to extract RNA using Trizol Reagent (GIBCO-BRL), first-strand cDNA synthesis with oligo(dT) or random hexamer primers using the SuperScript First-Strand Synthesis System (Invitrogen), and PCR with Expand High-Fidelity polymerase (Roche). Amplification was performed for 30 cycles of 30 sec at 94°C, 1 min at 56°C, and 2 min at 72°C using the following primers: *btbd6a1*, GAAGCGGG CAAGCAAGCA and TTCACCAGGAGGTCCAAC; *btbd6a2*, CACAAGTCCAGCCCTCGT and AATACGACTCACTATAG GGCTCAGATTTTGTGGGTTAGT.

Zebrafish maintenance

Zebrafish embryos were obtained by natural spawning and were raised as described (Westerfield 1994). The stage was determined by the number of somites and hours post-fertilization (Kimmel et al. 1995).

MO and RNA injections

MOs were purchased from Gene Tools. MO (0.8–6.5 ng) was injected into the yolk of one- to four-cell-stage embryos. The following MOs were used: *btbd6a1*, CGCAGCGGGCATGAGA ACGAGCGAG; *btbd6a2*, GTACAGTTCCGCCGCCATCCTC TTC; *neurog1*, ATACGATCTCCATTGTTGATAACCT; *plzfa*, TTCCCATTTTAGTCAAATCCATAAC; Control, CCTCTTAC CTCAGTTACAATTTATA.

For overexpression experiments, capped RNA was synthesized out as described (Xu et al. 1999) and 5–350 pg were injected into one cell at the one- or two-cell stage.

In situ hybridization

In situ hybridization was performed as described (Xu and Wilkinson 1998). Double detection with DIG- and fluorescein-labeled probes was carried out as described (Jowett 1998), except that the first probe was detected with BM Purple, followed by inactivation of alkaline phosphatase with 100% methanol for 20 min, and the second probe was detected with Fast Red (Roche). For double detection of RNA and β-galactosidase, embryos were fixed in 4% paraformaldehyde in PBS for 15 min and dechorionated, and, after washing several times in PBS, β-galactosidase was detected by staining in X-gal. Embryos were refixed in 4% paraformaldehyde in PBS for 35 min, and in situ hybridization was carried out using BM Purple substrate. Plasmid templates or PCR products that include a T7 RNA polymerase site were used to generate RNA probes. The following probes were used: *krox20* (Oxtoby and Jowett 1993); *neurog1* (Blader et al. 1997); *pax2a* (Krauss et al. 1991); *deltaA* and *deltaB* (Haddon et al. 1998); *isl1* (Korzh et al. 1993); *btbd6a* and *plzfa,* full-length cDNAs; *plzfb,*

PCR product using AGGATGTTGAAGACCGCAG and AAT ACGACTCACTATAGGGGCAGAACTCACAGCCAAAG.

DAPT treatment

DAPT treatment was performed as described (Geling et al. 2002) using 0.01–0.02 mM DAPT (Calbiochem) in Danieau solution applied to zebrafish embryos from 4 h post-fertilization (hpf) to the 3–5s stage.

Yeast two-hybrid screen (Poliakov et al. 2008)

A yeast two-hybrid screen was performed by Hybrigenics (http://www.hybrigenics.com) on an 18- to 20-h zebrafish cDNA library using full-length Btbd6a1 as bait.

Cell culture, immunoprecipitation, and Western blotting

HEK293 cells were grown as described (Poliakov et al. 2008). Stable cell lines expressing myc epitope-tagged Btbd6a1, Btbd6a1ΔBTB, or Btbd6a1ΔPHR were generated by cotransfection of clones in pCS2 + MT with pcDNA3 vector (Invitrogen). Cells were selected in the presence of 900 μg/mL G418 for 2 wk, and expression was confirmed by Western blotting. Coimmunoprecipitation, SDS-PAGE, Western blotting, and immunocytochemistry of HEK293 cell lines were carried out as described (Poliakov et al. 2008). For immunocytochemistry of zebrafish embryos, they were fixed in 4% paraformaldehyde for 45 min, washed in PBS, dechorionated, dehydrated in methanol, and rehydrated in PBS/0.1% Tween. After blocking with 10% goat serum, embryos were incubated with primary antibodies in 2% goat serum overnight at 4°C, then washed and incubated with secondary antibodies for 2 h at room temperature.

Ubiquitination assay

HEK293 cells transfected with different combinations of HA-tagged *plzfa* and myc-tagged *btbd6a* cDNA were incubated for 24 h and then treated with 10 μM MG-132 for 4 h before lysis. Cell lysates were incubated with anti-HA Affinity matrix (Roche Diagnostics) for 3 h at 4°C under rotation. Bound proteins were eluted from the matrix and subjected along with total cell lysates to SDS-PAGE and Western blotting.

Antibodies

Antibodies were from the following sources: monoclonal 9E10 anti-myc, rabbit polyclonal anti-myc, goat polyclonal anti-Cul3, anti-Plzf, anti-ubiquitin, and anti-actin (Santa Cruz Biotechnologies); rabbit polyclonal anti-ERK1/2 and rabbit polyclonal anti-MAPK (Sigma); rat anti-HA monoclonal antibodies (Roche); rabbit anti-Cul3 (Abcam); Alexa Flour 488- and 594-conjugated goat anti-rat and goat anti-mouse (Molecular Probes); donkey anti-mouse CY2 (Jackson ImmunoResearch).

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