The splicing co-factor Barricade/Tat-SF1 is required for cell cycle and lineage progression in Drosophila neural stem cells

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ABSTRACT
Stem cells need to balance self-renewal and differentiation for correct tissue development and homeostasis. Defects in this balance can lead to developmental defects or tumor formation. In recent years, mRNA splicing has emerged as an important mechanism regulating cell fate decisions. Here we address the role of the evolutionarily conserved splicing co-factor Barricade (Barc)/Tat-SF1/CUS2 in Drosophila neural stem cell (neuroblast) lineage formation. We show that Barc is required for the generation of neurons during Drosophila brain development by ensuring correct neural progenitor proliferation and differentiation. Barc associates with components of the U2 small nuclear ribonucleoprotein (snRNP) complex, and its depletion causes alternative splicing in the form of intron retention in a subset of genes. Using bioinformatics analysis and a cell culture-based splicing assay, we found that Barc-dependent introns share three major traits: they are short, GC rich and have weak 3′ splice sites. Our results show that Barc, together with the U2 snRNP complex, plays an important role in regulating neural stem cell lineage progression during brain development and facilitates correct splicing of a subset of introns.

KEY WORDS: Drosophila, Splicing, Intron retention, Brain development, Barricade, Cell cycle

INTRODUCTION
Stem cells have the unique ability to generate differentiating daughter cells while maintaining their stem cell fate. This process needs to be tightly balanced for correct tissue development (Morrison and Kimble, 2006; Shenghui et al., 2009; Weissman, 2000). During brain development, failure to limit stem cell proliferation or to establish a differentiated state can result in ectopic stem cells that proliferate unrestrictedly and cause tumorigenic overgrowth, as in the case of pediatric brain tumors (Eberhart, 2007; Hemmati et al., 2003; Wang and Wechsler-Reya, 2000). During brain development, Barricade, Cell cycle

and differentiation is crucial for tissue development and the prevention of pathological states.

The Drosophila larval brain harbors neural stem cells called neuroblasts (NBs) that divide asymmetrically into a self-renewing NB and a differentiating daughter cell. Per central brain lobe, there are ∼100 type I NBs (expressing the markers Miranda (Mira), Deadpan (Dpn) and Asense (Ase)), and eight type II NBs (expressing Mira and Dpn but not Ase). Upon asymmetric cell division, type I NBs produce a ganglion mother cell [GMC; expressing Ase and Prospero (Pros)], which divides into two neurons or glias cells (Doe, 2008; Homem and Knoblich, 2012; Knoblich, 2008; Reichert, 2011).

Type II NBs produce intermediate neural progenitors (INPs), which mature and undergo four to six asymmetric cell divisions, generating a new INP and a GMC each time (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). While all INPs express Mira, the immature INPs are initially Ase−Dpn−, but as they mature they re-express first Ase, then Dpn (Fig. 1A) (Bayraktar et al., 2010; Bowman et al., 2008). As they contain a transient amplifying population of INPs, type II NBs generate more than twice as many progeny as a type I NB (Bello et al., 2008) and they are often compared to mammalian neural stem cells (Homem and Knoblich, 2012).

It has been demonstrated that INPs pass through different windows of competence as they age. This temporal patterning is achieved by sequential expression of the transcription factors Dichaete, Grainy head and Eyeless, and contributes to increasing the neural diversity in the brain (Bayraktar and Doe, 2013).

Limited cell migration and distinctive marker combinations present in NBs and their progeny make the Drosophila larval brain a good system with which to study stem cell self-renewal and differentiation (Dumstrei et al., 2003; Kang and Reichert, 2015; Reichert, 2011; Spindler and Hartenstein, 2010; Weng and Lee, 2011; Weng et al., 2010). Several studies have identified factors and mechanisms that regulate the self-renewal and differentiation of NBs and their progeny (Homem et al., 2015; Kang and Reichert, 2015).

In a genome-wide RNAi screen for regulators of NB self-renewal, we identified a lineage regulator, barricade (barc) (Neumüller et al., 2011). Knockdown of barc induced a differentiation block resulting in an accumulation of INPs, a phenotype distinct from those caused by knockdown of other INP regulators (Neumüller et al., 2011). Barc is the Drosophila homolog of mammalian Tat-specific factor 1 (Tat-SF1; HTATSF1) and yeast CUS2. Tat-SF1 was first identified as a co-factor stimulating Tat-directed HIV-1 transcriptional elongation (Zhou and Sharp, 1996) and was later found to be a general transcriptional elongation factor (Chen et al., 2009; Li and Green, 1998; Parada and Roeder, 1999). Consistent with the yeast homolog CUS2, which has a well-characterized role in splicing (Perriman and Ares, 2000, 2007; Perriman et al., 2003; Rodgers et al., 2016; Yan et al., 1998), Tat-SF1 has also been suggested to

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play a role in splicing (Fong and Zhou, 2001; Miller et al., 2011, 2009; Yan et al., 1998; Zhou et al., 2002). However, its specific role in this process has not been characterized. In addition, there are no in vivo studies of Tat-SF1 in higher eukaryotes, nor are there studies addressing its role in the context of stem cell self-renewal and differentiation.

Fig. 1. Knockdown of barc increases progenitor cell numbers. (A) Schematic of a Drosophila larval central brain lobe with type I and II neuroblast (NB) lineages and expressed markers. (B, C) Representative wild-type and barc RNAi type II NB lineages, labeled by coexpressed UAS-mCD8::GFP. RNAi was expressed using insc-Gal4. (B) Overview and magnification of type II lineages among barc knockdown. Mira labels progenitor cells. Asterisks, type II NBs. Compared with separated control lineages, barc RNAi type II lineages are often close together. (C) barc RNAi type II lineages display an accumulation of immature Ase⁺ Dpn⁻ Pros⁻ INPs and fewer GMCs and neurons compared with control. Asterisks, type II NBs; white arrowheads, immature INPs (Ase⁻ Dpn⁺ Pros⁻); yellow arrowheads, GMCs (Ase⁻ Dpn⁻ Pros⁺). (D) Quantification of total INP numbers per dorsomedial type II lineage. Control: n=14 lineages, 3 brains. barc RNAi: n=25 lineages, 6 brains. (E) Quantification of immature (Ase⁻ Dpn⁻ Pros⁻ and Ase⁺ Dpn⁻ Pros⁻) and mature (Ase⁺ Dpn⁺ Pros⁻) INPs in wild-type and barc RNAi type II lineages. Based on the same dataset as in D. (F) Model depicting the altered type II lineage composition upon barc knockdown. (G) Type I NB lineages in the central brain. RNAi was expressed using ase-Gal4. Asterisks, type I NBs; yellow arrowheads, Ase⁺ Pros⁺ GMCs; white arrowheads, neurons (Ase⁻ Pros⁻). (H) Quantification of GMC numbers per type I lineage. barc knockdown results in increased GMC numbers. Control: n=64 lineages, 4 brains. barc RNAi: n=70 lineages, 4 brains. Quantification based on control and barc RNAi expressed using ase-Gal4. Data are mean±s.d. Student’s t-test (D, H). Two-way ANOVA (E). ns, non significant; *P<0.05, ***P<0.001. Scale bars: 20 µm in B, C; 10 µm in G.
Here, we employed the *Drosophila* NB system to investigate the role of Barc in neural stem cell differentiation in vivo. We show that loss of Barc results in a cell cycle defect in the neural progenitor cells, and in an accumulation of INPs and GMCS at the expense of neurons. We further show that Barc associates with members of the U2 snRNP complex and plays a crucial role to ensure proper and efficient splicing of short, GC-rich introns with weak 3′ splice sites.

**RESULTS**

**barc loss of function causes an accumulation of neural progenitor cells at the expense of neurons**

We have previously identified *barc* as a regulator of *Drosophila* neural stem cell lineage progression, and revealed that Barc is a nuclear protein expressed in all cells of type I and II NB lineages (Neumüller et al., 2011). Expression of a UAS-*barc* RNAi construct using *insc*-Gal4 (the *insc*-Gal4::UAS-mCD8::GFP driver line is used throughout the present study unless otherwise stated), resulted in an accumulation of *Mira*− INPs in dorsomedial type II NB lineages (Fig. 1B) (Neumüller et al., 2011). Quantification confirmed that the total number of INPs per lineage was increased by 20% upon *barc* RNAi [38.2±2.2 (±s.e.m.) upon *barc* RNAi versus 31.8±1.4 in control] (Fig. 1D).

Analysis of the INP composition showed that, on average, Barc-depleted dorsomedial type II lineages exhibited no difference in *Ase*− *Dpn*− immature INP numbers but a 300% increase (11.7±1.3 upon *barc* RNAi versus 2.9±0.2 in control) in immature *Ase*− *Dpn*− INP numbers (Fig. 1C,E). No significant difference (24.2±3.3 upon *barc* RNAi versus 26.3±1.5 in control) was observed in mature *Ase*− *Dpn*− INP numbers (Fig. 1C,E). There were also fewer GMCS and *Pros*− neurons in the *barc* RNAi type II lineage clusters (Fig. 1C,F). This suggests that the increase in INP numbers upon Barc depletion may be due to impaired INP maturation and differentiation.

In order to test if the *barc* RNAi type I lineages also displayed an accumulation of progenitor cells we counted GMC numbers. Whereas control type I lineages contained 4.8±0.1 GMCs per lineage, *barc* RNAi resulted in 6.1±0.2 GMCs per lineage upon *ase*-Gal4-mediated *barc* knockdown (Fig. 1G,H). A similar accumulation was observed upon *insc*-Gal4-mediated *barc* knockdown (Fig. S1B). Similarly to the type II lineages, staining for the neuronal markers *Pros* and *Elav* showed a decreased neuronal output upon *barc* RNAi (using *ase*-Gal4) (Fig. 1G, Fig. S1A).

To ensure that the observed *barc* RNAi phenotype is specific to the loss of Barc, we overexpressed an RNAi-resistant *barc* coding sequence in the *barc* RNAi background. This rescued both the type II and type I lineage phenotypes (Fig. S2A,C,D; type I rescue not shown) (Neumüller et al., 2011). Taken together, these data suggest that Barc is required for neuronal progenitors to both proliferate and differentiate into neurons.

**Barc** mutant clones confirm immature INP accumulation and underproliferation

To gain more insight into the *barc* loss-of-function phenotype, we generated a *barc* loss-of-function allele using CRISPR. *barc*^mut1,2^ harbors a deletion in exon 2, which causes a frameshift and premature stop (Fig. 2A). As the homozygous mutant is early lethal, we used the MARCM system (Lee and Luo, 1999) to generate homozygous mutant NB clones in a heterozygous mutant background. Similar to the RNAi phenotype, 90-96 h *barc* mutant type II NB clones consisted of *Mira*− *Ase*− *Dpn*− INPs and comparatively few neurons (Fig. 2B,C). In contrast to the control clones, *barc* mutant clones contained many *Mira*− *Ase*− *Dpn*− INPs (Fig. 2B,C). Careful quantification confirmed the accumulation of immature *Ase*− *Dpn*− *Pros*− INPs and revealed a decrease in mature *Ase*− *Dpn*− *Pros*− INPs (Fig. 2E, Fig. S3). Overall there was no difference in total INP numbers (Fig. 2D). Thus, the *barc* mutant clones display a phenotype similar, but not identical, to the *barc* RNAi phenotype. This can be explained by different *barc* depletion efficiency, different timing of the phenotype analysis or fewer analyzed type II clones compared with RNAi depleted type II lineages. Consistent with the *barc* RNAi phenotype, the type I NB clones displayed an increase in GMC numbers (Fig. 2F,G). Quantification revealed a severe decrease in total cell numbers in both type I and II NB lineages, predominantly due to loss of neurons (Fig. 2F,H,I). Overall, key phenotypes were consistent between the *barc* mutant clones and the RNAi phenotype, and further insights were derived from the MARCM clones confirming the underproliferation of the *barc* RNAi phenotype (Fig. 2J,K).

**Overexpression of mouse Tat-SF1 rescues the *barc* RNAi phenotype**

To test if the Barc function is evolutionarily conserved, we generated a myc-tagged mouse Tat-SF1 transgene and overexpressed it in a *barc* RNAi background. Interestingly, the mammalian homolog also rescued the *barc* RNAi phenotype, indicating that the fly and mammalian homologs indeed have conserved functions (Fig. S2B-D).

**barc** RNAi alters INP temporal patterning and causes neuroanatomical defects in adult brain morphology

INPs sequentially express the young INP marker *Dichaeta* (D), the middle-age INP marker *Grany* (Grh) and the old INP marker *Eyeless* (Ey) (Bayraktar and Doe, 2013) (Fig. S4A). As inhibition of *barc* by RNAi affected INP maturation, we tested whether it also affects their temporal patterning. Indeed, INPs appeared stuck at the D− stage due to maintained D expression but no re-expression of Grh or Ey (Fig. S4B,C). This suggests that Barc is important for both the maturation and temporal patterning of INPs.

INP progeny generated during larval stages contribute strongly to the adult brain structure called the central complex (CCX) (Bayraktar and Doe, 2013; Bayraktar et al., 2010; Izergina et al., 2009; Viktorin et al., 2011). As *barc* RNAi has a strong INP phenotype, we knocked down *barc* using *PntP1-Gal4*, which is expressed in all type II NB lineages in larval brains (Zhu et al., 2011) and in a small subset of type II NB-derived neurons that innervate the CCX in adults (Fig. S5). This resulted in adult brains with neuroanatomical defects in the CCX and a decreased number of type II NB-derived neurons, which localize in the posterior brain region (Fig. S5). Thus, Barc-depleted type II lineages fail to produce sufficient neurons for the correct development of adult brain structures.

**Barc-depleted neural progenitor cells display an extended cell cycle with a G2/M delay**

The accumulation of progenitors and their increased lifespan could be caused by defects in cell division, differentiation or the cell cycle. Our initial characterization revealed larger type I NBs upon *barc* RNAi (Neumüller et al., 2011), a phenotype consistent with a cell cycle delay (Neufeld et al., 1998). We confirmed this phenotype in type I NBs as well as in Barc-depleted GMCS, type II NBs and INPs (Fig. S6A-D).

To address the effect of *barc* RNAi on cell cycle progression, we quantified the cell cycle length in control and *barc* RNAi conditions.
Fig. 2. barc mutants confirm GMC and immature INP accumulation at the expense of neurons. (A) The barc genomic locus. The barcm4-2 mutant allele results from a one nucleotide deletion in exon 2, leading to a frameshift and premature stop codons. (B,C) Dorsomedial control (B) and barcm4-2 mutant (C) type II NB MARCM clones 90-96 h after clone induction. Three separate z-planes are included to show the NBs, INPs and neurons. Asterisks, NBs; yellow arrowheads, Mira+ Ase− Dpn− INPs; white arrowheads, Mira+ Ase+ Dpn− INPs; arrows, Mira+ Ase+ Dpn+ INPs. (D) Total INP numbers per control or barcm4-2 mutant type II NB MARCM clone. Control: n=9 clones, 8 brains. barcm4-2: n=7 clones, 6 brains. (E) Quantification of immature (Ase− Dpn− Pros− and Ase+ Dpn− Pros−) and mature (Ase+ Dpn+ Pros−) INPs in control or barcm4-2 mutant type II NB MARCM clones. Based on the same dataset as in D. (F) 90-96 h control and barcm4-2 type I NB MARCM clones. Asterisks, NBs; arrows, GSCs. (G) Quantification of GMC numbers per control or barcm4-2 mutant type I NB clone. Control: n=17 clones, 7 brains. barcm4-2: n=30 clones, 12 brains. (H) Total cell numbers per control or barcm4-2 mutant type II NB MARCM clone. Control: n=9 clones, 7 brains. barcm4-2: n=5 clones, 4 brains. (I) Total cell numbers per control or barcm4-2 mutant type I NB MARCM clone. Control: n=17 clones, 7 brains. barcm4-2: n=17 clones, 6 brains. (J,K) Models depicting the altered type II (J) and type I (K) lineage composition in barc mutants. Data are means±s.d. Student’s t-test (D,G,H,I). Two-way ANOVA (E). ns, non significant; *P<0.05, **P<0.01, ***P<0.001. Scale bars: 10 µm.
using live cell imaging (Homem et al., 2013). Indeed, Barc-depleted type II NBs (RNAi expressed using wor-Gal4, axe-Gal80) divided on average 35% (32 min) slower than controls (Fig. 3A,B). Likewise, the INP maturation time (from cell formation to first division) was extended on average by 58% (3 h) upon loss of Barc (Fig. 3C). barc knockdown (using axe-Gal4) extended the cell cycle of type I NBs on average by 23% (18 min; Fig. 3E,F) and of GMCs by 25% (1 h; Fig. 3G). Thus, loss of Barc extends the cell cycle of all three types of neural progenitor cells (Fig. 3D,H).

Using FACS analysis we assessed the DNA content in INPs and GMCs (as they are more abundant than NBs), which revealed a higher proportion of cells in G2/M phase upon barc RNAi (Fig. 3I,J). Consistently, Barc is also required for proper cell cycle progression in S2 cells (Andersen and Tapon, 2008).

Thus, Barc is necessary for proper cell cycle progression in both neural progenitors and S2 cells.

**Barc associates with the U2 snRNP complex**

To understand the molecular function of Barc, we analyzed its binding partners using a transgenic HA-tagged Barc protein (Fig. S8A-C). Upon immunoprecipitation of Barc from larval brains and subsequent MS analysis we identified 15 binding partners (Table S1). Using STRING (Szklarczyk et al., 2015) we analyzed the relationships of the interactors (Fig. 4A). Barc strongly interacted with several splicing factors, which are important for both prespliceosome formation and the U2 small nuclear ribonucleoprotein (U2 snRNP) complex, one of several multiprotein complexes that bind to introns during pre-mRNA splicing (Herold et al., 2009; Mount and Salz, 2000; Will and

![Fig. 3. barc loss of function results in increased cell cycle duration with a G2/M phase delay.](image-url)
Lührmann, 2011) (Fig. 4B,C). Nuclease treatment of the larval brain extract suggested that most of the interactions between Barc and the splicing factors are dependent on DNA or RNA (Table S1). Additionally, rescue experiments using a transgenic Barc protein with a mutated RNA recognition motif (RRM) suggested that the interaction of Barc with the splicing factors is mediated via RNA (Fig. S9A,B). Thus, Barc associates with U2 snRNP (Fig. 4D) in a nucleic acid-dependent manner, consistent with the described functions of its homologs CUS2 and Tat-SF1 (Fong and Zhou, 2001; Yan et al., 1998; Zhou et al., 2002).

To test whether the interaction of Barc with the U2 snRNP complex is functionally relevant, we depleted four of the most abundant U2 snRNP-related interaction partners. RNAi of the most abundant interactor, CG6227 (dPRP5), a prespliceosome RNA
RNAi expression, we depleted resulted in a phenotype similar to that of using a temperature-sensitive resulted in an almost complete loss of type I lineages (Fig. S10). We conclude that the temperature-sensitive Barc knockdowns displayed phenotypes of different strength, with (Fig. S11) (Neumüller et al., 2011). In general, the specific subunit from relatively normal type II lineages to their complete loss and observed a gradient of underproliferation phenotypes ranging (NEUMÜLLER ET AL., 2011). Barc is important for the proper splicing of a subset of introns. As depletion of various snRNP subunits results in cell cycle defects in the G2/M phase (Andersen and Tapon, 2008; Hofmann and Burge, 2001; Mount et al., 1992) and from a control splice site (Controlexpressed) based on all introns expressed in NBs and S2 cells. We further observed occasional Ase upregulation in the type II NBs and is in line with previous observations (Brooks et al., 2015). To understand the specificity of the barc loss-of-function splicing defects, we analyzed the characteristics of the 282 introns retained in both NBs and S2 cells. Barc-dependent introns were significantly shorter than control introns: median Barc-dependent intron (retained), 60 nt; median wild-type expressed intron (expressed), 70 nt; median D. melanogaster intron (all), 93 nt (Fig. 5D). We also characterized their GC content. Since short Drosophila introns (<81 nt) have different characteristics to long introns (≥81 nt) (Mount et al., 1992), we analyzed the retained introns against (1) the introns from all genes expressed in both the cell types and (2) a random subsample of 282 introns with the same length distribution as the retained intron dataset (Fig. S13). The Barc-dependent introns had a significantly higher GC content than the control introns [median Barc-dependent intron (retained), 0.40; median wild-type expressed intron (expressed), 0.36; median short intron (short introns), 0.33] but still lower than the average exon GC content (0.53) (Zhu et al., 2009) (Fig. 5E). Overall, Barc-sensitive introns tend to be short and GC rich. In addition to GC content and length, splice site strength is another feature that influences splicing outcome (Sakabe and de Souza, 2007; Shepard et al., 2011). We analyzed the 5’ and 3’ splice site motifs in all common, retained introns from the NB and S2 RNAseq datasets and compared them with the same controls used for the GC content analysis. The Barc-sensitive introns had 5’ and 3’ splice site motifs [including the polypyrimidine tract (PPT)] deviating both from known splice site consensus sequences (Lim and Burge, 2001; Mount et al., 1992) and from a control splice site motif (Controlexpressed) based on all introns expressed in NBs and S2 cells (Fig. 5F). It was previously demonstrated that short Drosophila introns have a weaker PPT (containing fewer pyrimidines) than long introns (Mount et al., 1992), but our retained intron dataset displayed an even weaker PPT (Controlshort versus Retained) as well as a weak intron-exon junction sequence, resulting in a 3’ splice site predicted to be even weaker (Fig. 5F). Thus, Barc-sensitive introns tend to be short, GC rich and have weak splice sites. Barc-sensitive introns have a weak 3’ splice site To test whether Barc sensitivity was conveyed by intron sequence or by the context, we designed a minigene assay to study splicing in...
S2 cells. We used the Pcmt gene, which displayed clear retention of its first, short (<81 nt) and GC-rich (47.5%) intron but not of its second, long intron (>81 nt) of average (36.9%) GC content. We restricted our analysis to the endogenous sequence between exon 1 and exon 3 (first 282 nt; minigene Ppcmt) (Fig. 6A,B). Upon Barc depletion, splicing of the minigene Ppcmt recapitulated the previously observed Pcmt splicing defects (Fig. 6B′) and thus provided a good system with which to study Barc-dependent splicing. Replacing the first, Barc-sensitive intron with a Barc-independent, short, average GC content (37.5%) intron (intron 11 from mus205; minigene Pmus205) completely rescued the splicing defect (Fig. 6B,B′, Fig. S14), indicating that Pcmt intron 1 splicing is Barc dependent due to the intronic sequence.

This effect could be the result of lower splice site strength in the Barc-sensitive introns. As we identified U2 snRNP as the main interactor of Barc, it is plausible that Barc deficiency acts via U2 snRNP by affecting its stability, conformation or composition. As U2 snRNP binds to the 3′ end of the intron (Will and Lührmann, 2011), we focused on the difference in 3′ splice site strength and tested whether our minigene sequences recapitulated the above 3′ splice site motif analysis. The Barc-sensitive intron (Pcmt i-1) had an A-rich 3′ splice site, in contrast to the T-rich 3′ splice site of the Barc-independent intron (mus205 i-11) (Fig. 6C), and their differential strength was further confirmed by the Berkeley Drosophila Genome Project (BDGP) splice site predictor (Reese et al., 1997) (mus205 i-11, 0.95; whereas no Pcmt i-1 3′ splice site could be identified). Given the decreased 3′ splice site strength in Barc-sensitive introns, we tested whether the Barc-dependent splicing was influenced by the strength of the 3′ splice site. Indeed, enhancing the weak 3′ splice site by replacing parts of the Pcmt i-1 sequence with 6-48 bp sequence stretches from mus205 i-11 restored efficient splicing in Barc-depleted cells (minigenes a-e; Fig. 6D).
Conversely, we tested whether a Barc-independent intron (mus205 i-11 in the Pmus205 minigene) could become Barc dependent by weakening its 3′ splice site. Replacing parts of the mus205 i-11 sequence with 6-48 bp sequence stretches from Pcmt i-1 (minigenes f-j; Fig. 6E) was indeed sufficient for the splicing of this intron to become Barc dependent. However, instead of resulting in intron retention, we observed Barc-dependent skipping of exon 2 (Fig. 6E), which is another splicing decision influenced by splice site strength or differential GC content between intron and exons (Amit et al., 2012; Ma et al., 2011; Shepard et al., 2011).

As splicing of a terminal intron has been coupled to 3′ UTR formation (Kaida, 2016; Rigo and Martinson, 2008), we tested...
whether the exon skipping outcome shown in Fig. 6E was due to the original terminal location of mus205 i-11. We repeated parts of the minigene assay using another Barc-independent mus205 intron, namely intron 6. Again, minigenes containing hybrid introns between Pcmnt i-1 and mus205 i-6 underwent exon skipping upon Barc depletion (Fig. S15). As mus205 i-6 is a middle intron, and not a terminal intron like i-11, we conclude that the exon skipping outcome shown in Fig. 6E is not due to mus205 i-11 being a terminal intron. Taken together, these results suggest that a strong 3′ splice site is required for Barc-independent splicing.

We conclude that Barc is a splicing regulator that associates with U2 snRNP and is required for efficient splicing of short, GC-rich introns with weak 3′ splice sites (Fig. 7).

**DISCUSSION**

Our data show that the *Drosophila* CUS2 and Tat-SF1 (HTATSF1) homolog Barricade (Barc) acts as a cell cycle regulator and is required for neural stem cell lineage specification in the larval brain. Loss of Barc leads to neural progenitor accumulation and insufficient neuronal output. Furthermore, we have shown that Barc is a splicing co-factor, facilitating the splicing of a subset of introns in genes acting in the cell cycle and DNA damage response processes.

Our previous analysis indicated that Barc regulates type II NB lineage progression and that its loss causes INPs to accumulate (Neumüller et al., 2011). In this study, we show that the ectopic INPs predominantly consist of immature Ase+ Dpn+ INPs, although the exact ratios of immature to mature INPs vary for each lineage. As immature INPs seem to be arrested in G2 phase (Bowman et al., 2008), it is possible that Barc plays a role in timely re-activation of INP proliferation and thereby facilitates their maturation. In recent years, the transcription factors PntP1 (PntP1), Earmuff (Erm) and Buttonhead (Btd) have been shown to play important and distinct roles in INP maturation. PntP1 ensures type II identity and INP generation, Erm restricts the developmental potential of INPs, and Btd ensures that the INPs do not undergo premature differentiation (Komori et al., 2014; Weng et al., 2010; Xie et al., 2014; Zhu et al., 2011). Our results suggest that Barc is likewise an INP regulator required for proper INP maturation and temporal patterning. However, it remains to be elucidated if and how these processes are interconnected.

We have also observed that type II lineages contain few GMCs, in contrast to type I lineages, which display an accumulation thereof. However, it is not surprising that the effect of Barc depletion on GMCs might vary between type I and type II NB lineages as they are generated from different cell types in the different lineages, and loss of Barc strongly affects INPs whereas it only mildly affects type I NBs. Therefore, it is more meaningful to compare the effect of Barc depletion in NBs and their immediate progeny cell type (NB I and GMCs, versus NB II and INPs), which in both lineage types are accumulated upon Barc depletion.

An interesting observation is the lack of separation between Barc-depleted type II NB lineages (Fig. 1B,C,F). It is unclear whether the lineages simply lie in close proximity to one another (due to few generated daughter cells pushing them apart), or if the lineages are actually fused together. If the lineages were indeed fused, it could suggest that barc is important for the formation and/or maintenance of cortex glia chambers, which surround the NB, GMCs and neurons of individual lineages (Hartenstein, 2011; Pereanu et al., 2005). In the future, it would be interesting to determine whether Barc-depleted type II lineages are fused, and elucidate a potential role of barc in cortex glia formation. A better understanding of this process could shed light on the importance of cortex glia for NB lineage development.

We have shown that Barc is in a complex with U2 snRNP and is required for efficient splicing of a subset of introns. Our data are consistent with a model in which splicing of a set of genes is crucial for proper NB lineage progression. In this model, loss of Barc would result in the retention of Barc-sensitive introns in cell cycle, DNA repair and mRNA processing genes. This would result in degraded, truncated or altered proteins, affecting those processes during NB lineage progression.

In this model, two scenarios could explain the barc RNAi phenotype. First, Barc could be crucial for adequate splicing of one or a limited number of key target genes. To assess this hypothesis, we picked a few genes, which were highly misspliced upon barc knockdown, and performed single-gene rescue experiments. However, none of these genes was able to rescue the barc RNAi phenotype when expressed individually (data not shown). Second, the barc knockdown phenotype might be caused by a global effect on splicing. This scenario seems particularly plausible since as many as 479 genes were misspliced in the Barc-depleted type I NBs. Although we cannot exclude that, among these inefficiently spliced genes, there are a few that strongly contribute to the phenotype, we currently favor a model whereby the cumulative effect of mild changes in gene expression of many cell cycle, DNA repair and differentiation genes manifest in the observed phenotype.

Although it would be interesting to determine the direct targets of Barc in INPs, the rarity of this cell type meant that we could not collect sufficient material to prepare RNAseq data of good quality. Instead, our RNAseq was performed using the abundant type I NBs.
and although it cannot be employed to identify crucial Barc targets in INPs, it revealed that Barc functions as a splicing co-factor that is important for the proper splicing of a subset of introns in many genes, which suggests that it has a global effect on splicing.

Why are these introns affected in the first place? We have shown that the Barc-sensitive introns in general are short, GC rich and have weak 3’ splice sites, features that impair intron definition by the spliceosome and are likely to make them more sensitive to spliceosomal alterations (Ge and Porse, 2014). An impaired intron-exon distinction by the spliceosome could explain why barc RNAi can cause both intron retention and exon skipping, as observed in the minigene assay. This is further supported by previous studies (Ma et al., 2011; Romano et al., 2001; Sakabe and de Souza, 2007; Shepard et al., 2011; Ward and Cooper, 2010; Wickramasinghe et al., 2015; Amit et al., 2012; Galante et al., 2004; Wong et al., 2013).

Interestingly, not only do the retention-prone introns share similar characteristics, but they are also sometimes conserved or located in genes acting in similar processes (e.g. cell cycle, DNA repair, mRNA processing) or in equivalent tissues across different organisms (e.g. the nervous system) (Boutz et al., 2015; Braunschweig et al., 2014; Burns et al., 2002; Dvinge and Bradley, 2015; Galante et al., 2004; Jia et al., 2012; Lareau and Brenner, 2015; Mamon et al., 2013; Sakabe and de Souza, 2007; van der Leij et al., 2014; Wickramasinghe et al., 2015; Wong et al., 2013). This suggests that retention-prone introns might be biologically significant and could have regulatory functions, an exciting hypothesis in line with previous observations (Boutz et al., 2015; Galante et al., 2004; Ge and Porse, 2014; Wong et al., 2016). Such a biological function of intron retention could be controlled by changes in spliceosome composition due to varying splicing factor levels, which have been shown to vary across cell types and development, and to influence splicing outcomes (Grosso et al., 2008; Papasakias et al., 2015; Park et al., 2004; Wong et al., 2013).

While mammalian Tat-SF1 has mainly been described to act in transcriptional elongation, our data suggest a stronger functional similarity of Barc with yeast CUS2. Similar to barc knockdown, RNAi of CG6627 (dPRP5), the most abundant Barc interactor, also resulted in additional Ase’ Dpn’ immature INPs, although the total number of INPs was not affected. As RNAi of core splicing factors causes strong underplication phenotypes (Fig. S11), it is possible that the discrepancy between the barc and CG6627 RNAi phenotypes is due to varying splicing factor levels, which have been shown to vary across cell types and development, and to influence splicing outcomes (Grosso et al., 2008; Papasakias et al., 2015; Park et al., 2004; Wong et al., 2013).

MATERIALS AND METHODS

Fly strains, husbandry and clonal analysis
Fly strains are described in the supplementary Materials and Methods. Fly crosses were set up at 25°C, UAS transgenes were expressed at 29°C, and wandering L3 larvae were dissected. For experiments with the temperature-sensitive tub-Gal80<sup>a</sup>, the crosses were set up and maintained at 18°C for 6 days, followed by 2.5 days at 29°C, then wandering L3 larvae were dissected. For adult stainings, females were dissected at 4-6 days post eclosion. MARCM clones were generated using elav-Gal4 (C155), the FLP/ FRT system (Lee and Luo, 1999), FRT2A and barc<sup>mt2</sup> (this study). Larvae were heat shocked for 1 h at 37°C and dissected as wandering L3 larvae (90-96 h post heat shock).

Y287D mutagenesis

The QuikChange Lightning Site-directed Mutagenesis Kit (Agilent Technologies) and primers (Table S4) were used to generate the Y287D substitution in pUAS<sup>T</sup>-barcRNAi-resistant (Neumüller et al., 2011), resulting in pUAS<sup>T</sup>-barcm<sup>Y287D</sup>.

Generation of barc shmiR

The barc-targeting short hairpin, artificial miRNA (shmiR; Haley et al., 2008) was designed according to Berger et al. (2012). The oligos (Table S4) were annealed and cloned into pValium20 according to the Transgenic RNAi Project protocol (Ni et al., 2011).

Generation of barc<sup>mt2</sup> mutant

The barc<sup>mt2</sup> mutant was generated using CRISPR/Cas9 in FRT2A flies (BL1997) as described previously (Gokcezade et al., 2014). The gRNA used was 5’-GGCGGTGTAGAAGAAGTTGA-3’.

The predicted barc<sup>mt2</sup> peptide (amino acid sequence: MSDEGGCKS-EQLKEKSEEAEKKGDAEGEAKAPILPSVPEVDKTPKONPHS- HNADKTDTPEPSQDFAAAYEEHMTYGADGGAIYTDPSKTOKYKWC- ATGNWQPGWQVDMDRLKIPQKTTSTTSVTGPENPQCNKRNKLRK- RSTSGMTSISRGSSCPSTRKLGRFACWMASALTPTRMASSSFQG- MQPRVLGSQRRSMMMISLWAK) contains no new domains or repeats, as determined using the InterPro tool (https://www.ebi.ac.uk/interpro), although the secondary structure might consist of two new alpha helices at the C-terminal end of the peptide, as predicted with the PSIPRED tool (http://biasinf.cs.ucl.ac. uk/psipred/).

Antibodies and immunohistochemistry

Antibodies are listed in the supplementary Materials and Methods. Larval stainings were performed according to Eroglu et al. (2014) and adult stainings according to Jiang and Reichert (2012). Details are provided in the supplementary Materials and Methods. Confocal images were acquired on a Zeiss LSM780 or LSM510 or a Leica TSC-SP5 microscope.

Brain dissociation, primary cell culture and live cell imaging

Primary cultures from larval brains were prepared as described (Homem et al., 2014). Live cell imaging was performed according to Homem et al. (2013). For details, see the supplementary Materials and Methods.

Image processing and statistics

Images were processed using Fiji software (fiji.sc) by adjusting brightness and contrast through the minimum and maximum levels. Unless otherwise stated, all images within an experiment were processed in the same way.

Unpaired two-tailed Student’s t-test, ANOVA or Wilcoxon rank-sum were used for statistical analysis. P<0.05 was considered significant. Scatter dot plots display the mean with s.d. Box plots display the median, the first to the third quartile (the box) and 1.5x the interquartile range (the whiskers). Sample sizes were estimated based on previous experience.

DNA content analysis

Larval brains were dissected, dissociated enzymatically as previously described (Berger et al., 2012) and incubated with Hoechst 33342 (1:1000, Thermo Fisher Scientific) for 45-60 min at room temperature.
dsRNA-treated S2 cells were harvested on day 7, pelleted, washed in PBS and fixed in cold 70% ethanol at least overnight at 4°C. The next day, the fixed cells were washed in PBS and resuspended in PBS with 200 μg/ml RNase A (Thermo Fisher Scientific) and 40 μg/ml propidium iodide (PI) (Thermo Fisher Scientific, P1304MP) and incubated for 1 h at room temperature. The samples were analyzed using FACS (Aria III or LSRFortessa, BD Biosciences), and the data plots were created using FlowJo software.

**Immunoprecipitation and mass spectrometry (MS) analysis**
Details are provided in the supplementary Materials and Methods. Briefly, L3 brains were homogenized and lysed in lysis buffer [50 mM Tris-HCl pH 8, 100 mM NaCl, 10% glycerol, 0.5% Triton X-100, 1 mM DTT, 1 mM PMSF, 1× Complete EDTA-free protease inhibitor cocktail (Roche)], cleared by centrifugation, snap frozen and stored at −80°C. Thawed lysates were precleared and incubated overnight at 4°C with mouse anti-HA antibody or peptide block (10%). The immunocomplexes were precipitated using Protein G PLUS-agarose beads (Santa Cruz Biotechnology, sc-2002), washed, and eluted by boiling in 2× Laemmli buffer. The proteins were run on 4-12% gradient Bis-Tris gels (NuPAGE, Invitrogen) and subjected to western blotting or a modified version of the Blum silver staining protocol. Nuclease digestion was performed using benzonase (0.1 U/μl, Novagen) before the preclearing.

Protein bands visualized with silver staining were cut out from the gel and processed for MS using in-gel trypsin digest. NanoLC-MS analysis was performed according to Köcher et al. (2012) with slight modifications.

Cutoffs determining significant interactors: minimum ten peptides recovered and minimum 10-fold enrichment over control. Functional groupings and relationships were determined using STRING (Szklarczyk et al., 2015). GO term enrichment was analyzed using Flymine (Lyne et al., 2007).

**Western blotting**
After SDS-PAGE, the proteins were transferred to nitrocellulose membranes (Hybond ECL, GE Healthcare), blocked with 5% milk solution and incubated overnight at 4°C with rabbit anti-Barc (1:200) (Neu miller et al., 2011), followed by secondary antibody (ECL HRP-conjugated whole antibodies, GE Healthcare) and detected using Pierce ECL Plus (Thermo Scientific).

**FACS, library preparation and RNA sequencing**
NB sample preparation and FACS analysis and sorting were performed according to Berger et al. (2012).

For RNAseq, L3 brains were used from UAS-dcr2; asc-Gal4, UAS-mCD8::GFP crossed to control (VDRC, TID60100) or barc RNAi (VDRC, TID107013). Type I NBs were collected into TRIzol LS (Invitrogen) and stored at −80°C until enough material was collected. Two biological replicates per genotype were collected and sequenced.

mCherry or barc dsRNA-treated S2 cells were collected after 7 days. RNA isolation was carried out using TRIzol. Samples were collected and sequenced from two independent experimental runs, each from two wells (n=2 experiments, 4 wells). Preparation of mRNA and cDNA for RNAseq was performed as described (Harzer et al., 2013) with slight modifications. Libraries were prepared using the Illumina KAPA Library Preparation Kit or Illumina NEB Ultra Kit. The samples were UDGase treated and subjected to strand-specific paired-end sequencing. See the supplementary Materials and Methods for details.

**Computational transcriptome analysis**
Differential expression analysis was performed according to Berger et al. (2012). Intron retention was determined using DESeq (Anders et al., 2012) as described in the supplementary Materials and Methods. GC content was extracted with bedtools (Quinlan and Hall, 2010). Logos and entropy calculation were performed with WebLogo (Crooks et al., 2004). The GO term analysis was performed using Flymine (Lyne et al., 2007).

**Tissue culture**
S2 cells were cultured in Schneider’s Drosophila Medium (Life Technologies) supplemented with 10% FBS ( Gibco). dsRNA was generated using MEGAscript T7 (Ambion), using the primers listed in Table S4. Transfections were performed using Effectene Transfection Reagent (Qiagen) according to Zhou et al. (2013) with slight modifications.

Gene knockdown and minigene transfection experiments were performed as described (Malone et al., 2014) with slight modifications (see the supplementary Materials and Methods). Minigene sequences are provided in Table S4.

**RNA isolation, cdNA synthesis and RT-PCR**
RNA was isolated using Trizol, subjected to DNA digestion (TURBO DNA-free Kit, Ambion) and first-strand cdNA synthesis with random primers (Invitrogen) or oligo(dT) primers (Invitrogen) according to the SuperScript III reverse transcriptase (Invitrogen) protocol.

RT-PCR was performed using Go Taq Green Master Mix (Promega), Taq DNA polymerase (NEB) or Phusion High-Fidelity DNA polymerase (Thermo Scientific) on a C1000 Touch Thermal Cycler (Bio-Rad). Primers are listed in Table S4.

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**Competing interests**
The authors declare no competing or financial interests.

**Author contributions**


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**Data availability**
The RNAseq data are available at the NCBI Gene Expression Omnibus through series accession number GSE96549. The mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD006068.

**Supplementary information**

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.152199.supplemental

**References**


