Review

Neural stem cell derived tumourigenesis

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Abstract: In the developing *Drosophila* CNS, two pools of neural stem cells, the symmetrically dividing progenitors in the neuroepithelium (NE) and the asymmetrically dividing neuroblasts (NBs) generate the majority of the neurons that make up the adult central nervous system (CNS). The generation of a correct sized brain depends on maintaining the fine balance between neural stem cell self-renewal and differentiation, which are regulated by cell-intrinsic and cell-extrinsic cues. In this review, we will discuss our current understanding of how self-renewal and differentiation are regulated in the two neural stem cell pools, and the consequences of the deregulation of these processes.

Keywords: *Drosophila*; stem cell; neuroblast; neuroepithelia; proliferation; differentiation; self-renewal

1. Introduction

The brain is the body's decision and communication centre, and is responsible for functions such as learning, thought and memory. Generating a correct sized brain, with the right number of neurons and glia is of fundamental importance for neural function and this process is tightly regulated by neural stem cell self-renewal and differentiation. While precocious differentiation can lead to an undersized brain, excessive stem cell expansion leads to neural tumours such as gliomas and neuroblastomas [1]. Many aspects of neural stem cell control such as mode of division, proliferation and differentiation are evolutionarily conserved between vertebrates and invertebrates [2]. Neurogenesis in *Drosophila* occurs in two distinct phases, the first during embryogenesis and the second during larval and pupal development. In this review, we will focus mainly on the postembryonic phase of *Drosophila* neurogenesis, during that 90% of the adult neurons are generated by different neural stem cell pools of the *Drosophila* CNS. We will discuss recent progress made in understanding how deregulation of these stem cells and their surrounding niche contributes to brain

tumours.

Two main pools of neural stem cells generate the *Drosophila* CNS: 1) the symmetrically-dividing progenitors of the NE give rise to the optic lobes (OL) that comprise the lateral half of each of the two brain hemispheres, and give rise to the adult visual processing centres and 2) the asymmetrically-dividing NBs which are located throughout the CNS, and give rise to the neurons and glia with distinct cellular fates and functions, which form most of the cells of the adult brain (Figure 1).
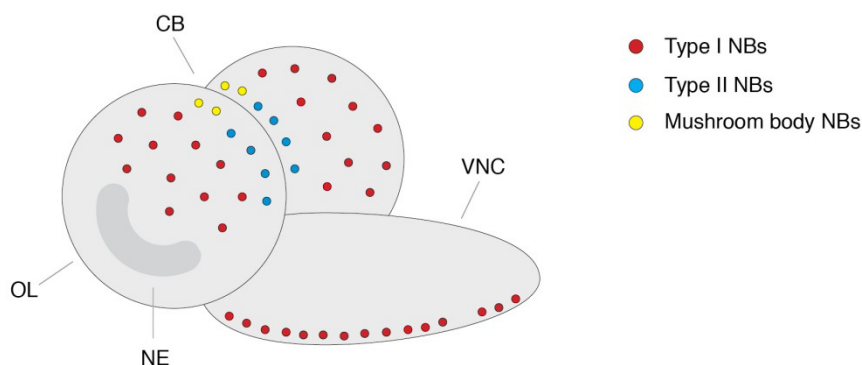

Figure 1. Schematic representation of the Drosophila larval CNS. The brain consists of the optic lobes (OL), the central brain (CB) and the ventral nerve cord (VNC). The OLs will give rise to the adult visual system and are generated from the neuroepithelium (NE, gray). The majority of NBs of the CB and all the NBs of the VNC are type I progenitors (red); the CB also contains type II NBs (blue) and mushroom body NBs (yellow).

2. Two progenitor pools, NE cells and NBs in the developing optic lobes give rise to the adult visual centres

Symmetrically-dividing NE cells generate asymmetrically dividing NBs via a carefully balanced process to control the number of neurons of the OLs. This wave of differentiation starts from the medial edge of the NE sheet and moves laterally during the third instar stage of larval development; and is mainly regulated by Notch, Epidermal Growth Factor Receptor (EGFR) and JAK/STAT signalling pathways [3-6]. The Hippo pathway, a recently discovered pathway that controls organ growth, is also required cell-autonomously in the NE, to regulate its proliferation as well as its differentiation [7-10]. Mutation of tumor suppressor genes within the pathway, or ectopic expression of Yorkie, a downstream activator of the pathway, promotes overgrowth of NE cells and delays their differentiation [10]. Furthermore, it has been shown that the atypical cadherin Fat and the Hippo pathway regulate the EGFR/Ras signalling along the NE field and, thus, ensure the progression of NB differentiation [9]. Non-cell-autonomous glia-derived signals, regulated by the microRNA *mir-8* also modulate EGFR/Ras signalling and optic lobe growth. *mir-8,* expressed in a subpopulation of optic-lobe-associated cortex glia that ensheath the NE, promotes the secretion of Spitz, a TGF-alpha-like ligand, which in turn induces EGFR activation to promote NE proliferation.

Loss of *mir-8* results in excess proliferation of the NE, as well as ectopic NE to NB transition [11], suggesting that communication between the glia niche and the NE stem cell pool is important for proliferation and differentiation of the NE.

3. Adult neuronal numbers are specified by the regulation of division mode and length of proliferation of type I and type II NBs

In contrast to the optic lobe NBs that form during larval neurogenesis, the other types of NBs (type I, II and mushroom body NBs) located in the central brain (CB) and the ventral nerve cord (VNC) are first formed during the embryonic stages of *Drosophila* development. NBs delaminate from the embryonic NE and are specified by a process called lateral inhibition [12]. NBs undergo repeated, self-renewing asymmetric divisions [13], giving rise to another NB and a smaller ganglion mother cell (GMC), which divides once to produce neurons and glial cells. Embryonic NB divisions produce the bulk of the larval CNS but only 10% of the cells in the adult CNS [14]. At the end of embryonic neurogenesis, NBs arrest their cell cycle and exit from G1 into a G0-like quiescent state. Around 8–10 hours after larval hatching, during the late 1st instar stage, the NBs start exiting quiescence and re-enter mitosis [15]. Neurogenesis continues throughout larval and early pupal stages, at which point the NBs exit from the cell cycle and terminally differentiate [15,16,17]. Most of the NBs (type I) follow patterns of proliferation similar to those of embryonic NBs (Figure 2). However, a smaller group (type II NBs) has a different proliferative mode that involves intermediate progenitors with transit-amplifying cell divisions. Type II NBs are distinguished from type I NBs by the lack of expression of the transcription factor Asense (Ase). They divide asymmetrically to self-renew and generate smaller intermediate neural progenitors (INPs) [18,19], which undergo a maturation process of approximately 6 hours [20] during which they initially turn on Ase expression but are still incapable of divisions. Coinciding with the onset of the expression of markers such as transcription factors Deadpan (Dpn) and Earmuff (Erm) [21], they begin to divide asymmetrically to give rise to another INP and a GMC (Figure 2). Through this trans-amplifying mode of division, type II NBs form larger lineages than type I NBs, and give rise to around 5000 neurons in the adult brain [22].

Another parameter which determines the final size of the CNS is the timing when NBs enter the cell cycle at the beginning of larval neurogenesis, and when they finally and irreversibly stop dividing at the end of neurogenesis [15]. Recently it has been demonstrated that nutrition regulates NB exit from quiescence by activating the insulin receptor (InR) and target of rapamycin (TOR) pathways. These signalling events induce an increase in NB size and subsequently their entry into the cell cycle [23,24]. At the end of neurogenesis, changes in energy metabolism coordinated by the Mediator complex—consisting of 26–30 subunits that connects transcription factors to RNA Polymerase II and the steroid hormone Ecdysone trigger a switch from glycolysis to oxidative phosphorylation, leading to the progressive reduction in NB size and, terminal differentiation [17]. Together, these newly emerged studies suggest that regulation of neural stem cell activation, termination and proliferation are all likely to be closely coordinated with the metabolic status of the animal, and that multiple signals from other organs regulate NB growth, and determine its proliferative ability. So far it has been thought that neural proliferation does not occur in the mature adult brain, however, a recent study showed that upon injury, a population of neural progenitors in the optic lobes possess surprising ability to undergo self-renewal, posing the interesting possibility that *Drosophila* adult brains are capable of regeneration [25].
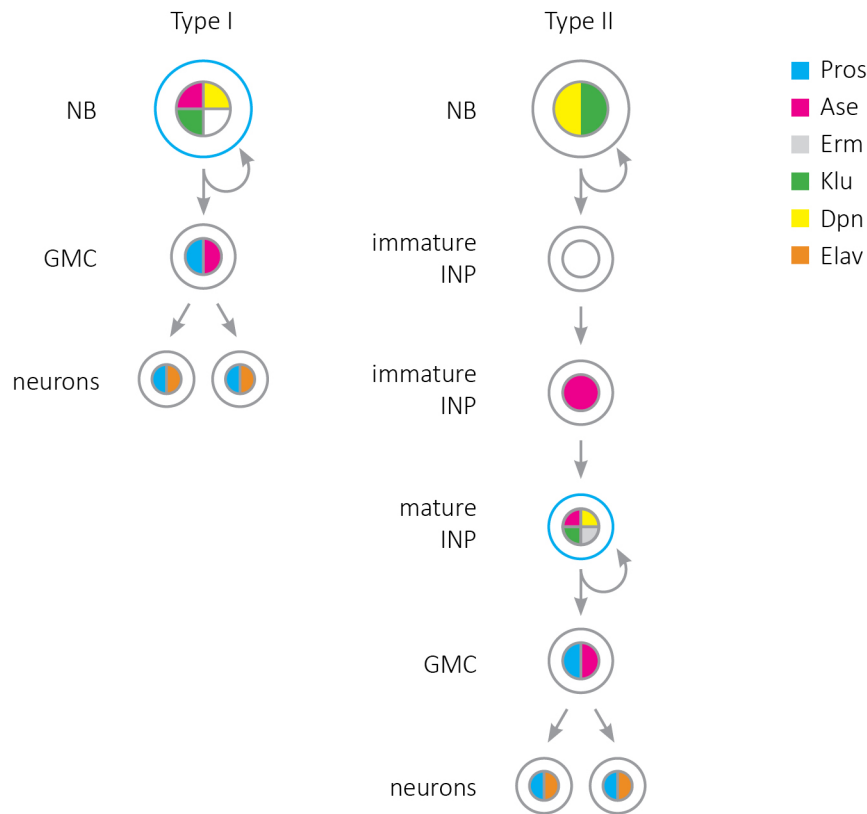
Figure 2. Cell division mode of type I and type II NBs. Type I NBs (left) divide asymmetrically to self-renew and to generate a GMC which will divide once to generate differentiation-committed progeny. Upon cell division, type II NBs (right) produce another NB and an immature INP. The INPs undergo a maturation process that involves the sequential expression of specific factors. Mature INPs divide a few times to self-renew and to produce a GMC that will in turn divide once to generate neurons or glia.
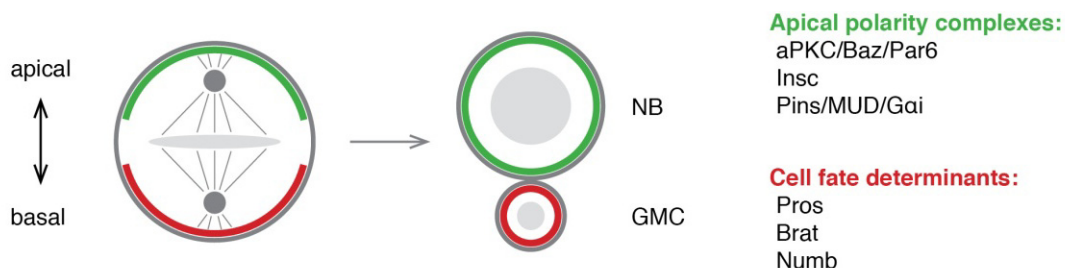

Figure 3. Asymmetric cell division. In the dividing NB, the aPKC/Baz/Par6 complex is localized to the apical domain and interacts with the Pins/ MUD/Gαi/ complex via Insc to properly orient the mitotic spindle. The cell fate determinants Pros, Brat and Numb are basally localized so that upon cell division they are segregated into the GMC where they will inhibit self-renewal and promote differentiation.

The major strategy to expand neuronal progeny number, while maintaining the number of neural stem cell pool, is asymmetric cell division (ACD, Figure 3): the process by which key cell fate determinants are unequally segregated into one daughter cell to promote its differentiation while the other daughter retains stem cell properties. NB ACD involves four major steps: 1) setting up of an axis of polarity, 2) asymmetric localization of cell fate determinants, 3) orientation of the mitotic spindle and 4) differential segregation of cell fate determinants between the two daughter cells.

4. Establishment of apical-basal polarity and spindle orientation in NBs

The establishment of an apical-basal (A-B) polarity axis in the NB is an important first step in discriminating between the stem cell and its differentiating daughter cell. The A-B axis is initially established by the apically localized Par complex consisting of atypical Protein Kinase C (aPKC), Partition Defective 6 (Par6) and Bazooka (Baz) [26-29] which functions by antagonizing the activity of the Lethal giant larvae (Lgl), Discs large (Dlg) and Scribble (Scrib) complex. As a result, Lgl promotes localization of cell fate determinants only in the basal NB cortex, where the Par complex is absent [30]. Upon establishment of apical-basal polarity, the adaptor protein Inscuteable (Insc) is recruited to the apical cortex by Baz and activates partner of inscuteable (Pins) [31,32,33]. Pins then binds to the heterotrimeric G protein subunit Galphai, and Mushroom body defect (Mud) [34-37], which recruits the Dynein/Dynactin complex to the apical cortex, and pulls on the astral microtubules in order to correctly align the mitotic spindle [38]. Together, the Par complex, that specifies A-B polarity and the heterotrimeric G-protein complex that controls spindle orientation, ensure basal localization of cell fate determinants, and the creation of two daughter cells of unequal size and content (Figure 3). Disruption to either process can lead to supernumerary NBs, and transplantable tumours. As an example, expression of an activated form of aPKC or loss of aPKC inhibitors disrupts the cortical localisation of the cell fate determinants, prevents the GMC from receiving the appropriate differentiation signals and leads to a dramatic increase in NB numbers [39-42]. Similarly, spindle misorientation defects in NBs such as in brains mutant for *mud* and *pins* also cause ectopic NB formation, despite a correct cortical polarity being present [35,36,37,43]. Finally, some of the kinases that regulate A-B polarity are also tumour suppressors. Larval brains mutant for *aurA* and *polo* have supernumerary NBs, as these proteins are necessary to constrain aPKC to the apical cortex and are also required for spindle alignment [44,45,46].

5. Segregation of cell fate determinants

The main result of ACD is the exclusive segregation of the key cell fate determinants Prospero (Pros), Brain tumour (Brat) and Numb to the daughter cell that will undergo differentiation. Failure in GMC formation also leads to supernumerary NBs. Pros is an atypical homeodomain transcription factor expressed in all neuronal lineages [47]. In NBs Pros is cortically localized and asymmetrically segregated into the GMC [48,49] through binding to the adaptor protein Miranda (Mira) [50]. Following cell division, Pros is released from the cortex and translocates to the nucleus of the GMC where it binds to several hundred promoters repressing genes involved in self-renewal and cell cycle progression and activating genes involved in terminal differentiation [51]. Brat is an evolutionary conserved translational repressor, and, similarly to Pros, a Mira binding partner that is unequally segregated into one daughter cell upon cell division [52]. Numb is a transmembrane protein that is

best known by its activity as a regulator of Notch signalling [53,54], a key signalling pathway in the specification of NB identity in the larval nervous system [18,21,44,45,55-59]. Following its segregation into the GMC, Numb promotes differentiation and inhibits NB self-renewal [44,45].

6.   Loss of cell fate determinants results in tumour growth

Larval brains mutant for the cell fate determinants *pros*, *numb* or *brat* develop malignant tumours that can be serially transplanted into the abdomen of naïve hosts [43]. These tumours are composed of undifferentiated cells that express NB-specific markers. Prospero is required for GMC specification and differentiation in both type I and type II lineages. Type I *pros* mutant lineages are devoid of differentiated cells and consist almost entirely of actively dividing, progenitor-like cells [52,60]. In type II lineages, the *pros* overgrowth phenotype is more subtle, as GMCs revert to INPs (which undergo few rounds of divisions by comparison to NBs) at the expense of differentiated neurons, without full reversion to highly proliferative NBs [18,21]. Loss of function of *numb* also causes tumours in both lineages, as a consequence of Notch hyperactivation but, contrary to that of *pros* it elicits a stronger effect in type II lineages. In these, loss of *numb* or hyperactivation of Notch results in INP to NB reversion, and a dramatic accumulation of ectopic NBs, as INPs fail to turn on Ase, and are unable to progress through maturation [18,21,44,45,55-59]. Type I lineages appear to be more resistant to Notch signalling manipulations and demonstrate less penetrant phenotypes [18,55,56,58,61]. One possible explanation was offered by the notion that Notch signalling acts redundantly with the transcription factor Dpn in specifying NB identity and indeed double knock-outs of *dpn* and members of Notch pathway show dramatically enhanced phenotypes in type I lineages [59]. Differently to *pros* and *numb* loss of function, *brat* mutation induces tumorous growth only in type II lineages, giving rise to large numbers of ectopic progenitors that are incapable of exiting the cell cycle [52,60]. In these lineages, Brat has been shown to act in parallel to Numb in promoting the maturation of INPs. Similar to *numb,* loss of *brat* blocks INP transition from an Ase$^-$ to an Ase$^+$ state and causes reversion of these INPs to NBs [18].

How Brat and Numb prevent INPs from reverting to type II NBs is still not entirely clear but several lines of recently emerged evidence are beginning to address the mechanism. One of the studies showed that dedifferentiation and proliferation of Notch-dependent tumours are reliant on Eukaryotic translation initiation factor 4E (eIF4E) and the growth regulator Myc. Notch directly activates Myc expression, which forms a feedback regulatory loop with eIF4E in sustaining growth. Cell growth regulation downstream of Notch signalling thus appears to be critical in controlling the proliferative potential of NBs [57]. Other studies have shown that Brat and Numb can antagonize the activity of Zn-finger transcription factor Klumpfuss (Klu). Klu overexpression causes the formation of transplantable tumours characterized by reversion of INPs to ectopic type II progenitors. Conversely, loss of Klu causes NB premature differentiation suggesting that downregulation of Klu is an essential step in INP maturation [58,62]. Furthermore, Klu can suppress ectopic NB formation due to loss of *brat* or Notch hyperactivation, and its overexpression can also cooperate with Notch signalling to promote reversion of GMCs into type I NBs [58], suggesting that Klu must play an important role in both type I and type II NB lineages. More recently, it was shown that Brat specifies INP identity by attenuating the transcriptional activity of β-catenin/Armadillo (Arm). The authors found that decreased Arm activity suppressed the number of ectopic NBs induced by overexpression of Klu, and Arm activity is capable of modulating the ectopic NB phenotype in *brat* mutants, leading

them to suggest that Brat might antagonize Klu via an Arm-mediated mechanism [63].

7.   Epigenetic factors restrict self-renewal of neural progenitors

Following specification of INPs by Brat and Numb, the conserved C_2H_2 Zn-finger transcription factor dFez/Earmuff (Erm) acts in the immature INPs to restrict their proliferation potential. In *erm* mutants, INPs revert to fully functional type II NBs, despite the fact that INPs are correctly specified, and exhibit correct apical and basal polarity [21]. Genetic evidence led to the proposal that Erm prevents tumour formation by two means, it restricts INP proliferation through promoting Pros nuclear localization and prevents INP dedifferentiation by antagonizing Notch signalling [21]. Several recent studies showed that in fact Erm acts directly and synergistically with chromatin remodelling complexes such as the BAP complex [64], Brahma complex and Histone deacetylase 3 (HDAC3) [65] leading to attenuated competence of INPs to respond to NB self-renewal factors and preventing their dedifferentiation into type II NBs. Another axis of control that limits INP self-renewal is the SWI/SNF complex, which activates a temporal transcription factor cascade that confers temporal identity to neuronal subsets generated at different times during neurogenesis. Failure to do so results in lineage reversion and consequent tumour formation [66]. Chromatin remodelling factors are not just involved in restricting the self-renewing capability of INPs, but also in the NE of the OLs [67,68] *lethal brain malignant tumour* (*lmbt*) encodes for a member of the dREAM-MMB complex, implicated in chromatin repression and is a close homolog of Polycomb group proteins, well-known promoters of epigenetic silencing (reviewed in [69]). Its mutation causes malignant growth in the larval brain. Interestingly, genome-wide expression profiling of *lmbt* tumours showed upregulation of germline-specific genes. Furthermore, inactivation of these genes is able to suppress overgrowth, suggesting that tumour formation in this context is a consequence of extensive genomic reprogramming [67].

8.   Post-mitotic neurons: a new tumour cell of origin

So far, most of the brain tumours were shown to be caused by reversion of GMCs or INPs to NBs, however, more recently postmitotic neurons have been shown to be a new tumour cell of origin. Mutation in the BTB-Zinc finger transcription factor Lola (which is a co-factor of Pros) is capable of causing supernumerary NBs derived from post-mitotic neurons in the optic lobes [70]. Like Pros, Lola binds to genes involved both in stem cell self-renewal and differentiation [70], but unlike Pros, which primarily acts in the GMCs, Lola is required to maintain neurons in a differentiated state. Similarly, the RNA splicing factor Midlife crisis (Mlc), a regulator of *pros* expression, has also been implicated in neuronal differentiation maintenance. Intriguingly, though reverted *mlc* mutant neurons express stem cell genes, they do not divide or form tumours [71]. Finally, the zinc finger transcription factor, Nerfin-1 also maintains neuronal differentiation in both type I and type II lineages, and its loss results in Myc- and Tor-dependent neuron to NB reversion, and tumourigenesis in both larval and adult CNS [72]. Together, these findings suggest that differentiated, post-mitotic cells can be the source of tumour growth, and support the model of bidirectional inter-convertibility between stem cells and differentiated cells.

9. *Drosophila* models of glioma

Besides the neurons, the other main population of cells in the CNS are glia cells. Tumours associated with over-proliferation of glia cells (gliomas) include the most common and deadly type of brain tumour in adults (glioblastoma) and the most common solid tumours in children. Several *Drosophila* models of glioma have recently emerged. Constitutive co-activation of EGFR-, Ras- and Phosphoinositide 3-kinase (PI3K) pathways in *Drosophila* glia has been found to cause neoplastic, invasive and transplantable tumours that closely mimicked human glioma [73]. Genetic analyses demonstrated that EGFR and PI3K initiate malignant neoplastic transformation via Tor, Myc, G1 Cyclins-Cdks, and Rb-E2F pathways, which are commonly mutated or activated in human glioma [73]. Furthermore, a kinome-wide RNAi screen revealed that RIO kinases mediate this phenotype, and human orthologs of novel kinases uncovered by these screens were functionally assessed in mammalian glioblastoma models and human tumours [74]. In a separate study, it was found that glial overexpression of activated EGFR resulted in enhanced proliferation and migration of larval glial cells, tumour-like enlargement of the optic stalk, and metastatic glial cells along the optic nerve [75]. The EGFR/ PI3K phenotype was partially reverted by the administration of the EGFR tyrosine kinase inhibitor *gefitinib* and completely rescued by the PI3K inhibitor *wortmannin* and the Akt inhibitor *triciribine*, demonstrating that *Drosophila* models are useful not only to decipher signalling cascades that mediate glioma formation, but also for experimental therapy purposes.

10. Conclusions

Over the last 10 years, studies in *Drosophila* neural progenitors and their surrounding glia cells have lent us great insight into the mechanism of ACD, and regulation of stem cell proliferation. As a result of these studies, we have gained deeper understanding in how neural progenitors such as NBs and INPs become increasingly limited in their proliferative potential to allow terminal differentiation, and how defects in asymmetric cell division or cell fate specification can lead to brain tumours. Excitingly, studies on chromatin-remodelling in the context of stem cell self-renewal are beginning to appear, offering mechanistic insight into how the deregulation of this process often observed in human cancers, can lead to tumorigenesis. Furthermore, dedifferentiation of postmitotic cells is emerging as another means by which tumours can arise, and elucidating the mechanisms behind these events will help us answer fundamental questions such as tumour cells of origin. Finally, non-cell autonomous cues are becoming increasingly relevant as important regulators of the timing as well as proliferative abilities of NBs both in physiological and pathological conditions. Further studies will be required to better understand how NB proliferation is coordinated with extrinsic nutritional and hormonal signals, and how these signals are integrated to specify NB self-renewal and differentiation.

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Conflicts of interest

The authors declare no conflicts of interest in this paper.

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