Using *Drosophila* to study regulation of neural stem cell quiescence by nucleocytoplasmic transport

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Abstract
Cellular quiescence is a reversible non-dividing state. Subsets of adult mammalian stem cells, namely neural stem cells, spend the majority of their time in quiescence. The ability of stem cells to adopt the quiescent state appears to be crucial for long-term maintenance of the stem cell compartment. Tumour cells can also become quiescent and this renders them resistance to most chemotherapeutics, which target proliferating cells. Despite its importance, the molecular mechanisms that regulate the quiescent state still remain to be fully elucidated. In particular, the use of *Drosophila melanogaster* as a genetic model organism has provided important insights into various molecular mechanisms and cellular processes, including neurogenesis and neural stem cell quiescence. *Drosophila* neural stem cells, called neuroblasts, undergo two waves of neurogenesis separated by a period of quiescence. This project aimed to study the role of nucleocytoplasmic transport in regulating neuroblast quiescence. Here, we show evidence of a role for key components of nuclear transport machinery in the regulation of neuroblast quiescence. Furthermore, we investigate nucleocytoplasmic partitioning of key regulators of growth and/or cell-cycle progression. This work provides preliminary data implicating nucleoporins and casein kinase II as determinants of the quiescent state.

Keywords
quiescence, stem cells, neuroblasts, nucleocytoplasmic transport, nuclear localisation signal, nucleoporin, casein kinase II

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Introduction

Cell cycle-arrest, also known as G0, is an inactive, non-dividing state. This state is exemplified by differentiated cells, namely neurons; senescent cells, whose are dysfunctional and are irreversibly out of the cell cycle [1]; and quiescence, which cardinal feature is being reversible. In 1974, Arthur Pardee demonstrated that cells can be shifted between G0 and G1 before the restriction point (R-point) [2]. After this point cells are committed to enter the mitotic cell cycle [3].

Stem cells are defined as an undifferentiated population of cells capable of producing both self-renewing and differentiating daughter cells. These cells can be subdivided into a long-term subset that is capable of self-renewing for an indefinite period of time, and a short-term subset that divide for a defined interval [4]. A subset of tissue-specific adult stem cells, namely neural stem cells (NSCs), reside predominantly in the quiescent state [5, 6]. It is believed that the ability of stem cells to adopt the quiescent state is crucial to preserve key functional properties [7, 6], contributing to long-term maintenance of the stem cell compartment. Quiescence is controlled by both intrinsic regulatory mechanisms and extrinsic signals from the microenvironment [8]. In particular, studies have suggested that NSC division is promoted by signals that enhance production of new neurons, namely running [9] and seizures [10]. Hence, NSC quiescence is thought to provide a reserved pool for supplying new neurons when needed. Moreover, different studies have suggested a correlation between abrogation of quiescence and exhaustion of NSCs [11, 12]. More recently, Furutachi and colleagues demonstrated that long-term quiescence abrogation led to excessive reduction of NSCs and impaired neurogenesis in mice [13].

From a clinical point of view, better understanding of quiescence is of great importance, as tumour recurrence can occur from reactivation of surviving cells [14]. Quiescent cells are more resistant to traditional chemotherapy regimens, as these usually target proliferating cells [14, 15]. For instance, in 1994, Cindi M. Morshead and colleagues found that killing of the constitutively proliferating cells in the adult mouse forebrain had no effect on quiescent stem cell ability of complete repopulation [16]. Moreover, in 2007, Fumihiko Ishikawa et al. showed that the majority of human leukaemia stem cells were quiescent and resistant to cell cycle-dependent cytotoxic therapies [17]. Moreover, in 2013, C. Lutz and coworkers provided further evidences for the existence of quiescent stem cells in leukemia and suggested that quiescence contributes mechanistically to enhanced chemoresistance [18]. This demonstrates how ineffective conventional cytotoxic therapies can be and it might help explaining why tumours, even when they seem to fully regress, can recur. However, to date, the molecular mechanisms that underlie regulation of quiescence still remain to be fully elucidated. Our understanding of the characteristics of quiescent cells is greatly limited by the lack of knowledge on their cells markers and molecular signatures; consequently the role of stem cell quiescence in tumour progression and recurrence is still not completely clear.

Recent advances in genetic approaches and high-throughput analyses of different stem cell populations have provided important information to understand the regulatory pathways and specific determinants of the quiescence state [7]. In particular, recent studies have also shed light on the metabolic regulation of the quiescent state (reviewed in reference [19]) and it seems that the phosphatidylinositol-3-kinase (PI3K)/target of rapamycin (TOR) pathway has emerged as one of the most conserved pathways in the regulation of the metabolic status between quiescence and proliferation, including *Saccharomyces cerevisiae* [20], *Drosophila melanogaster* [21], and mammals [22, 5]. Moreover, studies of the FoxO (Forkhead box subgroup O) family of transcription factors have shown to be a functionally important pathway in regulating stem cell quiescence and survival [21, 23, 24, 25]. However, how these pathways specifically regulate and control the various molecular and cellular changes unique to the quiescent state still remains unknown. Given evolutionary conservation of the regulatory pathways and molecular mechanisms previously mentioned, this project aims to study quiescence regulation of neural stem cells found in the developing *Drosophila* central nervous system (CNS), called neuroblasts (NBs).

NBs divide asymmetrically, giving rise to another NB and an intermediate progenitor. This asymmetric cell division (ACD) is accomplished through differential localisation and segregation of cell fate determinants between the two daughter cells [26, 27], namely Miranda (Mira), Prospero (Pros) and Brain tumour (Brat). Dysregulation of ACD of stem/progenitor cells has been found in cancers, namely leukaemia [28], mammary tumours [29] and brain tumours [30]. If asymmetry is disrupted, such that the daughter cell that was meant to follow a differentiation path fails to do so, this can result in tumour formation. The molecular mechanisms that underlie and regulate asymmetric cell division have been extensively studied (mostly in *Drosophila*) and numerous components involved in the process have been identified (reviewed by Knoblich [31]). Importantly, even though mammalian and *Drosophila* brains are morphologically very different, many basic aspects found in fly development are surprisingly conserved in mammals (reviewed in [27, 32]).

At the end of embryogenesis, most NBs in the abdominal region are eliminated through programmed cell death [33], while most NBs in the cephalic and thoracic regions undergo cell-cycle arrest, exiting from G1 to a G0 quiescent state [34]. Around 8 to 10 hours after larval hatching, food intake activates the Insulin Receptor (InR) and Target of Rapamycin (TOR) pathways in dormant neuroblasts, triggering exit from quiescence and allowing for neuroblasts size increase [21, 35]. In order to ensure that a sufficient variety of neuronal lineages have been generated before NBs undergo cell-cycle arrest, entry into quiescence has to be regulated by both extrinsic and intrinsic stimuli. Quiescent neuroblasts extend a primary cellular process toward the neuropil and also occasionally toward the ventral surface or other neuroblasts [36, 35, 37]. These processes are present until neuroblasts start to reactivate [38], but their function is not yet known.

In a forward genetic screen, Rita Sousa-Nunes recovered a mutant (*2V327*) in which late larval NBs (actively cycling in wild-type animals) anachronically undergo cell-autonomous cell-cycle arrest (unpublished). These mutant NBs extend a cellular process (data not shown), characteristic of a quiescent NB. Deficiency-mapping revealed that the gene responsible for the 2V327 phenotype (CG14712) seems to encode a nucleoporin (Nup) (RSN unpublished; data not shown). Nups are the constituents of the nuclear pore complexes (NPCs), which penetrate the nuclear envelope (NE), allowing for the exchange of molecules between the cytoplasm and the nucleus. NPCs are highly selective and are responsible for the nuclear transport of an enormous amount of proteins. Due to their crucial role in controlling the nucleocytoplasmic transport, their correct synthesis and function are essential for cell homeostasis and survival [39, 40]. Based on their approximate location within the NPC, Nups can be grouped into three different functional classes [41, 42]: integral membrane proteins that anchor the NPC in the NE, also called pore membrane proteins (Poms), structural Nups that stabilise the NE curvature at nuclear pores and provide scaffolding, nuclear basket Nups and phenyalanine-glycine rich, called FG Nups. Transport of macromolecules into and out of the nucleus occurs through NPCs , which allow passive diffusion of ions and small proteins ($<$40 kDa), and active transport of larger molecules that harbour specific targeting signals [43]. The active transport of macromolecules is facilitated by carrier proteins called karyopherins, which are termed importins [44, 45] when involved in cargo import and exportins [46] when involved in cargo export from the nucleus. The classical nuclear import pathway involves the recognition of a specific sequence, called nuclear localisation signal (NLS), in the protein to be transported by importinα. Classical NLSs, recognized by importinα, are classified into two major classes: monopartite and bipartite. Monopartite NLSs contain a single cluster of basic residues, being exemplified by simian virus 40 (SV40) large T antigen NLS (^{126}PKKKRRV^{132}) [47]. In their turn, bipartite NLSs contain two stretches of basic aminoacids separated by 10-12 amino acids linker. This type of classical NLSs is exemplified by the nucleoplasmin NLS (^{155}KRPAATKKAGQAKKKK^{170}) [48]. Due to the abundance of known classical NLS-containing proteins, it is believed that this nuclear import pathway is the most prevalent in the cell [49].

Interestingly, Rita Sousa-Nunes observed that reporters carrying the SV40 large T-antigen (called NLS2 in Flybase) fail to enter the nucleus of early larval neuroblasts still in quiescence. However, these reporters robustly localise to the nuclei of late larval neuroblasts, which are actively cycling (data not shown). In contrast, reporter proteins containing NLS5 (amino acids 28-41 of the fly transformer gene, which sequence contains a nucleoplasmin-like bipartite NLS and is enriched in RS domains) always localise to neuroblast nuclei, whether they are in a quiescent state or actively cycling (data

not shown).

Additionally, Casein Kinase II (CKII), a ubiquitous, highly pleiotropic and constitutively active Ser/Thr protein kinase, has been identified as the determining factor in the enhancement of the nuclear transport of proteins carrying the SV40 large T antigen NLS [50] (classical monopartite) and of nucleoplasmin, which carries a classical bipartite NLS [51].

Our main goal with this study is to establish the role of nucleocytoplasmic transport in NSC quiescence, by establishing the role of key regulators of protein transport, such as nucleoporins and casein kinase II; and by investigating nucleocytoplasmic partitioning of key regulators of growth and/or cell-cycle progression and determine if they are involved in the control of the quiescent state, using candidate cargo. Candidates included the transcription factors Forkhead box O (FoxO), Nuclear factor I (NFI), Prospero (Pros), Deadpan (Dpn), Tailless (Tll), Yorkie (Yki), Myc, Notch intracellular domain receptor (N^{intra}); and cell-cycle Cyclin E (CycE). In this study, we also generated novel molecular tools to study NB quiescence: rescue constructs (with and without a Venus tag) for the gene responsible for the *2V327* phenotype; and an unprecedented construct to label cells with spatial and temporal reproducibility and in a GAL4/*UAS* independent manner.

1. Materials and Methods

Fly husbandry Fly stocks were maintained at 18°C. GAL4-*UAS* experiments [52] were carried out at 29°C for optimal GAL4 activity; other experiments8 were carried out at 25°C. Stock keeping and virgin female collection were performed as described by Ralph J. Greenspan [53]. Animals were raised on our standard cornmeal medium (8% (m/v) glucose, 2% (m/v) cornmeal, 5% (m/v) Brewer's yeast, 2% (v/v) ethanol, 0.22% (v/v) methyl-4-hydroxybenzoate, 0.38% (v/v) propionic acid, 0.8% (m/v) agar in water). For timed larval collections crosses were set-up in cages with grapefruit juice plates (25% (v/v) grape-juice, 1.25% (m/v) sucrose, 2.5% (m/v) agar) supplemented with yeast paste, which were changed daily. Other crosses were set-up on either rich food (6% (m/v) glucose, 3% (m/v) sucrose, 6.6% (m/v) cornmeal, 8% (m/v) Brewer's yeast, 2% (m/v) yeast extract, 2% (m/v) peptone, 1% (v/v) methyl-4-hydroxybenzoate, 0.6% (v/v) propionic acid, 0.05% (m/v) magnesium sulphate, 0.05% (m/v) calcium chloride, 0.8% (m/v) agar in water) or standard cornmeal medium supplemented with active dried yeast.

Fly stocks Fly stocks used in this study were: *grh-GAL4,UAS-CD8::GFP/CyO,Dfd-YFP* from A. Brand [35] (recombined and rebalanced);*nab-GAL4/(TM6B,Sb,Dfd-YFP)* from DGRC (rebalanced); *nab-GAL4,UAS-Dcr2* (unknown source); If/CyO-;UAS-Dpn from J. Knoblich; *UAS-yki* from J.P. Vincent; *UAS-yki::GFP* and *UAS-yki[S168A]::GFP/CyO,Dfd-YFP* from K. Irvine [54]; *FRT19A;;UAS-cycE* from L. Cheng; *hsFLP;UAS-dMyc* from L. Johnston; *UAS-YFP::pros* from A. Brand [55]; *UAS-N[intra]* (unknown source); *UAS-foxo* from BL; *UAS-tll* from DGRC; from *yw;UAS-CKII*α from BL; *UAS-CK2*α*KM/-CyO,Dfd-YFP* from J. Jia [56] (rebalanced); *UAS-CKII*β*[EY21591]* from Bloomington Stock Center; *w-;;UAS-CK2*β*-VIIb[3]*, *w-;;UAS-CK2*β*-VIIc[1]* and g*w-;;UAS-CK2*β*-VIId-VI*^{2} from T. Raabe [57]; *CKII*α*[TikR]/TM6B,Sb,Dfd-YFP* from BL (rebalanced); *Mi{ET1}Nup54[MB03363]/CyO,Dfd-YFP* from BL (rebalanced); *P{PZ}Nup214[10444] cn[1]/CyO,Dfd-YFP* from (rebalanced); *Nup98-96[339]/TM6B,Sb,Dfd-YFP* from BL (rebalanced); *UAS-Nup214[RNAi]* from BL (TRiP collection); *UAS-Nup98-96[RNAi]* from BL (TRiP collection); *UAS-CG14712[RNAi];FRT82B,pros[17]/TM6B,Sb,Dfd-YFP* original from VRDC; *w;Sco/CyO,Dfd-YFP* from BL; and *w;;ry,Dr/-TM6B,Sb,Dfd-YFP* from BL.

Stock rebalancing Balancer chromosomes and genetic markers used are described in references [58, 59, 60]. In order to genotype animals of any stage, strains with lethal mutations were rebalanced over *Dfd-YFP* marked balancers [59]. To rebalance a mutation on the second chromosome, flies were crossed with the stock (*Sco*)/*CyO*,*Dfd-YFP*; when rebalancing a lethal mutation on the third chromosome, were crossed with the stock (*Dr*)/*TM6B,Sb,Dfd-YFP*. Male and virgin progeny carrying the desired balancer were selected to generate rebalanced stocks.

Genotyping Larvae from rebalanced stocks were genotyped using a Stereomicroscope (Zeiss SteREO Discovery.V8), by the presence or absence of *Dfd-YFP* (yellow fluorescent pattern in head region [61, 59]). Balanced animals were used as controls. Larvae that did not present the fluorescent pattern carried the genes of interest for the experiments. *CyO* and *TM6B* balancers are not found in homozigosity, as they are embryonic lethal.

Staging of *Drosophila larvae* For reactivation assays, crosses were performed in cages, as described by Sousa-Nunes, *et al.* [21]. From the moment each cross was set-up, cages were incubated at 25°C for 24 hr and then transferred into a 29°C incubator with a fresh plate. After 17 hr, plates were collected and cleared from yeast paste and any hatched larvae, then incubated at 29°C for further 2 hr. Larvae that hatched within this 2 hr time window were genotyped and transferred to Petri dishes containing mashed-up standard cornmeal food and incubated in humidified chambers for 24 hr at 29°C to reach a mixed moulting population of first and second instar larvae (L1 and L2, respectively). Wandering third instar (wL3) were simply selected from the sides of the tubes.

Dissections Dissections were performed in phosphate-buffered saline (PBS). L1 and L2 dissections were performed using forceps (no. 5) and tunsgten needles and CNSs were immobilised on poly-L-lysine coated slides; L3 larvae were dissected using forceps only. Tungsten needles were made from 0.5mm diameter, 2cm long tungsten wire (Goodfellow Cambridge Ltd.) bent to be held in needle holders. Tips were electrolytically sharpened with a 3-12V AC current passing through 2M NaOH.

5-ethynyl-2'-deoxyuridine (EdU) incorporation and staining All steps in this section were performed at room temperature. Dissected CNSs were incubated for 1 hr in 10 μM

EdU (Life Technologies) diluted in PBS and fixed with 4% Formaldehyde in PBS for 15 min. Tissue was then permeabilised for 20 min with 0.1% PBT (PBS with 0.1% (v/v) Triton X-100 from Fluka Biochemika), followed by addition of Alexa Fluor Azide colour reaction mix according to the manufacturer's instructions (Click-iT, EdU Imaging Kit, Life Technologies). From this point onwards, samples were kept light-tight. After 30 min of colour reaction, samples were washed with 0.1% PBT (at least 4 times for 20 min each) and mounted in Vectashield (Vector laboratories).

Mosaic analysis with a repressible cell marker (MARCM) MARCM crosses were incubated at 25°C and transferred into a new vial every day. Larvae were submitted to 37°C heat-shocks for 1 hr and 30 min at L1 and L2 stages. Wandering L3 larvae were selected for the genotypes carrying clones.

Immunostaining Antibody staining was perfomerd according to standard protocols. Primary antibodies used in this study were: mouse anti-Mira 1/50 [62]; rabbit and mouse anti-GFP 1/1000 (Life Technologies); rabbit anti-RFP 1/1000 (Rocklands); guinea pig anti-Deadpan 1/1000 (J. Skeath); rabbit anti-FoxO 1/500 (P. Leopold); mouse anti-dMyc 1/200 (Abcam - ab32); mouse anti-Nintra (DSHB - C17.9C6); mouse anti-Prospero 1/50 (DSHB); rat anti-Prospero 1/50 (C. Doe); rabbit anti-Tailless 1/500 (J. Reinitz); rabbit anti-Yorkie 1/400 (K. Irvine). Secondary antibodies used were: 488, 555 and/or 633 Alexa-Fluorophore conjugated anti-mouse, rabbit, rat and/or guinea pig 1/400 (Life Technologies); and cross-adsorbed 488, 555, Cy3 and/or 633 anti-mouse cross-adsorbed against rat and/or anti-rat cross-adsorbed against mouse 1/200 (Jackson).

Image acquisition and processing Confocal images were acquired using a Zeiss LSM 510. Z-series scans were obtained using a 1.5 μm steps. Images were processed using ImageJ (National Institutes of Health), Adobe® Photoshop® CS3 and Adobe® Illustrator® CS6.

EdU Quantification Volume of EdU incorporation was quantified for the 'thoracic' region of the CNS, defined as the area of the ventral nerve cord that extends between the brain lobes to the segments A1/A2 (easily distinguished from more posterior neuromeres due to drastic reduction in neuroblast density), as described by Sousa-Nunes, et al. [21]. Given that absolute numbers of reactivated neuroblasts can vary with small differences in temperature, humidity and genetic background, sibling control experiments were carried out in parallel for each genotype. EdU volume in the described region of each CNS was quantified using Volocity software (PerkinElmer) and normalised to control for each experiment.

Statistical analysis Statistical analyses were performed with Prism 6 (GraphPad Software). Data from each experiment were submitted to unpaired *t-test*s, assuming a Gaussian distribution.

DNA cloning and Gateway cloning Synthesis of GAL4-miRNA was outsourced from GenScript [63]. Plasmid constructs habouring components for GAL4-independent cell-non autonomous studies were generated in the RSN laboratory by Ana Mateus (backbone plasmid was kindly provided by Cyrille Alexandre). pCasper3 containing *hs-mFLP5* was kindly provided by Iris Salecker [64]. The plasmid carrying an *attB* sequence was kindly provided by Cirylle Alexandre. pOT2 vector carrying CG14712 cDNA clone [65] was obtained from the Drosophila Genomics Resource Center (stock no. LD43047, GOLD collection) on a Whatman® FTA® disc. To extract the DNA, 75μl of Tris-EDTA (TE) buffer was added, followed by incubation overnight at room temperature. Gateway entry vector pENTR™ was obtained from Life Technologies (pENTR™/D-TOPO® Cloning Kit); destination vectors were pTW and pTVW [66]. Plasmids were extracted and purified QIAprep Spin Miniprep Kit and Plasmid Midi Kit (Qiagen). *hs-mFLP5* and CG14712 coding sequence (cds) were amplified by Polymerase chain reaction, using Fast Start High Fidelity PCR System, dNTPack (Roche, product no. 04738292001) [67]. Plasmid containing GAL4-miRNA and plasmid constructs habouring components for GAL4-independent cell-non autonomous studies were digested with AvrII (New England Biolabs). Amplified *hs-mFLP5* fragment and the plasmid carrying an *attB* sequence were digested with BamHI and NheI (New England Biolabs). Digested plasmid backbones and inserts were ligated according to the ligation protocol with T4 DNA Ligase [68] (New England Biolabs). PCR-amplified cds for CG14712 was cloned into the entry vector (pENTR™/D-TOPO® Cloning Kit, Invitrogen) by a topoisomerase-catalyzed reaction, following the manufacturer's user guide [69]. The LR recombination reaction was performed to transfer the gene of interest into a destination vector to create an expression clone, using Gateway® LR Clonase™ II Enzyme Mix (Invitrogen), as described in the manual [70].

Inverse PCR The protocol to perform inverse PCR was essentially the one described in [71].

Bioinformatics Potential nuclear localization signals were predicted using three softwares: NucPred [72, 73], WoLF PSORT [74, 75, 76] and cNLS Mapper [77, 78]. Prediction and mapping of casein kinase II specific phosphorylation sites was carried out using Group-based Prediction System (GPS 2.1) [79, 80, 81]. CLC Sequence Viewer 7 software (CLC Bio) [82] was used to align multiple amino acid sequences in this study. Protein Basic Local Alignment Search Tool (BLASTp) [83] was used to predict a function for CG14712 and to search for related nucleoporin sequences in the *Drosophila* genome. Nucleotide BLAST against *Drosophila melanogaster* genome [84] and GBrowse [60] were used to determine the cytogenetic location of Inverse PCR resulting fragments. Gene Ontology terms and protein family were searched using AmiGO [85] and Panther Classification System [86]. Sequencing results were analysed with ApE and Sequence Scanner Software 2 (Applied Biosystems, Life Technologies).

2. Results

2.1 Downregulation of CG14712 phenocopies *2V327*

We confirmed that downregulation CG14712 leads to the presence of L3 neuroblasts with abnormal morphology, extending cell processes (Fig. 1**a**) - features of a quiescent neuroblast [38]. We never observed this phenotype in wild-type (WT) animals of the same stage (data not shown). This result strongly suggests that CG14712 is the gene mutated in the *2V327* stock.

2.2 Generation of *UAS-CG14712* constructs

Since CG14712 is a strong candidate for the gene responsible for the *2V327* phenotype, we generated *UAS* rescue constructs. We employed gateway cloning with destination vectors carrying a *UAS* promoter and either untagged or N-terminus tagged cds with a Venus tag.

2.3 CG14712 seems to encode a nucleoporin (Nup)

To predict a molecular/cellular function for CG14712, we performed a BLAST search with its protein sequence with the National Center for Biotechnology Information (NCBI) database. The first hit with a described function corresponded to a Nucleoporin from *Wickerhamomyces ciferrii*(data not shown). Searching for CG14712 Gene Ontology (GO) terms we found that for its molecular function it is predicted to bind RNA and for its biological process it is predicted to be involved in the intracellular/nuclear protein transport, belonging to a nuclear pore complex protein family (PTHR23193) [85, 86].

2.4 Nup98-96 and Nup214 regulate quiescence

Since it is likely that CG14712 encodes a nucleoporin, we investigated the possible role of other fly nucleoporins in quiescence regulation. The rationale to find other nucleoporins to study in this project consisted of doing a BLAST search to look for similarity between protein sequences. Using CG14712 protein sequence as query to perform BLAST against *Drosophila melanogaster* genome, we found nucleoporin 98-96 (Nup98-96) as the most similar(data not shown). Subsequently, we used Nup98-96 protein sequence as query to do a BLAST also against the fruit fly genome, from which we retrieved nucleoporins 214 and 54 (Nup214 and Nup54), followed by CG14712 (data not shown). The latter BLAST search reinforced our conviction that CG14712 indeed encodes a Nup.

We assessed the cell shape of wL3 neuroblasts, with downregulated and/or mutated Nup98-96, Nup214 and Nup54. Since we did not have a RNAi line to downregulate Nup54, we decided to use homozygous late larvae (wL3) from a L3 lehtal GAL4 enhancer trap in the Nup54 *locus* (Fig. 1**b**, **c**, and **d**). Downregulation of Nup98-96 and Nup214 results in the presence of neuroblasts extending cell processes (Fig. 1**b** and **c**). This phenotype was not observed in *Nup54-GAL4* animals (Fig. 1**d**).

Furthermore, the EdU assay revealed delayed reactivation in homozygous $Nup214^{10444}$ mutants relative to heterozygous sibling controls, whilst *Nup54-GAL4* showed no significant difference (Fig.2**a**). We conclude that at least some Nups affect NB reactivation.

2.5 Casein kinase II regulates quiescence

Given the role of CKII in the subcellular localisation of proteins involved in signal transduction, cell cycle regulation and cell proliferation [87], we investigated its involvement in the regulation of NB quiescence.

First, we looked for the presence of NB fibers in wL3 mutants for CKIIα ($CKII\alpha^{TikR}$). Interestingly, this was the case (Fig. 1**e**). We next performed reactivation assays following overexpression of WT CKIIα and its inactive form *CKIIαKM*, as well as of CKIIβ isoforms ($CKII\beta^{EY21591}$, CKIIβ-VIIb^3, CK2β-VIIc^1, CK2β-VIId-VI^2), using either *nab*-GAL4 or *grh*-GAL4 NB drivers(Fig. 2**b**).

Reactivation phenotypes obtained when overexpressing different casein kinase II forms are puzzling. Surprisingly, overexpression of CIIαKM driven by *nab* triggers precocious neuroblast reactivation, while using *grh* as driver does not alter reactivation timing. Since both drivers have been established as competent neuroblast drivers, this outcome was not expected. However, it was difficult to maintain a healthy and expanded *nab*-GAL4 stock and so we mostly employed *grh*-GAL4. Nevertheless, using *grh* to drive overexpression of CKIIα, CKIIβ-VIIb^3 and CK2β-VIId-VI^2 all inhibited reactivation from quiescence.

2.6 Regulation of quiescence by candidate proteins

In order to study the nucleocytoplasmic protein partitioning in quiescent neuroblasts, we took a candidate approach. As aforementioned in the Introduction, the list of candidates comprises: Pros, Dpn, Yki, NFI, FoxO, Tll, Myc, N^{intra} and CycE.

2.6.1 Candidate prediction of nuclear localisation signals and casein kinase II target sites

Selected candidates have all been previously reported to be able to localise to the nucleus [88, 89, 60, 90, 91, 92, 93, 94, 95]. However, with the exception of Pros [90] and N^{intra} [91], sequences that mediate their import into the nucleus (NLSs) have not been functionally mapped. Hence, we examined their amino acid sequences searching for motifs that may constitute NLSs and CKII target sites, using web-based prediction softwares. We found that all candidates are predicted to harbour classical NLSs (with the exception of Tll) and potential CKII target sites (data not shown). It is also clear that output from distinct softwares do not always match, which means that their algorithms are based on different assumptions, highlighting the idea that the definition of a NLS sequence is still not fully elucidated.

2.6.2 Candidate subcellular localisation

To investigate the nucleocytoplasmic partitioning of candidate nuclear factors and test our primary hypothesis that proteins

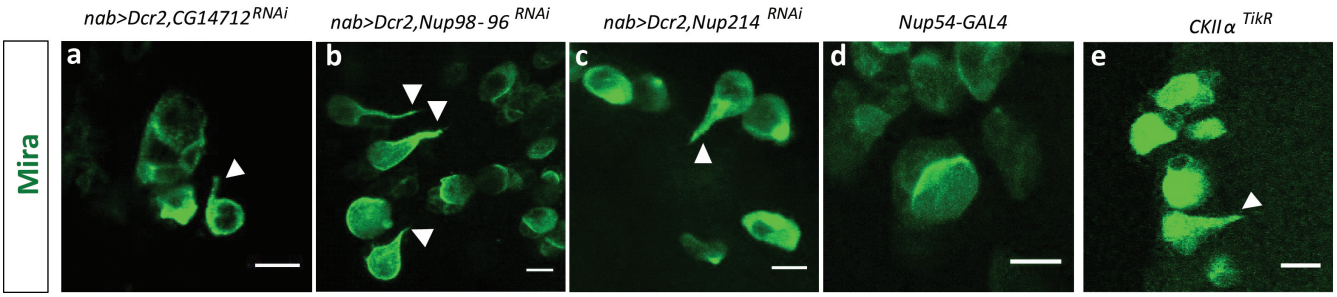

Figure 1. **Nuclear transport machinery regulates NB quiescence**. Third instar larval neuroblasts stained for Miranda, when downregulating **a** CG14712, **b** Nup98-96, **c** Nup214, and with mutation in the **d** Nup54 and **e** CKIIα *loci*. Arrowheads depict cell fibers. Scale bars: 10μm.

harbouring classical monopartite NLSs would be excluded from the nucleus of quiescent neuroblasts, we started by determining the endogenous subcellular localisation of each protein in early first instar larval neuroblasts. It was not possible to do this experiment for NFI, because there is no antibody available. We tested an antibody against mouse Nfix, but, unfortunately, it did not work. Staining of L1 *grh<CD8::GFP* revealed that Pros and Dpn localise to the nucleus of GFP-labelled cells (data not shown). Yki, Myc, N^{intra} and CycE localised to cytoplasm (data not shown). Endonegous Tll was undetectable (data not shown). FoxO localised to the nuclei of subsets of non-identified cells in the VNC (data not shown) and central brain (data not shown). We hypothesize that the latter correspond to the brain median neurosecretory cells (mNSCs), which are an important source of insulin-like peptides (ILPs) [96, 97, 98], but this was not confirmed.

Next, we overexpressed each candidate in NBs with the aim of complementing functional studies, as negative results might be due to failure in nuclear localisation. Early larvae overexpressing Pros died immediately after hatching, consequently no image could be acquired. Upon overexpression, Tll was detected in the nucleus (data not shown) and, interestingly, these larva die at late L1. Subcellular localisation of Myc, Yki, CycE and N^{intra} following overexpression (data not shown) was fairly similar to their endogenous one.

2.6.3 Overexpression of candidates delays neuroblast reactivation when driven by *grh*

We determined whether overexpression of any of these nuclear regulators, using *grh* as a NB driver, led to a reactivation phenotype (not possible for Pros or Tll due to lethality). Surprisingly, overexpression of each candidate tested delayed NBs reactivation (Fig. 2**c**). Note that driving *yki::GFP* and *yki*^{S168A}*::GFP* leads to a reduction in EdU incorporation, whilst *yki* does not alter reactivation timing (see Discussion).

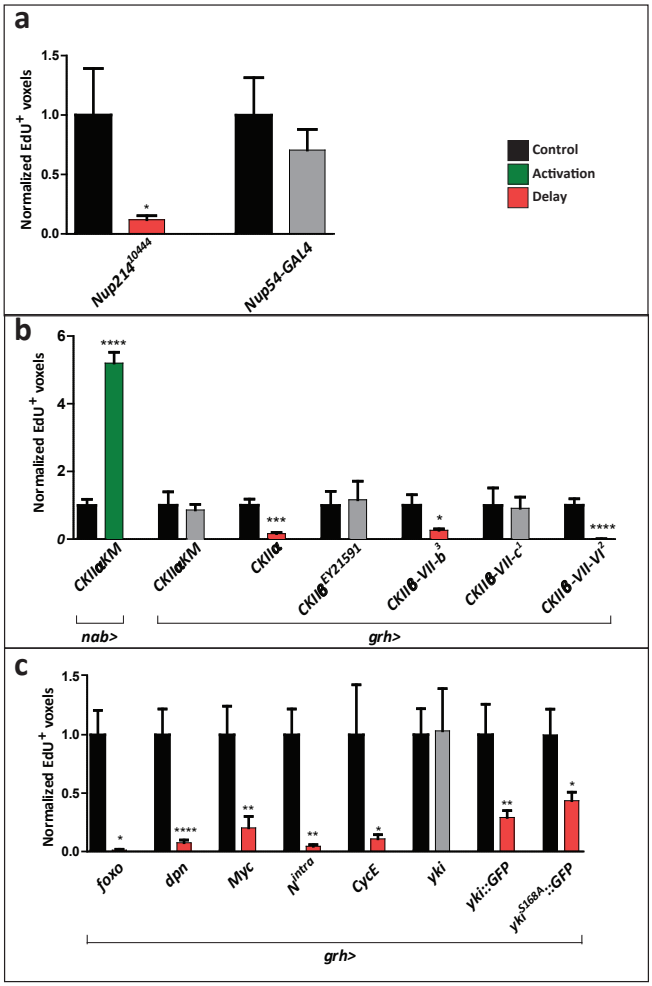

Figure 2. **Nuclear transport machinery regulates NB reactivation**. Histograms of EdU$^+$ voxels from the thoracic region of larval CNSs, normalized to sibling controls, involving the role of **a** Nups, **b** CKII isoforms, and **c** candidate cargo. Error bars are represented as SEM; significance was determined using *t-tests* (*0.01≤p<0.05; **0.001≤p<0.01; ***0.0001≤p<0.001; ****p<0.0001).

2.7 Is a GFP reporter carrying a classical monopartite NLS excluded from the nucleus of *2V327* mutant NBs?

2V327 mutant neuroblast clones had only been labelled with the membrane reporter *CD8::GFP*. We wished to determine if the hypothetical nucleoporin encoded by the gene responsible for the *2V327* phenotype was required for the nuclear import of reporters carrying classical monopartite NLSs. To test if this was the case, we wished to generate *2V237* clones using a MARCM stock containing *NLS2::GFP* [99]. Unfortunately,

at the time of this work, the only living stock that enabled us to generate *2V327* mutant clones had been recombined with the $mira^{L44}$-null allele. As expected, Miranda was absent from GFP-labelled clones (data not shown); Deadpan was found in the nucleus of neuroblast clones (data not shown); and Prospero was absent from GFP-labelled cells (data not shown). Unexpectedly, *NLS2::GFP* appears to robustly localise to nuclei. However, we do not know to what extent $mira^{L44}$ mutation is affecting the generation of *2V327* mutant clones and the nucleocytoplasmic partitiong. In fact, *2V327* mutation is known to frequently generate single cell clones (Rita Sousa-Nunes, personal communication), and, using this stock, large cell masses were observed (data not shown), indicating that double-mutant clone cells proliferate more than single *2V327* clones so they are unlikely to be quiescent. This experiment thus needs to be repeated with the *2V327* single mutant (now generated but not in time for this report).

2.8 *Nup98-96*$^{F1.13}$ clones are rare and small

We decided to investigate whether if Nup98-96 mutant neuroblasts would also anachronically enter quiescence and exclude *NLS2::GFP* from the nucleus. We found that these clones are rare and small (data not shown), which suggests that this is a strong and, probably, cell lethal mutation.

2.9 Construct to study cell non-autonomous effects

The Sousa-Nunes laboratory has designed a GAL4/*UAS* independent construct that allows labelling of genetically manipulated cells in a spatiotemporal controlled manner. This tool thus permits GAL4 manipulation of the microenvironment to study its effects on labelled cells. One of the goals of this project was to contribute towards this genetic tool.

The construct has two transcriptional termination (*STOP*) cassettes each flanked by FRT and mFRT71 sites, which are specifically recognised by FLP1 and mFLP5, respectively [64]. To achieve spatiotemporal control, we can provide a lineage-specific expression to one Flippase and heat-shock control to the other. However, it is known that mFLP5 is capable of inducing recombination of FRT sites with a low efficiency (1%), whilst FLP1 does not act on mFRT71 sites [64]. Therefore, we decided to constantly express FLP1 in a spatial controlled manner (with lineage-specific enhancers) and express mFLP5 downstream of a heat-shock promoter, which allows temporal control. The publically available *hs-mFLP5*, which was inserted on the second chromosome, is lethal. In order to have site-specific insertion [100] into a viable and convenient site on the second chromosome, we inserted *hs-mFLP5* into an *attB*-carrying plasmid. Transgenic flies carrying this construct were successfully generated and heat-shock control was functionally validated (data not shown).

To assure GAL4 silencing in the cells labelled with the construct, we inserted GAL4-microRNA into the sequence.

In order to efficiently manipulate expression of target genes through the RNAi pathway, the introduction of an *UAS-Dcr-2* element is helpful. To do so, it was necessary to generate recombinant flies carrying several exogenous inserts at various genomic locations. To determine probability of obtaining the desired recombination events [58]. Given that the genomic location of *UAS-Dcr2* is not known, we used inverse PCR to sequence fragments that are flanking its P-elements. Based on sequencing results, we found that the cytogenetic location of *UAS-Dcr-2* is 19A2.

3. Discussion

3.0.1 A role for Nups in regulating NB quiescence

Deficiency mapping (RSN, data not shown) and RNAi phenocopy (Fig. 1**a**), suggests that the gene responsible for the *2V327* phenotype is CG14712. This gene encodes a putative Nup previously implicated in neurogenesis by a genome-wide study of *Drosophila* neural lineages using transgenic RNAi performed by Neumüller and coworkers [101]. They found that knocking-down CG14712 leads to abnormal NB morphology, and underproliferation of neural lineages – all of which are consistent with the phenotype seen in *2V327* mutants. Together, these studies suggest that this Nup regulates quiescence, at least in NBs. We now hypothesise that this putative NPC protein is involved in the nuclear transport of key cell-cycle regulators.

Results obtained with MARCM for the *2V327* mutant were not conclusive in regard to the nuclear exclusion of *NLS2::GFP*, since the only available stock was a $mira^{L44}$ recombinant. *2V327* has now been isolated from the *miranda* mutation, although the originally planned experiment could not be performed in time for this project.

Successful generation of flies carrying the inducible *UAS-CG14712* constructs, either untagged or with a N-terminus Venus tag, will enable cell-type specific rescue of the mutation and assessment of phenotype reversibility. Since we can use temperature to control GAL4 drivers (e.g. TARGET system that combines the conventional GAL4/*UAS* system and a temperature-sensitive GAL80 molecule, which represses GAL4 transcriptional activity at permissive temperatures), anachronic NB quiescence can be studied in a flexible manner, with control over the exact stage and duration of *UAS* expression. Furthermore, these animals can be used for over-expression studies, assessing the effect of increased CG14712 levels on quiescence or cell-cycle speed.

We also showed that downregulation of other Nups leads to a similar phenotype. This suggests that Nups are involved in the regulation of quiescence, namely Nup98-96 (Fig. 1**b**) and Nup214 (Fig. 1 **c**). Nikos Xylourgidis and colleagues [102] demonstrated that Nup214 is responsible for sequestering part of the CRM1 pool to the nuclear pore, making it unavailable to mediate nuclear export, and that it modulates NF-κB proteins activation, involved in many regulatory mechanisms. The extremely conserved *nup98-96* locus encodes two alternatively spliced mRNA variants: one containing an open reading frame for Nup98 and the other for Nup98-96. The latter is then processed by a proteolytic cleavage, which subsequently separates the two functional units, Nup98 and Nup96. Human Nup214 and Nup98 have been described to

be implicated in acute forms of leukaemia, following chromosome translocation that leads to the fusion of these Nups with other proteins [103, 104]. It is plausible that the fusion of these proteins leads to aberrant nuclear transport in leukaemia. Together, these results indicate an important role of Nup214 and Nup98 in the regulation of cell cycle progression. Generated *Nup98-96$^{F1.13}$* clones were rare and small, suggesting that this mutant is cell lethal. On the other hand, other stocks mutated for Nup98-96 are viable until, at least, third instar. To eliminate the possibility of having maternal contribution to the phenotype, we want to find the best transheterozygous combination to determine if this gene is involved in the NB reactivation timing. Possibly, this could be done by combining *Nup98-96$^{F1.13}$* with another allele. Given the strength of the Nup98-96$^{F1.13}$ allele, MARCM experiments could be performed using hypomorphic alleles, such as *Nup98-96^{2288}* or *Nup98-96^{G2120}*, to better understand if Nup98-96 is crucial for the nucleocytoplasmic partitioning of key regulators during quiescence. We also wish to be able to label Nup214 mutants using MARCM to more specifically study their role in nucleocytoplasmic partitioning during quiescence.

We did not observe any phenotype that relates Nup54 with quiescence regulation. However, we can hypothesise that maternal contribution of gene product may mask the effect of the mutation. Hence, it would be interesting to investigate this Nup using other stocks (mutant and RNAi).

Nup98, Nup214, Nup54 and the putative CG14712 Nup are all FG-enriched Nups [41] (and data not show). We would like, therefore, to explore if only FG Nups lead to this phenotype, or if it is a combination of different types of Nups. Furthermore, Martynoga et al. [105] performed transcript profiling of quiescent and proliferating mouse NSCs and determined which genes were being differently expressed when comparing both cell states. Their results demonstrated that several Nups are downregulated in quiescence. We wish to assess the level of conservation of these Nups in *Drosophila* to study their role in regulation of quiescence.

In this project, we only assessed cell morphology to find anachronically quiescent cells. However, we would like to perform similar experiments using different RNAi lines, but also doing co-stains for the mitotic marker Phospho-Histone H3 (PH3). The absence of this marker in cells extending cellular fibers would provide stronger evidence that these cells are indeed quiescent.

3.0.2 CKII regulates NB quiescence

CKII is pleiotropic and its activity can enhance or inhibit DNA binding capacity of some of its substrates, even if we are only accounting positive regulators of cell proliferation [87]. Nevertheless, CKII activity is often increased in cells that are actively proliferating [87, 106]. CKII experiments performed in this study led to puzzling observations, since the loss-of-function *CKIIα^{TikR}* mutant presented presumably anachronically quiescent neuroblasts (in late larval stages, Fig. 1**e**), whereas overexpression of active CKIIα led to delayed NB reactivation using *grh-GAL4* (Fig. 2**b**). Overexpression of kinase-dead CKIIα with the same GAL4 driver did not alter reactivation timing (Fig. 2**b**). Intriguingly, it has been demonstrated that increased expression of active forms of CKIIα in human osteosarcoma U2-OS cells resulted in modest decrease in cell proliferation, whereas induction of kinase-inactive CKIIα-KM did not significantly affect proliferation rate [107], which corroborates the results obtained in this project using *grh-GAL4* as a NB driver (Fig. 2**b**). However, evidence regarding CKII and cell proliferation are far from being consensual, as high levels of CKII are often associated with tumour progression and other studies have suggested that specific downregulation of CKII catalytic unit might be useful for therapeutic elimination of tumours [108]. It is plausible to suggest that optimal levels of CK2α are required for optimal proliferation. Concerning the regulatory subunit CKIIβ, it is highly relevant to mention that studies performed in *Xenopus laevis* oocytes reported that this subunit is an inhibitor of c-Mos serine/threonine kinase and showed that, via its inhibitory interactions with c-Mos, CKIIβ negatively regulates progesterone-induced maturation [109]. Moreover and consistent with these results, overexpression of CKIIβ in yeast resulted in growth inhibition [110]. Additionally, ectopic expression of CKIIβ in mouse 3T3-L1 adipocytes and in CHO (Chinese hamster ovary) cells led to attenuated proliferation [111]. Therefore, results obtained in previous studies in different systems are in accordance to what has been observed in the present study. Indeed, although it did not happen for all the CKIIβ isoforms, overexpression of *CKIIβ-VII-b^3* and *CKIIβ-VII-VI^2* resulted in a significant delay in NB reactivation from the quiescent state (Fig. 2**b**).

Altogether, these experiments, which aim was studying the role of CKII in the nucleocytoplasmic transport in quiescent NBs, not only established that indeed CKII has a determining role in the control of the quiescent state, but also emphasised its complex nature associated with different aspects of the cell-cycle regulation.

3.0.3 Candidate prediction of NLSs and CKII target sites

Regarding the study of quiescence regulation by candidate proteins involved in proliferation and cell-cycle progression (Pros, Dpn, Yki, NFI, FoxO, Tll, Myc, N^{intra} and CycE), we showed that these proteins harbour putative classical NLSs (with the exception of Tll) and putative CKII target sites. However, when looking at their subcellular localisation during quiescence, we observed that some of these candidates localised to quiescent NB nuclei in spite of carrying classical monopartite NLSs (data not shown), refuting the hypothesis that proteins containing classical monopartite NLSs would be excluded from the NB nucleus during quiescence.

3.0.4 Functional role of candidates on NB quiescence

We will now analyse the results obtained for each one of the aforementioned candidates:

Prospero, which is known to repress genes required for self-renewal and to activate genes for terminal differentiation [55], was shown to localise to the nucleus of quiescent

NBs (data not shown). Interestingly, it has been very recently described that levels of Pros are intimately related with progenitors fate [112]: high levels lead to differentiation, low levels induce quiescence and its absence is associated with self-renewal. We did not assess the phenotype of Pros overexpression due to its lethality at 29°C. Hence, this experiment will be repeated at lower temperatures.

Deadpan was found in the nucleus of quiescent NBs both endogenously and upon overexpression of its protein in NBs (data not shown). Unexpectedly, we observed a delayed reactivation from quiescence following overexpression of Dpn, as compared to its sibling controls (Fig. 2**c**). In fact, it has been demonstrated that overexpression of Dpn leads to overproliferation and failure to terminate self-renewal in both type I and type II NBs [113].

Yorkie subcellular localisation, endogenously and upon overexpression, seems to be mainly cytoplasmic (data not shown). In its native form, Yki, a transcription factor supressed by the Hippo pathway, is negatively regulated by the Warts (Wts) kinase, which promotes its cytoplasmic localization [88, 114]. On the other hand, activation of Yorkie by mutating a key Wts phosphorylation site (S168A) [115] drives cell growth, as demonstrated by Binnaz Kucuk Staley and Kenneth D. Irvine [116] in the case of intestinal stem cells. It is not surprising that Yki overexpression does not produce a phenotype in NB reactivation timing (Fig. 2**c**), since its native form is susceptible to be constantly inactivated by Wts. The delayed NB reactivation phenotype following overexpression of *yki*^{S168A}*::GFP* and *yki::GFP* contradict recent unpublished data implicating Yki as a positive regulator of NB reactivation (Christian Berger, personal communication). Due to the complexity of the pathways involved in growth regulation, it is possible that cell non-autonomous effects are responsible for these unexpected results. We must, therefore, repeat these reactivation assays using different NB drivers.

Forkhead box O (endogenous) was undetectable in quiescent NBs (data not shown). In addition, FoxO overexpression driven by *grh-GAL4* resulted in delayed NB reactivation, which is consistent with what was previously described using *nab-GAL4* [21]. Indeed, overexpression of FoxO inhibits PI3K/Akt pathway [21], which regulates NB reactivation.

Tailless is involved in maintenance of NSC self-renewal. Zhe Zhu and colleagues [117] showed that Tlx (Tll mammal homolog) positive cells in mouse primary brain tumours are quiescent. When knocking-down Tlx they observed loss of self-renewal of brain tumour stem cells and prolonged animal survival. We were intrigued by its possible role in NB quiescence/reactivation, however, just like for Pros, Tll overexpression at 29°C was lethal (at late L1). Hence, this experiment will be repeated at lower temperatures. Regarding its subcellular localisation, while endogenous Tll was undetectable (data not shown), upon overexpression we saw nuclear Tll presumably in quiescent NBs (data not shown). Possibly, Tll is expressed endogenously, but below detectable levels. It is plausible that Tll might be involved in the mechanism that leads cells to shift from a proliferative state, in which cells are continually self-renewing, to the quiescent state and also in the maintenance of that state.

Myc subcellular localisation seems to be cytoplasmic and not restricted to GFP-labelled cells (data not shown). Importantly, mammalian oncogene *c-myc* is involved in a variety of tumours [118]. It is known to be a downstream effector of the TOR signalling pathway, having a role in the control of cell growth, in part by regulating ribosome biogenesis [119, 120]. Previous studies showed that the amount of Myc might be crucial for cell fate decisions, such as quiescence (when it is absent) and apoptosis (when it is abundant). Indeed, since the early 1990s it is known that Myc overexpression can drive cells to undergo apoptosis (reviewed in [121]). The ability of Myc to induce apoptosis has been hypothesised to have a tumour suppressive role: increased Myc activity results in apoptotic elimination of mutant cells, instead of allowing them to proliferate and lead to tumour formation [122]. Like mammalian Myc, *Drosophila* Myc (or dMyc) controls growth and cell cycle progression [123] and its physiological function regarding control of apoptosis has been demonstrated [124, 125]. In addition, very recently, its transient overexpression has been shown to be sufficient to activate slow-dividing NBs [126]. This clearly demonstrates that Myc possesses a determining role in cell fate decision, which might depend on different factors, namely levels of this protein in the cell. Our results show that Myc overexpression leads to delay in NB reactivation (Fig. 2**c**). However, due to its role in the cell programmed death pathway, it would be interesting to also look at apoptosis markers (Dcp-1 [127]).

Notch intracellular domain localises to the cytoplasm of NBs both endogenously and when overexpressed and, like Myc, its expression is not restricted to NBs (data not shown). It is not surprising that we cannot observe N^{intra} localising to the nucleus, considering that all attempts to obtain direct evidence for its ligand-dependent nuclear access were unsuccessful [128]. It is known that Notch signalling is pleiotropic and either promotes or represses cell-cycle progression depending on the cellular context [129]. Notch signalling involved binding of Delta-like (Dll) and Jagged ligands, followed by Notch cleavage by γ-secretase complex, allowing its intracellular domain (N^{intra}) to be translocated into the nucleus, where it associates with CBF1 (also called RBP-J or CSL) and Mastermind-like (Maml) proteins to activate transcription of target genes [130]. Interestingly, it was demonstrated in zebrafish that adult NSCs balance between the quiescent and the proliferative state according to Notch activity levels: inducing Notch signalling drives them into quiescence, whereas blocking it massively reinitiates NSC division [131]. Furthermore, it was shown that Notch ligand Dll1 is required to maintain quiescent NSCs in the adult mouse SVZ [132]. By overexpressing N^{intra} with *grh* NB driver, we observed a delay in NB reactivation (Fig. 2**c**), which is not surprising if we take into consideration the aforementioned studies. Nevertheless, regulation of Notch signalling is puzzling, as there are

evidences that its canonical signalling pathway is not required for maintenance HSCs in mouse [133].

Cyclin E is an important G1 cyclin and its downregulation arrests the cell cycle. In addition, it has been shown that ectopic expression of CycE can induce re-entry into the S-phase in larval eye imaginal epithelium and embryo dorsal epidermis terminally mitotic cells, resulting in additional cell cycles [134, 135]. Furthermore, degradation of CycE by Cul3 has been implicated in the maintenance of quiescence in mammalian cells [136]. Interestingly, it has been demonstrated that induced expression of CycE rescues G1 arrest of *trol* NB mutants, being *trol* a regulator of the timing of *Drosophila* NB proliferation[137]. CycE is, therefore, pivotal for the regulation of the cell-cyle. Here CycE was found to localise to the cytoplasm of neuroblasts (and other cells), even when overexpressing CycE or not (data not shown). On the other hand, overexpression of CycE with *grh-GAL4* resulted in delayed NB reactivation (Fig. 2**c**), which is not in agreement with the aforementioned studies.

Nuclear factor I is a candidate to be involved in the regulation of NB quiescence, given the role of mouse Nfix to mouse NSC quiescence [105]. However, it was neither possible to assess subcellular localisation of endogenous NFI during NB quiescence, due to the nonexistence of an antibody specific for *Drosophila* NFI, nor to obtain functional data (i.e. NB reactivation phenotype) upon overexpression. There is an available *UAS-NFI* from FlyORF and we want to generate a dominant-negative NFI *UAS* construct, employing the engrailed fusion strategy [138] analogous to what has been done for mammal Nfix. Having these constructs we will be able to test functional implications of NFI in NB quiescence.

3.0.5 *nab* and *grh* drivers can lead to different results in reactivation assays

Using *grh* as a NB driver led to some results that contradict what has been described in the literature and what can be obtained using the *nab* driver. It is not possible, therefore, with the experiments performed in this project, to draw conclusions or even hypotheses, regarding the specific role of CKII isoforms and the candidate proteins. Overall, it would be thus important to repeat this experiments using, at least, two drivers in parallel: *grh* and *nab*. If dubious results are obtained, a third driver should also be used, such as *inscutable* (*insc*). Furthermore, we wish to compare sites of expression of both drivers throughout development.

3.0.6 GAL4 protein perdurance

GAL4 protein perdures in descendants of NBs, and, therefore, observed GFP-labelled cells (data not shown) might be neurons born from proliferating NBs prior to their entry into quiescence. To ensure that GFP-labelled cells with nuclear stainings are indeed quiescent NBs, we need to perform co-stainings with Dpn, which is a well-established NB marker.

3.0.7 Flexible tool to study cell non-autonomous effects

The CNS hosts a heterogeneous population composed of, not only NSCs, but also other progenitors at various stages towards differentiation, plus neurons, glia, blood vessels (in mammals)/trachea (in insects), and the blood-brain barrier that creates an exclusive molecular and cellular environment. We believe that it is plausible that the cellular extension of quiescent NBs is sensing cues from the environment. The novel molecular tool generated in RSN laboratory, which is GAL4/*UAS* system independent and drives spatially and temporally controlled expression of reporters, will allow manipulation of the microenvironment, whilst being able to visualise and analyse NB behaviours, such as growth and cellular extension morphology *in vivo*. In this project, we successfully inserted GAL4-miRNA sequence into the construct, generated a plasmid carrying *hs-mFLP5*, and determined the genomic location of *UAS-Dcr-2*, which is going to contribute to enhance the RNAi efficiency. Therefore, we are building, step-by-step, our way towards having an extremely flexible tool to study different types of interactions between specific cell populations and their surroundings.

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