The SCF^{Slimb} E3 ligase complex regulates asymmetric division to inhibit neuroblast overgrowth

Song Li^{1,2}, Cheng Wang¹, Edwin Sandanaraj^{3,4}, Sherry S Y Aw^{1,†}, Chwee T Koe^{1,2}, Jack J L Wong^{2,5}, Fengwei Yu^{1,2,5}, Beng T Ang^{3,4,6}, Carol Tang^{4,6,7} & Hongyan Wang^{1,2,6,*}

Abstract

Drosophila larval brain neuroblasts divide asymmetrically to balance between self-renewal and differentiation. Here, we demonstrate that the SCF^{Slimb} E3 ubiquitin ligase complex, which is composed of Cul1, SkpA, Roc1a and the F-box protein Supernumerary limbs (Slimb), inhibits ectopic neuroblast formation and regulates asymmetric division of neuroblasts. Hyperactivation of Akt leads to similar neuroblast overgrowth and defects in asymmetric division. Slimb associates with Akt in a protein complex, and SCF^{Slimb} acts through SAK and Akt to inhibit neuroblast overgrowth. Moreover, Beta-transducin repeat containing, the human ortholog of Slimb, is frequently deleted in highly aggressive gliomas, suggesting a conserved tumor suppressor-like function.

Keywords asymmetric division; neuroblasts; polarity; the SCF complex

Subject Categories Development & Differentiation; Cell Adhesion, Polarity & Cytoskeleton

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Introduction

The neural stem cells, or neuroblasts (NBs), of the *Drosophila* larval brain have emerged as an important model for studying stem cell self-renewal and tumorigenesis [1–4]. There are at least two NB lineages with distinct spatial positions and intrinsic properties in the *Drosophila* larval central brain [5–7]. In both lineage types, NB overproliferation can be triggered by perturbation of asymmetric divi-

sions [2,4]. Asymmetric division ensures the polarized distribution of “proliferation factors,” including atypical protein kinase C (aPKC), and “differentiation factors,” including basal proteins Numb, Miranda (Mira), Brain tumor (Brat), and Prospero (Pros), to the daughter NB and GMCs, respectively [2]. The failure of asymmetric division in NBs can result in their hyperproliferation and in the induction of brain tumors [2]. Correct asymmetric protein segregation also relies on mitotic spindle orientation. Inscuteable (Insc), the heterotrimeric G proteins G α i and G β γ , and their regulators, Partner of Insc (Pins), Ric-8, and Mushroom body defect control mitotic spindle orientation in NBs [4]. Several centrosomal proteins, including Aurora A, Polo, Anastral Spindle 2, also regulate spindle orientation and NB self-renewal [8–12].

The ubiquitination step is mediated by a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3). However, the functions of these enzymes during NB self-renewal and differentiation were almost completely unknown. Here, we have demonstrated that all three enzymes suppress NB overproliferation and regulate the asymmetric division of NBs.

Results and Discussion

The SCF^{Slimb} E3 ligase complex suppresses NB overproliferation in larval brains

Knockdown (KD) of *cullin1* (*cul1*) using the NB driver *insc*-Gal4 resulted in a prominent NB overproliferation in larval brains (supplementary Fig S1B). Approximately 300 NBs that expressed the NB marker Deadpan (Dpn) were observed in a pupal lethal *cul1*^{EY11668} mutant at 96 h after larval hatching (ALH) (supplementary Fig S1C; *n* = 20) compared with approximately 100 NBs in wild-type (WT) central brains (supplementary Fig S1A; *n* = 20). Animals that were

1 Neuroscience & Behavioral Disorders Program, Duke-National University of Singapore Graduate Medical School Singapore, Singapore City, Singapore

2 NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore, Singapore City, Singapore

3 Singapore Institute for Clinical Sciences, A*STAR, Singapore City, Singapore

4 National Neuroscience Institute, Singapore City, Singapore

5 Temasek Life Sciences Laboratory and Department of Biological Sciences, National University of Singapore, Singapore City, Singapore

6 Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore City, Singapore

7 Division of Medical Sciences, Humphrey Oei Institute of Cancer Research, National Cancer Centre, Singapore City, Singapore

*Corresponding author. Tel: +65 6516 7740; Fax: +65 6557 0729; E-mail: hongyan.wang@duke-nus.edu.sg

†Institute of Molecular and Cell Biology, Singapore City, Singapore

Figure 1. The SCF^{slimb} E3 ligase complex suppresses NB overproliferation in larval brains.

A–D Dpn and EdU are labeled in WT and *cul1*⁻ larval brains. The central brain (CB) is to the left of the white dotted line, which marks the border between the CB and the optic lobe. Scale bar, 5 μ m.
 E Quantification of larval brain NBs per brain hemisphere.
 F–O Type I and type II NB clones are labeled for Ase, CD8 and Dpn in control (driver), *cul1*^{Ex}, *skipA*¹, *roc1a*^{G1}, and *slimb*⁰⁰²⁹⁵. NBs (Dpn⁺ Ase⁺ in type I and Dpn⁺ Ase⁻ in type II lineages) in the clones are indicated by arrows and clones are outlined by white dotted lines. Scale bar, 5 μ m.

trans-heterozygous for *cul1*^{EY11668} and a null allele *cul1*^{Ex} (henceforth referred to as *cul1*⁻) displayed 473 ± 100 NBs (Fig 1B and E; $n = 23$), which was fully rescued by a *Flag-cul1* transgene (supplementary Fig S1D–F). In a time course experiment, *cul1*⁻ exhibited a dramatic increase in NB numbers at late larval stages (Fig 1E). The numbers of cells labeled by phospho-Histone H3 (pH3), dMyc, and EdU were significantly increased in *cul1*⁻, compared with WT brains (Fig 1C–D; supplementary Fig S1G–J). A dramatic decrease in the number of neurons labeled by Embryonic lethal abnormal vision (Elav) or nuclear Pros was observed in *cul1*⁻ brains (supplementary Fig S1K–N). There was only one NB in each WT type I (Fig 1F; 100%, $n = 32$) or type II (Fig 1J; 100%, $n = 19$) NB MARCM (Mosaic Analysis with Repressible Cell Marker [13]) clone. In contrast, *cul1*^{Ex} clones contained ectopic NBs in both type I (Fig 1G; 40%, $n = 20$) and type II (Fig 1K; 44%, $n = 18$) lineages. *cul1* KD using a “type II driver” (see Materials and Methods) also resulted in

the generation of multiple NBs (supplementary Fig S3B; 54.7%, $n = 75$).

We next assessed the function of two other *Drosophila* SCF subunits, SkpA and Roc1a. Clones derived from *skipA*¹, a loss-of-function allele, displayed ectopic NBs in both type I (Fig 1H; 50%, $n = 16$) and type II lineages (Fig 1L; 62%, $n = 21$). SkpA KD also resulted in 450 ± 83 NBs (supplementary Fig S3H; $n = 20$), in contrast to 117 ± 15 NBs in the control (supplementary Fig S3G; $n = 20$). *skipA* KD under the type II driver also resulted in NB overproliferation (supplementary Fig S3C; 33.3%, $n = 66$). A different *skipA* RNAi line resulted in NB underproliferation in a RNAi screen [14], likely due to the effect of unknown positional insertion. Likewise, *roc1a*^{G1} clones possessed extra NBs in both type I (Fig 1I; 56%, $n = 18$) and type II (Fig 1M; 64%, $n = 22$) NB clones. *roc1a* KD under the type II driver also caused the formation of ectopic NBs (supplementary Fig S3D; 56%, $n = 33$).

Figure 2. The SCF^{Slimb} complex regulates asymmetric division of NBs.

A-F aPKC and Numb localization in control (MACRM driver), *cul1^{Ex}*, *roc1a^{G1}*, *skpA¹* and *slimb⁸* metaphase NBs in clones. DNA is in blue, and insets are enlarged views of single NBs. Arrow, ectopic apical Numb.

G, H WT and *cul1*⁻ NBs at telophase are labeled for aPKC, Numb and DNA.

I-K WT, *cul1*⁻, and *cul1*⁻; *insc*-Gal4 UAS-Flag-Cul1 (*insc* > Flag-Cul1) NBs are labeled by Insc, α -tubulin and DNA. Mitotic spindle orientation is quantified in (I'-K').

L, M Live-imaging stills of control and *cul1⁻* expressing Ubi- α -tubulin-GFP. Time is shown as minutes: seconds. The cartoon illustrations are shown at the right corner.

Data information: Scale bars, 5 μm .

Figure 3. The UPS regulates NBs self-renewal and asymmetric division.

A–H Type I and type II clones from control (driver), *uba1*^{s3484}, *eff*^{D73}, and *nedd8*^{AN105} are labeled for Dpn, Ase and CD8-GFP. Arrow, NB.
 I–L aPKC and Numb, CD8 and DNA are labeled in control (MACRM driver), *uba1*^{s3484}, *eff*^{D73}, and *nedd8*^{AN105} metaphase NBs in clones.
 M–O Baz, α -tubulin and phospho-Histone H3 (pH3) are labeled in control (driver) and *uba1*^{s3484}, and *nedd8*^{AN105} metaphase NBs in clones. Insets are enlarged views of NBs in the clones.
 P–S Dpn is labeled in WT, *cul1*^{EY11668}, *sak*^{C06612} and *cul1*^{EY11668} *sak*^{C06612} at 84 h ALH.
 T Quantification of NB numbers per hemisphere. The central brain is to the left of the white dotted line.
 Data information: Scale bars, 5 μ m (A–H), 2 μ m (I–O) and 20 μ m (P–S).

F-box proteins confer substrate specificity to various SCF E3 ubiquitin ligases [15]. Among 35 F-box proteins we identified Supernumerary limbs (Slimb) through RNAi targeting and mutant analyses (supplementary Table S1). Clones of the loss-of-function allele *slimb*⁰⁰²⁹⁵ exhibited a NB overgrowth in both type I (Fig 1N; 42%, $n = 24$) and type II (Fig 1O; 30%, $n = 23$) lineages. In the stronger allele *slimb*⁸, ectopic NBs were also observed in both type I (supplementary Fig S2B, B'; 45%, $n = 20$) and type II lineages (supplementary Fig S2D, D'; 50%, $n = 24$). Likewise, *slimb* KD under *insc*-Gal4 resulted in 271 ± 21 NBs per brain hemisphere compared with control brains (101 ± 8 NBs; supplementary Fig S2E–G). *slimb* KD under the type II driver also generated supernumerary NBs (supplementary Fig S3F). Loss of three other F-box proteins, Skp2, Nutcracker (Ntc) or Ago, did not result in NB overgrowth (supplementary Fig S3I–O).

The SCF^{Slimb} complex regulates asymmetric division of NBs

In *cul1*^{Ex} clones, 55% of metaphase NBs displayed either uniformly cortical or diffused cytoplasmic aPKC localization (Fig 2B; $n = 31$), in contrast to WT NBs (Fig 2A; 100%, $n = 18$). Consistently, 39% of *cul1*^{Ex} metaphase NBs exhibited delocalized Numb (Fig 2C; $n = 31$), in contrast to control NBs (Fig 2A; 100%, $n = 18$). The localization of other polarized proteins, including Bazooka (Baz), Par6, Insc, and Pins, was mildly disrupted as cortical distribution or misoriented crescents in the *cul1*^{Ex} NBs (supplementary Fig S4A–H). *cul1*^{Ex} NBs also displayed a spindle misorientation at metaphase (Fig 2J, J'; 40%, $n = 95$; WT, Fig 2I, I') with 13% of NBs showed a 90° misalignment (orthogonal division) of the mitotic spindle with the apicobasal axis (Fig 2J, J'; $n = 95$). This defect in *cul1* mutants was fully rescued by the expression of Flag-tagged Cul1 (Fig 2K, K'; $n = 31$). aPKC (5.4%, $n = 205$) and Numb (5.4%, $n = 205$) were missegregated into both daughter cells during telophase in *cul1*^{Ex} NBs (Fig 2H) compared with the control (Fig 2G; $n = 136$).

In time-lapse experiments on living whole-mount brain explants expressing α -tubulin-GFP, control NBs always divide asymmetrically (Fig 2L; $n = 21$). In contrast, 22% of *cul1*^{Ex} NBs divided to generate two similar-sized daughters (Fig 2M; $n = 23$), a remarkable phenotype in asymmetric division. Although *cul1*^{Ex} NBs showed a delay in mitosis, cell division defects are unlikely a major cause of NB polarity defects in mutants for the SCF complex, because several known cell division mutants did not affect cell polarity [16–18].

roc1a^{G1} clones also exhibited delocalization of aPKC (Fig 2D; 28.6%, $n = 14$) and Numb (Fig 2D; 28.6%, $n = 14$) at metaphase. Similarly, in *skpA*¹ clones, 37.5 and 12.5% of metaphase NBs displayed delocalized aPKC and Numb, respectively (Fig 2E; $n = 16$). Interestingly, aPKC (Fig 2F; 71%, $n = 7$) and Numb (Fig 2F; 57%, $n = 7$) were dramatically delocalized in metaphase NBs

derived from *slimb*⁸ clones. Spindle misorientation defects were also observed in *skpA*¹ (supplementary Fig S4J; 43%, $n = 14$) and *slimb*⁰⁰²⁹⁵ (supplementary Fig S4K; 29%, $n = 28$) clones. Therefore, we conclude that the SCF^{Slimb} complex plays an important role in regulating NB asymmetric division.

In *aPKC*⁰⁶⁴⁰³ *cul1*^{Ex} double mutant, the NB number was significantly reduced at 70 h ALH (supplementary Fig S5C and D; 119 ± 42 , $n = 22$), compared with the *cul1*^{Ex} control (supplementary Fig S5B and D; 200 ± 20 , $n = 21$). Further, the NB overgrowth in *cul1*^{Ex} was largely suppressed by *notch*^{ts1} at 84 h ALH at 29°C (supplementary Fig S5E–H). These data suggest that Cul1 may function upstream of aPKC and Notch signaling or redundantly with them to inhibit NB overgrowth.

Uba1, Eff and Nedd8 suppress NB overproliferation and regulate asymmetric division

Uba1 is the only E1 enzyme in *Drosophila* and in a loss-of-function *uba1*^{s3484} allele, ectopic NBs were observed in both type I (Fig 3B; 40%, $n = 42$) and type II clones (Fig 3F; 26%, $n = 31$). Among 16 genes encoding E2 enzymes (supplementary Table S2), RNAi targeting of two of them, *effete* (*eff*; also called *ubcD1*) (supplementary Fig S6B, B') and *ubcD10* (data not shown), resulted in type II NB overgrowth. Ectopic NBs were observed in both type I (Fig 3C; 33%, $n = 36$) and type II (Fig 3G; 40%, $n = 32$) *eff*^{D73} clones, but not in clones of *ubcD10*^{BG00902} (supplementary Fig S6E–F). Given that the *ubcD10* KD construct has predicted off-target sites, the phenotype caused by *ubcD10* KD was unlikely specific. Clones for two other E2 mutants, *ubcD2* and *ubc9*, did not obviously change NB numbers (supplementary Table S2 and data not shown). Collectively, Eff is a specific E2 enzyme that regulates NB self-renewal cell-autonomously. Neddylation, a process of Nedd8 conjugation at a conserved lysine residue of Cullins, is essential for Cullin-based E3 ligase activities [19]. Ectopic NBs were observed in both type I (Fig 3D; 29%, $n = 28$) and type II clones (Fig 3H; 40%, $n = 25$) that were derived from a *nedd8* hypomorphic allele, *nedd8*^{AN015}.

In *uba1*^{s3484} metaphase NBs, aPKC was delocalized throughout the cell cortex and became cytoplasmic (Fig 3J; 43%, $n = 35$), and Numb was partially delocalized (Fig 3J; 29%, $n = 35$). Similarly, in *eff*^{D73} NBs, aPKC (Fig 3K; 59%, $n = 29$) and Numb (Fig 3K; 21%, $n = 29$) were no longer asymmetrically localized at metaphase. In *nedd8*^{AN105} clones, both aPKC (Fig 3L; 42%, $n = 24$) and Numb (Fig 3L; 25%, $n = 24$) proteins were often delocalized. Furthermore, spindle misorientation was observed in both *uba1*^{s3484} (Fig 3N) and *nedd8*^{AN015} (Fig 3O) NBs at metaphase. Interestingly, NBs from either *uba1*^{s3484} or *eff*^{D73} clones formed multiple centrosomes (supplementary Fig S6H–I), similar to those from *slimb* mutants [20,21]. Therefore, Uba1, Eff and Nedd8, similar to the SCF^{Slimb} E3 ligase,

Figure 4. Over expression of Myr-Akt results in NB overgrowth and defects in asymmetric division.

A–L Dpn, pH3, CycE, EdU, Elav and Pros are labeled in larval brains of control (UAS-CD8) or Myr-Akt under *insc*-Gal4, tub-Gal80^{ts}. The central brain is to the left of the white dotted line which marks the border between the central brain and the optic lobe.

M–T aPKC, Numb, Mira, and Pon are labeled in metaphase NBs of control and *insc* > Myr-Akt.

U, V Insc, α -tubulin and DNA are labeled in control and *insc* > Myr-Akt NBs. U', V' present quantifications of spindle orientation.

Data information: Scale bars, 20 μ m (A–L) and 1 μ m (M–V).

regulate asymmetric division/self-renewal of NBs as well as centrosome numbers.

SAK is a target of the SCF^{Slimb} complex during NB self-renewal

The SCF^{Slimb} complex targets the SAK kinase (Polo-like kinase 4) for degradation during centriole formation [20,21]. We therefore assessed whether the SCF^{Slimb} complex controls NB self-renewal through SAK. SAK-GFP overexpression led to spindle misorientation in NBs (supplementary Fig S7B, B'; 24%, $n = 63$; [22]), but not NB overproliferation, likely due to the small proportion of cells undergoing orthogonal divisions (supplementary Fig S7B, B'; 5%, $n = 63$). Neither did it cause any apparent delocalization of aPKC, Numb or Mira in metaphase NBs (supplementary Fig S7O–P and data not shown). SAK overexpression under *insc*-Gal4 with concomitant *cul1* KD in larval brains resulted in a significant increase in the number of NBs compared with the control (supplementary Fig S7C, D and G), suggesting a genetic enhancement. Similarly, NB overgrowth in *skpA* KD was exacerbated by overexpressing SAK (supplementary Fig S7E–G). Moreover, defects of NB overgrowth (supplementary Fig S3P–T) and multiple centrosomes (supplementary Fig S7H–L) were remarkably suppressed in the *cul1*^{EY11668} *sak*^{C06612} double homozygotes. Thus, the SCF^{Slimb} complex controls NB self-renewal through SAK and additional unknown targets.

Akt hyperactivation leads to the formation of supernumerary NBs and asymmetric division defects

Akt is a critical regulator of cell proliferation, growth and metabolism [23] and is required for NBs to exit from quiescence at early larval stages [24,25]. Akt KD was found to give less NBs by a RNAi screen [14]. Overexpression of a myristoylated, constitutively active form of Akt (Myr-Akt) in central brain NBs, starting from 24 h after egg laying (AEL) using *insc*-Gal4 under the control of a *tub*-Gal80^{ts} element, resulted in 659 ± 169 NBs at 96 h ALH at 29°C (Fig 4B; $n = 21$) compared with 91 ± 8 NBs in control brains (Fig 4A; $n = 20$). Upon Akt hyperactivation, the number of cells labeled by pH3, CycE and EdU were significantly increased while the number of neurons labeled by Elav or Pros was strongly decreased compared with the control (Fig 4C–L). Myr-Akt overexpression under the type I-specific driver *ase*-Gal4 (supplementary Fig S8B, B'; 31%, $n = 75$) or the type II driver (supplementary Fig S8D, D'; 55%, $n = 53$) resulted in NB overgrowth.

In Myr-Akt NBs, aPKC (Fig 4N; 55%, $n = 51$), Numb (Fig 4P; 45%, $n = 51$), Mira (Fig 4R; 50%, $n = 32$) and Pon (Fig 4T; 29%, $n = 45$) were delocalized at metaphase. Furthermore, Myr-Akt expression resulted in mitotic spindle misorientation in 36% of NBs with 9% orthogonal divisions (Fig 4V, V'; $n = 53$). Thus, Akt hyperactivation significantly disturbs asymmetric protein localization and mitotic spindle orientation during NB division. However, hyperacti-

vation of Akt does not affect centrosome number in neuroblasts (supplementary Fig S8F), suggesting that Akt and SAK likely affect spindle orientation through different mechanisms.

The SCF^{Slimb} complex inhibits ectopic NB formation, in part through Akt

We next assessed whether Akt functions downstream of the SCF^{Slimb} complex. In *cul1*^{EY11668}; *akt*¹/*akt*³ double mutant, the NB number was restored to a number close to that of the WT control (Fig 5E and G; 100 ± 15 ; $n = 20$), in contrast with either the *cul1*^{EY11668} (Fig 5B and G; 307 ± 21 ; $n = 20$) or *akt*^{1/3} (Fig 5D and G; 38 ± 5 ; $n = 20$) single mutants. Partial suppression was even observed via the heterozygous *akt*¹ mutation (Fig 5C, F and G; 186 ± 25.6 , $n = 20$). Knocking down of *akt* by RNAi also dramatically suppressed neuroblast overproliferation in *cul1* RNAi but not *brat* RNAi background (supplementary Fig S5I–L). Moreover, using an antibody against phosphorylated Akt at Serine 505, a significant increase in the phosphorylated Akt signal was observed in *cul1* brains compared with the control (Fig 5H–J). Consistently, p505-Akt signal was dramatically increased in both *slimb* RNAi brains (Fig 5G–H) and *slimb*⁸ mutant clones (Fig 5K–L'), but decreased in both *akt*³ clones and *akt* KD (supplementary Fig S8I–L).

We showed recently that Akt associates with Slimb and can be ubiquitinated by Slimb (Fig 5L; [26]). Another human E3 ligase tetratricopeptide repeat domain 3 facilitates the ubiquitination and degradation of Akt [27]. Next, we determined which domain of Akt is important for its association with Slimb. The central region of Akt, which contains its protein kinase domain (T2), but not the N-terminus (T1, containing a pleckstrin homology domain) or the C-terminus (T3, containing an AGC kinase domain), interacted with Slimb in S2 cells by co-immunoprecipitation (Fig 5K–L). Taken together, our biochemical and genetic data suggest that the SCF^{Slimb} complex inhibits ectopic NB formation in part through an association with Akt (Fig 5M).

Conserved function of human BTRC in gliomas

Beta-transducin repeat containing (BTRC/ β -TrCP; human homologue of Slimb) showed a significant loss in 72.5% of glioma patients (supplementary Fig S9A; $n = 261$) in the glioma patient database REMBRANDT [28]. Its copy number was an independent predictor of prognosis in a multivariate analysis ($P = 0.0434$). *BTRC* copy loss was observed in patients with glioblastoma (82%) and oligodendroglioma (68%). It was also observed in patients with mesenchymal and proliferative (85 and 88%, respectively) (Table 1), which are frequently associated with activated AKT signaling, a central oncogenic pathway regulating glioblastoma (GBM) growth and survival [29]. The *BTRC* functional module was derived by mining mRNA expression databases and mapped to 544 mRNA transcripts

Figure 5. SCF^{Slimb} functions upstream of Akt in NB self-renewal.

- A–E WT, *cul1*, *cul1*; *akt1/akt1*^Δ and *cul1*; *akt1/akt1*^Δ larval brain NBs at 96 h ALH are labeled by Dpn. Scale bar, 20 μm.
- F Dpn staining of *cul1*; *akt1/akt1*^Δ larval brain NBs at 120 h ALH.
- G Quantifications of NBs (n = 20).
- H, I WT and *cul1*[−] larval brains are labeled by p505-Akt.
- J Quantification of mean intensity of p505-Akt in single neuroblasts from WT (n = 138) and *cul1*[−] (n = 151).
- K, L Control (driver) and *slimb*⁸ clones are labeled by Dpn, p505-Akt and CD8.
- M An illustration of the Akt constructs.
- N Co-IPs of S2 cells co-expressing Flag-Slimb and Myc-Akt (Full length; FL), Myc-Akt T1 (1–131aa), T2 (131–442aa), or T3 (443–530aa). PH, pleckstrin homology. Arrows, the detected proteins. *IgG heavy chains.
- O Model.

Table 1. Distribution of Phillips glioma molecular subclasses in BTRC high and low groups. The BTRC copy number aberrations were significantly correlated with histological grades and molecular subclasses among glioma patients (Fishers exact test; P = 0.0003 and 1.74E-07, respectively). Consistently, the BTRC functional subtypes (High/Low) were significantly correlated with molecular subclasses of glioma patients (Table 1; P < 2.2E-16 in both REMBRANDT and Gravendeel)

Phillips molecular classes	High BTRC	Low BTRC	Fishers exact test P-value
Mesenchymal (%)	0.00 ^a , 6.78 ^b	100.00 ^a , 93.22 ^b	<2.2e-16
Proneural (%)	83.33 ^a , 100.00 ^b	16.67 ^a , 0.00 ^b	
Proliferative (%)	2.86 ^a , 41.79 ^b	97.14 ^a , 58.21 ^b	

^aREMBRANDT
^bGravendeel

(supplementary Table S3). This module was able to stratify patients into two subgroups and was significantly associated with survival (supplementary Fig S9B). A multivariate Cox Regression model confirmed that the BTRC functional subgroup was independently associated with survival (supplementary Table S4). Furthermore, BTRC copy changes have a significant inverse correlation with the gene expression of AKT2 and PIK3CD (supplementary Fig S9C).

β-TRCP, the mammalian Slimb homolog, is speculated to play a greater role as an oncogene than as a tumor suppressor [30]. Here we demonstrate that the SCF^{Slimb} complex plays an important role in NB self-renewal and asymmetric division. It acts at least in part through the oncogenic protein Akt, a key player that regulates NB self-renewal and asymmetric division. The activation of Akt in NBs appears to be independent of Phosphatase and tensin homologue (PTEN), a negative regulator of PI3K/Akt signaling, because no NB overgrowth were observed in two loss-of-function alleles, *PTEN*^{C494} and *PTEN*¹ (data not shown). A recent report suggested a role for Target of rapamycin (TOR) signaling in restraining tumorigenesis in larval brains [31]. PI3K/TOR also interacted genetically with *Pins* in the suppression of tumor growth in larval brains [32], suggesting the complex role of PI3K/Akt and the interconnected TOR pathway in NB homeostasis.

Materials and Methods

Clonal analysis

MARCM clones were generated as previously described [13]. Briefly, larvae were heat-shocked at 37°C for 90 mins at 24 h ALH and at

10–16 h after the first heat shock. Larvae were further aged for 3 days at 25°C, and larval brains were dissected and processed for immunohistochemistry. For gene KD under the type II driver (w; UAS-Dicer 2, *wor*-Gal4, *ase*-Gal80/CyO; UAS-mCD8-GFP/TM3, Ser [14]), UAS lines were crossed to type II NB driver at 25°C and shifted to 29°C at 24 h ALH for 3 or 4 days.

Live imaging recording

Live imaging recording was performed as previous described [33]. NBs of the central brain were sampled using a 40× objective on a LSM 700 confocal microscope. Pictures and videos were exported and processed with Adobe Photoshop and ImageJ respectively.

Supplementary information for this article is available online: <http://embor.embopress.org>

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Author contribution

HW, CT, BTA and FY designed the experiments and analyzed the data. LS, WC, ES, SA, CTK and JW conducted the experiments. LS, CT and HW wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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