Roles of PINK1, mTORC2, and mitochondria in preserving brain tumor-forming stem cells in a noncanonical Notch signaling pathway

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The self-renewal versus differentiation choice of *Drosophila* and mammalian neural stem cells (NSCs) requires Notch (N) signaling. How N regulates NSC behavior is not well understood. Here we show that canonical N signaling cooperates with a noncanonical N signaling pathway to mediate N-directed NSC regulation. In the noncanonical pathway, N interacts with PTEN-induced kinase 1 (PINK1) to influence mitochondrial function, activating mechanistic target of rapamycin complex 2 (mTORC2)/AKT signaling. Importantly, attenuating noncanonical N signaling preferentially impaired the maintenance of *Drosophila* and human cancer stem cell-like tumor-forming cells. Our results emphasize the importance of mitochondria to N and NSC biology, with important implications for diseases associated with aberrant N signaling.

Supplemental material is available for this article.

Received June 22, 2013; revised version accepted November 6, 2013.

Maintaining a delicate balance between self-renewal and differentiation is a hallmark of all stem cells (Morrison and Kimble 2006; Doe 2008; Zhong and Chia 2008). Impairments of such balance can lead to lineage depletion or tumorigenesis. The self-renewal versus differentiation decision of mammalian neural stem cells (NSCs) and *Drosophila* neuroblasts (NBs), an excellent model for NSC biology (Doe 2008; Knoblich 2010; Sousa-Nunes et al. 2010), requires Notch (N) signaling (Wang et al. 2006; Artavanis-Tsakonas and Muskavitch 2010; Andersson et al. 2011). In the type II NB lineages of the *Drosophila* larval central brain, which contain transit-amplifying intermediate progenitors (IPs) and are similar to mammalian NSCs in lineage hierarchy (Fig. 1A), inhibition of N signaling leads to NB loss, whereas N activation causes the dedifferentiation of IPs into ectopic NBs (Bowman et al. 2008; Weng et al. 2010; Song and Lu 2011), reminiscent of the cell of origin of brain tumors in mammals (Dirks 2010; Liu and Zong 2012). The mechanism by which N signaling maintains NB lineage homeostasis is not well defined. N can signal through Suppressor of Hairless [Su(H)] to transcriptionally regulate its target gene, Myc, whose regulation of cell growth is critical for the maintenance of NSCs and cancer stem cell (CSC)-like cells in *Drosophila*. However, overexpression of Myc alone is insufficient to mimic the effect of N in promoting ectopic NSC formation (Song and Lu 2011), suggesting the involvement of other pathways.

Here we show that a novel noncanonical N signaling pathway is involved in N-directed NSC regulation. In this pathway, N interacts with PTEN-induced kinase 1 (PINK1) to influence mitochondrial function, activating mechanistic target of rapamycin complex 2 (mTORC2)/AKT signaling and enhancing NB growth and proliferation. Importantly, attenuating the noncanonical N signaling pathway preferentially impaired the maintenance of *Drosophila* and human brain CSC-like cells. Canonical N signaling, which promotes nucleolar growth, acted together with the newly identified noncanonical N signaling pathway to maintain normal NBs. Moreover, coactivation of canonical and noncanonical N signaling was sufficient to induce the dedifferentiation of IPs into ectopic NBs, recapitulating the effect of N activation. Our results identify a noncanonical N signaling pathway preferentially required by brain CSC-like cells, emphasize the underappreciated importance of mitochondria in N and stem cell biology, and have important implications for cancer and other diseases caused by aberrant N signaling.

Results and Discussion

To test whether canonical N signaling is sufficient to account for the full effect of N on NB lineage homeostasis, we used the NB-specific *1407-Gal4*, type II NB-specific *Pnt-Gal4*, or IP-specific *Erm-Gal4* drivers to overexpress Su(H) and mastermind (Mam), key genes in the canonical N pathway, and Myc, a transcriptional target of Su(H) (Song and Lu 2011). Compared with the controls, there was no significant change in the number of central brain NBs after these genetic manipulations (Fig. 1B,C; data not shown). Since overexpression of Su(H) or Mam was sufficient to activate canonical N signaling, as indicated by up-regulation of *E(spl)* expression (Supplemental Fig. S1A), these results suggest that activation of canonical N signaling under these conditions is in-

[*Keywords*: Notch; PINK1; mTORC2/AKT signaling; mitochondria; neural stem cells; cancer stem cells]
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Article is online at http://www.genesdev.org/cgi/doi/10.1101/gad.225169.113.

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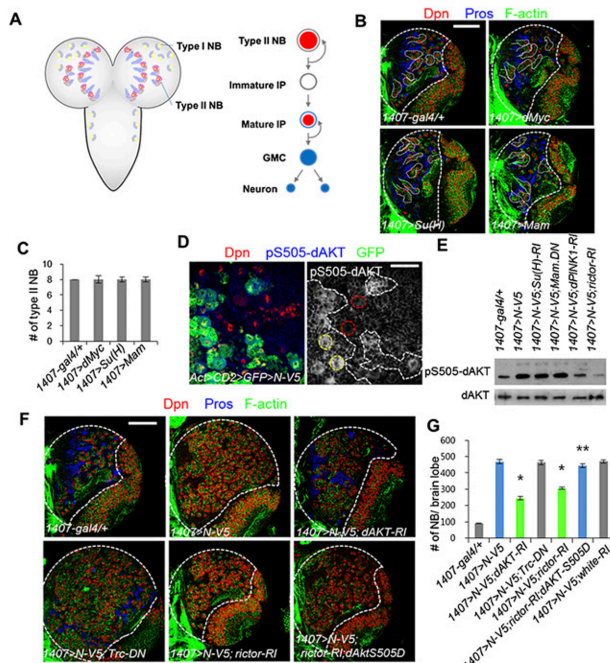

Figure 1. Notch regulates the mTORC2/AKT pathway in NBs. (*A*) Diagram of *Drosophila* larval CNS showing type I and type II NBs in the central brain area (*left*) and the lineage hierarchy of type II NBs (*right*). (*B*) The effects of NB-specific overexpression of canonical Notch pathway components Su(H), Mam, and dMyc. Larval brains at 120 h after larval hatching (ALH) were stained for Dpn (NBs), Pros (differentiated cells), and F-actin (cell cortex). Type II NB lineages are marked with fine white lines. In this and all subsequent figures, the central brain area is outlined with a bold white dashed line, and the Dpn^+ NBs within this area are quantified. (*C*) Quantification of data from *B*. (*D*) pS505-dAKT staining of GFP-marked NB flip-out clones overexpressing full-length N (N-v5). NBs within clones (circled in yellow) show an increased pS505-AKT signal compared with those outside of clones (circled in red). (*E*) Assay of mTORC2 activity by pS505-dAKT Western blot analysis of larva brain extracts after RNAi (RI) or dominant-negative (DN) transgene expression. Total dAKT was used as a loading control. (*F*) N-induced NB expansion is blocked by inhibition of key components of the mTORC2/AKT pathway but not Trc-DN, and the effect of Rictor inhibition is restored by AKT-S505D. (*G*) Quantification of data from *E*. (*) $P < 0.0003$ (vs. *1407>N-V5/+*); (**) $P < 0.002$ (vs. *1407>N-V5; rictor-RI*) in Student's *t*-test; $n = 10$. Bars: *A*,*E*, 100 µm; *C*, 20 µm.

sufficient and that additional pathways are needed for the induction of ectopic NBs by N gain of function (GOF), as observed previously (Song and Lu 2011).

We next searched for other signaling events that may act together with the canonical N signaling pathway to mediate the effect of N. We found that in the N GOF condition, there was a significant increase in the p-AKT(S505) level, as measured by immunostaining and Western blot analyses (Fig. 1D,E). Since mTORC2 is the primary kinase responsible for AKT(S505) phosphorylation (Sarbassov et al. 2005; Hietakangas and Cohen 2007), this result indicated that mTORC2 is activated in the N GOF condition. Conversely, mTORC2 is inhibited in the N loss-of-function (LOF) condition (Supplemental Fig. S1B). No obvious change of p-AKT level was observed when Wingless or Hh signaling was altered (Supplemental Fig. S2), indicating specificity of the p-AKT response to N signaling. To assess the functional significance of mTORC2 activation, we inhibited Rictor (Hietakangas and Cohen 2007), a key component of mTORC2. Knockdown of *rictor* but not the control *white* (*W*) gene significantly attenuated ectopic NB induction by N (Fig. 1F,G; Supplemental Fig. S3A). These data support that activation of mTORC2 contributes significantly to N-induced NB overproliferation.

We next asked which mTORC2 effector is critically involved in NSC regulation by noncanonical N signaling. Intriguingly, in contrast to post-mitotic neurons in which Tricornered (Trc), but not AKT, acts as a key effector of mTORC2 to promote neuronal maintenance (Wu et al. 2013), NBs use AKT as the key mTORC2 effector for homeostasis control, since inhibiting AKT, but not Trc, prevented N GOF-induced ectopic NB formation (Fig. 1F,G), and a phospho-mimetic, constitutively active AKT (AKT-S505D) rescued the mTORC2 LOF effect on NSC homeostasis (Fig. 1F,G). These results support a critical role of the mTORC2/AKT axis in N-directed NSC homeostasis. Since canonical N signaling had no obvious effect on p-AKT(S505) level (Supplemental Fig. S1C,D), mTORC2 activation appears to be a specific effect of noncanonical N signaling.

We next sought to elucidate the signaling mechanism of the noncanonical N pathway that leads to mTORC2 activation. In flies and mammals, TOR kinase forms at least two complexes: mTORC1 and mTORC2. Compared with mTORC1, little is known about how mTORC2 is regulated by upstream signals (Zoncu et al. 2011) and its function in NSCs. Recent studies suggest that the functional state of mitochondria maintained by PINK1, a gene associated with Parkinson's disease (PD) and cancer (Devine et al. 2011), critically regulates mTORC2 activity and influences mitochondrial dynamics and function (Wu et al. 2013). Interestingly, *dPINK1*-null mutants exhibited defects in NB maintenance (Supplemental Fig. S4A,B). Furthermore, partial inhibition of PINK1 by RNAi could block the activation of mTORC2 and the formation of ectopic NBs induced by N GOF (Figs. 1E, 2A), although normal NBs were largely unaffected. RNAi-mediated inhibition of several other genes implicated in mitochondrial regulation, including ND-75 (respiratory chain complex-I [RCC-I] 75-kD subunit), PGC-1α (biogenesis), and Drp1 (fission), also rescued the ectopic NB formation induced by N GOF without affecting normal NBs. By comparison, RNAi of *W* or an RCC-III component failed to rescue the N GOF effect (Fig. 2A,B; Supplemental Fig. S3A). Genetic manipulations of PINK1 and the mitochondria-related genes also rescued the larval lethality induced by N GOF (Supplemental Fig. S4C). Treatment of N-GOF larvae with a small molecule inhibitor of Drp1 (Cassidy-Stone et al. 2008) induced mitochondrial fusion/aggregation (Supplemental Fig. S5A) and partially prevented ectopic NB formation and brain tumor formation (Fig. 2E,F). The role of mitochondrial fission was further evaluated by analyzing *drp1*-null mutant NB clones in which normal NBs were maintained, but activated N-induced ectopic NBs were largely abolished (Fig. 2C,D).

To test the relevance of these findings to humans, we turned to glioblastoma multiforme (GBM), where altered N and mTOR signaling has been implicated (Dirks 2010; Cloughesy et al. 2013). Using patient-derived GBM lines that exhibited elevated N and PINK1 expression (Fig. 3K), N and mTORC2 signaling (Supplemental Fig. S6A), and RCC assembly (Supplemental Fig. S6B), we pharmacolog-

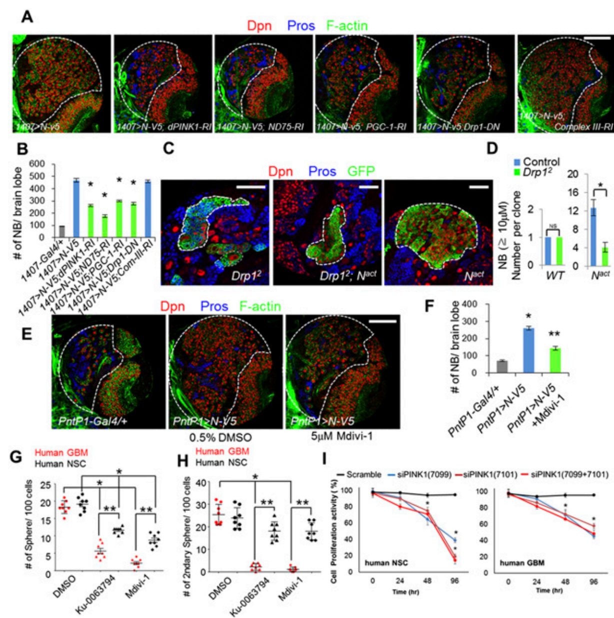

Figure 2. Involvement of PINK1 and other mitochondria-related genes in N-induced NB overproliferation. (*A*) N-induced NB expansion is blocked by inhibiting genes involved in mitochondrial regulation. Larval brains at 120 h ALH were immunostained. (*B*) Quantification of data from *A*. (*) $P<0.0001$ versus *1407>N-V5/+*; n = 10. Complex-III RNAi served as a specificity control. (*C*) Clonal analysis of NBs in the *Drp1* mutant ($Drp1^2$), $Drp1^2$; N^{act}, and N^{act} backgrounds. MARCM clones are marked with GFP and outlined with white dashed lines. (*D*) Quantification of data from *C*. (NS) Not significant; (*) $P<0.001$; n = 5. (*E*) N-induced NB expansion is attenuated by Mdivi-1 treatment. (*F*) Quantification of data from *E*. (*) $P<0.001$ versus *PntP1-Gal4/+*; (**) $P < 0.005$ versus *PntP1>N-V5*; n = 5. (*G*,*H*) Primary (*G*) and secondary (*H*) neurosphere-forming activity of human GBM CSCs and normal fetal NSCs after chemical inhibition of Drp1 (Mdivi-1) or mTORC2 (Ku-0063794). (*) $P < 0.0001$ versus DMSO control; (**) $P < 0.001$ human NSC versus human GBM. (*I*) Effects of lentiviral delivery of *PINK1* shRNA (either singularly or with two shRNAs combined) on the proliferation of human NSCs and GBM cells. (*) $P < 0.001$ versus scrambled shRNA control in Student's *t*-test. Bars: *A*,*E*, 100 μm; *C*, 20 μm.

ically inhibited Drp1 (Cassidy-Stone et al. 2008), mTORC2 (Garcia-Martinez et al. 2009), or RCC-I. We found that growth and self-renewal of GBM CSCs were preferentially impaired by these treatments (Fig. 2G,H; Supplemental Fig. S5B,C), supporting a critical and conserved role of the newly identified noncanonical N signaling pathway in preserving CSCs. We also examined the effect of genetically inhibiting PINK1 in human NSC and GBM cell cultures. Consistent with PINK1 function being required in both normal NSCs and CSCs, shRNA-mediated knockdown of PINK1 inhibited cell proliferation in normal NSC and GBM CSC cultures (Fig. 2I).

We next tested the relationship between N and PINK1 in regulating the mTORC2/AKT axis. As reported before (Clark et al. 2006; Park et al. 2006; Yang et al. 2006), PINK1 LOF altered mitochondrial morphology and impaired indirect flight muscle integrity, as measured by the wing posture assay. These defects were effectively rescued by N GOF (Fig. 3A,B). Consistent with N playing an important role in regulating mitochondrial function, N LOF resulted in impaired RCC assembly and reduced ATP production (Fig. 3F; Supplemental Fig. S7A), and, as in the case of PINK1, N LOF in DA neurons caused mitochondrial aggregation and neuronal loss (Supplemental Fig. S7B,C), features associated with PD (Park et al. 2006; Yang et al. 2006). NB-specific knockdown of RCC-I 75 kD also resulted in reduced NB number at the larval stage and loss of DA neurons in surviving adults (Supplemental Fig. S7D). These results suggested that mitochondrial function regulated by PINK1 and N is important for maintaining the NSCs during development and differentiated DA neurons in adults. Moreover, *N* mutant mitochondria manifested morphological defects and reduced membrane potential in adult flight muscle (Fig. 3C–E). Importantly, N GOF efficiently rescued the PINK1 LOF effect on RCC assembly and ATP production, whereas PINK1 GOF failed to rescue the mitochondrial defects caused by N LOF (Fig. 3G; Supplemental S7A). In keeping with mitochondrial function being a key determinant of mTORC2 activation (Wu et al. 2013), PINK1 and N had similar effects on mTORC2 activity, as measured by p-AKT level, and N GOF restored mTORC2 activity in the PINK1 LOF condition (Fig. 3G). Intriguingly, PINK1 GOF also restored mTORC2 activity in the N LOF condition (Fig. 3G), and the coactivation of PINK1 and N had additive effects on mTORC2 activity (Supplemental Fig. S8A). Thus, although the functional assays support that N may act downstream from PINK1, the biochemical assays indicate that PINK1 and N have additive effects on mTORC2/AKT activation. PINK1 and N may have signaling branches that act independently to influence mTORC2 activity. Alternatively, PINK1 and N may act at the same level (e.g., by working in a protein complex rather than in a linear pathway) to regulate mTORC2 activation.

We further explored the mechanisms by which N and PINK1 interact to regulate mitochondrial function. By immunostaining with anti-N, the specificity of which was confirmed using the *N* mutant (Supplemental Fig. S8B), we found that endogenous N colocalized with the mito-GFP reporter (Supplemental Fig. S8B), and a N-GFP reporter expressed from the endogenous *N* promoter colocalized with the mitochondrial marker Tom20 (Supplemental Fig. S8C). We also found that full-length N was present in Percoll gradient-purified mitochondria, whose purity was confirmed by the absence of various membrane markers (Fig. 3H; Supplemental Fig. S9A), and that N resided mostly at the outer membrane, as shown by fractionation studies (Supplemental Fig. S9B). These results support that N exerts its effect directly at the mitochondrial surface. Mammalian N was also found to localize to mitochondria (Supplemental Fig. S6D,E). Given that PINK1 can recruit proteins to the mitochondrial surface (Narendra et al. 2010), we next tested whether the mitochondrial localization of N is dependent on PINK1. The amount of mitochondria-bound N, but not total N, was reduced in the *dPINK1* mutant but increased in the PINK1 GOF condition (Fig. 3I), consistent with PINK1 playing an active role in recruiting N to mitochondria. Intriguingly, although PINK1 can become stabilized on CCCP-damaged mitochondria and recruit proteins such as Parkin to these damaged organelles (Narendra et al. 2010), the amount of mitochondrial N was not affected by damage (Supplemental Fig. S9E). Importantly, in coimmunoprecipitation assays, PINK1 and N were found to physically associate in vivo (Fig. 3J; Supplemental Fig. S9C). Endogenous N–PINK1 in-

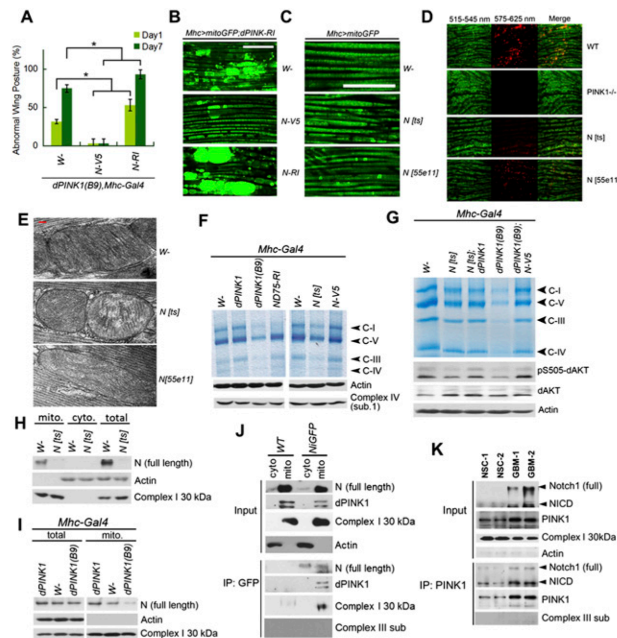

Figure 3. Genetic and biochemical interactions between N and PINK1. (*A*,*B*) Effects of N overexpression or N RNAi on PINK1 LOF-induced abnormal wing posture (*A*) and aggregation of muscle mitochondria (*B*). (*) $P < 0.0001$ versus w^{-} control in Student's *t*-test. (*C*) Effects of N LOF on muscle mitochondrial morphology monitored with mito-GFP. The uneven and enhanced mito-GFP signals suggest mitochondrial aggregation. (*D*) Effects of *N* mutations on mitochondrial membrane potential (indicated by JC-1 signal at 575–625 nm). The *PINK1* mutant was used as a control. (*E*) Transmission electron microscopy analysis of mitochondrial cristae morphology in *N* mutants. Bar, 100 nm. *N[55e11]* refers to *N[55e11]*/+ heterozygous females in *C*–*E*. (*F*) Blue native gel (BNG) and Western blot analyses of RCC assembly in PINK1 and N LOF and GOF backgrounds. Actin and the complex IV subunit 1 served as loading controls. (*G*) Effects of N GOF on RCC assembly and pS505-dAKT level changes caused by PINK1 LOF. (*H*,*I*) Fractionation assay showing enrichment of full-length N at mitochondria (*H*) and increased mitochondrial N in the PINK1 overexpression condition (*I*). (*J*) Coimmunoprecipitation assays using *NiGFP* fly head tissue to demonstrate N interaction with PINK1 and complex I 30 kD. The complex III subunit served as a negative control. (*K*) The interaction between hNotch1 and PINK1 in the mitochondria of human NSCs and GBM cells. The complex III subunit served as a negative control. GBM cells show elevated expression of PINK1 and both the full-length and the intracellular domain of Notch1.

teraction was also observed in human GBM cells (Fig. 3K). Moreover, we detected N association with a RCC-I 30-kD subunit, which was previously shown to interact with PINK1 (Wu et al. 2013), but no N association with the RCC-III or RCC-IV subunits (Fig. 3J). These results reveal a conserved noncanonical mechanism whereby N regulates mitochondrial function and mTORC2 activity through direct interaction with PINK1 and specific RCC subunits under physiological conditions.

We next tested the relationship between the newly identified noncanonical N signaling pathway and the canonical N pathway in N-directed NB homeostasis. Although knocking down the canonical or noncanonical N signaling pathway individually only partially rescued the N GOF-induced brain tumor phenotype, combined knockdown of both pathways resulted in nearly complete rescue (Fig. 4A,B). Strikingly, while RNAi-mediated inhibition of either pathway alone had little effect on normal NB maintenance, their combined knockdown significantly reduced the number of normal NBs (Fig. 4C,D), while their combination with control *W RNAi* had no effect (Fig. 4C; Supplemental Fig. S3C), supporting the importance of both pathways in maintaining normal NBs. Moreover, although the GOF of genes in the noncanonical pathway alone had no obvious effect on NB number, they strongly enhanced NB expansion induced by N GOF (Fig. 4E,F). Furthermore, combining the inhibition of mitochondrial fusion regulator Marf (Deng et al. 2008), which impinges on mitochondria and activates the noncanonical pathway (Supplemental Fig. S9D), with the GOF of dMyc, a key target of the canonical pathway (Song and Lu 2011), resulted in full rescue of N LOF-induced type II NB loss (Fig. 4G,H). By comparison, the coexpression of GFP or Parkin, another gene involved in mitochondrial regulation, failed to modify the rescuing effect of dMyc (Fig. 4H; Supplemental Fig. S9F). Thus, canonical and noncanonical N signaling pathways both play important and specific roles in mediating the effects of N on NSC homeostasis.

Finally, we tested whether coactivation of the canonical and noncanonical N signaling pathways is sufficient to induce ectopic NBs, hence recapitulating the effect of N GOF. Activation of the canonical pathway via Su(H) GOF promoted nucleolar growth of IPs (Supplemental Fig. S1D), which is likely mediated by dMyc, a known master regulator of nucleolar growth (Song and Lu 2011). However, no ectopic NB was formed when Su(H) was overexpressed using the IP-specific *Erm-Gal4* driver (Fig. 4I,J). In contrast, coexpression of Su(H) and AKT-S505D under the control of *Erm-Gal4* promoted the dedifferentiation of IPs into ectopic NBs resembling those found in the N GOF condition (Fig. 4I,J), although AKT-S505D overexpression alone did not affect nucleolar growth (Supplemental Fig. S1E) and had no obvious effect on type II NB number (Fig. 4I,J). Similar results were obtained when Su(H) or Mam were coexpressed with PINK1 (Supplemental Fig. S10A,C). We also used a NΔcdc10 construct defective in Su(H) binding and thus canonical N signaling (Lawrence et al. 2000) but capable of noncanonical N signaling (Supplemental Fig. S1A). Coexpression of Su(H) and NΔcdc10 also induced ectopic NBs (Supplemental Fig. S10B,D). These results suggest that noncanonical and canonical N signaling mediate distinct aspects of N function and act coordinately to regulate NSC behavior.

Our results uncover a novel mechanism of N in regulating NSC self-renewal and maintenance through a noncanonical signaling pathway involving PINK1, mTORC2, and AKT. A central feature of this noncanonical N signaling pathway is specific mitochondrial roles of N in regulating RCC function through direct interactions with PINK1 and select RCC subunits and in activating mTORC2. N could act through a number of possible mechanisms, such as facilitating the import or assembly of RCC components (Ades and Butow 1980), as suggested by its interaction with complex I subunits (Fig. 3J), or directing the quality control of mitochondria, as has been implicated for PINK1 (Rugarli and Langer 2012). Future studies will determine the exact domains of N involved in PINK1 interaction and whether noncanonical N signaling is ligand-dependent. Although the exact mechanism remains to be determined, our results will help us to

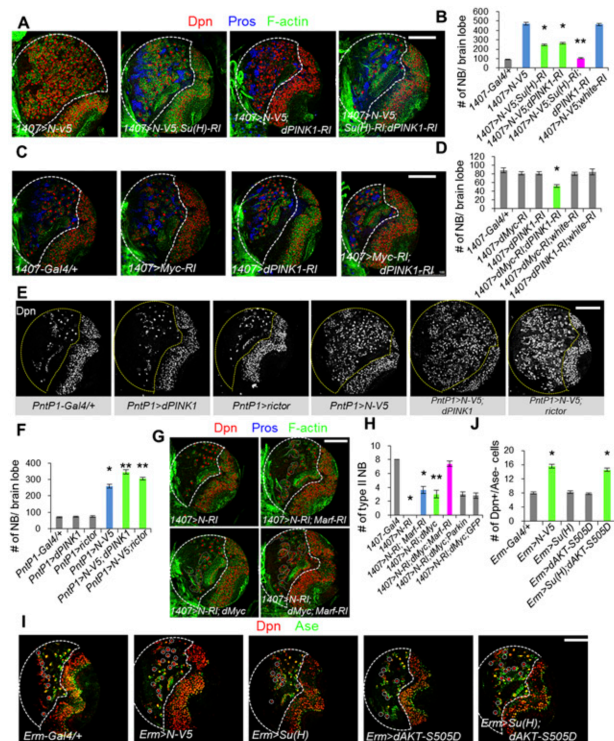

Figure 4. The interaction between canonical and noncanonical N signaling on NB homeostasis. (*A*) The effects of combined RNAi of Su(H) and PINK1 on N GOF-induced NB expansion. (*B*) Quantification of data from *A*. (*) $P < 0.0002$ versus *1407>N-V5/+*; (**) $P < 0.0005$ versus *1407>N-V5; Su(H)-RI* or *1407>N-V5; dPINK1-RI*; $n = 5$. (*C*) The effects of combined RNAi of Myc and PINK1 on normal NB number. (*D*) Quantification of data from *C*. (*) $P < 0.001$ versus *1407>dMyc-RI* or *1407>dPINK1-RI*; $n = 5$. (*E*) The effects of coexpression of PINK1 and Rictor on N GOF-induced NB expansion. (*F*) Quantification of data from *E*. (*) $P < 0.0001$ versus *PntP1-Gal4/+* control; (**) $P < 0.05$ versus *PntP1>N-V5/+*; $n = 5$. (*G*) Synergy between dMyc overexpression and Marf-RI in rescuing N LOF-induced type II NB loss. (*H*) Quantification of data from *G*. (*) $P < 0.005$ versus *1407>N-RI/+*; (**) $P < 0.05$ versus *1407>N-RI;dMyc* or *1407>N-RI;Marf-RI*; $n = 5$. Parkin or GFP coexpression served as a specificity control. (*I*) The effects of coexpression of Su(H) and dAKT-S505D in mature IPs (driven by *Erm-gal4*). The ectopic NBs dedifferentiated from mature IPs are identified by marker expression (Dpn$^+$ and Ase$^-$) and marked by white dashed circles. (*J*) Quantification of data from *I*. (*) $P < 0.0002$ versus *Erm-Gal4/+* control in Student's *t*-test; $n = 5$. Bars, 100 μm.

understand earlier observations in *Drosophila* that mutations in *N* affected mitochondrial respiration (Thorig et al. 1981) and data from mammalian systems implicating N in mitochondrial and metabolic regulation (Dotti et al. 2004; Landor et al. 2011). The physiological significance of this newly defined noncanonical N pathway is also underscored by the phenotypes of various diseases associated with N dysregulation. For example, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), a disease caused by mutations in *NOTCH3* (Joutel et al. 1996), is associated with mitochondrial impairment (Dotti et al. 2004). On the other hand, GOF mutations of Notch1 are implicated in over half of human T-cell acute lymphoblastic leukemia (T-ALL) (Weng et al. 2004), in which a pathogenic role of mTORC2 has been proposed, although how N impinges on mTORC2 in this setting is unknown (Lee et al. 2012). Mammalian N has been shown to act through AKT and mitochondria to promote T-cell survival (Perumalsamy et al. 2010), although the mechanism is distinct from the one uncovered here. Finally, mTORC2 was shown to be required for the self-renewal and maintenance of CSCs but dispensable in normal stem cells (Zoncu et al. 2011). Our findings that CSC-like brain tumor-forming cells are particularly dependent on the noncanonical N pathway in flies and humans identify the newly discovered noncanonical N signaling pathway as a potential target for disease intervention.

Materials and methods

Fly genetics

Fly culture and crosses were performed according to standard procedures and were raised at the indicated temperatures. *Drosophila* stocks used in this study were *dPINK1*^{B9} (J.K. Chung), *Drp1*^{2} (H. Bellen), *N*^{55e11}*;NiGFP/+* (F. Schweisguth), *1407-Gal4* (L. Luo), *PntP1-Gal4* (Y.N. Jan), *Erm-Gal4* (C.Y. Lee and G. Rubin), *Mhc-Gal4* (T. Littleton), and *UAS-N-V5* (M. Fortini). *dPINK1*, *dPINK1-RNAi*, and *Parkin* transgenes were described previously (Yang et al. 2006). The other transgenes were *dMyc* (F. Demontis and B. Edgar), *rictor*, *dAKT-S505D* (S. Cohen), *mitoGFP* (W. Saxton), *Marf-RNAi* (M. Guo), *white-RNAi* (D. Smith), *NECN* (deletion of the entire intracellular domain of N), *NΔcdc10* [deletion of the intracellular domain of N comprising the Su(H) interacting region] (E. Giniger), *ND75-RNAi* (2286-R-3) (National Institute of Genetics Fly Stock Center, Japan), *Complex III-RNAi* (v33015), *dMyc-RNAi* (v106066), *dMyc-RNAi-2* (v17487), *N-RNAi* (v1112 and v27229), and *Dicer2* (v60008; Vienna *Drosophila* RNAi Center). *Mam-WT* (B27743), *Su(H)myc* (B5814), *Mam-DN* (dominant negative) (B26672), *Trc-DN* (B32086), *Su(H)-RNAi* (B28900), *rictor-RNAi* (B31388), *rictor-RNAi* (B31527), *dAKT-RNAi* (B33615), *dPGC1α-RNAi* (B33914), *Notch*^{ts} (B2533), *Shaggy-DN* (B5255), *TCF-ΔN* (B4784), *Smoothened-RNAi* (B27037), and all other stocks were obtained from Bloomington *Drosophila* Stock Center. To enhance the efficiency of N knockdown, *Dicer2* was coexpressed with *N-RNAi* (Song and Lu 2011).

MARCM and flip-out clonal analysis

To generate NB MARCM clones and overexpression clones, 24 h after larval hatching (ALH), larvae were heat-shocked for 90 min at 37°C and further aged for 72 h at 25°C before dissection. MARCM analyses were performed essentially as described (Song and Lu 2011). For making overexpression clones, *w, hsFLP; Actin 5c>CD2>Gal4, UAS-GFP-NLS* was crossed with the indicated *UAS* lines, and, 24 h ALH, larvae were heat-shocked for 90 min at 37°C and further aged for 72 h at 25°C before dissection.

Abnormal wing posture

Abnormal wing posture was analyzed as described (Yang et al. 2006). Briefly, the number of flies with abnormal wing posture (either held up or drooped) was scored after male flies of the indicated genotypes were aged for 14 d at 29°C. For each experiment, at least 60 flies in three separate vials were scored, and the percentage of flies with abnormal wing posture was presented for each genotype.

ATP measurement

The ATP level in *Drosophila* thoracic muscle was measured essentially as previously described (Wu et al. 2013) using a luciferase-based bioluminescence assay (ATP Bioluminescence assay kit HS II, Roche Applied Science).

Statistical analysis

The statistical significance of all data was evaluated by unpaired Student's *t*-tests.

Acknowledgments

We are grateful to Dr. J.K. Chung, Dr. H. Bellen, Dr. E. Giniger, Dr. F. Schweisguth, Dr. L. Luo, Dr. Y.N. Jan, Dr. C.Y. Lee, Dr. G. Rubin, Dr. T. Littleton, Dr. M. Fortini, Dr. F. Demontis, Dr. B. Edgar, Dr. S. Cohen, Dr. W. Saxton, Dr. M. Guo, Dr. J. Skeath, Dr. L. Pallanck, University of Iowa Developmental Studies Hybridoma Bank, Bloomington *Drosophila* Stock Center, National Institute of Genetics Fly Stock Center Japan, and Vienna *Drosophila* RNAi Center for fly stocks and reagents. We thank J. Gaunce and G. Silverio for technical assistance, and members of the Lu laboratory for discussions and help. This work was supported by National Institutions of Health grants (R01 NS043167 and R01 AR054926) to B.L., and a Stanford University School of Medicine Dean's Post-doctoral Fellowship to Z.W.

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