Minibrain and Wings apart control organ growth and tissue patterning through down-regulation of Capicua

Liu Yang[a], Sayantanee Paul[a], Kenneth G. Trieu[a], Lucas G. Dent[b], Francesca Froldi[b], Marta Forés[c], Kaitlyn Webster[a], Kellee R. Siegfried[a], Shu Kondo[d], Kieran Harvey[b], Louise Cheng[b], Gerardo Jiménez[c,e], Stanislav Y. Shvartsman[f], and Alexey Veraksa[a,1]

[a]Department of Biology, University of Massachusetts, Boston, MA 02125; [b]Cell Growth and Proliferation Laboratory, Peter MacCallum Cancer Centre, East Melbourne, VIC 3002, Australia; [c]Instituto de Biología Molecular de Barcelona, Consejo Superior de Investigaciones Científicas, 08028 Barcelona, Spain; [d]Laboratory of Invertebrate Genetics, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka, Japan; [e]Institució Catalana de Recerca i Estudis Avançats, 08010 Barcelona, Spain; and [f]Department of Chemical and Biological Engineering and Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ 08544

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The transcriptional repressor Capicua (Cic) controls tissue patterning and restricts organ growth, and has been recently implicated in several cancers. Cic has emerged as a primary sensor of signaling downstream of the receptor tyrosine kinase (RTK)/extracellular signal-regulated kinase (ERK) pathway, but how Cic activity is regulated in different cellular contexts remains poorly understood. We found that the kinase Minibrain (Mnb, ortholog of mammalian DYRK1A), acting through the adaptor protein Wings apart (Wap), physically interacts with and phosphorylates the Cic protein. Mnb and Wap inhibit Cic function by limiting its transcriptional repressor activity. Down-regulation of Cic by Mnb/Wap is necessary for promoting the growth of multiple organs, including the wings, eyes, and the brain, and for proper tissue patterning in the wing. We have thus uncovered a previously unknown mechanism of down-regulation of Cic activity by Mnb and Wap, which operates independently from the ERK-mediated control of Cic. Therefore, Cic functions as an integrator of upstream signals that are essential for tissue patterning and organ growth. Finally, because DYRK1A and CIC exhibit, respectively, prooncogenic vs. tumor suppressor activities in human oligodendroglioma, our results raise the possibility that DYRK1A may also down-regulate CIC in human cells.

minibrain | capicua | organ growth | DYRK1A | tissue patterning

The high mobility group-box transcriptional repressor protein Capicua (Cic) has been identified as a key regulator of tissue patterning and organ growth in multiple developmental contexts (1, 2). In *Drosophila*, Cic controls anteroposterior and dorsoventral embryonic polarity, the subdivision of the lateral ectoderm, and pattern formation in several tissues (1, 3–6). In addition, Cic negatively regulates the growth of imaginal discs and the midgut (7, 8). In humans, a single Cic ortholog (CIC) has been implicated in the neurodegenerative disease spinocerebellar ataxia 1 (SCA1) (9), and recently mutations in CIC have been found in the majority of oligodendroglioma cases, suggesting that CIC is a tumor suppressor (10–12).

In both *Drosophila* and mammals, Cic functions as a primary sensor of signaling downstream of the receptor tyrosine kinase (RTK)/extracellular signal-regulated kinase (ERK) pathway (2, 5–8, 13–16). According to the current model, activation of RTK signaling results in the accumulation of doubly phosphorylated activated ERK, which directly binds to and phosphorylates Cic (5). ERK-mediated Cic phosphorylation leads to a rapid relief of repression of Cic target genes, followed by a slower export from the nucleus and eventual cytoplasmic degradation (13, 17). The molecular details of these processes are unknown, although apparently each of them contributes to the overall down-regulation of Cic activity. Cic is also involved in a mutual regulatory relationship with the Hippo pathway, although regulation of Cic in this context appears to take place at the RNA level (18).

Here, we present the identification of the kinase Minibrain (Mnb) (19, 20) and an adaptor protein, Wings Apart (Wap) (20, 21), as Cic regulators that cooperate to phosphorylate Cic and restrict its repressor activity. We show that Mnb/Wap and ERK target different regions of the Cic protein for phosphorylation, and that inhibition of Cic activity by Mnb and Wap is required for the growth of several organs and for correct patterning of the wing. Our data suggest that Mnb/Wap-dependent down-regulation of Cic occurs in parallel to the RTK/ERK and Hippo signaling pathways. We propose that Cic functions as an integrator of upstream developmental signals, which together tightly control its activity. This mechanism is necessary for the proper execution of tissue patterning and regulation of organ growth.

Results

Wap and Mnb Interact with Cic. To identify Cic regulators, we used affinity purification/mass spectrometry (AP-MS) (22) to study the Cic protein interactome in *Drosophila* S2 cells and embryos. Embryonic *cic-Venus* was expressed at endogenous levels as part of a genomic rescue construct (13). We successfully recovered most of the known interactors of Cic, such as the *Drosophila* ERK ortholog Rolled (5), Ataxin-1 (9), and 14-3-3 proteins (16) (Fig. 1*A*). One of the prominent Cic interactors identified with high confidence in both cultured cells and embryos was Wap (also known as Riquiqui) (Fig. 1*A* and Dataset S1) (20, 21). Wap binds to the kinase Mnb (19, 20), and this interaction is

Significance

The transcriptional repressor protein Capicua (Cic) is a conserved regulator of organ growth and tissue patterning, and mutations in the CIC gene in humans result in the brain cancer oligodendroglioma. Cic activity is controlled by the receptor tyrosine kinase (RTK) signaling pathway. Here, we identify the kinase Minibrain (Mnb) and its adaptor Wings apart (Wap) as Cic regulators. Mnb and Wap bind to and phosphorylate the Cic protein, and inhibit the ability of Cic to repress gene expression. Mnb-dependent down-regulation of Cic is necessary for the proper growth of multiple organs and correct the patterning of tissues. Our results uncover a previously unknown mechanism of Cic regulation that acts in parallel to other growth-controlling pathways.

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[1]To whom correspondence should be addressed. Email: alexey.veraksa@umb.edu.

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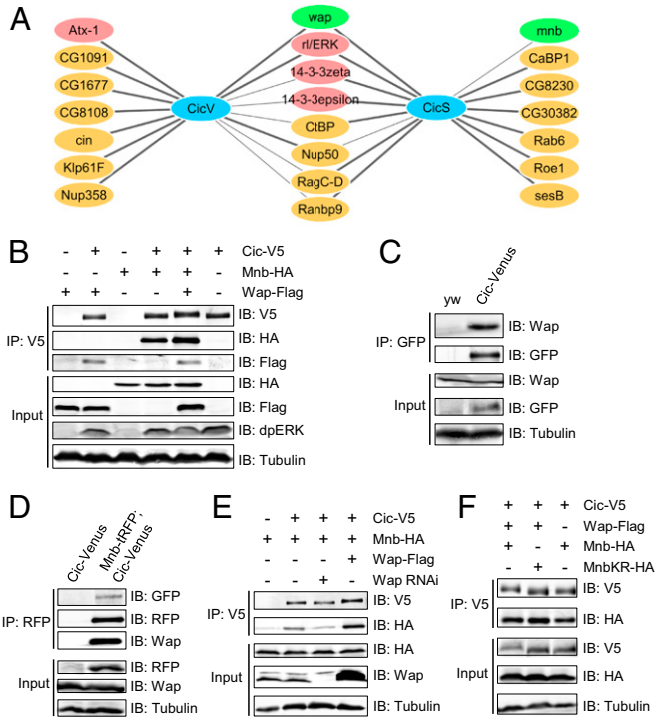

Fig. 1. Mnb and Wap physically interact with Cic, and Wap promotes the binding of Mnb to Cic. (*A*) The Cic protein interactome identified in *Drosophila* S2 cells (CicS) and embryos (CicV). Thick lines, highly significant interactions. A complete dataset is in Dataset S1. (*B*) Western blots showing coimmunoprecipitation of Cic, Mnb, and Wap in S2 cells. Endogenous dpERK is stabilized by Cic expression. (*C* and *D*) Coimmunoprecipitation of Cic, Mnb, and Wap in vivo using embryo lysates from *yw* (control), *cic-Venus*, or *cic-Venus* crossed with *mnb-tRFP*. (*E*) Wap is required and sufficient to bridge Cic and Mnb. (*F*) Cic mobility was changed when Cic was coexpressed with Wap and wild-type Mnb, but not kinase-dead Mnb (MnbKR).

conserved in mammals, because the Wap ortholog DDB1 and CUL4 associated factor 7 (DCAF7) forms a stable complex with the dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A), which is an ortholog of Mnb (23). We have shown that Wap functions together with Mnb to regulate wing and leg tissue growth through the Hippo pathway (20). Our AP-MS experiments also identified four peptides of Mnb in the Cic-streptavidin binding peptide (SBP) pulldown in S2 cells (Fig. 1*A* and Dataset S1), suggesting that Wap, Mnb, and Cic form a protein complex. Coimmunoprecipitation in S2 cells using overexpressed proteins confirmed that Cic binds to both Wap and Mnb (Fig. 1*B*). To study the interactions between proteins expressed at endogenous levels in vivo, we generated tagged *mnb-tagRFP-T* (*mnb-tRFP*) and *wap-Venus* alleles by CRISPR/Cas9-mediated homologous recombination. Successful targeting was confirmed by RNAi (Fig. S1), and we found that both Mnb–tRFP and Wap–Venus were expressed throughout imaginal discs and the larval brain (Fig. S2). Endogenous Wap was detected in the Cic–Venus complex by using an anti-DCAF7 antibody (Fig. 1*C*), and both Wap and Cic–Venus were present in Mnb–tRFP complexes isolated from embryos in which Mnb–tRFP and Cic–Venus were coexpressed (Fig. 1*D*). Next, we asked whether Wap could serve as a bridge for the interaction between Mnb and Cic. RNAi depletion of *wap* in S2 cells led to a reduction in the binding of Cic to Mnb, whereas overexpression of Wap promoted the interaction (Fig. 1*E*). Collectively, these data suggest that Wap, Mnb, and Cic form a protein complex, with Wap likely serving as a bridging adaptor between Cic and Mnb.

Mnb Phosphorylates the Amino-Terminal Third of Cic in S2 Cells. Given that Mnb/DYRK1A is a kinase (19, 20), we asked whether Mnb could phosphorylate Cic. Cic mobility on an SDS/PAGE was reduced when Cic, Mnb, and Wap were coexpressed (Fig. 1 *B*, *E*, and *F*). Notably, the levels of activated ERK (dpERK) did not increase in this condition (Fig. 1*B*). In contrast, a kinase-dead mutant of Mnb (MnbKR) (20) failed to reduce Cic mobility (Fig. 1*F*), suggesting that the kinase activity of Mnb is required to reduce Cic mobility and that this modification is likely to be phosphorylation.

To determine which region of Cic was phosphorylated by Mnb, three Cic fragments (Cic1–3; Fig. 2*A*) were coexpressed with Wap in the presence or absence of Mnb in S2 cells. Only the amino-terminal fragment of Cic (Cic1, representing amino acids 1–453) was found to interact with Mnb (Fig. 2*B*). In addition, Mnb decreased the electrophoretic mobility of Cic1, but not Cic2 or Cic3 (Fig. 2*B*). Phos-tag gel analysis confirmed phosphorylation of Cic1 by wild-type but not kinase-dead Mnb (Fig. 2*C*). Next, we asked which residue(s) in Cic1 are phosphorylated by Mnb. Threonine 28 is part of a motif in Cic1 (RSATP) that closely matches the DYRK1A phosphorylation consensus RP(X)(S/T)P (24). Surprisingly, mutation of this residue (T28A) did not alter the phosphorylation pattern of Cic1 (Fig. 2*C*). To identify Cic residues that are phosphorylated by Mnb, Cic1 and Wap were coexpressed in S2 cells either with Mnb or MnbKR, Cic1 was purified, and its phosphorylation was analyzed by mass spectrometry. Four Cic1 residues (S41, S49, T89, and S91) were more highly phosphorylated by Mnb compared with MnbKR, with T89 and S91 phosphorylations found exclusively in the wild-type Mnb sample (Fig. 2*A* and Fig. S3). The S41 and S49 residues were also found to

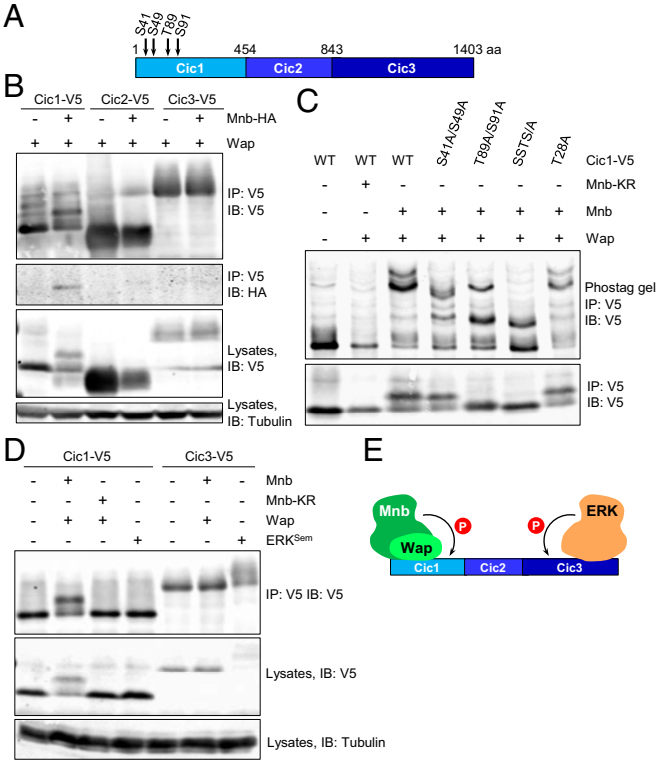

Fig. 2. Mnb and ERK target different regions of Cic for phosphorylation. (*A*) Schematic diagram of the three Cic fragments (Cic1, Cic2, and Cic3) with locations of phosphorylation sites. (*B*) Mnb interacts with and phosphorylates only the amino-terminal Cic fragment, Cic1. (*C*) Phos-tag gel analysis of Cic1 phosphorylation. (*C Bottom*) Regular SDS/PAGE. (*D*) Mnb phosphorylates region Cic1, whereas activated ERK (ERK^{Sem}) phosphorylates region Cic3. (*E*) Summary of Cic binding and phosphorylation data.

be phosphorylated in an unbiased global phosphoproteomic study in *Drosophila* embryos (25). Alanine substitutions of the four residues resulted in a reduction of phosphorylation of Cic1, with the most pronounced effect observed for a quadruple mutant, Cic-SSTS/A (Fig. 2*C*).

Our previous studies showed that region Cic3 includes an ERK docking site and is subject to ERK-mediated phosphorylation (5, 14, 26). To compare the activities of Mnb and ERK, we coexpressed Cic1 or Cic3 with Mnb or a constitutively active *Drosophila* ERK, ERK^{Sem} (27). Mnb could only reduce the electrophoretic mobility of Cic1 but not Cic3, whereas ERK^{Sem} only reduced the electrophoretic mobility of Cic3 but not Cic1 (Fig. 2*D*). Collectively, these results suggest that Mnb and ERK target different regions of Cic for phosphorylation: Wap facilitates Mnb-dependent phosphorylation of the amino-terminal third of Cic, whereas ERK targets the carboxyl-terminal region (Fig. 2*E*).

Mnb and Wap Reduce Cic Repressor Activity. Previous studies have shown that phosphorylation of Cic by ERK can result in down-regulation of Cic by lowering its repressor activity, protein level, or nuclear localization (5, 6, 17). We hypothesized that Mnb may exert similar effects. First, we used the CoinFLP-GAL4 system (28) to generate RNAi-depletion clones in the eye imaginal discs (Fig. S4*A*). As expected, we observed reduced levels of Cic protein in *UAS-cic-RNAi* clones (Fig. S4*B*). However, no obvious increase in Cic protein level or change in subcellular localization was found in CoinFLP-generated *UAS-mnb-RNAi* clones (Fig. S4*C*). Therefore, Mnb is unlikely to control Cic at the level of protein turnover or nuclear access.

We have shown that the relief of Cic repressor function by ERK does not necessarily require reduction in Cic protein levels (17). To assess whether Mnb could similarly affect Cic repressor activity, we used a reporter, *CUASC-lacZ*, which contains five GAL4 binding sites flanked on either side by two Cic binding motifs (Fig. 3*A*) (6). This reporter is only responsive to GAL4 in areas where Cic activity is inhibited, e.g., by RTK signaling (Fig. 3*B*). Uniform induction of GAL4 expression in the wing pouch under the control of the *C5-GAL4* driver (29) resulted in a localized activation of LacZ expression in prospective veins (Fig. 3*C*). This pattern results from epidermal growth factor receptor (EGFR)/ERK-mediated inactivation of Cic in these regions (Fig. 3*B*) (6). RNAi depletion of *cic* or overexpression of ERK^{Sem} throughout the wing pouch led to a much broader expression of LacZ (Fig. 3*D* and Fig. S5*B*), confirming that the normal restriction of the expression pattern of *CUASC-lacZ* to prospective veins is Cic-dependent. In contrast, overexpression of Cic resulted in the loss of LacZ expression in the vein L5 region (Fig. 3*E*, open arrowhead). Overexpression of Mnb or Wap induced a broader LacZ expression in the wing pouch (Fig. 3*F* and Fig. S5*C*). Conversely, RNAi depletion of *mnb* or *wap* under the control of *C5-GAL4* resulted in reduced LacZ expression, particularly in vein L5 (Fig. 3*G* and Fig. S5*D*). These data suggest that Mnb and Wap limit Cic repressor function in the wing disc. This contribution likely complements the regulation by ERK, which appears to be insufficient on its own, at least for vein L5 (Fig. 3*B*).

Mnb and Wap have been shown to phosphorylate and inhibit Warts, which results in elevated Yki activity (20). To test whether *CUASC-lacZ* expression was affected by Hippo signaling, we depleted the levels of the Yki-interacting transcription factor Scalloped (Sd), which is required for the activation of Yki targets (30). We observed that knockdown of *sd* using RNAi had no obvious effect on the expression of *CUASC-lacZ* (Fig. S5*E*), suggesting that Hippo signaling is not involved in the regulation of Cic repressor activity in this context. To further assess whether Mnb and Wap engage RTK/ERK signaling to control Cic, we analyzed dpERK levels in wing pouches expressing *mnb-RNAi* or *wap-RNAi*. We found that RNAi depletion of *wap* or *mnb* did not alter the dpERK pattern in wing discs (Fig. S5 *F*–*H*). This result is in agreement with the observation that overexpression of Mnb did not increase dpERK levels in S2 cells (Fig. 1*B*). We conclude that Mnb and Wap down-regulates Cic repressor activity independently from the RTK/ERK and the Hippo pathways.

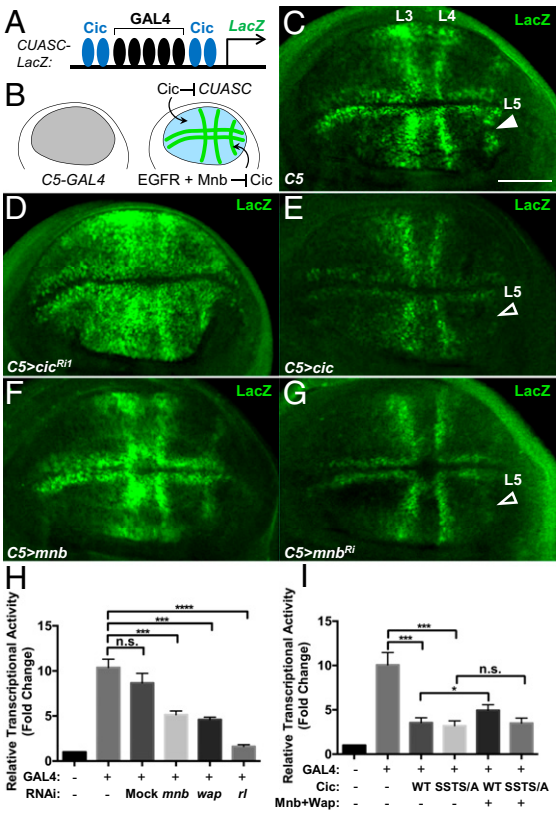

Fig. 3. Mnb reduces Cic repressor activity. (*A*) Diagram of the *CUASC-lacZ* reporter. (*B*) Summary diagram of expression patterns. (*C*–*G*) LacZ expression pattern resulting from *C5-GAL4*–directed activation of *CUASC-lacZ* in wing discs from control (*C*), *UAS-cic*^{RNAi1} (*D*), *UAS-cic* (*E*), *UAS-mnb* (*F*), and *UAS-mnb*^{RNAi} (*G*) larvae. (Scale bar: 50 μm.) (*H* and *I*) Luciferase assays using *CUASC-Luc* reporter in S2 cells. (*H*) *mnb*, *wap*, and *rl* (ERK) are required to limit the activity of Cic. (*I*) Mnb and Wap reduce transcriptional repressor activity of wild-type Cic, but not of the phosphorylation site mutant, Cic-SSTS/A. n.s., not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, statistical significance was analyzed by using unpaired Student's *t* test. Error bars represent SD.

To directly address how Mnb and Wap affect Cic function as a transcriptional repressor, we studied the activity of a reporter, *CUASC-Luc*, which is controlled by GAL4 and Cic, in S2 cells (Fig. S5*I*). Transfection of GAL4 activated this reporter ~10-fold, and this activation was repressed by coexpression of Cic in a dose-dependent manner (Fig. S5*J*). Depletion of endogenous *mnb*, *wap*, or *rl* (ERK) by RNAi resulted in a reduction of reporter activity (Fig. 3H), suggesting that Mnb, Wap and ERK are required to limit the activity of Cic. We next tested whether Mnb and Wap could reduce the capacity of Cic to repress *CUASC-Luc* expression, and found that cotransfection of Cic with Mnb and Wap partially relieved Cic-mediated repression of this reporter (Fig. 3*I*). Whereas the Cic-SSTS/A mutant repressed the reporter gene expression to a similar level as wild-type Cic, coexpression of this mutant with Mnb and Wap did not affect its ability to repress *CUASC-Luc* (Fig. 3*I*). Collectively, these results indicate that Mnb and Wap reduce the activity of Cic as a transcriptional repressor, likely via Mnb-mediated phosphorylation of residues located in the amino terminus of the Cic protein.

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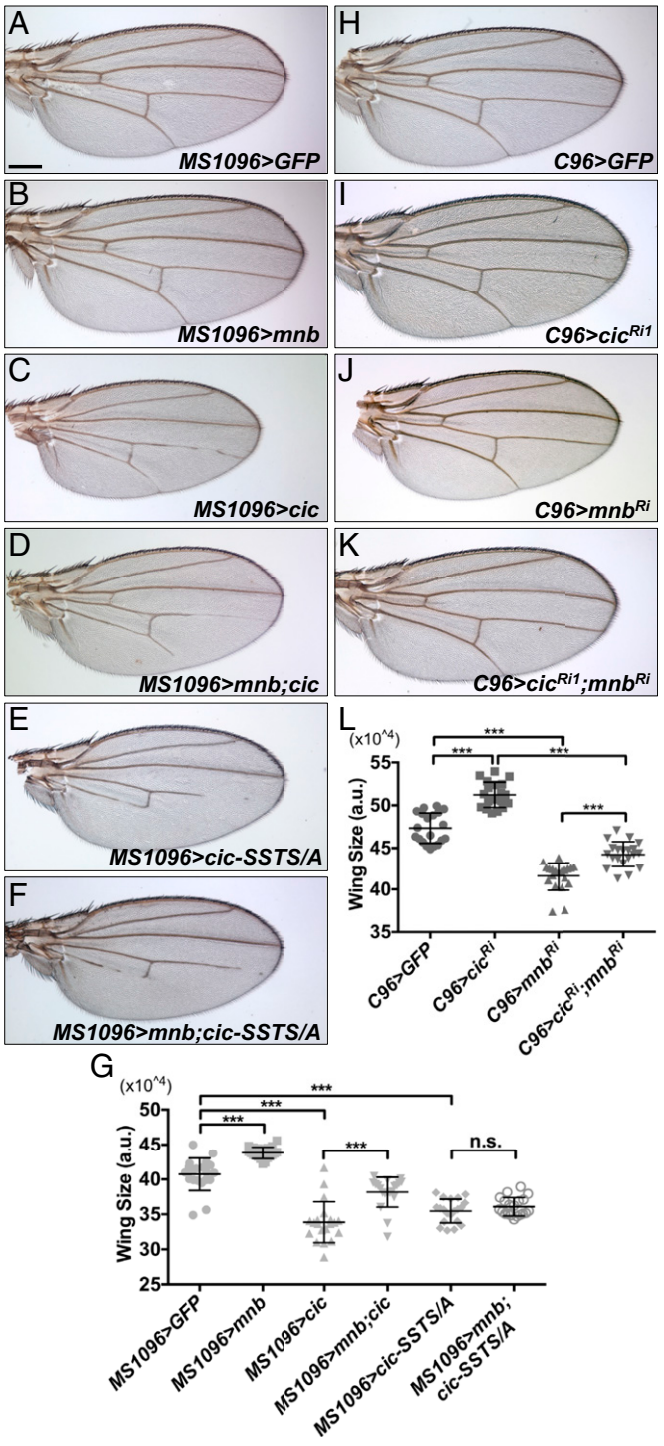

Fig. 4. Mnb opposes Cic function in controlling wing growth. (*A–F*) Wings from adult female flies expressing *UAS-GFP* as a control (*A*), *UAS-mnb* (*B*), *UAS-cic* (*C*), *UAS-mnb* together with *UAS-cic* (*D*), *UAS-cic-SSTS/A* (*E*), and *UAS-mnb* together with *UAS-cic-SSTS/A* (*F*) using the *MS1096-GAL4* driver. (*G*) Quantification of the wing areas for the genotypes shown in *A–F* ($n = 20$ for each genotype). (*H–K*) Wings from adult female flies expressing *UAS-GFP* as a control (*H*), *UAS-cic^{RNAi1}* (*I*), *UAS-mnb^{RNAi}* (*J*), and *UAS-cic^{RNAi1}* together with *UAS-mnb^{RNAi}* (*K*) using the *C96-GAL4* driver. (*L*) Quantification of the wing areas for the genotypes shown in *H–K* ($n = 20$ for each genotype). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Statistical significance was analyzed by using Student's *t* test. Error bars represent SD. (Scale bar: 200 μm.)

Mnb Opposes Cic Function in Controlling Wing and Eye Growth. We next investigated whether the inhibitory effects of Mnb/Wap on Cic were involved in the control of organ growth. Overexpression of Mnb using the wing pouch *MS1096-GAL4* driver (31) promoted wing growth (Fig. 4 *B* and *G*). Conversely, overexpression of Cic or the Cic-SSTS/A mutant resulted in a reduction of wing size (Fig. 4 *C*, *E*, and *G*). Whereas coexpression of Mnb with Cic suppressed the smaller wing size associated with Cic overexpression (Fig. 4 *C*, *D*, and *G*), coexpression of Mnb with Cic-SSTS/A did not modify this phenotype (Fig. 4 *E–G*). These data suggest that Mnb regulates Cic function at least in part through the phosphorylation of the SSTS residues. We also asked whether *mnb* and *cic* would display opposing effects on growth in a reduction-of-function context. RNAi depletion of *cic* using the *MS1096-GAL4* driver caused a severe defect in wing development. We thus used a weaker driver, *C96-GAL4*, which is expressed primarily around the wing margin (32), to study the effects of reduced levels of *mnb* and *cic*. Knockdown of *cic* caused wing overgrowth (Fig. 4 *I* and *L*), and RNAi depletion of *mnb* resulted in an opposite effect (Fig. 4 *J* and *L*). Importantly, RNAi depletion of *cic* partially rescued the small wing phenotype induced by expression of *mnb-RNAi* (Fig. 4 *K* and *J*). Mutually antagonistic effects of Mnb and Cic on growth were also observed in the eye (Fig. S6). Collectively, we conclude that Mnb and Wap promote wing and eye growth by antagonizing the growth-restricting function of Cic.

Reduction of *cic* Level Restores Adult Brain Size in *mnb* Mutants. Mnb was originally identified in a genetic screen for mutants with altered brain structure (19). Mutant *mnb* adult animals have smaller brains, with the optic lobes (OLs) most significantly affected (19). In *Drosophila* development, the size of the central brain (CB) is determined by the proliferative ability of the neuroblasts (NBs) that are of embryonic origin, whereas the OLs are generated by the neuroepithelium (NE), which gives rise to the OL NBs during the larval stages (33). To identify the tissue origins of the reduction in adult brain size, we studied the larval and pupal brains from the wild-type and *mnb^{d419}* animals (*mnb^{d419}* is a null allele; ref. 34). The volumes of the larval and pupal brains in the *mnb^{d419}* mutants were significantly smaller than controls (Fig. S7), suggesting that the effects of loss of *mnb* can be traced to these developmental stages. We asked whether the smaller OLs in *mnb* mutants could result from altered proliferation in the NE and/or NB regions during the larval stages. The widths of both the NB and NE regions in the larval brains from *mnb^{d419}* animals were significantly reduced, compared with controls (Fig. 5 *A–D*, *G*, and *H*). Conversely, overexpression of Mnb in MARCM clones resulted in an increase of the width of NE specifically in the clone area (Fig. 5 *E* and *F*). These results suggest that Mnb is required for the proper growth of both the NE and NB regions in the OL. Additionally, Mnb may be involved in controlling the timing of NE to NB differentiation (35).

We next asked whether interactions among *cic*, *mnb*, and *wap* were involved in the control of adult brain size. RNAi knockdown of *mnb* or *wap* with a ubiquitous *da-GAL4* driver (36) resulted in a smaller adult brain, especially in the optic lobes (Fig. 5 *K*, *L*, and *O*). This result suggests that both Mnb and Wap are required for normal brain growth. Knockdown of *cic* resulted in an increased adult brain size (Fig. 5 *J* and *O*). Strikingly, depletion of *cic* strongly suppressed the small brain phenotype caused by the knockdown of *mnb* (Fig. 5 *K*, *M*, and *O*), suggesting that Mnb promotes brain growth via down-regulation of Cic. Similarly, RNAi depletion of *cic* rescued the smaller brain phenotype of *wap-RNAi* (Fig. 5 *L*, *N*, and *O*). Overall, our results implicate Cic, Mnb, and Wap in a common pathway controlling organ growth and suggest that at least some of the growth-promoting functions of Mnb and Wap are mediated via their inhibition of Cic activity.

Mnb and ERK Have Additive Effects on Cic Activity. Our results so far have shown that Mnb is required for inhibiting Cic activity in various tissue contexts, which is also how ERK transmits signals

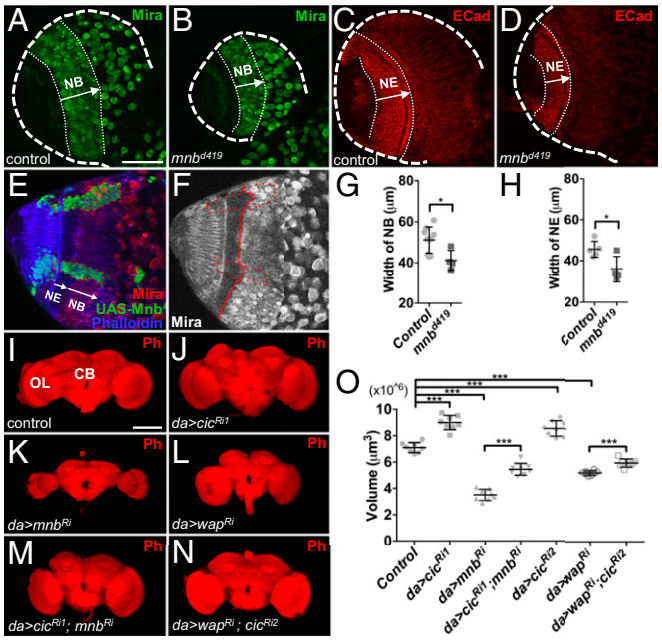

Fig. 5. Reduction in *cic* level restores adult brain size in *mnb* mutants. (*A* and *B*) Neuroblast (NB) regions (Mira-positive cells) in larval CNS from control (w^{1118}) (*A*) and mnb^{d419} animals (*B*). (*C* and *D*) Neuroepithelium (NE) regions (E-cad positive cells) in larval CNS from control (w^{1118}) (*C*) and mnb^{d419} animals (*D*). (*E* and *F*) NE region is expanded cell-autonomously in *UAS-mnb* overexpression clones (marked in green in *E*). Dotted red line, clone areas; solid red line, boundary between NB and NE. (*G*) Quantification of results in *A* and *B* ($n = 9, 4$). (*H*) Quantification of results in *C* and *D* ($n = 5, 4$). (*I–N*) Brains from adult female flies with the indicated genotypes. *da-GAL4* driver was used to drive the expression of *UAS-GFP* (*I*), *UAS-*cic^{RNAi1} (*J*), *UAS-*mnb^{RNAi} (*K*), *UAS-*wap^{RNAi} (*L*), *UAS-*cic^{RNAi1} together with *UAS-*mnb^{RNAi} (*M*), or *UAS-*cic^{RNAi2} together with *UAS-*wap^{RNAi} (*N*). Ph, phalloidin stain. (*O*) Quantification of brain volumes for the genotypes shown in *I–N* ($n = 8$ for each genotype). (Scale bars: *A–H*, 50 μm; *I–N*, 100 μm.) $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Error bars represent SD. Statistical significance was analyzed by using Student's *t* test.

Discussion

Our knowledge of upstream signals controlling Cic activity has been largely limited to its regulation by the RTK/ERK pathway (2). This study identifies a previously unknown mechanism for the regulation of Cic by the kinase Mnb and its adaptor Wap. Wap facilitates Mnb-dependent phosphorylation of Cic in the amino-terminal region, which is necessary for down-regulation of Cic activity. We found that the primary mechanism of Cic down-regulation by Mnb is through the relief of Cic-dependent transcriptional repression. Given that the DYRK family kinases autoactivate themselves soon after translation (37), it is likely that the effects of Mnb and Wap on Cic are constitutive.

Inhibition of Cic activity by Mnb/Wap has two developmentally important consequences (Fig. 6*E*). First, this regulation is important for the proper growth of several organs, such as the wings, eyes, and the brain. Second, down-regulation of Cic activity by Mnb/Wap is required for proper tissue patterning. Given the broad expression patterns of Cic, Mnb, and Wap (Fig. S2), the inhibitory mechanism we describe appears to operate in most, if not all, cells. In relation to ERK, the contribution from Mnb and Wap to Cic down-regulation depends on the tissue context and includes three possible scenarios: In some cells (e.g., developing vein L5 in the wing), both pathways are required for complete inhibition of Cic and operate additively. In other cells, ERK is the primary inhibitory signal, whereas the contribution of Mnb/Wap is less prominent (e.g., veins L2 and L3). Finally, in yet other cells in which ERK is not active, the function of Mnb and Wap to limit Cic activity would be dominant.

In addition to the RTK/ERK pathway, Cic was also shown to be regulated by Hippo signaling (18), and we have previously implicated Mnb and Wap as Hippo pathway regulators downstream of Dachsous (20). In this study, we found that knockdown of *sd*, a required component of Hippo signaling, did not affect the pattern of expression of *CUASC-LacZ*, and that knockdown of *mnb* or *wap* did not alter the pattern of ERK activation (Fig. S5), suggesting that Mnb and Wap control Cic activity independently from ERK and Hippo signaling. Altogether, current evidence suggests that Cic functions as an integrator of upstream developmental signals from RTKs to control growth and patterning. We next asked whether the effects of ERK and Mnb on Cic are additive by first individually and then simultaneously reducing their ability to inhibit Cic. ERK-mediated down-regulation of Cic depends on the conserved C2 motif located in region Cic3, which serves as the ERK docking site (5). Deletion of the C2 motif abrogated Cic–ERK interaction (5), and a single amino acid substitution, F1054A, in the C2 motif (QQFILAPTPAQLG) reduced the binding of Cic to ERK (26). Importantly, deletion of the C2 domain did not affect the binding of Cic to Mnb (Fig. S8). Using CRISPR/Cas9-mediated mutagenesis, we generated a *cic* allele (cic^3) lacking residue F1054, which is predicted to specifically disrupt the interaction of Cic with ERK. Most of the cic^3 mutant animals showed normal wing vein pattern (Fig. 6 *A* and *B*); however, a partial loss of vein L5 was observed in ~30% of adult flies, indicating that this is a gain-of-function mutation. RNAi depletion of *mnb* using *C5-GAL4* resulted in a partial loss of veins L4 and L5 (Fig. 6*C*, arrowheads), which is in agreement with our observation that *CUASC-lacZ* expression was lost in the L5 region in this background (Fig. 3*G*). We reasoned that if Mnb and ERK had additive effects on Cic activity, reduction of *mnb* level in the cic^3 background would cause a more severe vein loss phenotype compared with depletion of *mnb* alone. Indeed, we observed not only a more severe loss of veins L4 and L5, but also partial loss of veins L2 and L3 in the *C5 > mnbRi;* cic^3 animals (Fig. 6*D*). We conclude that Mnb and ERK function additively to regulate wing tissue patterning via inhibition of Cic activity.

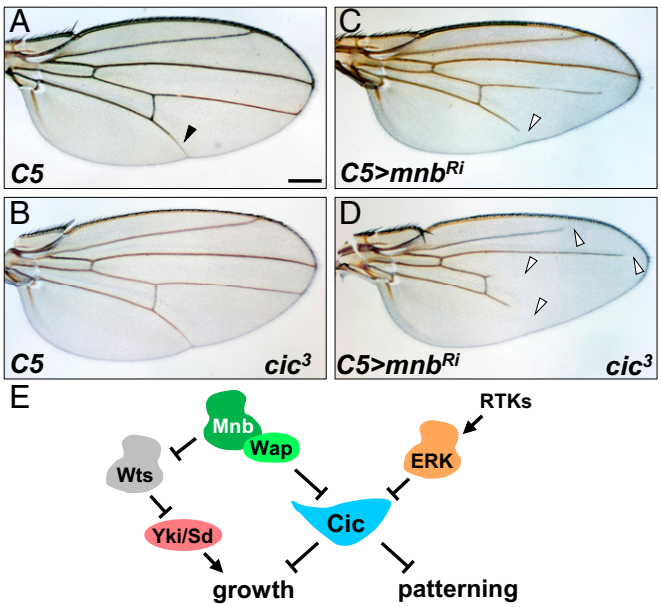

Fig. 6. Mnb and ERK function additively to inhibit Cic. Wings from adult female flies of the following genotypes: *C5-GAL4* (*A*), *C5-GAL4* cic^3/+ (*B*), *C5-GAL4/UAS-*mnb^{RNAi} (*C*), *C5-GAL4* cic^3/*UAS-*mnb^{RNAi} (*D*). (Scale bar: 200 μm.) (*E*) Cic integrates upstream signals to control organ growth and tissue patterning.

that converge on Cic to limit its activity, which is necessary for the proper execution of developmental programs responsible for tissue patterning and organ growth (Fig. 6*E*). Cic controls these developmental programs by direct binding to the enhancers of the genes encoding regulators of tissue patterning and cell proliferation in *Drosophila* and mammals (6, 8, 38, 39).

Interactions between Mnb/Wap and Cic in the brain have interesting parallels in human biology. The majority (>70%) of oligodendrogliomas, which are aggressive brain tumors, have been recently shown to harbor loss-of-function mutations in CIC, suggesting that it functions as a tumor suppressor (11, 12). Higher expression of DYRK1A was also found in a subset of oligodendroglioma patient samples (40), raising a possibility that DYRK1A may suppress Cic activity in human cells, much like Mnb does in *Drosophila*. A connection between DYRK1A and Cic in controlling brain development may extend even deeper, because both proteins have been implicated in neurodegenerative diseases (9, 41–43).

Materials and Methods

Experimental procedures are provided in *SI Materials and Methods*. They include a description of *Drosophila* stocks; information on antibodies, expression plasmids, and cell culture; procedures for luciferase reporter assays and mass spectrometry; and methods used for quantification of wing and brain size.

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