

# Bimodal regulation of RAF by CNK in *Drosophila*

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**Connector enhancer of KSR (CNK) is a multidomain-containing protein previously identified as a positive regulator of the RAS/MAPK pathway in *Drosophila*. Using transfection experiments and an RNAi-based rescue assay in *Drosophila* S2 cells, we demonstrate that CNK has antagonistic properties with respect to RAF activity. We show that CNK's N-terminal region contains two domains (SAM and CRIC) that are essential for RAF function. Unexpectedly, we also report that the C-terminal region of CNK contains a short bipartite element that strongly inhibits RAF catalytic function. Interestingly, CNK's opposite properties appear to prevent signaling leakage from RAF to MEK in the absence of upstream signals, but then transforms into a potent RAF activator upon signal activation. Together, these findings suggest that CNK not only participates in the elusive RAF activation process, but might also contribute to the switch-like behavior of the MAPK module.**

**Keywords:** CNK/RAS-MAPK module/RNAi /signal transduction

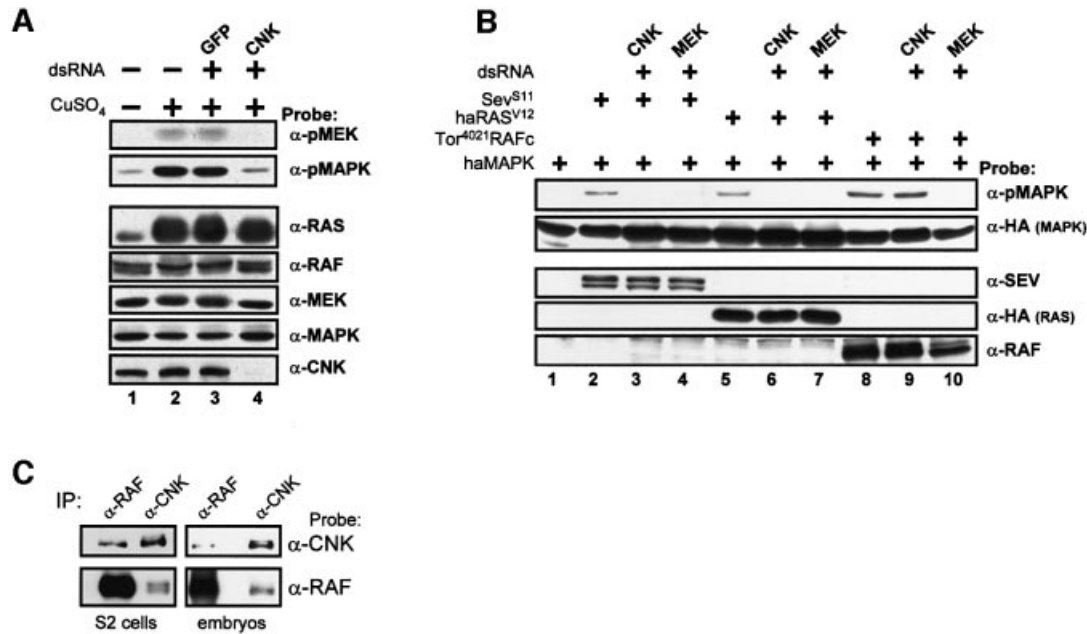
## Introduction

The extracellular-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) module, herein referred to as the MAPK module, is defined as a group of three kinases that is comprised of specific isoforms of the serine/threonine kinase RAF, the dual-specificity MAPK/ERK kinase (MEK) and the proline-directed serine/threonine kinase ERK/MAPK. This module transmits signals mainly received from the small GTPase RAS to control a number of critical cellular events such as proliferation, differentiation and survival (for review, see English *et al.*, 1999). Early characterization of this signaling pathway identified a simple relationship among the core components, whereby upon RAS activation, RAF is recruited to the plasma membrane by RAS, which in turn triggers a phosphorylation cascade from RAF to MAPK. In-depth investigations of each individual step, however, are now unraveling a surprisingly complex process that involves additional proteins whose respective role is either partially or not understood (for review, see Kolch, 2000).

RAS was recognized early on as a major player in RAF activation, principally by its ability to recruit RAF to the

plasma membrane through an interaction between its effector loop region and the RAS-binding domain (RBD) on RAF (for review, see Avruch *et al.*, 1994). However, as this event did not appear sufficient to activate mammalian Raf-1 *in vitro*, additional molecules were predicted to participate in RAF activation. A search for proteins that could bind and modify RAF activity identified the 14-3-3 protein family as potential RAF regulators (for review, see Morrison and Cutler, 1997). These abundant proteins bind as dimers a wide range of targets through the recognition of specific sequence motifs, some of which require threonine or serine phosphorylation for binding (for review, see Tzivion and Avruch, 2002). RAF proteins contain two evolutionarily-conserved 14-3-3 binding sites; one surrounding phospho-serine 259 (pS259) and the second at phospho-serine 621 (pS621) in Raf-1 (Muslin *et al.*, 1996). Growing evidence now suggests that 14-3-3-binding to these sites has opposite effects on RAF. Whereas pS621 occupancy seems critical for RAF activity (Thorson *et al.*, 1998; Yip-Schneider *et al.*, 2000), pS259 binding correlates with inactive RAF (Dhillon *et al.*, 2002; Light *et al.*, 2002), possibly by forcing RAF to adopt an inactive conformation and/or by sequestering RAF in the cytoplasm. Displacement of 14-3-3 from pS259, an event apparently triggered by RAS-binding and accompanied by pS259 dephosphorylation, appears to be one of the critical events leading to RAF activation. Despite its importance, this event does not fully account for RAF activation since mutations disrupting the pS259 site modestly enhance RAF catalytic function (Dhillon *et al.*, 2002; Light *et al.*, 2002).

Genetic and yeast two-hybrid screens conducted over the years have identified additional putative components of the RAS/MAPK pathway (for review, see Kolch 2000). As some of these appear to modulate RAF function, their molecular characterization might unveil key aspects to solve at last the mystery surrounding RAF activation. For instance, genetic screens in *Drosophila* and *Caenorhabditis elegans* identified *kinase suppressor of Ras* (*ksr*), an evolutionarily conserved gene encoding a putative protein kinase structurally related to RAF (Kornfeld *et al.*, 1995; Sundaram and Han, 1995; Therrien *et al.*, 1995). Functional studies revealed that KSR facilitates signaling from RAF to MAPK essentially by its ability to bring together the three kinases of the MAPK module (for reviews, see Morrison, 2001; Raabe and Rapp, 2002; Roy and Therrien, 2002). Besides its importance for efficient MEK and MAPK activation, KSR also appears to control RAF activity since depletion of endogenous KSR by RNA interference (RNAi) impaired RAF catalytic function in *Drosophila* S2 cells (Anselmo *et al.*, 2002). It is unclear, however, whether this effect depends on KSR's scaffolding property as recently



**Fig. 1.** CNK activity is required downstream of RAS, but upstream of RAF. **(A)** Untreated (–) or CuSO<sub>4</sub>-treated (+) RAS<sup>V12</sup> cells were either incubated in the absence (–) or in the presence (+) of the indicated dsRNAs. pMEK and pMAPK levels, as well as endogenous RAS, RAF, MEK, MAPK and CNK levels were assessed by immunoblot analysis using the antibodies indicated to the right. The results shown here and thereafter are representative of at least three similar experiments. **(B)** S2 cells were transfected with the haMAPK reporter construct (0.3 μg) either alone (lane 1) or together (+) with the indicated combinations of SEV<sup>S11</sup> (0.4 μg), haRAS<sup>V12</sup> (0.4 μg) or Tor<sup>4021</sup>RAFc (0.2 μg) constructs and the dsCNK or dsMEK RNAs (0.5 μg). Cells were lysed 16 h post-induction of expression and pMAPK levels were determined. Protein levels were determined as indicated. **(C)** Three milligrams of protein from plain S2 cells or 0–14 h *Drosophila* embryos were immunoprecipitated (IP) using either α-RAF or α-CNK antibodies.

suggested (Roy *et al.*, 2002) or is mediated by another mechanism.

Another potential RAF regulator is connector enhancer of KSR (CNK), a multidomain-containing protein conserved among metazoans, which was originally identified in a KSR-dependent genetic screen in *Drosophila* (Therrien *et al.*, 1998). As for other bona fide components of the RTK/RAS/MAPK pathway in *Drosophila*, CNK is required for photoreceptor cell differentiation, wing vein formation as well as for imaginal disc cell proliferation and/or survival (Therrien *et al.*, 1998). Genetic epistasis experiments positioned CNK downstream of RAS, but upstream or in parallel to RAF, thereby suggesting that CNK might be regulating RAF activity (Therrien *et al.*, 1998). Consistent with that possibility, CNK was found to associate with the catalytic domain of RAF (Therrien *et al.*, 1998) and depletion of endogenous CNK by RNAi in S2 cells abolished insulin-induced RAF activation (Anselmo *et al.*, 2002). The role of CNK with respect to RAF is probably not restricted to *Drosophila* since a rat homolog, named Maguin, has recently been found to associate with Raf-1 in rat brain extracts (Yao *et al.*, 2000).

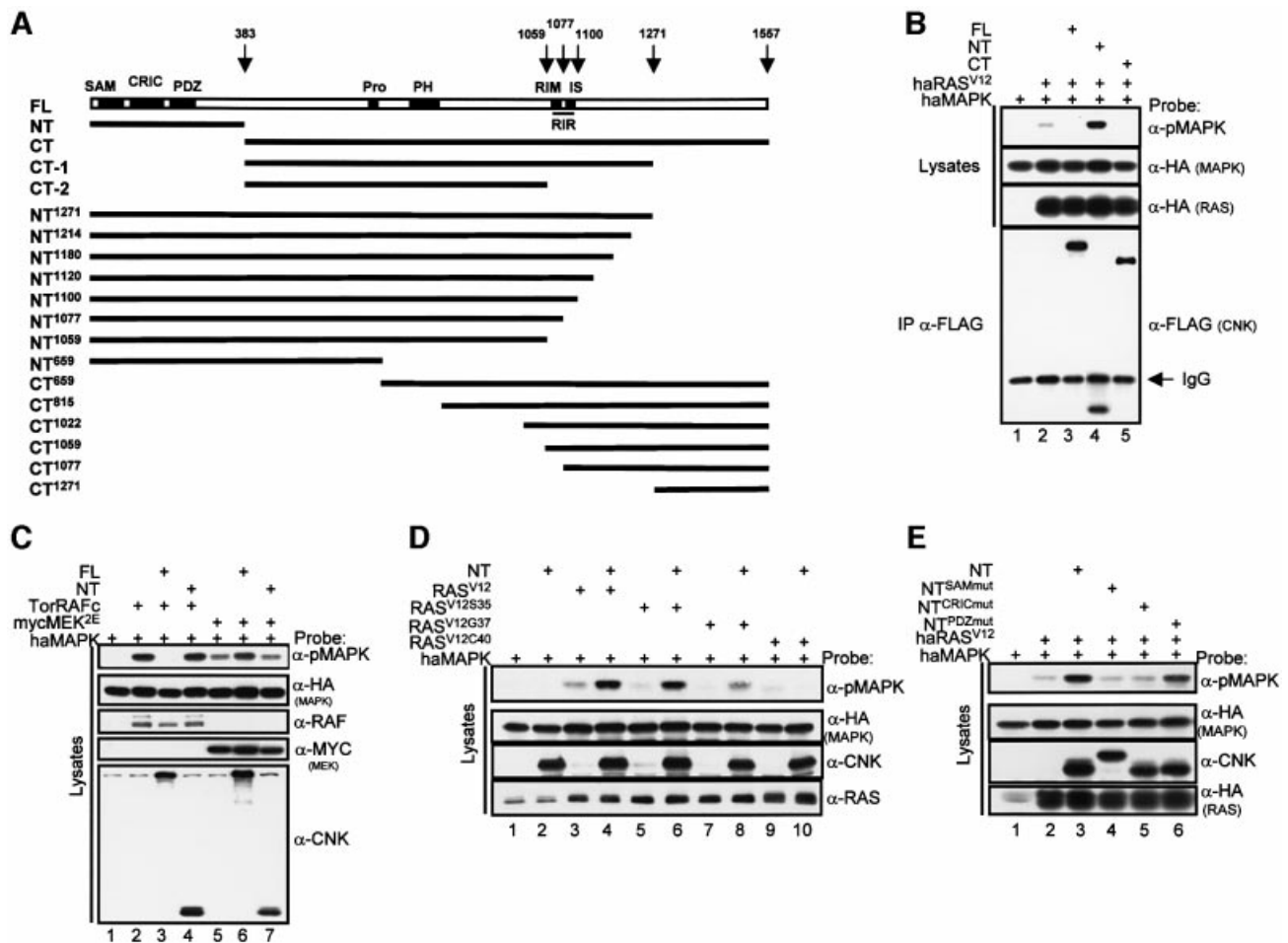
Here, using a CNK-dependent MAPK activation assay in S2 cells combined to a novel RNAi-based rescue protocol, we show that CNK has both a positive and a negative impact on RAF function. We found that CNK, through two of its N-terminal domains, integrates RAS signals to control MEK phosphorylation by RAF. In contrast, we found that CNK's ability to associate with RAF is mediated by a short bipartite element that acts as an inhibitor of RAF catalytic function. Finally, we present evidence that the opposite functions of CNK amplify

signaling difference between the off and on states of a KSR/RAF/MEK complex, which might contribute to the switch-like behavior of the MAPK module. Together, these findings identify CNK as a novel type of signal regulator that specifically controls RAF function.

## Results

To delineate biochemically the position of CNK with respect to the components of the RAS/MAPK module, we depleted endogenous CNK by RNAi in a stable RAS<sup>V12</sup>-expressing S2 cell line and assessed its effect on endogenous MEK and MAPK activation. As shown in Figure 1A, reduction of CNK by the addition of double-stranded (ds) CNK RNA specifically abrogated MEK and MAPK activation, as revealed by the decrease in phosphorylated (activated) MEK and MAPK. These results demonstrated that CNK is required downstream of RAS for activation of the MAPK module. We next examined the effect of removing CNK on activated RAF-induced MAPK activation. Compared to the activated receptor tyrosine kinase Sevenless (Sev<sup>S11</sup>) or RAS<sup>V12</sup>, which did not activate MAPK upon CNK or MEK depletion (Figure 1B, lanes 3, 4, 6 and 7), activated RAF (Tor<sup>4021</sup>RAFc) was still fully capable of activating MAPK upon CNK depletion, but not when MEK was eliminated (Figure 1B, lanes 9 and 10). Together, these results strongly suggest that CNK is acting between RAS and RAF.

Overexpression of CNK has been found previously to associate with endogenous RAF in S2 cells. Furthermore, a C-terminal fragment of CNK has also been reported to



**Fig. 2.** Opposite behavior of CNK in the RAS/MAPK pathway. (A) Schematic representation of *Drosophila* FL–CNK (top open box) with its various domains/elements (black boxes): sterile alpha motif (SAM); conserved region in CNK (CRIC); PSD-95/DLG-1/ZO-1 (PDZ); proline-rich stretch (Pro); pleckstrin homology (PH); RIR; RIM; IS. Numbers on top correspond to amino acid positions frequently referred to in the text. Solid lines, labeled to the left, denote the various CNK deletion constructs used in this study. Every CNK construct contains one copy of the Flag epitope at the N-terminus. (B) S2 cells were transfected with haMAPK alone (lane 1) or with the indicated combinations of haRAS<sup>V12</sup> (0.25 μg), FL–CNK (FL; 1.65 μg), NT–CNK (NT; 0.1 μg) and CT–CNK (CT; 1.65 μg) constructs. Cells were lysed 36 h post-induction and pMAPK levels were determined. Owing to a poor detection of Flag-tagged CNK constructs directly from cell lysates using the anti-Flag antibody, their levels were examined by immunoprecipitation (IP). (C) S2 cells were transfected with haMAPK alone (0.3 μg) or with the indicated combinations of Tor<sup>4021</sup>RAFC (0.2 μg), mycMEK<sup>2E</sup> (0.3 μg), FL (0.6 μg) or NT (0.1 μg) constructs. Cells were lysed 16 h post-induction. The flag-tagged CNK variants were examined using anti-CNK. (D) S2 cells were transfected and analyzed as in (C) using the indicated combinations of haMAPK (0.6 μg), RAS<sup>V12</sup> (0.6 μg), RAS<sup>V12S35</sup> (0.1 μg), RAS<sup>V12G37</sup> (0.25 μg), RAS<sup>V12C40</sup> (0.6 μg) and NT (0.25 μg). Various amounts of the RAS constructs were used to adjust for their apparent difference in expression levels. The RAS proteins were not tagged and thus were monitored using an anti-*Drosophila* RAS monoclonal antibody, which also detected endogenous RAS as seen in lanes 1 and 2. (E) S2 cells were transfected using the indicated combinations of haMAPK (0.6 μg), haRAS<sup>V12</sup> (0.25 μg), and wild-type or mutant NT constructs (0.25 μg). The mutated SAM domain (SAMmut) has a two amino acid change in conserved residues (amino acids W17S and I18S) that are critical for structural integrity (Stapleton *et al.*, 1999). For unclear reasons, this NT–CNK mutant migrates differently from the other NT constructs (lane 4). We have generated another mutant version of the SAM domain (L71K), which changes an amino acid shown to be critical for dimer formation of the EphA4 receptor SAM domain, but does not appear to alter the structural integrity of the domain (Stapleton *et al.*, 1999). This mutated SAM domain (NT<sup>L71K</sup>–CNK) migrates normally, and like the SAM<sup>W17S–I18S</sup> mutant, it does not cooperate with RAS<sup>V12</sup> (data not shown). NT<sup>CRICmut</sup> has a three amino acid deletion (A162–H163–R164) in the CRIC region similar to the mutation found in a *Drosophila* cnk loss-of-function allele (Therrien *et al.*, 1998). Finally, NT<sup>PDZmut</sup> has a two amino acid change (G217S and F218S) in highly conserved residues of the PDZ domains (Ponting *et al.*, 1997).

interact directly with the catalytic domain of RAF (Therrien *et al.*, 1998). To demonstrate that a CNK/RAF complex does exist *in vivo*, we immunoprecipitated plain S2 cell or *Drosophila* embryo extracts using either anti-RAF or anti-CNK antibodies and probed immunoblots with either antibodies to look for co-immunoprecipitation. As shown in Figure 1C, the anti-RAF antibodies brought down endogenous CNK (~10% of total NP-40-soluble CNK) and, likewise, the anti-CNK monoclonal antibody co-immunoprecipitated endogenous RAF (~5% of total

NP-40-soluble RAF) in both S2 cells and embryos. These results thus demonstrate the existence of a CNK/RAF complex *in vivo*. Together with the fact that CNK activity appears to be required upstream of RAF, these findings strongly suggest that CNK directly regulates RAF function.

#### **CNK has opposite effects on RAF function**

To characterize the molecular event(s) within the RAS/MAPK pathway that is/are regulated by CNK activity, we

examined whether forced expression of CNK could modulate MAPK activation in S2 cells. For this purpose, we used three Flag epitope-tagged CNK constructs (Figure 2A), namely, full-length (FL), N-terminal (NT) and C-terminal (CT) that had previously been shown to modulate RAS<sup>V12</sup>-mediated signaling in the developing *Drosophila* eye (Therrien *et al.*, 1999).

We assayed for MAPK activation by monitoring the phosphorylated levels of HA-tagged MAPK as performed above for endogenous MAPK. When expressed alone, none of the CNK constructs elevated phospho-MAPK (pMAPK) levels (data not shown and Figure 2D, lane 2 for NT-CNK). However, compared to HA-tagged RAS<sup>V12</sup> expressed alone (Figure 2B, lane 2), co-expression of FL-CNK and CT-CNK inhibited MAPK activation (Figure 2B, lanes 3 and 5, respectively), whereas NT-CNK stimulated MAPK activation (Figure 2B, lane 4). Therefore, these results indicate that forced expression of CNK affects RAS-mediated MAPK activation and also suggest that CNK comprises both positively- and negatively-acting regions.

Because CNK appears to be required between RAS and RAF (Figure 1B), we reasoned that the opposite effects of CNK could be due to a modulation of RAF function. To investigate this possibility, we examined the ability of FL-CNK and NT-CNK to alter MAPK activation induced by activated RAF or activated MEK (myc-tagged MEK<sup>2E</sup>). If FL-CNK blocked a positive step upstream of RAF, there should be no effect on MAPK activation induced by activated forms of RAF or MEK. In contrast, if CNK blocked a step downstream of RAF, it should either inhibit RAF or both RAF and MEK activities depending on the position of the inhibitory event. Strikingly, we found that FL-CNK (like CT-CNK, data not shown), completely prevented MAPK activation induced by activated RAF (Figure 2C, lane 3), but not by activated MEK (lane 6) [the apparent slight positive effect of FL-CNK on pMAPK levels induced by MEK<sup>2E</sup> (lane 6) was not reproducible]. These results therefore indicate that the negative influence of CNK occurs at a step between RAF and MEK. We applied the same logic to position the positive effect of NT-CNK and concluded that NT-CNK exerts its positive effect in a RAS-dependent manner between RAS and RAF as NT-CNK was inert on its own and did not cooperate with either activated RAF or MEK (Figure 2C, lanes 4 and 7, respectively).

Therrien *et al.* (1999) previously reported that NT-CNK cooperated in the *Drosophila* eye not only with RAS<sup>V12</sup>, but also with RAS<sup>V12G37</sup>, which is a RAS effector loop mutant that has a much reduced capacity to send signals through the MAPK pathway owing to its impaired association with RAF (White *et al.* 1995). They concluded that either NT-CNK augments RAS signaling through a RAS<sup>V12G37</sup>-dependent, but MAPK-independent pathway or that, if NT-CNK functions between RAS and RAF, it could rescue or compensate to some extent the defect caused by this particular effector loop mutation thereby permitting RAF activation. To distinguish between these possibilities, we co-expressed NT-CNK with RAS<sup>V12</sup> or the three RAS effector loop mutations that had been tested. These included RAS<sup>V12S35</sup>, which interacts normally with RAF, and RAS<sup>V12G37</sup> and RAS<sup>V12C40</sup>, which no longer interact with RAF (data not shown). As shown in

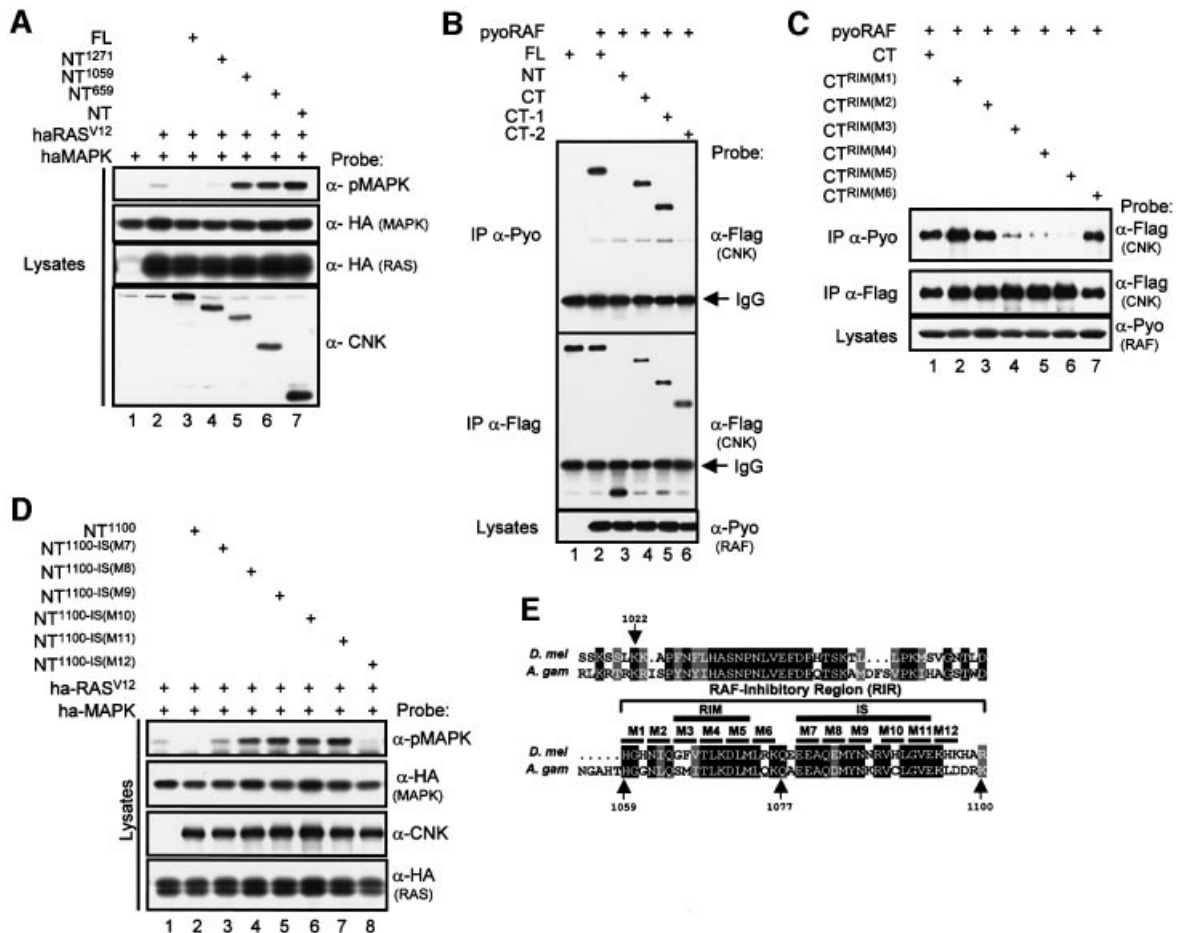
Figure 2D, NT-CNK strongly augmented pMAPK levels induced by either RAS<sup>V12</sup> or RAS<sup>V12S35</sup> (lanes 3–6) and surprisingly, it also allowed RAS<sup>V12G37</sup>, which is inert on its own, to activate MAPK (compare lanes 7 and 8). These data therefore suggest that NT-CNK exerts its effect not through an alternate pathway, but largely within the RAS/MAPK pathway. The fact that NT-CNK appears to compensate to some extent the inability of RAS<sup>V12G37</sup> to activate MAPK, but not for RAS<sup>V12C40</sup> (Figure 2D, compare lanes 9 and 10), indicates that these two effector loop mutations are not equivalent with respect to their defect in activating RAF.

NT-CNK comprises three conserved regions (SAM, CRIC and PDZ domains; Figure 2A). To determine which of these is required for the positive effect of NT-CNK on the MAPK module, we tested the activity of NT-CNK mutant constructs affecting each domain individually. When co-expressed with RAS<sup>V12</sup>, the mutated SAM and CRIC domain variants failed to cooperate with RAS (Figure 2E, lanes 4 and 5). In contrast, the PDZ domain mutant still retained activity (Figure 2E, lane 6). These results thus indicated that the SAM and CRIC domains are critical for the ability of NT-CNK to stimulate MAPK activation by RAS. Since the CRIC mutation used in this assay corresponds to the lesion found in a *cnk* loss-of-function allele (Therrien *et al.*, 1998), it strongly suggests that our assay mimics a genuine functional property of CNK (see below).

#### **Two short amino acid sequences in CNK define a 'RAF-inhibitory region' that blocks MEK phosphorylation by RAF**

Interestingly, in addition to its positive role on the MAPK module, CNK can block RAS- or RAF-dependent MAPK activation (Figure 2). We investigated this property by first mapping the region(s) of CNK that has a negative influence on the pathway. We generated a series of C-terminal deletions of CNK (Figure 2A) and tested their ability to inhibit RAS-dependent MAPK activation. As FL-CNK, the first deletion construct (NT<sup>1271</sup>-CNK) also blocked RAS<sup>V12</sup> activity (Figure 3A, lane 4). In contrast and similar to NT-CNK, two other deletion constructs (NT<sup>1059</sup>- and NT<sup>659</sup>-CNK) no longer inhibited, but instead cooperated with RAS<sup>V12</sup> (Figure 3A, lanes 5–7). These data indicated that the C-terminal boundary of an inhibitory region, hereafter called RAF-inhibitory region (RIR), lies between amino acid position 1059 and 1271. Finer deletion constructs were then similarly tested, which positioned the RIR to a short area of ~40 amino acids between positions 1059 and 1100 (see Supplementary figure S1, available at *The EMBO Journal* Online).

As CNK associates with RAF, it could be responsible for the negative effect of CNK. To address this possibility, we mapped the RAF binding site(s) on CNK to determine whether it corresponds to the RIR. CT-CNK, but not NT-CNK, was previously found to interact with RAF (Therrien *et al.*, 1998). We first tested whether we could reproduce these findings using a transient expression assay. A polyoma (pyo) epitope-tagged RAF construct was co-expressed with either FL-, NT- or CT-CNK in S2 cells. Cell lysates were immunoprecipitated using an anti-pyo antibody and co-immunoprecipitated CNK variants were detected by probing immunoblots with an anti-flag

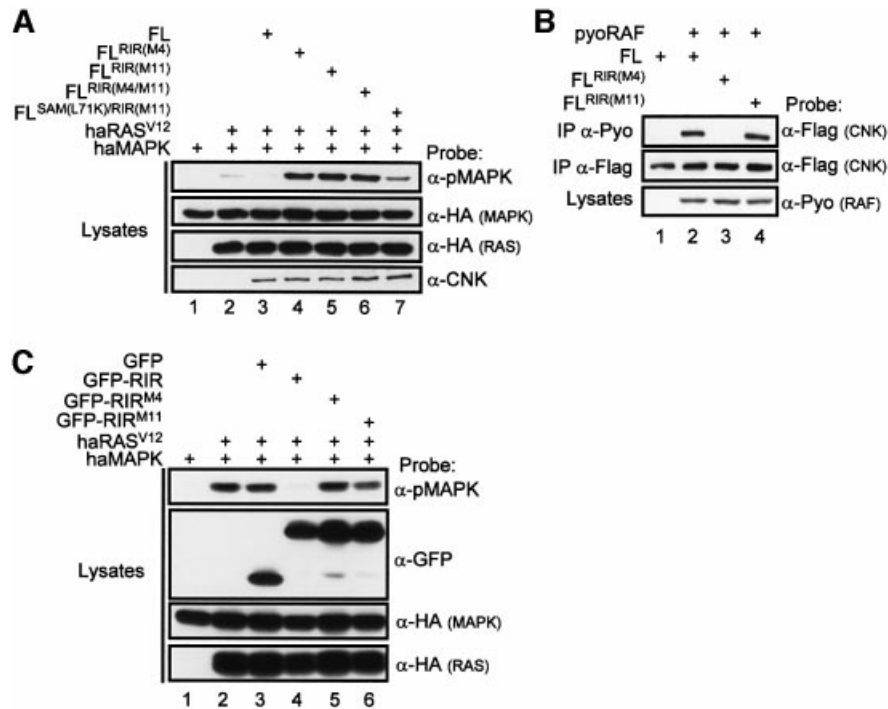


**Fig. 3.** Functional mapping of the RIR on CNK. (A) S2 cells were transfected with the indicated combinations of haMAPK (0.6  $\mu$ g), haRAS<sup>V12</sup> (0.25  $\mu$ g), FL and NT constructs (0.1–1  $\mu$ g). Cell lysates were prepared 16 h post-induction of expression. (B) S2 cells were transfected with the indicated combinations of pyoRAF (0.7  $\mu$ g), FL and CT constructs (1–1.8  $\mu$ g) and NT construct (0.08  $\mu$ g). Cells were lysed 36 h post-induction. For these experiments and below, pyoRAF was immunoprecipitated (IP) from cell lysates using the  $\alpha$ -Pyo antibody and co-immunoprecipitated CNK proteins were detected using the  $\alpha$ -Flag antibody. (C) S2 cells were transfected with the indicated combinations of pyoRAF (0.7  $\mu$ g) and CT constructs (1–1.8  $\mu$ g). (D) S2 cells were transfected with the indicated combinations of haMAPK (0.3  $\mu$ g), haRAS<sup>V12</sup> (0.125  $\mu$ g), NT constructs (0.6  $\mu$ g). (E) Amino acid comparison of the *Drosophila* (*D. mel*) CNK RIR (pos. 1022–1100) to the equivalent region of *A. gambiae* (*A. gam*) CNK. Identical and conserved residues are in black and grey boxes, respectively. Positions of the ‘alanine-scanning’ mutations (M1–M12) are depicted as a solid line over the amino acid sequence. Minimal amino acid sequence for the RIM and the IS are also highlighted by a solid line over the relevant area. Although sequences within the 1022–1059 interval also appear to participate in RAF-binding (Supplementary figure S2B), these are not essential.

antibody. As shown in Figure 3B (lanes 2–4), FL- and CT–CNK, but not NT–CNK, associated with RAF (~25% of RAF is associated with FL–CNK, and ~50% of FL–CNK is associated with RAF in these conditions). Two C-terminal deletions of CT–CNK (CT-1 and CT-2, Figure 2A) were also included in that experiment. CT-1, which ended at position 1271, still bound to RAF, whereas CT-2, which ended at position 1059, no longer interacted (Figure 3B, lanes 5 and 6). These results thus placed the C-terminal border of the RAF-binding region, hereafter called the RAF-interacting motif (RIM), in the 1059–1271 interval. The finer C- and N-terminal truncations used above to map the RIR were then used to delineate more accurately the RIM. This analysis showed that sequences in the 1059–1077 interval are critical for RAF binding (see Supplementary figure S2). Finally, we narrowed down the RIM to a nine amino acid stretch (positions 1065–1073) by testing for RAF interaction, a series of ‘alanine scanning’ mutants within the 1059–1077 interval (M1–M6,

Figure 3C and E). Together, our data thus revealed that the RIM is part of the RIR, which strongly suggests that the binding of RAF by CNK is responsible for the inhibitory effect of CNK. In support of this conclusion, the three point mutations (M3–M5) that impeded RAF binding (Figure 3C) also abrogated the inhibitory effect of CNK (data not shown and see below).

Our mapping data showed that the RIR comprises additional sequences after the C-terminal end of the RIM (Figure 3E). This indicated that other sequences that are not required for RAF interaction have an inhibitory effect on RAS signaling. To define more precisely the position of these sequences, we tested a set of alanine scanning mutants within the 1077–1100 interval (M7–M12, Figure 3E) and found that mutants M7–M11 relieved the inhibitory effect of CNK (Figure 3D) but, as expected, did not prevent RAF binding (data not shown and see below). These results therefore confirmed that the RIR contains at least two distinct negative elements: the RIM that interacts



**Fig. 4.** The negative effect of CNK is mediated by two co-required elements. (A) S2 cells were transfected with the indicated combinations of haMAPK (0.3  $\mu$ g), haRAS<sup>V12</sup> (0.14  $\mu$ g) and FL (0.5–0.8  $\mu$ g) constructs. (B) S2 cells were transfected with the indicated combinations of pyoRAF (0.7  $\mu$ g) and FL–CNK (1.5  $\mu$ g) constructs. (C) S2 cells were transfected with the indicated combinations of haMAPK (0.3  $\mu$ g), haRAS<sup>V12</sup> (0.2  $\mu$ g) and GFP constructs (0.5  $\mu$ g).

with RAF and an adjacent inhibitory sequence (IS), which is required along with the RIM to inhibit signal transmission within the MAPK module.

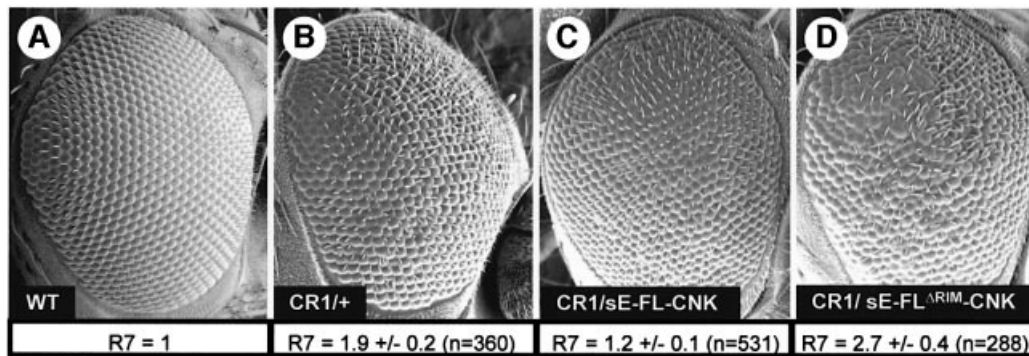
To verify whether the two negative elements (RIM and IS) also functioned accordingly in full-length CNK, we introduced the M4 mutation (affects the RIM) or the M11 mutation (affects the IS) in FL–CNK and examined their behavior with respect to RAS-induced MAPK activation and RAF-binding. Compared to FL–CNK (Figure 4A, lane 3), FL<sup>RIR(M4)</sup>–CNK and FL<sup>RIR(M11)</sup>–CNK no longer inhibited MAPK activation, but instead strongly cooperated with RAS<sup>V12</sup> (lanes 4 and 5). Furthermore, a double FL<sup>RIR(M4/M11)</sup>–CNK mutant cooperated to the same extent as either single mutants (lane 6), thus indicating that the two elements are co-required for the negative effect. As for NT–CNK (Figure 2), the ability of those mutants to cooperate with RAS appears to depend on the N-terminal domains of CNK as a double mutant version that affects both the SAM domain and the RIR barely cooperated with RAS<sup>V12</sup> (Figure 4A, lane 7). We next examined the ability of the M4 and M11 mutants to associate with RAF. As predicted, FL<sup>RIR(M4)</sup>–CNK no longer interacted with RAF (Figure 4B, lane 3), whereas FL<sup>RIR(M11)</sup>–CNK interacted normally with RAF (lane 4), thus confirming that only the RIM is essential for RAF-binding. Taken together, these results demonstrate that the RIM and IS elements, which constitute the RIR, are jointly required and sufficient to explain the inhibitory effect of CNK on the MAPK module.

Finally, to determine whether the RIR functions autonomously, we fused it (position 1059–1100) to the C-terminal end of GFP (GFP–RIR) and examined whether

this was sufficient to transpose CNK's negative effect on GFP. As shown in Figure 4C, GFP–RIR strongly inhibited RAS-induced MAPK activation (lane 4), whereas GFP alone (lane 3) or two inactivated versions (lanes 5 and 6) of the RIR (RIR<sup>M4</sup> or RIR<sup>M11</sup>) did not affect MAPK activation. These results therefore indicate that the RIR acts as an independent negative unit.

#### The RIR of CNK antagonizes RAS signaling during eye development

We wanted to determine whether the RIR of CNK also negatively influenced RAS signaling during *Drosophila* eye development. Intriguingly, in contrast to the data presented above, previous work showed that FL–CNK cooperated with RAS<sup>V12</sup> in the *Drosophila* eye (Therrien *et al.*, 1998). We found, however, that this cooperation greatly depended on RAS<sup>V12</sup> signaling strength as well as FL–CNK expression levels, that is, FL–CNK inhibited RAS<sup>V12</sup> phenotype when a weaker RAS<sup>V12</sup> line was used (see below) or when FL–CNK levels were increased (data not shown). Nonetheless, the ability of FL–CNK to cooperate with RAS<sup>V12</sup> in the developing eye, a phenomenon not detectable in S2 cells by simply co-expressing various amounts of either protein, suggests that S2 cells might be missing a critical signal and/or factor. Although not mutually exclusive, another possibility is that there is a close relationship between the amount of activated RAS molecules and available N-terminal domains that ultimately determine RAF activation level. At low doses of RAS activity, endogenous CNK levels are probably not limiting and thus can fully mediate RAS signals for optimal RAF activation. Consequently, increasing CNK



**Fig. 5.** CNK's RIR antagonizes RAS signaling *in vivo*. (A–D) Scanning electron micrographs of adult *Drosophila* eyes. The genotypes are indicated on the figures, as well as the average number of R7 cells per ommatidium ( $\pm$  SD). *n* denotes the number of ommatidia analyzed and three eyes were analyzed per genotype. A *t* test applied on the difference in mean number of R7 cells/ommatidium between the CR1 and CR1/sE-FL–CNK genotypes or the CR1 and CR1/sE-FL<sup>RIM</sup>–CNK genotypes confirmed their statistical significance:  $P = 0.006$  and  $0.036$ , respectively.

levels in that context is only providing additional RIR sequences that in turn block signal transmission. In contrast, when high levels of RAS activity are provided, endogenous CNK levels are probably limiting and thus could not fully transmit available RAS signals to RAF. Artificially increasing CNK levels in that context would provide missing CNK molecules, thereby generating greater RAF activity.

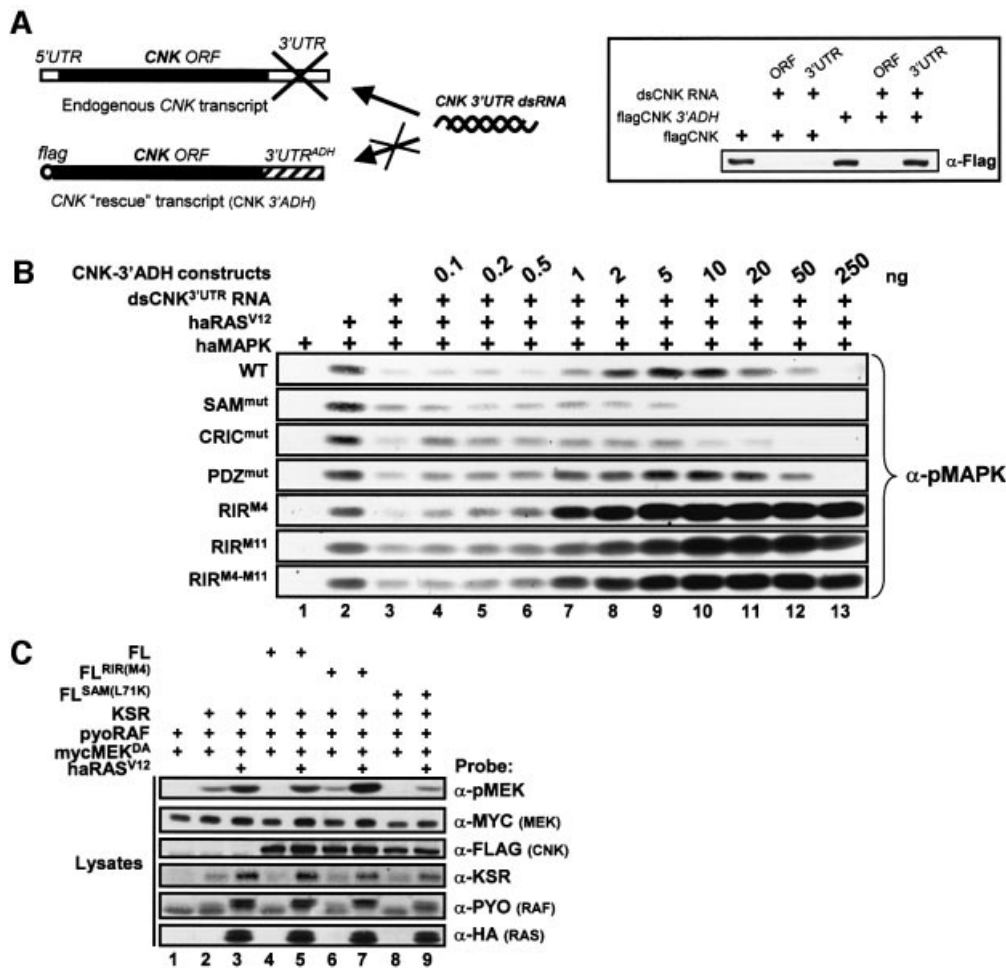
To determine whether the RIR had any inhibitory effect on RAS<sup>V12</sup> signaling *in vivo*, we compared the ability of FL–CNK or a CNK transgene with no RIM (FL<sup>RIM</sup>–CNK) to modulate RAS<sup>V12</sup> effects in the developing *Drosophila* eye. We crossed transgenic flies expressing the FL–CNK constructs to a strong RAS<sup>V12</sup> transgenic line (named CR2; Maixner *et al.*, 1998). As observed previously, FL–CNK cooperated with RAS<sup>V12</sup> (data not shown). Strikingly, however, co-expression of FL<sup>RIM</sup>–CNK and RAS<sup>V12</sup> resulted in a complete synthetic lethality at the pupal stage with pupae presenting highly disorganized eye tissues (data not shown). This phenomenon probably resulted from a particularly strong cooperation between RAS<sup>V12</sup> and FL<sup>RIM</sup>–CNK. Given that both constructs are probably expressed to low levels in tissues other than the eye, the observed lethality is likely a consequence of detrimental MAPK activation in tissues required for viability. To circumvent this problem, we crossed the CNK transgenic lines to a weaker RAS<sup>V12</sup> line (CR1; Maixner *et al.*, 1998). As expected, instead of cooperating with RAS<sup>V12</sup>, FL–CNK slightly suppressed the CR1 rough eye phenotype (compare Figure 5B and C). To confirm this, we determined the average number of R7 cells per ommatidium for each genotype as a read-out for RAS/MAPK signaling (Fortini *et al.*, 1992). The eye section results are summarized at the bottom of each SEM (Figures 5A–D). As shown in Figure 5B, CR1 had an average of 1.9 R7 cells per ommatidium. Co-expression of FL–CNK reduced this number to 1.2 (Figure 5C), which confirmed its ability to suppress RAS<sup>V12</sup> in this context. In contrast to FL–CNK, the FL<sup>RIM</sup>–CNK construct cooperated with RAS<sup>V12</sup> as the eyes were rougher than the CR1 parents and the average number of R7 cell per ommatidium increased to 2.7 (Figure 5D). Therefore, as in S2 cells, the RIR has a negative effect on RAS signaling during eye development. Identical results using the CR1

and CR2 lines were obtained with CNK lines that had a mutated IS element (G.Laberge and M.Therrien, unpublished results). Furthermore, and importantly, we found that the  $\Delta$ RIM or IS mutant constructs were as competent as wild-type CNK at rescuing the lethality of *cnk* null alleles (data not shown), thus strongly suggesting that the RIR does not naturally function as a positive element (see below).

#### **An RNAi-based rescue assay uncovers CNK's natural opposite effects on the MAPK module**

The finding that CNK has a negative impact on the RAS/MAPK pathway is intriguing given the fact that CNK has been originally defined as a positive component for this pathway. One possibility to explain our results would be that CNK functions as a scaffold by bringing together, through independent associations, at least two signaling proteins. Therefore, as previously described for a hypothetical scaffold (Burack and Shaw, 2000), overexpression of CNK might uncouple proteins that normally require physical juxtaposition, thereby abrogating signal transmission. According to that model, the RIR might normally be required for higher order assembly of the RAS/MAPK module and thus for optimal signal transmission.

To assess unambiguously the natural effect of the RIR on the MAPK pathway, its activity needs to be monitored in non-overexpressed conditions. To that end, we devised a novel RNAi-based strategy in S2 cells that allowed us to deplete endogenous CNK levels and to restore them with exogenous, but non-targetable CNK transcripts. The ability to rescue a CNK knock-down phenotype, such as a MAPK activation defect, would indicate that relatively normal levels of exogenous CNK have been reached. This approach could then serve as a simple assay to evaluate the effect of specific CNK mutants in relatively normal stoichiometric conditions. To specifically remove endogenous CNK, we used a dsCNK RNA (dsCNK<sup>3'UTR</sup>) encompassing exclusively CNK's 3'UTR sequences (Figure 6A), which reduced endogenous CNK levels by >90% after 4 days of culture (data not shown). Conversely, to obtain dsCNK<sup>3'UTR</sup>-resistant CNK transcripts, we replaced the natural 3'UTR sequences in the FL–CNK construct by those of the *Drosophila alcohol dehydrogenase (ADH)* gene. The resulting chimeric transcripts (CNK–



**Fig. 6.** CNK has a natural antagonistic effect on signal transmission through the MAPK module. (A) Diagram representing the strategy used to deplete endogenous CNK by targeting its 3'UTR sequences. CNK 'rescue' is achieved by co-transfecting a CNK construct containing the ADH 3'UTR sequences. The specificity of the CNK 3'UTR dsRNA compared to a dsRNA targeting CNK's open reading frame (ORF) is shown on the right: FL-CNK (0.4  $\mu$ g) or FL-CNK-3'ADH (0.4  $\mu$ g) constructs were transfected in S2 cells along either CNK's 3'UTR or ORF dsRNAs (0.5  $\mu$ g). Cells were lysed 36 h post-induction. (B) S2 cells were transfected with the indicated combinations of haMAPK (0.3  $\mu$ g), haRAS<sup>V12</sup> (0.125  $\mu$ g), dsCNK<sup>3'UTR</sup> RNA (20  $\mu$ g) and the indicated amounts of the CNK-3'ADH variants. Cells were lysed 4 days post-induction of expression. Activated MAPK levels were detected ( $\alpha$ -pMAPK) as well as those for haMAPK, haRAS<sup>V12</sup> and CNK-3'UTR variants (not shown). (C) S2 cells were transfected with the indicated combinations of mycMEK<sup>DA</sup> (0.3  $\mu$ g), pyoRAF (0.03  $\mu$ g), KSR (0.1  $\mu$ g), RAS<sup>V12</sup> (0.6  $\mu$ g) and FL-CNK variant (1.1  $\mu$ g) constructs. Cells were lysed 36 h post-induction of expression.

3'ADH) were insensitive to the dsCNK<sup>3'UTR</sup> RNA (see Figure 6A; and data not shown).

Consistent with its ability to reduce endogenous CNK levels, dsCNK<sup>3'UTR</sup> RNA decreased RAS-induced MAPK activation (Figure 6B, compare lanes 2 and 3). We then determined whether introduction of a wild-type CNK-3'ADH (CNK<sup>WT</sup>-3'ADH) construct would rescue MAPK activation under those conditions. Given that CNK overexpression leads to an inhibition of RAS-induced MAPK activation (Figure 2), we conducted parallel co-transfections using a spectrum of plasmid quantities to narrow down optimal rescuing amounts. Strikingly, not only did this approach restore MAPK activation, but it also revealed a dose-dependent rescue profile identifying reproducible sub-optimal (0.1–1 ng), optimal (2–10 ng) and inhibitory (>20 ng) doses of plasmids (Figure 6B, WT panel; and data not shown). To determine whether this assay faithfully identifies CNK regions that are functionally relevant with respect to RAS-mediated MAPK

activation, we tested the effect of mutations affecting the SAM, CRIC and PDZ domains, respectively. Consistent with their implication in the cooperation observed above between NT-CNK and RAS<sup>V12</sup> (Figure 2E), we found that CNK<sup>SAMmut</sup>-3'ADH and CNK<sup>CRICmut</sup>-3'ADH behave as loss-of-functions, whereas CNK<sup>PDZmut</sup>-3'ADH is as competent as wild-type CNK in restoring MAPK activation (Figure 6B). The inability of the CRIC mutation to rescue MAPK activation is consistent with its previous identification as a *cnk* loss-of-function allele (Therrien *et al.*, 1998), thereby providing compelling evidence that this assay can identify bona fide functional domains/elements. In addition, these results indicate that the SAM domain is genuinely required for RAS-induced MAPK activation, whereas the PDZ domain is not involved.

Next, we tested the ability of mutations affecting the RIR to rescue MAPK activation. If the RIR normally has a positive function, its disruption should attenuate or preclude the rescue capability of a RIR mutant construct



at doses optimal for WT CNK. Markedly, the three tested RIR mutants (M4, M11 or M4/M11) did not behave as loss-of-function, but rather as gain-of-function alleles (Figure 6B). Therefore, these results demonstrate that the RIR does not provide a positive input, but functions as a true inhibitory module.

### **CNK's RIR prevents 'signaling leakage' within the KSR/RAF/MEK complex**

A model to explain the two opposite roles of CNK would be that they take place at different times. For example, the negative function could be required prior to signal activation to prevent RAS-independent MEK activation by RAF. We previously reported that overexpression of KSR, RAF and MEK in S2 cells induced an association between RAF and MEK, which also resulted in a significant activation of MEK (Roy *et al.*, 2002). Although RAS activity could increase further MEK activation, it did not appear to be critical for either KSR/RAF/MEK complex formation or MEK activation. We thus concluded that overexpression of KSR, RAF and MEK might bypass natural repressive mechanism(s) that are essential to prevent signaling in the absence of upstream signals. Given that CNK's negative property seems to affect precisely the RAF/MEK step, we examined whether CNK could attenuate KSR-induced MEK activation by RAF.

As previously reported (Roy *et al.*, 2002), co-expression of KSR with pyoRAF and kinase-inactivated mycMEK (mycMEK<sup>DA</sup>), resulted in MEK activation (Figure 6C, lane 2), which could be further enhanced by co-expressing RAS<sup>V12</sup> (lane 3). However, co-expression of FL-CNK along with KSR, RAF and MEK strongly blocked RAS-independent MEK activation (compare lanes 2 and 4). To determine whether the RIR was responsible for this effect, we tested the FL-CNK<sup>RIRM4</sup> mutant in similar conditions. As shown in lane 6, this mutation obliterated the repressive effect of CNK. Similar results were obtained with either the RIR<sup>M11</sup> or RIR<sup>M4-M11</sup> mutations (data not shown). Interestingly, when RAS<sup>V12</sup> was introduced in the presence of FL-CNK, RAS activity counteracted much of the inhibitory effect (compare lanes 4 and 5), presumably owing to the ability of the N-terminal portion of CNK to cooperate with activated RAS. Consistent with that possibility, FL-CNK<sup>SAMmut</sup> was far less active than wild-type FL-CNK (compare lanes 5 and 9). Taken together, these results are consistent with a model whereby CNK prevents signal transmission between RAF and MEK within a KSR/RAF/MEK complex in the absence of a signal, but then facilitates signaling upon upstream activation, thus increasing significantly the signal-to-noise ratio of this signaling complex.

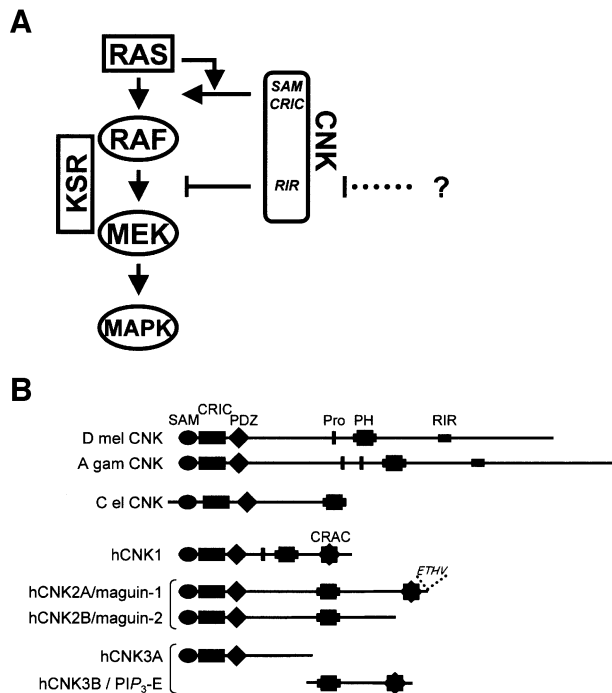
## **Discussion**

CNK was originally identified as a positive component of RAS/MAPK-mediated signaling in *Drosophila*. In this paper, we provide evidence that the primary role of CNK in the RAS/MAPK module is to regulate RAF function. Unexpectedly, in addition to its critical role for RAF activity, we also found that CNK negatively controls RAF's ability to phosphorylate MEK.

Three lines of evidence support the claim that CNK is essential for RAF activity. First, depletion of endogenous CNK prevented MAPK activation by RAS<sup>V12</sup>, but not by activated RAF (Figure 1B). Secondly, NT-CNK cooperated with RAS<sup>V12</sup> to activate MAPK (or MEK; data not shown), but not with activated RAF (Figure 2B and C). This result not only places CNK's positive effect between RAS and RAF, but it also suggests that this activity is RAS-dependent. Finally, NT-CNK rescued MAPK activation by RAS<sup>V12G37</sup> (Figure 2D). This finding is striking as it provides strong evidence that CNK function is intimately linked to the RAF activation mechanism. As for mammalian RAS (White *et al.*, 1995), *Drosophila* RAS G37 or C40 effector mutants no longer interact with *Drosophila* RAF (data not shown). Since only the G37 mutant is rescued by NT-CNK co-expression (a RAF-dependent event; data not shown), it suggests that it is either a weak loss-of-function with respect to RAF binding and/or that it retained another essential function that has been lost by the C40 mutant. The G37 mutant may thus prove useful to elucidate the role of CNK in RAF activation.

We have mapped CNK's inhibitory function to a 30 amino acid region named the RIR (see above). This region comprises at least two distinct, but co-required negative elements: the RIM and the IS elements (Figure 3E) that function together as an inhibitory unit (Figure 4A). Although its mechanism of action is unknown, the RIR appears to block signal transmission from RAF to MEK (Figures 2C and 6C) through an association between the RIM and the RAF catalytic domain. Indeed, an isolated RAF catalytic domain or the Torso-RAF catalytic domain fusion protein (Tor<sup>4021</sup>-RAFc) have been found to associate with CNK (Therrien *et al.*, 1998; and data not shown). The association appears to be direct as it is detectable using a yeast two-hybrid interaction assay (M.LeFrançois and M.Therrien, unpublished results). The role of the IS element is unknown. It is not required for RAF binding, but it is essential for the inhibitory effect of the RIR (Figure 4). Interestingly, since only a catalytically competent RAF kinase domain associates with CNK (data not shown), it is possible that the RIR works as a RAF pseudosubstrate to control MEK phosphorylation.

What could be the purpose of CNK's bimodal effect? Several scenarios can be envisioned to explain our data and two of these are presented here. In quiescent cells, CNK could function together with 14-3-3 in preventing signal-independent MEK activation by RAF. This negative role might be important to ensure that no signal leaks through prior to genuine upstream activation, which otherwise might be sufficient to initiate a biological response. Upon RAS activation, CNK's N-terminal domains would then integrate RAS signals and thus convert CNK into a positive regulator of signal transmission (Figure 7A). As CNK's opposite action appears to augment the signal-to-noise ratio of the RAS/MAPK module (Figure 6C), it might contribute to switch-like activation of the pathway (Ferrell, 1998). Alternatively, CNK's negative effect might have a similar role to RKIP, a recently described RAF inhibitor (Yeung *et al.*, 1999). Namely, it might work as a rheostat to finely adjust the amount of MEK molecules activated by RAF to satisfy cell-specific requirements. For that matter, it will be



**Fig. 7.** CNK, a bimodal regulator of RAF function. **(A)** Summary of the effects of CNK on the RAS/MAPK module. The SAM and CRIC domains are two critical regions integrating RAS's signals that then feed at a step between RAS and RAF. In contrast, the RIR inhibits MEK phosphorylation by RAF by binding to the RAF catalytic domain. The question mark and the dotted line illustrate the possibility that the RIR might itself be negatively regulated. KSR acts as a scaffold bridging RAF and MEK, thereby augmenting signal transmission (see Introduction). **(B)** Structural comparison of CNK homologs found in *D. melanogaster* (D mel; DDBJ/EMBL/GenBank accession No. AF100152), *A. gambiae* (A gam; DDBJ/EMBL/GenBank accession No. EAA15086), *C. elegans* (C el; DDBJ/EMBL/GenBank accession No. R01H10.8) and human (h). There are at least three separate genes in humans: hCNK1 (DDBJ/EMBL/GenBank accession No. AF100153), hCNK2A/B (DDBJ/EMBL/GenBank accession Nos AF418269 and AF418270, respectively) and hCNK3A/B (DDBJ/EMBL/GenBank accession Nos AK055911 and AJ310566, respectively). Although hCNK2A and hCNK2B are splicing variants, the situation is not clear for hCNK3A and hCNK3B/PIP<sub>3</sub>-E. Their respective ESTs were both mapped to the same chromosomal location (6q25.2; MapView NCBI), but are separated by ~100 kb and have no overlapping sequences. They might thus represent two separate genes. In addition to the domain composition previously described for the CNK homologs (Therrien *et al.*, 1998), mammalian and other CNKs found in chordates (not shown) have an additional conserved region of ~50 amino acids of unknown function. We named this novel region of homology conserved region among chordate (CRAC) CNKs and depicted it as a black star. Similar to the rat MAGUIN homolog (Yao *et al.*, 2000), hCNK2A contains a PDZ-binding motif (ETHV) at its C-terminus.

interesting to determine whether the RIR is itself negatively regulated to increase signaling flow from RAF to MEK (Figure 7A). This possibility is appealing given the large difference between the ability of WT CNK and RIR-inactivated CNK constructs to rescue the MAPK activation defect (Figure 6B).

CNK homologs are present in other metazoans (Figure 7B). This evolutionary conservation strongly suggests that ERK/MAPK modules in other species are also regulated by a CNK activity at the level of RAF. Although the SAM and CRIC domains are relatively well conserved, the sequence corresponding to the RIR

(Figure 3E) seems to be unique to *Drosophila melanogaster* and *Anopheles gambiae*. Nonetheless, rat CNK2/maguin isoforms (CNK's closest homologs; Figure 7B) have been shown to associate with c-RAF (Yao *et al.*, 2000), which suggests that they contain a region functionally similar to the RIR. If that were the case, it would be important to verify whether mutations disrupting its presumed negative function have any oncogenic properties. In addition, given the significance the RAS/MAPK module plays in tumor formation in humans, the identification of a short inhibitory peptide against RAF catalytic function might open new avenues for anticancer drug development.

## Materials and methods

### Plasmids

pMet-FL-CNK, NT-CNK and CT-CNK have been described previously (Therrien *et al.*, 1998) and were used as starting points to generate the various CNK mutant constructs used in this study. pMet-FL-CNK $\Delta$ <sup>RIM</sup> corresponds to an internal deletion from amino acids 1059 to 1077. The psE-FL-CNK $\Delta$ <sup>RIM</sup> P-element construct was generated by transferring the *Kpml/NotI* insert from the pMet construct into the psE P-element vector (Therrien *et al.*, 1998). Finally, the CNK-3'ADH constructs were generated by removing the natural CNK 3'UTR sequences from FL-CNK constructs, which juxtaposed downstream ADH 3'UTR sequences present in pMet.

pMet-haMAPK was generated first by inserting a PCR product corresponding to *Drosophila* MAPK into the *EcoRI/SacI* sites of pMet. A double-stranded oligonucleotide encoding three HA epitopes was then inserted in the *EcoRI* site of pMet-MAPK upstream of the first methionine. pMet-pyoTor<sup>4021</sup>RAF<sub>c</sub> was generated in three steps. First, an *EcoRI* fragment corresponding to Tor<sup>4021</sup>RAF<sub>c</sub> (Dickson *et al.*, 1992) was inserted in the pMet vector. An *AgeI* site was then introduced immediately downstream from Torso's signal peptide sequences and used to insert a PYO epitope. pMet-mycMEK<sup>2E</sup> was generated by replacing the serines 234 and 238 by glutamic acid residues.

pMet-RAS<sup>V12</sup> and effector loop mutants were described previously (Therrien *et al.*, 1999). pMet-haRAS<sup>V12</sup>, pMet-pyoRAF, pMet-KSR and pMet-mycMEK<sup>DA</sup> were described by Roy *et al.* (2002). pHS-SEV<sup>S11</sup> was described previously (Therrien *et al.*, 1998).

### Cell culture, transfection and protein analysis

Cell culture, transfection, RNAi and immunoprecipitation experiments in S2 cells were performed essentially as described by Roy *et al.* (2002).

Cell lysates or immunoprecipitated proteins were resolved on 8 or 10% SDS-PAGE and transferred to nitrocellulose membranes. Proteins were probed using appropriate primary antibodies from the following sources:  $\alpha$ -CNK monoclonal antibody (26A6A2) was generated by Elaine Kwan in the laboratory of Gerry Rubin.  $\alpha$ -RAS1,  $\alpha$ -SEV,  $\alpha$ -PYO epitope and  $\alpha$ -HA epitope (12CA5) mAbs were kindly provided by Gerry Rubin;  $\alpha$ -*Drosophila* RAF polyclonal antibody was a kind gift from Debbie Morrison;  $\alpha$ -MYC epitope mAb (9E10) was from Santa Cruz Biotechnology;  $\alpha$ -MEK-1&2 and  $\alpha$ -pMEK-1&2 polyclonal antibodies were from Cell Signaling; and  $\alpha$ -ERK-1&2,  $\alpha$ -dpERK-1&2 and  $\alpha$ -FLAG mAbs were from Sigma.

### *Drosophila* genetics and histology

Fly maintenance and crosses were conducted according to standard procedures. CR1 transgenic flies contain one copy of the *sev-Ras1<sup>V12</sup>* transgene (Fortini *et al.*, 1992) on the *Cyo* balancer. The *sE-FL-CNK* transgenic line was described previously (Therrien *et al.*, 1998). Multiple lines expressing the *sE-FL-CNK $\Delta$ <sup>RIM</sup>* transgene or the *IS* mutant version were analyzed. P-element-mediated germline transformation was performed as described by Rubin and Spradling (1982).

Adult *Drosophila* eye sections and scanning electron microscopy was conducted as previously described by Tomlinson and Ready (1987) and Kimmel *et al.* (1988), respectively.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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