The AF-6 Homolog Canoe Acts as a Rap1 Effector During Dorsal Closure of the Drosophila Embryo

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ABSTRACT

Rap1 belongs to the highly conserved Ras subfamily of small GTPases. In Drosophila, Rap1 plays a critical role in many different morphogenetic processes, but the molecular mechanisms executing its function are unknown. Here, we demonstrate that Canoe (Cno), the Drosophila homolog of mammalian junctional protein AF-6, acts as an effector of Rap1 *in vivo*. Cno binds to the activated form of Rap1 in a yeast two-hybrid assay, the two molecules colocalize to the adherens junction, and they display very similar phenotypes in embryonic dorsal closure (DC), a process that relies on the elongation and migration of epithelial cell sheets. Genetic interaction experiments show that Rap1 and Cno act in the same molecular pathway during DC and that the function of both molecules in DC depends on their ability to interact. We further show that Rap1 acts upstream of Cno, but that Rap1, unlike Cno, is not involved in the stimulation of JNK pathway activity, indicating that Cno has both a Rap1-dependent and a Rap1-independent function in the DC process.

Rap1 belongs to the Ras superfamily of small GTPases, which cycle between an inactive GDP-bound and an active GTP-bound state, eliciting distinct downstream responses in the active state. Rap proteins were originally identified as antagonists of oncogenic Ras (Kitayama *et al.* 1989; Cook *et al.* 1993; Okada *et al.* 1998; Mochizuki *et al.* 1999), but more recent studies suggest that the function of Rap1 is largely independent of Ras (reviewed in Zwartkruis and Bos 1999; Bos *et al.* 2001; Caron 2003). While Ras is mainly localized at the plasma membrane, Rap1 has been found in different membrane compartments, depending on the cell type. Further, Rap1 activation appears to be stimulated by numerous exchange factors that do not act on the prototypic Ras GTPases. Rap1 has been shown to act in a Ras-independent manner in the production of superoxide (Bokoch *et al.* 1991; Maly *et al.* 1994), in cAMP-induced neurite outgrowth (York *et al.* 1998), and, most recently, in the regulation of integrin-mediated cell adhesion and AMPA receptor trafficking during synaptic plasticity (Tsukamoto *et al.* 1999; Caron *et al.* 2000; Reedquist *et al.* 2000; Arai *et al.* 2001; Zhu *et al.* 2002; Rangarajan *et al.* 2003).

Perhaps the most important insights into the function of Rap1 are emerging from studies in Drosophila. Loss-of-function (lof) mutations in Drosophila *Rap1* cause severe morphogenetic abnormalities during embryonic development, while cell proliferation and cell fate determination, processes that rely heavily on regulation by Ras, appear to be unaffected. Specifically, the ventral invagination and migration of mesodermal precursors in the embryo are severely impaired, as are head involution, dorsal closure, and the migration of gonadal precursors (Asha *et al.* 1999). More recently, Rap1 has been shown to play a role in cell adhesion, specifically in the positioning of adherens junctions in proliferating epithelial cells (Knox and Brown 2002). These findings strongly suggest that Rap1 plays a largely Ras-independent role in cell migration and morphogenesis.

Little is currently known about the signaling pathways mediating the downstream effects of Rap1 in vertebrates or Drosophila. A number of molecules that were originally identified in vertebrates as Ras-interacting proteins, including B-Raf, members of the RalGEF family, and AF-6, were subsequently shown to associate with Rap1 as well (Linnemann *et al.* 1999; Quilliam *et al.* 1999; Zwartkruis and Bos 1999; Boettner *et al.* 2000; Bos *et al.* 2001). However, the relevance of these interactions for Rap1 function *in vivo* remains largely unknown; to date, none of these molecules have been shown to act as Rap1 targets in an *in vivo* context.

Here we report that Canoe (Cno), the Drosophila ortholog of AF-6, acts as an effector of Rap1 during dorsal closure (DC) of the Drosophila embryo. DC is a morphogenetic process that occurs during midembryo-

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genesis and involves the dorsalward movement of the lateral ectoderm over the amnioserosa, a transient structure that covers the dorsal aspect of the embryo, to enclose the embryo. This process relies entirely on the migration and elongation of ectodermal cells, without cell recruitment or proliferation, and is akin to the epithelial cell sheet movements that occur during wound healing (Stronach and Perrimon 1999). Among the genes identified as necessary for normal DC are proteins associated with the cytoskeleton and/or cell junctions and components of the Drosophila Jun N-terminal kinase (JNK) and Decapentaplegic (Dpp) pathways (Stronach and Perrimon 1999). *cno* is required for DC (Jürgens *et al.* 1984); its protein is localized to the adherens junction and feeds into the JNK pathway by an unknown mechanism (Takahashi *et al.* 1998). Apart from the fact that it interacts with the ZO-1 homolog Tamou (Takahashi *et al.* 1998), nothing is known about the regulation of Cno activity at the adherens junction.

We identified Cno as a protein that interacts with activated Rap1 in a yeast two-hybrid screen. To address the physiological relevance of this interaction, we undertook localization studies for the two proteins, a comparative phenotypic analysis, and genetic interaction experiments. We show that the *Rap1* and *cno* loci interact synergistically in DC and that the physical interaction between Rap1 and Cno is required for DC. We further show that the role of Canoe in promoting JNK pathway activity is independent of Rap1 and that Canoe therefore has two separate functions in DC.

MATERIALS AND METHODS

Yeast two-hybrid experiments, transgenes, and genetics: Drosophila Rap1^{wt}, Rap1^{V12}, and Rap1^{N17} were fused to LexA (pBTM116); Cno fragments encoding RA1 (amino acids 1–153) or RA2 (amino acids 255–396), the Cno N terminus with both RA domains (amino acids 1–396), and an N-terminally deleted version of Cno lacking the first 361 amino acids were inserted into pGAD.

Rap1 interacting proteins were identified by screening a Drosophila embryonic GAD-fusion library with pLexA-D-Rap1^{V12} as a bait, using previously described methods (Van Aelst 1998).

V12 and N17 versions of Drosophila Rap1 and Ras2 were generated by site-directed mutagenesis (QuickChange; Stratagene, La Jolla, CA). The Myc-epitope EQKLISEEDLNE was inserted between the second and third amino acid of Rap1 by PCR. The Cno N terminus was deleted using a primer that links codon 362 to a Kozak-embedded ATG. Wild-type and mutant Rap1, Ras2, and Cno cDNAs were inserted into pUAST (Brand and Perrimon 1993). Genotyping of embryos was based on the absence of markers carried by the balancers. For cuticle preparations, balancers carrying *ubiGFP* for immunohistochemistry and RNA *in situ* hybridization balancers carrying *lacZ* transgenes (*CyO, wglacZ* and *TM3, DfdlacZ*) were used. Germline clones of *Rap1^{CD5}* were generated as described (Asha *et al.* 1999). *Cno^{mis1},Rap1^{CD5}* and *cno^{2},Rap1^{CD5}* chromosomes were generated by meiotic recombination; recombinant genotypes were determined using *cno^{3}* and *Rap1^{B3}* alleles. *cno^{2}*, *cno^{3}*, *bsk^{2}*, *bsk^{3}*, and the *ptc-*, *dpp-*, *pnr-*, and *hs-GAL4* drivers as well as the *ubiGFP* balancers were kindly provided by the Bloomington Drosophila Stock Center, *Rap1^{B3}* and *Rap1^{CD5}* by I. Hariharan, *GFP-Rap1* by N. Brown, *cno^{mis1}* by D. Yamamoto, *UASbsk* by M. Mlodzik, *UASRas1* by D. Montell, and the *TM3, DfdlacZ* and *CyO, wglacZ* balancers by M. Baylies. Statistical significance of rescue experiments was assessed using the chi-square test.

Immunohistochemistry, RNA *in situ* hybridization, and cuticle preparation: Rat polyclonal antibodies against Cno (amino acids 729–1171) were generated using standard procedures (Covance). In lieu of DRap1-specific antibodies, a transgene encoding a GFP-Rap1^{wt} fusion protein was expressed under the control of the endogenous *Rap1* promoter (a gift from N. Brown); green fluorescent protein (GFP) was immunodetected with polyclonal anti-GFP antibodies (Molecular Probes, Eugene, OR). In addition, myc-tagged Rap1^{V12} and Rap1^{wt} transgenic fly lines were analyzed using monoclonal anti-Myc antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-Armadillo (Anti-Arm; Developmental Studies Hybridoma Bank) marks adherens junctions, anti-Neurexin (anti-Nrx; gift from M. Bhat) marks the lateral membrane compartment, and TRITC-phalloidin visualizes the actin cytoskeleton. Secondary antibodies were from Jackson Labs and Molecular Probes. Embryos were fixed with heat/methanol (Tepass 1996) and larval tissues as described in Takahashi *et al.* (1998). Confocal images were collected on a Zeiss LSM 510 laser scanning microscope. RNA *in situ* hybridizations were carried out as described in Tautz and Pfeifle (1989) and cuticle preparations were as described in Takahashi *et al.* (1998).

RESULTS

Rap1 binds Cno in a GTP-dependent manner and partially colocalizes with Cno *in vivo*: To identify molecules through which Rap1 exerts its effects in Drosophila morphogenesis, we carried out a yeast two-hybrid (YTH) screen using constitutively active Drosophila Rap1^{V12} as bait and a Drosophila embryonic cDNA library (0–24 hr) as the source of potentially interacting proteins (Van Aelst 1998). Among the clones, we found 37 cDNAs encoding *cno*. Cno is a multidomain protein that contains two predicted N-terminal Ras-binding domains (RA); FHA and DIL motifs that were initially described in microtubule- and actin-based motor proteins, respectively; and a PDZ domain, followed by an extended C-terminal tail with interspersed proline-rich patches (Figure 1a). Cno wild type as well as an N-terminal fragment that contains both RA domains (CnoN) binds strongly to activated Rap1^{V12}, but not to dominant negative Rap1^{N17}, as shown in two independent YTH reporter assays (Figure 1b), suggesting that Cno is a potential effector of Drosophila Rap1. This result is consistent with our previous finding that the vertebrate ortholog of Cno, AF-6, interacts in a similar fashion with mammalian Rap1 (Boettner *et al.* 2000, 2001) and indicates that the molecular mechanism of Rap1 function is conserved between flies and mammals. Further dissection of the interaction shows that both RA domains in Cno (RA1 and RA2) are able to bind to Rap1^{V12} (Figure 1b). Cno also binds to the H-Ras homolog Ras1, but not to the R-Ras homolog Ras2 (data not shown).

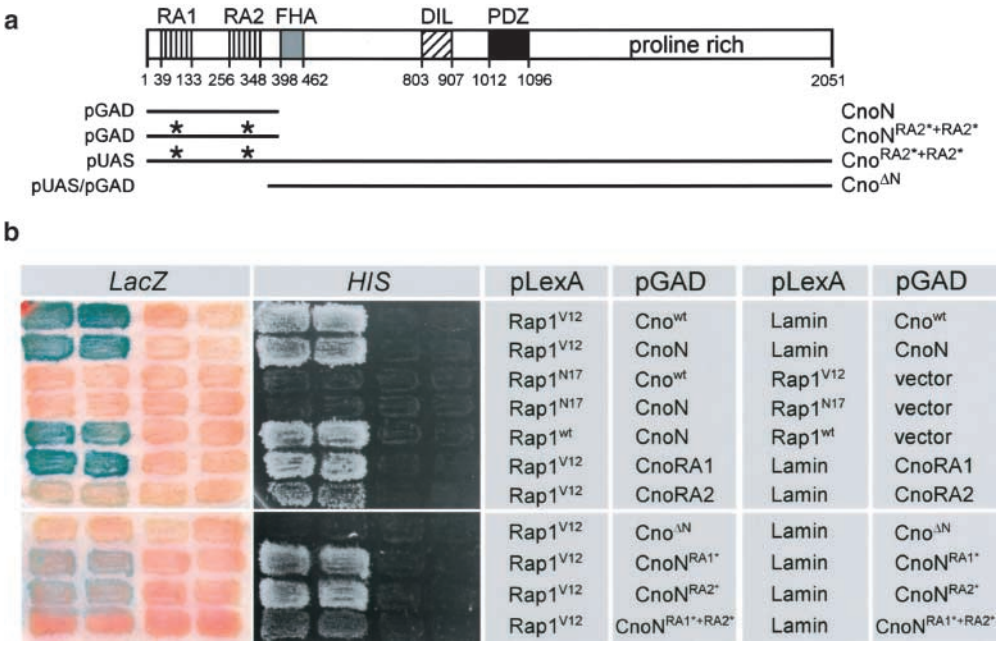


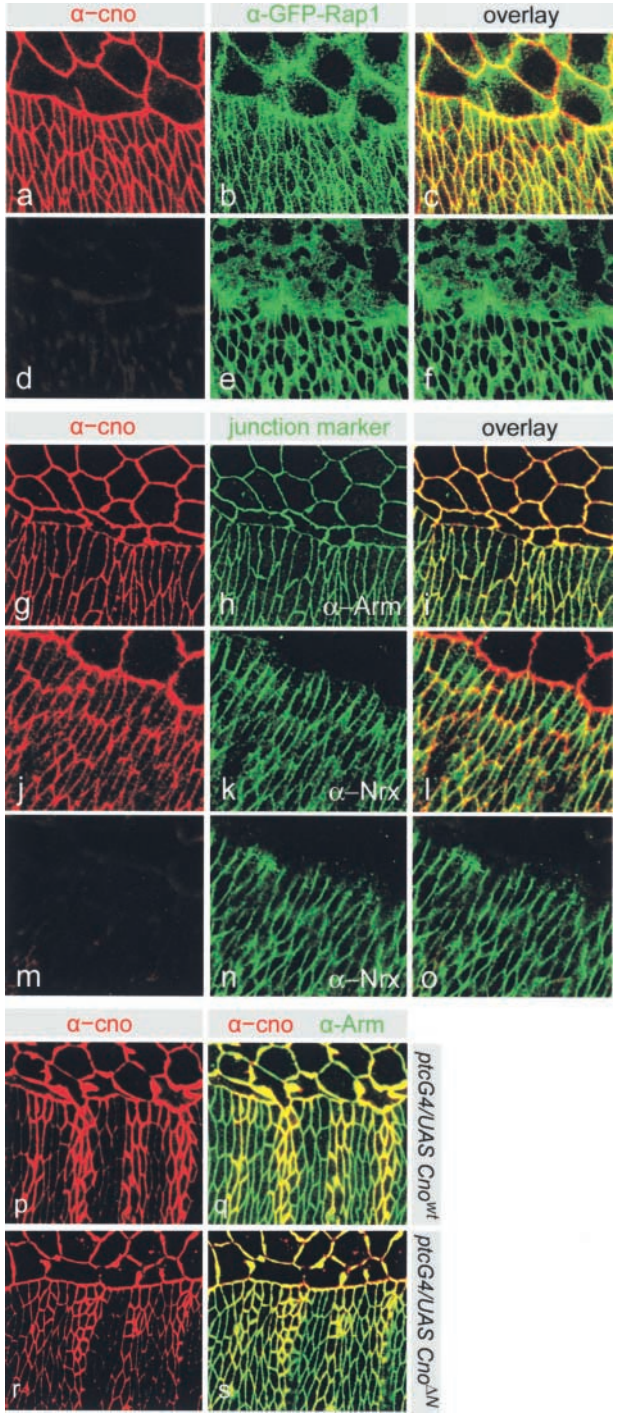
FIGURE 1.—(a) Scheme of the Cno protein domain structure and of mutant proteins used in this study. For YTH interactions, pGAD vectors were constructed containing full-length Canoe (Cno^{wt}), the N terminus harboring the two RA domains (CnoN), the individual RA domains (CnoRA1 and CnoRA2), and Canoe lacking the RA domains ($Cno^{\Delta N}$). The asterisk indicates K-L substitutions in the RA binding domains. For fly *in vivo* expression, pUAS vectors were constructed containing full-length Cno, either as wild type (Cno^{wt}) or with RA point mutations ($Cno^{RA1*+RA2*}$), and Cno lacking the N terminus ($Cno^{\Delta N}$). (b) YTH interactions between Rap1 and Cno using two independent reporter assays. The interaction-dependent activation of the *LacZ* (left) and *HIS* (right) genes in the L40 reporter strain was determined in duplicate after transformation with the indicated pLexA/pGAD plasmid combinations.

To examine the physiological relevance of the Rap1-Cno interaction, we conducted colocalization studies for the two proteins in the embryo at DC stages (see MATERIALS AND METHODS). We find that Cno colocalizes with the β-catenin homolog Armadillo (Arm), which is an integral component of the adherens junction, but is not present in the basolateral membrane compartment as marked by Nrx (Figure 2, g–o). GFP-Rap1 fusion protein is found in vesicular structures in the cytoplasm and on the lateral membrane, including the adherens and septate junctions. Confocal sectioning reveals an apically located membrane compartment that contains both Rap1 and Cno proteins (Figure 2, a–f). These subcellular distributions were observed in both the lateral ectoderm and the amnioserosa, *i.e.*, in both tissues participating in DC. We also found similar subcellular distributions for the two proteins in the wing disc epithelium using an Myc-Rap1 transgene (see MATERIALS AND METHODS; data not shown).

Thus, both proteins clearly colocalize at the adherens junction, lending support to the idea that they physically interact *in vivo*. The findings are consistent with our previous observation that vertebrate AF-6 and Rap1 partially colocalize at the plasma membrane in transfected epithelial MCF7 cells (BOETTNER *et al.* 2000).

Both *Rap1* and *cno* are required for dorsal closure: The first *cno* mutant alleles were isolated on the basis of their embryonic cuticle phenotype, which is characterized by defects in DC (JÜRGENS *et al.* 1984). The defects can be ordered into a phenotypic series, with head involution defects as weak, anterior holes of increasing size as intermediate, and complete "dorsal open" cuticles as the strongest phenotypes (Figure 3, b–d; Table 1). Even in the null allele cno^2, only 81% of the homozygous mutant embryos show a complete dorsal open phenotype, while the remaining 19% have anterior holes of varying size or even fully closed cuticles (Table 1). As indicated above, Cno protein is found at the adherens junctions of both amnioserosa and lateral ectoderm, suggesting that both epithelia require its function (Figure 2, a–c; TAKAHASHI *et al.* 1998). In contrast to *cno* mutants, *Rap1* zygotic mutant embryos can survive into larval stages (ASHA *et al.* 1999), due to the presence of maternally provided *Rap1*. ASHA *et al.* (1999) have shown that removal of maternal *Rap1* in germline clones leads to multiple and severe morphogenetic abnormalities, including defects in mesoderm invagination (50% of embryos), head involution (50%), and DC (10%); each of the defects increased in penetrance and severity when paternal/zygotic *Rap1* was removed as well. Consistent with these findings, we observe a variety of cuticle defects in maternal *Rap1* germline clones, ranging from ventral open (sometimes with an additional dorsal hole) to large anterior holes (Figure 3, e–f; see also Figure 4, n and o). The latter phenotype appears to represent a superimposition of head involution and ventral and dorsal closure defects. In many cases, however, the earlier defects in ventral closure and

concomitant defects in head involution may actually obscure the requirement for *Rap1* in DC. Specifically, tension in the lateral ectoderm is likely to be greatly reduced if the embryo is not closed ventrally, with the result that dorsal fusion requires a lot less stretching of ectoderm cells than is needed under wild-type conditions. When we examine maternal *Rap1* germline clones during DC, we indeed find that many embryos close dorsally with little or no stretching of the dorsal ectoderm (Figure 4n), supporting the idea that defective ventral closure may reduce the cellular (and thus the genetic) requirements for DC.



To sidestep any such masking of the role of Rap1 in DC by earlier defects and to study its function more specifically in the context of DC, we generated a dominant negative version of *Rap1*, $Rap1^{N17}$. We expressed it in the tissues that participate in DC, namely the lateral ectoderm and the amnioserosa, using the *UAS/GAL4* system with *patched* (*ptc*) and *pannier* (*pnr*) *GAL4* as drivers (see MATERIALS AND METHODS; BRAND and PERRIMON 1993). *ptcGAL4* promotes broad expression in the ectoderm and amnioserosa, with expression in the ectoderm resolving into two stripes per segment during DC, while *pnrGal4* drives expression more specifically in the dorsalmost cells of the lateral ectoderm and in the amnioserosa. We obtained similar results with both drivers (compare Figure 4, b, f, and j with Figure 4, e, g, and k; see below). Expression of $Rap1^{N17}$ in the ectoderm and amnioserosa leads to a strong but variable DC defect, similar to that of *cno* lof (Figure 3g). The DC defect of $Rap1^{N17}$ is completely rescued by coexpression of $Rap1^{wt}$ (Table 1). By contrast, the expression of dominant negative versions of Ras1 or Ras2, the Ras family members closest to Rap1, show little if any effect on DC, suggesting that the biochemically detectable interaction between Cno and Ras1 has no functional importance in DC [data not shown; HARDEN *et al.* (1999) report mild effects of $Ras1^{N17}$ and $Ras1^{Q13}$ on DC using *hsGAL4*, but we have not been able to reproduce these effects using $Ras1^{N17}$ and $Ras1^{V12}$ under *ptcGAL4* control]. Taken together, our findings indicate that the DC phenotype of $Rap1^{N17}$ results from a disruption of *Rap1* function rather than from promiscuous interference with another GTPase (*cf.* CARON *et al.* 2000; REEDQUIST *et al.* 2000).

Overactivity of *Rap1* and *cno* causes phenotypic defects as well: Expression of a dominant active $Rap1^{V12}$ transgene leads to very mild DC defects (Figure 3h). Overexpression of cno^{wt} does not affect DC, but causes a gain-of-function (gof) head involution defect (Figure 3m), which was not analyzed in greater detail.

To further compare the phenotypes of *Rap1* and *cno*, we carried out a cellular characterization of the DC defects of $Rap1^{N17}$, driven by either *ptcGAL4* or *pnrGAL4*,

FIGURE 2.—Colocalization of Rap1, Cno, and junction markers Arm and Nrx. Embryonic lateral ectoderm and amnioserosa are shown in apical or basal single confocal sections, stage 13. GFP-Rap1 colocalizes with Cno to the adherens junction of both embryonic lateral ectoderm and amnioserosa (a–c); Rap1 protein, but not Cno, is present in the entire basolateral membrane compartment, as well as in the cytoplasm (d–f). Cno and Arm colocalize at the adherens junction (apical section, g–i). Nrx, which at this stage marks the entire lateral membrane compartment, colocalizes with Cno in apical sections (j–l), but not in basal sections (m–o). The localization of Cno^{wt} (p and q) to the adherens junction is not affected by removal of the RA domains in $Cno^{\Delta N}$, as determined by colocalization with Arm (r and s). *cno* transgenes are expressed in stripes in the embryonic ectoderm using *ptcGAL4*.

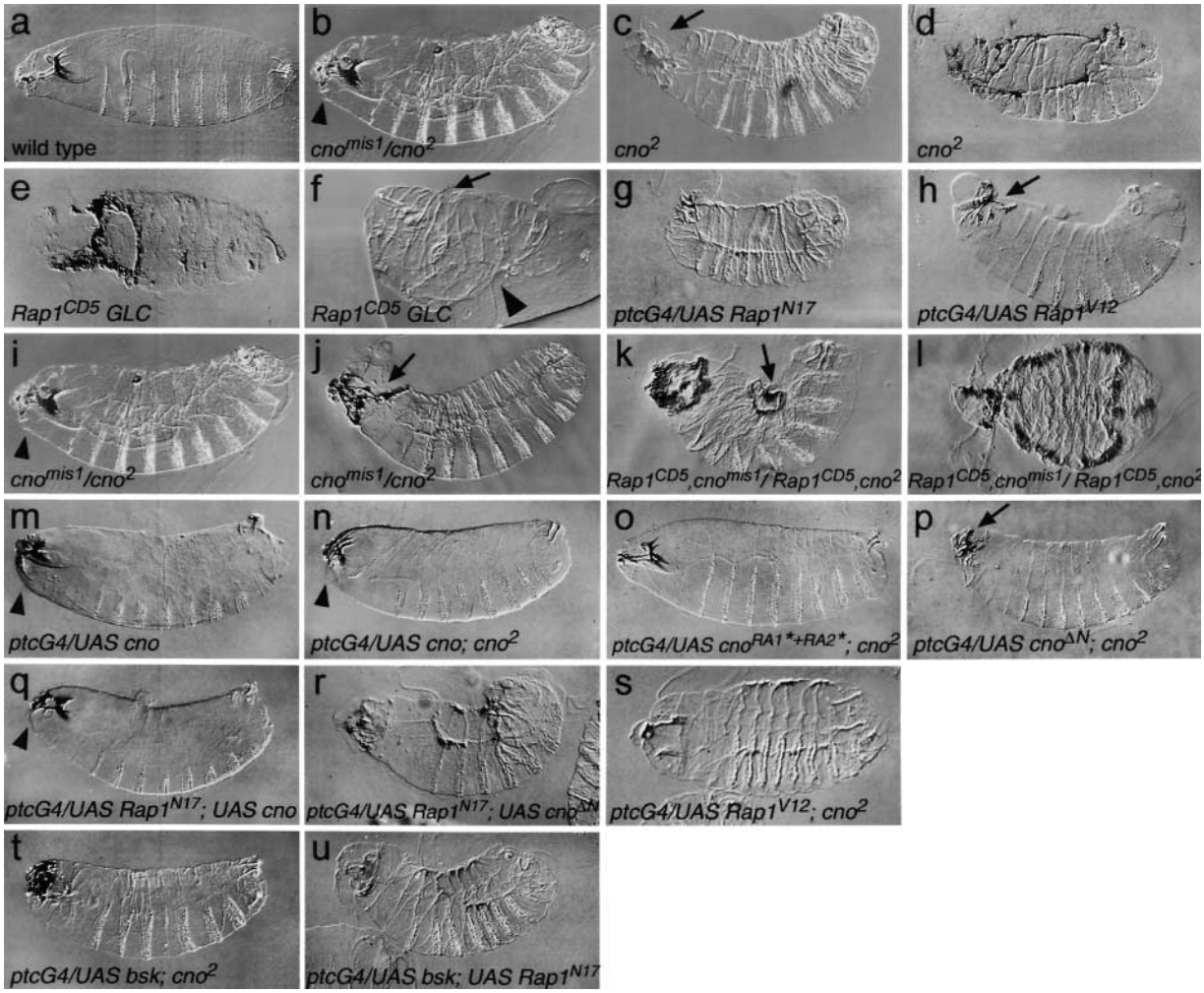


Figure 3.—Embryonic cuticle phenotypes. (a) Wild type. (b–d) *cno* lof allelic series. Phenotypes range from head involution defect (b, arrowhead) and small anterior hole (c, arrow) to large hole covering almost the entire dorsal aspect of the embryo (d). (e–h) *Rap1* lof and transgenic conditions. Maternal *Rap1* germline clones show large anterior holes (e) or are ventral open (f, arrowhead), sometimes with a small additional dorsal hole (arrow). $Rap1^{N17}$ embryos display large dorsal holes (g), similar to the strong *cno* lof phenotype. $Rap1^{V12}$ embryos show mild anterior defects (h, arrow). (i–l) Genetic interaction between *Rap1* and *cno*. Embryos heteroallelic for a weak and a strong *cno* allele (cno^{mis1}/cno^{2}) show, at worst, head involution defects (i, arrowhead) or small anterior holes (j, arrow). Removal of zygotic *Rap1* from this background leads to strongly exacerbated phenotypes: Most embryos either have large anterior holes, sometimes with additional smaller dorsal holes (k, arrow), or are completely dorsal open (l). (m–p) Rescue of *cno* lof with *cno* (mutant) transgenes. Expression of cno^{wt} almost completely rescues the *cno* lof defect (n); removal of the RA binding domains strongly reduces this rescue capacity (p, arrow indicates small anterior hole). Note that *cno* overexpression results in head involution defects (m and n, arrowheads). (q–s) Genetic interactions between *Rap1* and *cno*, using *Rap1* transgenes. Expression of *cno* provides substantial rescue of the $Rap1^{N17}$ phenotype (q, arrowhead indicates head involution defect), but removal of the RA binding domains abolishes this rescue capacity (r). $Rap1^{V12}$ is unable to rescue the *cno* lof phenotype (s). (t and u) Genetic interactions of *cno* and *Rap1* with *bsk*. *bsk* expression rescues the *cno* lof phenotype (t), but not $Rap1^{N17}$ (u). The genetic interaction and rescue experiments are quantified in Table 1.

and *cno* using several molecular markers. In both mutant situations, the leading edge (LE) cytoskeleton (an accumulation of actin, nonmuscle myosin, and phosphotyrosine-containing proteins in the dorsalmost row of ectodermal cells) is assembled largely as in wild type (Figure 4, i–l) and an initial stretching of ectodermal cells takes place (Figure 4, a–d). However, at later stages of DC, both types of mutant embryos show a detachment of the lateral ectoderm from the amnioserosa; the ectoderm retracts, with cells resuming a nonelongated shape, and the amnioserosa shrivels (Figure 4, e–h). This suggests that adhesion between the two structures is impaired or that the ectodermal cells are incapable of stretching sufficiently to maintain adhesion to the amnioserosa or, conversely, that the amnioserosa cells are incapable of changing shape appropriately to maintain adhesion to the ectoderm. Since Rap1 and Cno are coexpressed in both tissues, further experiments using specific drivers will be needed to determine whether their function is required in the ectoderm, in the amnioserosa, or in both tissues. Note that Cno is still found at the adherens junction in $Rap1^{N17}$ transgenic conditions, suggesting

TABLE 1

Quantitative analysis of cuticular phenotypes of genetic interaction and rescue experiments

Genotype	Phenotypes (%) Larvae hatched	Cuticle closed (but head involution defect)	Cuticle partially open	Dorsal open	*N*
cno^{mis1}/cno^{2}	54	23 (16)	22	1	318
Cno^{mis1},$Rap1^{CD5}/cno^{2}$,$Rap1^{CD5}$ (A)	3	2 (1)	33	62	258
Cno^{mis1},$Rap1^{CD5}/cno^{2}$,$Rap1^{CD5}$ (B)	2	2 (1)	37	59	316
ptcGAL4; cno^{2}	0	4 (2)	15	81	118
ptcGAL4/UAS cno^{wt}; cno^{2}	0	92 (86)	4	4	73
ptcGAL4/UAS $cno^{RA1*+RA2*}$; cno^{2}	0	75 (0)	18	7	85
ptcGAL4/UAS $cno^{\Delta N}$; cno^{2}	0	35 (19)	30	35	88
ptcGAL4/UAS bsk; cno^{2}	10	22	45	23	88
ptcGAL4/UAS $Rap1^{N17}$	0	0	7	93	175
ptcGAL4/UAS $Rap1^{N17}$; *UAS* $Rap1^{wt}$	100	0	0	0	103
ptcGAL4/UAS $Rap1^{N17}$; *UAS* cno^{wt}	10	23	22	45	132
ptcGAL4/UAS $Rap1^{N17}$; *UAS* $cno^{\Delta N}$	0	0	1	99	169
ptcGAL4/UAS bsk; *UAS* $Rap1^{N17}$	0	0	10	90	105
ptcGAL4/UAS $Rap1^{V12}$	0	90	10	0	100
ptcGAL4/UAS $Rap1^{V12}$; cno^{2}	0	1	1	98	102

The genetic interaction between *Rap1* and *cno* is examined by removing zygotic *Rap1* from a weak heteroallelic combination of *cno* (cno^{mis1}/cno^{2}); two independent recombinant chromosomes are tested (A and B). Genetic interactions between *Rap1*, *cno*, and *bsk* are examined by (co-)expression of transgenes using *ptcGAL4* in $Rap1^{N17}$ and *cno* lof (cno^{2}) mutant backgrounds. Rescue of *cno* loss of function by wild-type and mutant *cno* transgenes is assessed by expressing *cno* transgenes in a *cno* lof mutant background (cno^{2}) using *ptcGAL4* as a driver. The rescue analysis is complicated by the fact that expression of cno^{wt} using the *ptcGal4* driver causes head involution defects in wild type and in *cno* mutant backgrounds (*cf.* Figure 3, m and n), most likely due to inappropriately high levels of transgene expression. The ability to confer this gof effect is diminished in the mutant transgenes (*cf.* Figure 3, o and p). In all cases, embryos were genotyped using fluorescently marked balancers (see MATERIALS AND METHODS) and their cuticles were scored according to the severity of the observed DC defect. Embryos with a large dorsal hole (covering at least half the dorsal aspect) were considered "dorsal open"; embryos with smaller dorsal and/or anterior holes were scored as "partially open."

that Rap1 activity is not required for the localization of Cno. Overall, we observe strong phenotypic similarities between $Rap1^{N17}$ transgenic and *cno* lof conditions, at both the cuticular and the cellular level, lending further support to the idea that Rap1 and Cno participate in the same molecular mechanism.

Loss of zygotic *Rap1* enhances a mild *cno* phenotype: To test whether Rap1 and Cno act in the same process, we asked whether the *Rap1* and *cno* loci interact genetically. We made use of the previous finding that the *trans*-heterozygous combination of a weak and a strong *cno* allele (cno^{mis1}/cno^{2}), which shows only mild DC defects, provides a sensitive background for interacting loci (TAKAHASHI *et al.* 1998). In analyzing embryos with the heteroallelic combination, we find that 77% develop into larvae or have a completely closed cuticle, 22% have small anterior holes, and only 1% have a strong DC defect (see Figure 3, i and j; Table 1). *Rap1* zygotic null embryos survive into larval stages without externally visible defects, due to the presence of maternally provided *Rap1* (*cf.* ASHA *et al.* 1999). Removal of zygotic Rap1 from the heteroallelic *cno* background ($Rap1^{CD5}$, $cno^{mis1}/Rap1^{CD5}$, cno^{2}) results in a strong exacerbation of phenotypic defects: Only 4% of embryos develop into larvae or have a completely closed cuticle, 37% have (large) anterior holes, and 59% are completely open dorsally (Figure 3, k and l; Table 1). This pronounced synergy between the *Rap1* and *cno* loci in DC provides conclusive genetic evidence for the involvement of Rap1 in DC and argues that Rap1 and Cno act in the same pathway.

The interaction between Rap1 and Cno is required for dorsal closure: To directly assess the biological significance of the physical interaction between Rap1 and Cno, we decided to disrupt the ability of the two proteins to bind to each other and examine how this affects their function in DC. To generate Cno mutant proteins deficient in Rap1 binding, we introduced point mutations in the RA domains of Cno (K57L and K274L), since mutations at corresponding sites in AF-6 (K32L and K265L) abolish the binding of AF-6 to vertebrate Ras^{V12} *in vitro*. Individually, these mutations (Cno^{RA1*} and

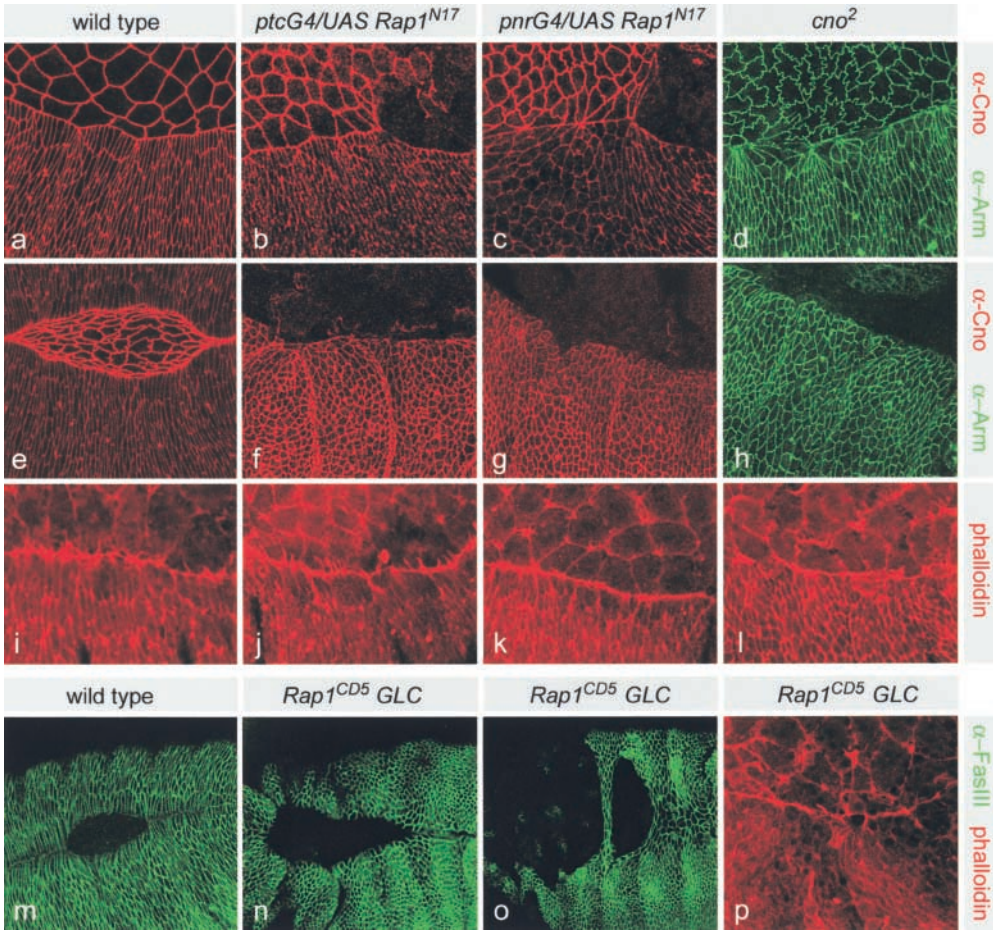


FIGURE 4.—Cellular phenotypes of wild-type, *Rap1*, and *cno* mutants during DC. Lateral and dorsolateral views of ectoderm and amnioserosa of wild-type (a, e, and i), *ptcGAL4; Rap1^{N17}* (b, f, and j), *pnrGAL4; Rap1^{N17}* (c, g, and k), and *cno* (d, h, and l) embryos during early (stage 13; a–d and i–l) and late (stage 15; e–h) DC, as visualized by staining with anti-Cno or anti-Arm antibodies and phalloidin, and of wild-type (m) and *Rap1* germline clone (GLC) embryos (n–p) in late DC, as visualized by anti-FasIII and phalloidin. In both *ptc/pnrGAL4; UASRap1^{N17}* and *cno* mutant embryos, DC begins normally, with accumulation of actin cytoskeleton at the LE (i–l) and stretching of the lateral ectoderm (a–d), but is followed by a severing of the connection between amnioserosa and lateral ectoderm and a relaxation of the cells in the lateral ectoderm (e–h). In *Rap1* GLC embryos, actin cytoskeleton accumulates at the LE (p); in many cases, DC does occur, but without stretching of the lateral ectoderm (n, compare with m; see text); in some embryos, DC defects are observed (o).

Cno^{RA2*}) lead to only a mild reduction in Rap1 binding in YTH assays (data not shown), while combining them (Cno$^{RA1*+RA2*}$) significantly reduces Rap1 binding (Figure 1b). However, if we remove the two RA domains located at the very N terminus of the protein (Cno$^{\Delta N}$), Rap1 binding is completely abolished in two independent YTH reporter assays (Figure 1b). This truncation leaves the other known functional domains of the protein intact. All four mutant Cno proteins can be expressed at high levels and still localize specifically to the adherens junction (Figure 2, r and s), suggesting that they are otherwise not detectably impaired in their function. The fact that the Cno$^{\Delta N}$ protein localizes to the adherens junction confirms that Rap1 input is not necessary for the localization of Cno.

We first asked whether expression of the *cno* transgenes can rescue the *cno* lof phenotype. As a baseline, we established that overexpression of a wild-type *cno* transgene with a *ptcGAL4* driver is sufficient to almost completely rescue the DC defects of *cno* lof embryos (Figure 3n; Table 1). We then compared the double mutant and deletion mutant proteins with the wild type. We find that the ability to rescue the *cno* lof defect is modestly reduced in *cno$^{RA1*+RA2*}$*: 75% of embryos show a closed cuticle, as opposed to 92% in embryos expressing *cno^{wt}* ($P < 0.02$). In contrast, it is strongly decreased in *cno$^{\Delta N}$*, with only 35% of embryos showing a closed cuticle ($P < 10^{-10}$). However, the rescue ability of *cno$^{\Delta N}$* is still considerable: 35% closed cuticle embryos compares to 4% in *cno* lof ($P < 10^{-10}$; Figure 3, o and p; Table 1). Thus, disruption of the Rap1-binding capacity of Cno results in reduction, but not elimination of the ability to restore Cno function. This partial rescue suggests that the role of Cno in DC is partially dependent on its ability to bind to Rap1, but also in part independent of it (see below).

In a second set of experiments we examined whether the function of Rap1 in DC shows a similar dependence on binding between Rap1 and Cno. We find that the DC phenotype of *Rap1^{N17}* is substantially rescued by concurrent expression of *cno^{wt}*. By contrast, expression of *cno$^{\Delta N}$* is completely unable to rescue *Rap1^{N17}* (Figure 3, q and r; Table 1), indicating that binding between Rap1 and Cno is indeed required for the function of Rap1 in DC. The mechanistic interpretation of these two experiments hinges on whether expression of *Rap1^{N17}* completely abolishes Rap1 activity: If it does, the rescue by *cno^{wt}* would have to be due to overactivity of the Rap1-independent aspect of Cno function, which, as shown above, possesses considerable ability to rescue the *cno*

lof phenotype. However, the fact that deletion of the Rap1-binding domains, under the same expression conditions, completely abrogates Cno's ability to rescue $Rap1^{N17}$ argues against this possibility. This leaves the following explanation: Expression of $Rap1^{N17}$ reduces Rap1 protein activity to a very low level that is insufficient to support DC when Cno protein is present at wild-type levels, but does allow a partial rescue when Cno protein is overexpressed. Thus, Cno's ability to rescue $Rap1^{N17}$ depends entirely on its ability to bind residual active Rap1. This indicates that the Rap1-Cno interaction is critical for the function of Rap1 in DC.

Taken together, our genetic experiments clearly demonstrate that Rap1 and Cno act in the same pathway and that their physical interaction is required for the function of both molecules in DC. What, then, is their epistatic relationship? The YTH experiments suggest that Rap1 acts upstream of Cno. In a typical signal transduction pathway, in which one component regulates the localization and/or activity of the other, one expects that expression of an independently localized/active version of the upstream component is unable to rescue a null condition of the downstream component, while expression of an independently localized/active form of the downstream component rescues the null condition of the upstream component. In the case of Rap1 and Cno, the first leg of this experiment is unproblematic, since we have both a *cno* null condition and a constitutively active version of *Rap1*. We find that $Rap1^{wt}$ and activated $Rap1^{V12}$ do not rescue *cno* lof, even though both are able to rescue $Rap1^{N17}$ (Figure 3s; Table 1); this is consistent with Rap1 acting upstream of Cno. The second leg of the epistasis experiment, however, is less straightforward due to the lack of suitable mutant proteins: As described above, the *Rap1* null condition is phenotypically complex and difficult to interpret, leaving only the $Rap1^{N17}$ transgenic condition, which most likely does not abolish Rap1 activity completely. Second, we have no constitutively active/localized version of Cno whose activity is independent of Rap1. In fact, the rescue experiments described earlier show that removal of the ability to bind Rap1 markedly reduces the DC functionality of Cno.

***Rap1* and *cno* differentially influence JNK pathway activity:** The fact that $Cno^{\Delta N}$ partially rescues *cno* lof indicates that a considerable portion of Cno's activity in DC is Rap1 independent. To investigate this further, we examined the events downstream of Cno. Cno has been reported to act upstream of the JNK pathway (Takahashi *et al.* 1998), which is an essential and the best-studied pathway involved in DC (Stronach and Perrimon 1999). The relationship between Drosophila Rap1 and the JNK pathway is largely unexplored. We therefore examined the influence of Rap1 and Cno on the JNK pathway by genetic interaction experiments and by assessing their effects on the expression of the secreted TGF-β homolog *dpp* in the LE. *dpp* is under JNK control and is thought to be essential for the elongation of the adjacent lateral ectodermal cells (Stronach and Perrimon 1999). Examination of *cno* lof mutants does not reveal any significant alteration in the early expression of *dpp* in the LE, but shows a consistent reduction of late *dpp* expression in ~50% of *cno* mutant embryos ($n > 100$; Figure 5, c and d). Overexpression of cno^{wt} has no discernible effect on *dpp* expression (data not shown), indicating that *cno* is partially required but not sufficient for *dpp* expression. Overexpression of *DJNK basket* (*bsk*) restores *dpp* expression to normal levels (Figure 5e) and results in a significant but partial rescue of the *cno* mutant DC defect (Figure 3t; Table 1). Together, these results indicate that Cno does indeed act upstream of the JNK pathway by permitting or stimulating signaling. In contrast, *dpp* expression is not altered by expression of $Rap1^{N17}$ or $Rap1^{V12}$ (Figure 5, f and g). Also, no obvious change in *dpp* expression is observed in *Rap1* null embryos, which lack both maternal and zygotic *Rap1* (Figure 5h). This is consistent with the previous finding that expression of *puc*, a second transcriptional target of the JNK pathway, is unaffected in embryos lacking maternal *Rap1* (Asha *et al.* 1999).

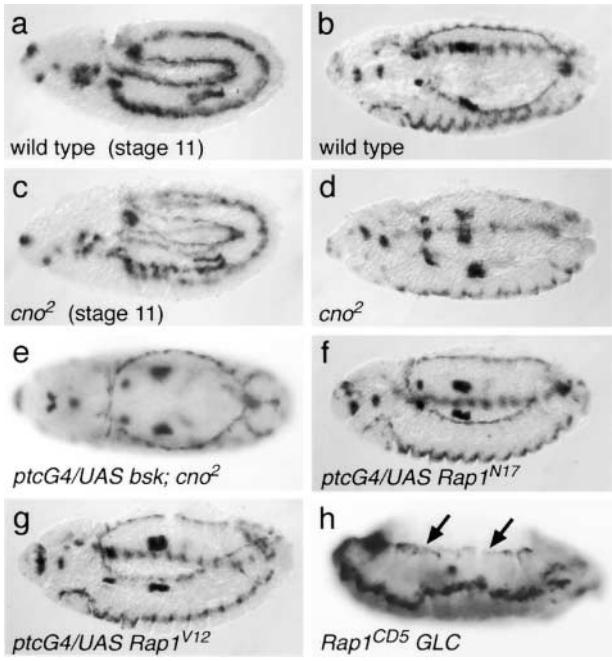


Figure 5.—*dpp* expression in the LE of wild-type, *Rap1*, and *cno* mutants, as indicated by RNA *in situ* hybridization. The genotypes of embryos were determined by scoring the absence of *lacZ*-marked balancers (see materials and methods). In wild type, LE cell-specific transcription of *dpp* begins in the extended germ band (stage 11, a) and is maintained during germ-band retraction (stage 13, b). In *cno* lof mutants, *dpp* levels are normal at stage 11 (c), but significantly reduced at stage 13 in ~50% of the animals (d); this effect is rescued by overexpression of *bsk* (e). *dpp* expression appears to be normal in $Rap1^{N17}$, $Rap1^{V12}$, and *Rap1* GLC embryos (f–h). Note that the overall *dpp* staining is stronger in h than in a–g.

Moreover, overexpression of bsk^{wt} does not rescue the DC defect of $Rap1^{N17}$ (Figure 3u; Table 1). Thus, while Cno plays a significant role in maintaining JNK activity during DC, Rap1 has no apparent effect on the JNK pathway. This finding is in line with the results of our genetic interaction experiments, which indicate that the function of Cno in DC is partially independent of its interaction with Rap1. Taken together, our data suggest that Cno has two separate functions during DC: The first is controlled by Rap1 and does not involve the JNK pathway; the second is independent of Rap1 and feeds into the JNK pathway.

DISCUSSION

Rap1 plays an important role in cell migration and morphogenesis in both vertebrates and invertebrates. In Drosophila, embryos lacking both zygotic and maternal *Rap1* display strong defects in diverse morphological aspects of embryogenesis, such as ventral invagination, migration of mesodermal precursors, head involution, and DC. A key question is which effector pathways mediate the morphogenetic functions of Rap1. We used the YTH system to identify Drosophila Rap1-specific effector molecules from an embryonic library and retrieved several cDNAs encoding Cno. We found that both N-terminal Ras-binding domains (RA1 and RA2) possess Rap1-binding potential and that they interact only with a constitutively active Rap1 mutant, $Rap1^{V12}$, but not with a dominant negative version of Rap1, $Rap1^{N17}$, suggesting that Cno may act as an effector for Rap1.

We have provided several lines of evidence confirming this hypothesis. Rap1 and Cno partially colocalize at the adherens junction in the two tissues that are involved in DC, the amnioserosa and the lateral ectoderm, with Rap1 being present at the entire lateral membrane and also showing vesicular expression throughout the cytoplasm. Moreover, loss of function of the two molecules leads to similar phenotypes, at both the cuticular and the cellular level. To directly address the question whether Rap1 utilizes Cno as an effector during DC, we conducted a series of genetic experiments. They demonstrate that the two molecules act in the same pathway and that their physical interaction is essential for their function in DC: (1) Removal of zygotic *Rap1* strongly enhances the phenotype of a weak heteroallelic *cno* combination; (2) removal of the RA-interaction domains and, thus, removal of the ability to bind Rap1, reduces the ability of *cno* transgenes to rescue the *cno* lof phenotype; and (3) removal of the RA-interaction domains eliminates the ability of *cno* to rescue $Rap1^{N17}$. Finally, our finding that activated $Rap1^{V12}$ fails to rescue the *cno* lof defects indicates that Rap1 acts upstream of Cno. Taken together, our YTH data, colocalization results, and genetic interaction experiments provide comprehensive evidence that Cno functions as a downstream effector of Rap1 in the DC process. To our knowledge, these findings represent the first demonstration of a protein acting as a Rap1 effector *in vivo*.

The events downstream of Rap1 and Cno, however, appear to be more complex. Several independent findings suggest that Cno's role in DC can be separated into Rap1-independent and Rap1-dependent functions: Removal of the RA-interaction domains does not affect the ability of the remainder of the protein to localize to the adherens junction, and the mutant protein retains the capacity to partially rescue the DC defect of a *cno* lof mutant. Further, Cno feeds into the JNK pathway, while Rap1 does not: *dpp* expression levels in the LE are significantly reduced in *cno* lof embryos at later stages of DC, but appear unaffected in *Rap1* mutants. In addition, *cno* lof is partially rescued by overexpressing *bsk* (*DJNK*), whereas the $Rap1^{N17}$ defect is not. Given the multidomain structure of Cno, it is not surprising that the molecule would participate in multiple pathways. Such a bifurcation of the pathway would also explain the lack of transitivity that we observe in our rescue experiments: *Rap1* lof is (partially) rescued by *cno* overexpression, *cno* lof is (partially) rescued by *bsk* overexpression, but *Rap1* lof is not rescued by *bsk* overexpression. The fact that both $cno^{\Delta N}$ and *bsk* are unable to rescue *Rap1* lof demonstrates that the Rap1-independent function of Cno cannot compensate for the loss of Rap1. This leaves the reciprocal question of whether Rap1 may have a second, Cno-independent function in DC. The fact that the DC phenotype of $Rap1^{N17}$ is as severe as that of *cno* lof without affecting JNK pathway signaling might suggest that Rap1 has additional effectors in DC (as does the fact that the phenotype of $Rap1^{N17}$ is more severe than that of cno^{2}*; ptcGAL4 UAS-*$cno^{\Delta N}$). However, we have no conclusive evidence to support this idea, since the additional effectors of Rap1 we identified in our YTH screen have not been investigated for their role in DC.

One obstacle in investigating the function of Rap1 is its pleiotropy. A detailed analysis of DC defects, in particular, is difficult to perform in *Rap1* null embryos, due to the severe disruption of multiple aspects of embryonic development prior to DC. We therefore had to make use of the dominant negative $Rap1^{N17}$ mutant. When expressed at appropriate stages in the epithelial cells that are involved in the DC process, this transgene results in robust DC defects. However, early *in vitro* studies appeared to show that the $Rap1^{N17}$ mutant does not compete well with normal Rap1 for the GEF C3G (van den Berghe *et al.* 1997), calling into question whether it can be regarded as a Rap1 dominant negative. But more recent *in vivo* studies by Caron *et al.* (2000) and Reedquist *et al.* (2000) and now our own clearly show that $Rap1^{N17}$ acts as a dominant negative mutant in Rap1 signaling. Our successful rescue of $Rap1^{N17}$ with a concomitantly expressed $Rap1^{wt}$ transgene demonstrates the specificity of the mutant. Further, dominant negative versions of Drosophila Ras1 and Ras2, the counter-

parts of the mammalian H, K, and N-Ras and of the R-Ras proteins, respectively, do not disrupt DC when they are examined under the same conditions. This shows that the interaction between DRas1 and Cno that has been detected *in vitro* by us and others (Matsuo *et al.* 1997) and the genetic interaction between *DRas1* and *Cno* that was found to influence cone cell formation in the Drosophila eye (Matsuo *et al.* 1997) have no role during DC.

Which cellular processes might Rap1 and Cno act on? Cno is a multidomain protein consisting of several known and putative protein-interaction domains, including the two RA domains and a PDZ domain, which targets proteins to specific cell membranes and assembles proteins into supramolecular signaling complexes, but no catalytic domain. Cno localizes to the adherens junction and may act by localizing and clustering signal transduction components at the junction (Buchert *et al.* 1999) or by modulating the mechanical resistance of the adherens junction, and thus, directly or indirectly, influence JNK signaling. Since Cno is found at the adherens junctions under *Rap1* lof conditions as well as in the absence of its RA domains, Rap1 cannot be required for the initial localization of the Cno protein, suggesting that Rap1 influences the activity of Cno by changing its conformation. However, another possibility is suggested by Knox and Brown (2002), who found that Rap1 function is required for evenly (re-)distributing adherens junction components in wing disc epithelial cells after mitosis. It is likely that the adherens junctions in the cells that undergo stretching in the embryonic ectoderm during DC are similarly subject to dynamic reorganization, which may in part be regulated by the Rap1/Cno complex. This idea would be consistent with our observation that in *Rap1* and *cno* lof mutants the lateral ectoderm begins its dorsal stretching, but is then unable to complete the process. Interestingly, Rap1 in mammalian cells has been shown to be activated in cell-stretching assays (Sawada *et al.* 2001). In this system, force initiation apparently results in the activation of the JNK kinase family member p38, suggesting the existence of a Rap1-dependent "mechanosensory" pathway. Our data fit this idea. Future studies using fluorescently tagged Rap1 and Cno proteins and live imaging will shed light on dynamic aspects of their localization and function during DC.

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