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Regulation of cone cell formation by Canoe and Ras in the developing *Drosophila* eye

Takashi Matsuo[1], Kuniaki Takahashi[1], Shunzo Kondo[1], Kozo Kaibuchi[2] and Daisuke Yamamoto[1,*]

[1]Mitsubishi Kasei Institute of Life Sciences, and ERATO Yamamoto Behavior Genes Project at Mitsubishi Kasei Institute of Life Sciences, 11 Minamiooya, Machida, Tokyo 194, Japan
[2]Nara Institute of Science and Technology, Division of Signal Transduction, Ikoma, Nara 630-01, Japan

*Author for correspondence (e-mail: daichan@fly.erato.jst.go.jp)

SUMMARY

Cone cells are lens-secreting cells in ommatidia, the unit eyes that compose the compound eye of *Drosophila*. Each ommatidium contains four cone cells derived from precursor cells of the R7 equivalence group which express the gene *sevenless* (*sev*). When a constitutively active form of Ras1 ($Ras1^{V12}$) is expressed in the R7 equivalence group cells using the *sev* promoter (*sev-*$Ras1^{V12}$), additional cone cells are formed in the ommatidium. Expression of $Ras1^{N17}$, a dominant negative form of Ras1, results in the formation of 1-3 fewer cone cells than normal in the ommatidium. The effects of Ras1 variants on cone cell formation are modulated by changing the gene dosage at the *canoe* (*cno*) locus, which encodes a cytoplasmic protein with Ras-binding activity. An increase or decrease in gene dosage potentiates the *sev-*$Ras1^{V12}$ action, leading to marked induction of cone cells. A decrease in cno^+ activity also enhances the *sev-*$Ras1^{N17}$ action, resulting in a further decrease in the number of cone cells contained in the ommatidium. In the absence of expression of *sev-*$Ras1^{V12}$ or *sev-*$Ras1^{N17}$, an overdose of wild-type *cno* (cno^+) promotes cone cell formation while a significant reduction in cno^+ activity results in the formation of 1-3 fewer cone cells than normal in the ommatidium. We propose that there are two signaling pathways in cone cell development, one for its promotion and the other for its repression, and Cno functions as a negative regulator for both pathways. We also postulate that Cno predominantly acts on a prevailing pathway in a given developmental context, thereby resulting in either an increase or a decrease in the number of cone cells per ommatidium. The extra cone cells resulting from the interplay of $Ras1^{V12}$ and Cno are generated from a pool of undifferentiated cells that are normally fated to develop into pigment cells or undergo apoptosis.

Key words: DHR/PDZ domain, fate determination, compound eye, cell death, morphogenesis, *Drosophila*, *canoe*, *sevenless*

INTRODUCTION

The *Drosophila* compound eye is an ideal system for studying cellular fate decision in development, since the ommatidium, the unit eye, is composed of only 20 identifiable cells (8 photoreceptors and 12 associated cells) whose fates are exclusively determined by cell-to-cell interactions regardless of their lineage (Ready et al., 1976; Wolff and Ready, 1991, 1993; Yamamoto, 1996). The most thoroughly studied event in cell-type specification in the *Drosophila* eye is formation of the R7 photoreceptor. In this process, the inductive signal Bride of sevenless (Boss), presented on the R8 cell membrane, stimulates the receptor tyrosine kinase Sevenless (Sev) in the R7 precursor, which then develops into an R7 photoreceptor as a result of Ras1 activation downstream of Sev (van Vactor et al., 1991; Basler et al., 1991; Gaul et al., 1992). Thus, constitutive activation of Ras1 in cells with the potential to develop into R7 photoreceptors (called collectively the R7 equivalence group; Dickson et al., 1992) drives these cells to adopt the R7 fate without involvement of Sev (Fortini et al., 1992; Peverali et al., 1996; Kramer et al., 1995; Brunner et al., 1994).

Ras1 also plays a crucial role in the development of other photoreceptor classes, based on the finding that the ommatidia of mutants exhibiting a complete loss of function of Ras1 are devoid of the R1-R6 photoreceptors, formation of which is regulated by the epidermal growth factor receptor (EGFR), a tyrosine kinase linked to the Ras1 cascade (Simon et al., 1991; Doyle and Bishop, 1993; Schweitzer et al., 1995).

In contrast to that for photoreceptor induction, the mechanism for non-neural retinal cell formation has attracted less attention. Freeman (1996) recently presented evidence that EGFR is required for the development of all retinal cells including cone cells and pigment cells; a dominant negative form of EGFR prevents cell differentiation whereas a secreted form of Spitz (Spi), the EGFR ligand, causes differentiation of supernumerary cells when expressed in the R7 equivalence group cells.

There are some mutations that uniquely affect the development of the cone cells. *canoe* (*cno*) (Miyamoto et al., 1995) and *rugose* (*rg*) (T. Venkatesh, personal communication) mutations are representatives of this class of mutations. For example, the flies homozygous for cno^{mis1}, a hypomorphic

mutation in the *cno* locus, have a variable number of cone cells per ommatidium, whereas eight photoreceptors are formed normally by the late third instar larval stage. Therefore the effect of the *cno* mutations on cone cell development cannot be interpreted in terms of cell fate transformation from/to photoreceptors, unlike in the case of mutations in the genes encoding the members of the Sev signaling cascade (Tomlinson and Ready, 1986; Basler et al., 1991; Buckles et al., 1992; Rogge et al., 1992; Dickson et al., 1992; Carthew and Rubin, 1990; Chang et al., 1995; Dickson et al., 1995).

Here we report on an attempt to elucidate the mechanism by which the *cno* product controls cone cell formation in the developing compound eye. We show that the phenotypic effect of the *cno* mutations on the cone cells depends critically on the state of Ras. In the absence of artificial activation of Ras1, the *cno* mutations result in a reduction in the number of cone cells in the ommatidia, whereas they strongly enhance the effect of a constitutively active Ras1 ($Ras1^{V12}$) so as to lead to marked induction of cone cells. Conversely, a dominant negative form of Ras1 ($Ras1^{N17}$) inhibits cone cell formation and the *cno* mutations strengthen the inhibitory effect of $Ras1^{N17}$. To explain these apparently conflicting observations, we propose the hypothesis that Cno is a protein which balances two counteracting pathways, one promoting and the other repressing cone cell formation.

The molecular structure of Cno suggests its direct association with Ras. It has significant sequence homology with a mammalian Ras-binding protein, AF-6 (Kuriyama et al., 1996; Ponting, 1995), which has been cloned as a fusion partner of All-1, a protein involved in acute myeloid leukemias in humans (Prasad et al., 1993; see also Taki et al., 1996). Cno and AF-6 share two putative Ras-binding domains (RA1 and RA2) (Ponting and Benjamin, 1996), a kinesin-like and a myosin-V-like domain (Ponting, 1995), and a Discs large homologous region (DHR; also known as the GLGF or PDZ motif) (Gomperts, 1996; Woods and Bryant, 1993; Sheng, 1996). As inferred from the structural similarity to AF-6, Cno binds to *Drosophila* Ras1. These findings suggest that Cno and AF-6 might be evolutionarily conserved regulatory components of Ras.

MATERIALS AND METHODS

Drosophila stocks

The cno^{10B1} mutant was originally isolated by Jürgens et al. (1984) and obtained from the Tübingen stock center. The cno^{mis1} mutant was isolated by P-element-mediated mutagenesis in our laboratory (Miyamoto et al., 1995). The *sev-*$Ras1^{V12}$ and *sev-*$Ras1^{N17}$ transformant lines were gifts of M. Simon. The raf^{C110} mutant was a gift from Y. Nishida.

Construction of the pUASTcno vector and germ line transformation

A 5.9 kb fragment of the *cno* cDNA was isolated from pBS-KS(-)cno (Miyamoto et al., 1995) and inserted into the pUAST vector (Brand and Perrimon, 1993). Inserted *cno* cDNA was sequenced for both DNA strands by the dideoxy chain termination method using an ABI 377 sequencer (Applied Biosystems). The resultant plasmid pUASTcno and a helper plasmid pUChspπΔ2-3 were injected into w^{1118} embryos for germ-line transformation as described by Rubin and Spradling (1982). Five independent homozygous viable transformant lines were obtained.

Scanning electron microscopy

For scanning electron microscopy (SEM), the flies were prepared for critical point drying and coated with a 2 nm layer of gold. Images were taken on a low-voltage prototype SEM.

Sections of adult compound eyes

Heads were removed from the bodies using razor blades and a longitudinal cut was made between the eyes. The tissue was fixed in 1% osmium tetroxide and 1% glutaraldehyde in phosphate-buffered saline (PBS) for 15 minutes on ice, fixed again in 2% osmium tetroxide in PBS for 1 hour on ice and dehydrated using three 10-minute incubations each in a series of ethanol solutions (50%, 70%, 80%, 90%, 95%, 99.5% and 100%). The tissue was then incubated in propylene oxide for 30 minutes, infiltrated overnight by a 2:1 mixture of propylene oxide and resin with desiccation, and subsequently desiccated again in pure resin for 4 hours. Then the tissue was embedded in pure resin at 60°C for 3 days.

Cobalt sulfide staining

Cobalt sulfide staining (Wolff and Ready, 1991) was used to visualize the apical surface of pupal retinae. Retina/brain complexes were dissected in PBS and then fixed in 2.0% glutaraldehyde in PBS for 10 minutes at room temperature. The complexes were washed for 5 minutes in PBS before being dipped in 2% $Co(NO_3)_2$ for 5 minutes. After a brief wash in distilled water, the complexes were incubated in 1% $(NH_4)_2S$ until the retinae turned black. After a rinse in distilled water, the retinae were removed from laminae and mounted in glycerol.

Anti-β-galactosidase and anti-Cut antibody staining

Retina/brain complexes dissected from staged pupae were fixed for 30 minutes at room temperature in 4% formaldehyde in PBS. Following two 5-minute washes in PBT (PBS supplemented with 0.3% Triton X-100), the retina/brain complexes were incubated for 1.5 hours on ice in 10% horse serum in PBT with an anti-β-galactosidase (Promega) or an anti-Cut antibody. After four 5-minute washes in PBT, the complexes were incubated for 1.5 hours on ice in 10% horse serum in PBT with biotinylated anti-mouse horse IgG. After four 5-minute washes in PBS, the complexes were treated with a Vectastain ABC kit (Vector Laboratories) for 30 minutes at room temperature. The complexes were then washed four times for 5 minutes each in PBS and treated with the substrate (0.1% 3,3′-diaminobenzidine (DAB) and 0.01% H_2O_2 in PBS). After two 5-minute washes in PBS, the discs were postfixed in 2% glutaraldehyde in PBS for 1 hour on ice.

Bromodeoxyuridine incorporation and visualization

An Amersham cell proliferation kit (RPN20) was used to label mitotic cells in the developing compound eye with bromodeoxyuridine (BrdU). Eye-antennal discs dissected out from the third instar larvae were incubated in 15 mg/ml BrdU in Schneider's medium for 30 minutes at room temperature (Truman and Bate, 1988). The discs were then fixed in Carnoy's fixative for 30 minutes at room temperature, rehydrated and washed twice in PBT. Subsequently, the discs were incubated in a nuclease and anti-BrdU antibody mixture solution for 16 hours at 4°C with agitation. After a series of washes to remove the primary antibody, the discs were exposed to a rabbit anti-mouse IgG antibody conjugated to peroxidase for 2 hours at room temperature with agitation. The peroxidase label was then visualized using DAB as a substrate. After this treatment, the discs were mounted in glycerol.

Acridine orange staining

Acridine orange was used to identify dead cells in pupal retinae

according to the method described by Spreij (1971) and Wolff and Ready (1991). White prepupae were collected and maintained at 20°C for 50 hours under constant illumination. Retina/brain complexes were dissected in PBS and soaked in acridine orange solution (1.6×10^{-6} M in PBS). The retinae were examined immediately after staining using a confocal microscope with a filter set for fluorescein isothiocyanate.

Yeast two-hybrid assay

For the yeast two-hybrid assay, *Drosophila Ras1* and *Ras1^{V12}* were subcloned into pAS2-1 (Trp maker) (CLONTECH). The DNA segments that encode different regions of *Drosophila* Cno peptides each includes either of the putative Ras-binding domains RA1 or RA2 (Ponting and Benjamin, 1996), or the DHR domain, were subcloned into pACT2 (Leu marker) (CLONTECH), resulting in pACT2 cno•NH (1-936 a.a), pACT2 cno•RA1 (31-162 a.a), pACT2 cno•RA2 (256-348 a.a) and pACT2 cno•DHR (843-928 a.a). Yeast strain Y187 (CLONTECH) was cotransformed with 100 ng of pAS2-1 Ras1 or pAS2-1 Ras1^{V12} and 100 ng of pACT2 cno•NH, pACT2 cno•RA1, pACT2 cno•RA2 or pACT2 cno•DHR. Transformants were selected for growth on SD-Trp/Leu medium. The plates were incubated at 30˚C for 4 days. Surviving yeast colonies were streaked on new SD-Trp/Leu medium. The plates were incubated at 30˚C for additional 3 days and then colonies were assayed for β-galactosidase activity.

RESULTS

The *cno* locus was defined by Jürgens et al. (1984) using several lethal mutations that result in defects in dorsal closure of the embryonic cuticle. Subsequently, we recovered, in our screen for rough eye mutations, a hypomorphic *cno* allele, *cno^{mis1}*, which survives to the adult stage (Miyamoto et al., 1995). Unlike the wild-type compound eye with a regular array of facets (Fig. 1A), the compound eye of *cno^{mis1}/cno^{10B1}* flies has a rough appearance since it is composed of ommatidia of variable size and shape (Fig. 1B). Fusion of adjacent ommatidia is a common feature of the *cno* mutant eye (Fig. 1B; Miyamoto et al., 1995). In tangential sections of the wild-type compound eye, seven round photoreceptor rhabdomeres are observed in constant positions (Fig. 1C). Similar sections prepared from the *cno* mutant eye reveal a variable number of rhabdomeres with distorted shapes in unusual positions in the ommatidia (Fig. 1D).

In spite of the rough appearance of the adult compound eye, the *cno* mutations do not affect the patterning of the photoreceptor clusters in the developing eye discs in the third instar larvae (Miyamoto et al., 1995). Abnormalities in the *cno* mutant discs are manifested at the pupal stage. In pupal retinae of flies heterozygous for *cno^{mis1}*, the R7 photoreceptors align along the ommatidial rows as in the wild-type pupal discs (Fig. 1E). The linear alignment of the R7 photoreceptors is disrupted in the pupal retinae of *cno^{mis1}/cno^{mis1}* flies (Fig. 1F) and *cno^{mis1}/cno^{10B1}* flies (Fig. 1G). Importantly, a single R7 photoreceptor is formed in each ommatidium in the *cno* mutant retinae without exception (Fig. 1F,G). These observations suggest that induction of photoreceptors, including the R7 photoreceptors, is unaffected by *cno* mutations. The R7 photoreceptors occupy unusual positions in pupal discs in *cno* mutants probably because the mutant discs fail to form a correct mesh of pigment cells that shape ommatidia into regular hexagonal structures (Cagan and Ready, 1989; Wolff and Ready, 1991).

For further investigation of the possible role of *cno* in R7 photoreceptor induction, the wild-type *cno* cDNA under the control of the *UAS* GAL4-binding sequence (*UAS-cno^{+}*) was expressed in R7 equivalence group cells using *sev-GAL4* flies (the strain generated by Konrad Basler; see Reiter et al., 1996) which are otherwise wild type. Overexpression of *cno^{+}* results in the absence of R7 photoreceptors in a fraction (≈10 %) of the ommatidia composing the compound eye (Fig. 1H). A few ommatidia are devoid of an outer photoreceptor (usually the one that is to occupy the R1 or R6 position) while the R7 photoreceptor is present (Fig. 1H). The rest of the ommatidia in the compound eye appear normal (Fig. 1H).

These facts indicate that the overexpression of *cno^{+}* perturbs the fate determination of photoreceptors, but decreasing the expression of *cno^{+}* has no effect on this process. The results also imply that neither an increase nor a decrease in *cno^{+}* expression results in cells of the R7 equivalence group developing as additional R7 photoreceptors.

The observation that the R7 cells are displaced in the pupal retinae (Fig. 1G) after their normal formation prompted us to examine other cell types produced in the pupal stage.

As in the wild-type flies, after the formation of R7 photoreceptors, four cone cells are recruited to the primordial ommatidium in flies heterozygous for *cno^{10B1}* (Fig. 1I). In the pupal retinae of flies homozygous for a weak allele *cno^{mis1}*, there are ommatidia having an additional cone cell or ones lacking 1-3 cone cells, although the majority of the ommatidia contain the correct number of cone cells (Fig. 1J). A strong mutation in the *cno* locus eliminates 1-3 cone cells from most of the ommatidia (Fig. 1K). In contrast, overexpression of *cno^{+}* by combination of *sev-GAL4* and *UAS-cno^{+}* results in the formation of supernumerary cone cells (Fig. 1L). These results indicate that an increase in *cno^{+}* expression promotes cone cell induction and a significant decrease in *cno^{+}* expression prevents cone cell precursors from differentiating into cone cells. A moderate decline in the level of *cno^{+}* expression appears to destabilize the fate determination mechanism, with variable phenotypic consequences. The effects of different levels of *cno^{+}* expression on cone cell formation suggest its role as a modulator of cell fate determination. If so, which mechanism is modulated by Cno in cone cell determination?

It is known that Spi and EGFR positively regulate cone cell formation (Freeman, 1996) and Argos (Aos) counteracts the Spi-EGFR action (Sawamoto et al., 1994; Freeman, 1994b; Freeman et al., 1992). Since Spi-EGFR initiates Ras signaling in developing *Drosophila* adult epidermis and wings (see Freeman, 1996), we examined the possibility of Ras involvement in cone cell formation and its modulation by Cno.

Expression of a constitutively active form of Ras1 under the control of the *sev* promoter (*sev-Ras1^{V12}*) causes the strong rough-eye phenotype (Fig. 2A) in keeping with previous observations by other researchers (Fortini et al., 1992; Therrien et al., 1995). Expression of Ras2 from the *sev-Ras2^{V14}* transgene results in slight roughness of the compound eye (Fig. 2D; see also Fortini et al., 1992). Interestingly, the rough-eye phenotype as a result of *sev-Ras1^{V12}* expression is dramatically enhanced in the *cno^{10B1}* heterozygous background (Fig. 2B), although *cno^{10B1}* is completely recessive and flies heterozygous for this allele exhibit no specific phenotype per se (Fig. 1I; Miyamoto et al., 1995). The *cno^{10B1}* also enhances the *sev-Ras2^{V14}* phenotype to a lesser extent (Fig. 2E). A weak

hypomorph *cno^{mis1}* is unable to modify the phenotype of *sev-Ras1^{V12}* (Fig. 2C) or *Ras2^{V14}* (Fig. 2F).

It is well known that *sev-Ras1^{V12}* ommatidia contain multiple R7 photoreceptors, which disrupt the overall organization of the retina leading to roughness of the eye surface (Fortini et al., 1992). The results of our analysis of tangential eye sections confirm the existence of extra R7-like photoreceptors in *sev-Ras1^{V12}* ommatidia (Fig. 3A). However, the number of R7-like photoreceptors in *sev-Ras1^{V12}* flies with a wild-type *cno* background (+/+) is similar to that in *sev-Ras1^{V12}* flies heterozygous for the *cno* locus (*cno^{10B1}*/+) (Fig. 3), despite the fact that the latter display a much more severe rough-eye phenotype than do the former (Fig. 2B versus 2E). Overexpression of *cno^{+}* in the R7 equivalence group cells similarly enhances roughness of the *sev-Ras1^{V12}* compound eye, without increasing the number of R7 cells beyond the level of *sev-Ras1^{V12}* flies that do not carry the *cno^{+}* transgene (data not shown). The proportion of the number of R7-like rhabdomeres to that of outer rhabdomeres (R7/outer = 1/6 ≈ 0.17 in wild-type flies) was calculated from four compound eyes for each genotype, giving the values: 471/1471 = 0.32 for *sev-Ras1^{V12}*/+, 445/1539 = 0.29 for *sev-Ras1^{V12}*/+;*cno^{10B1}*/+, and 523/2336 = 0.22 for *sev-Ras1^{V12}/sev-GAL4;UAS-cno^{+}*/+.

Since the *cno* mutations interfere with cone cell formation without affecting photoreceptor induction (see Fig. 1), it seems plausible that Cno interacts with Ras1 in the developing cone cells. For assessment of this possibility, the pupal retinae of *sev-Ras1^{V12}* flies were observed by cobalt sulfide staining (Fig. 4A). The number of cone cells is increased in most ommatidia although there are ommatidia with fewer than four cone cells

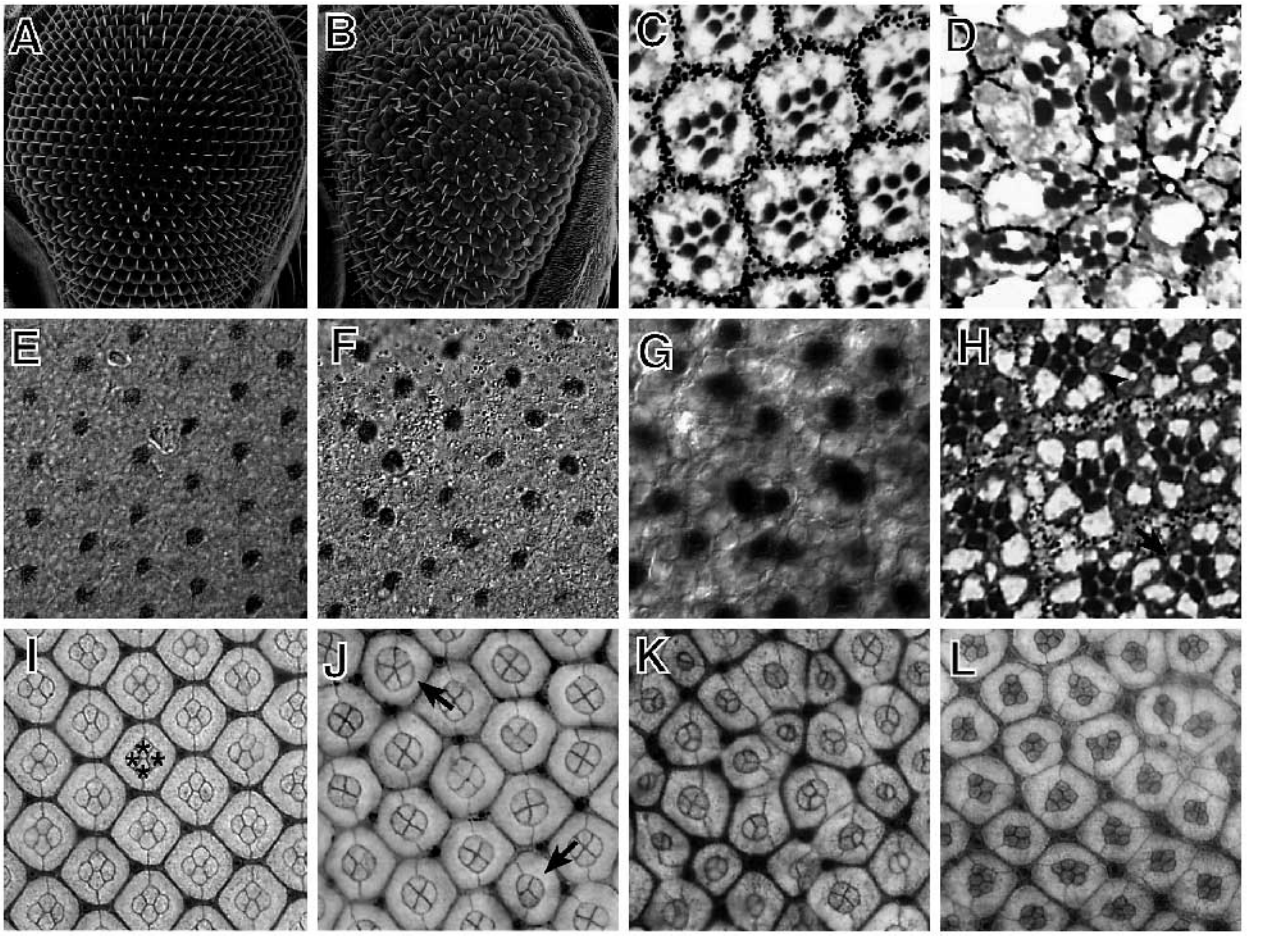

Fig. 1. The effect of Cno on compound eye development in *Drosophila melanogaster*. (A,B) SEM images of adult compound eyes. (A) Wild type, (B) *cno^{mis1}/cno^{10B1}*, (C,D,H) tangential sections of adult eyes. Constant shape and position of rhabdomeres in wild-type fly eyes (C) are strongly distorted in *cno^{mis1}/cno^{10B1}* eyes (D). Most ommatidia in *sev-Gal4/+;UAS-cno/+* flies exhibit normal morphology (H), with a few ommatidia lacking R7 (arrow) or outer photoreceptors (arrowhead). (E-G) Pupal retinae at 50 hours after puparium formation (APF) immunostained against β-galactosidase in a background of an XA12 enhancer trap. XA12 exhibits β-galactosidase expression in the nucleus of the R7 photoreceptor at this stage. *cno^{mis1}/+* retinae are completely normal with respect to number (one for each ommatidium) and position (constant intervals between each other) of R7 photoreceptors (E). Although the positions of R7 photoreceptors are slightly different, the number is normal in *cno^{mis1}/cno^{mis1}* flies (F). In *cno^{mis1}/cno^{10B1}* flies, the positions of R7 photoreceptors are markedly different (the depth of the nuclei in the retina is now not constant; some of them are out of the focal plane). However, the number of R7 photoreceptors per ommatidium is normal i.e. one (G). (I-L) Cobalt sulfide staining of pupal retinae at 50 hours APF. *cno^{10B1}/+* flies exhibit completely normal composition of cone cells and pigment cells (I). Note that each ommatidium has four cone cells (asterisks) without exception. Some ommatidia in *cno^{mis1}/cno^{mis1}* flies have an irregular number of cone cells (arrows) (J). Most ommatidia in *cno^{mis1}/cno^{10B1}* flies, which are viable transheterozygotes for the *cno* locus carrying a hypomorphic allele and a recessive lethal allele, have a decreased number of cone cells (K). In contrast, overexpression of *cno^{+}* in *sev-Gal4/+;UAS-cno^{+}/+* flies causes overproduction of cone cells (L). Anterior is to the left in all panels.

in the *sev-Ras1^{V12}*/+ compound eye (Fig. 4A). The number of primary pigment cells is also increased in some ommatidia. A further increase in the number of cone cells is achieved by addition of another copy of *sev-Ras1^{V12}* (Fig. 4B). Conversely, expression of a dominant negative form of Ras1 (by means of the *sev-Ras1^{N17}* transgene; Herbst et al., 1996) decreases the number of cone cells formed (Fig. 4C,D). The results are compatible with the idea that Ras1 activation is required for cone cell formation.

Induction of supernumerary cone cells by *sev-Ras1^{V12}* is markedly increased in the *cno* heterozygous background (Fig. 4E). Most parts of the retinal surface are covered by cone cells in *sev-Ras1^{V12}/+;cno^{10B1}/+* pupae. Essentially the same effect is obtained when a large deficiency (*Df(3R)6-7*) that removes the entire *cno* region is used in place of *cno^{10B1}* (Fig. 4F). We then examined the effect of an extra copy of *cno^{+}* on *sev-Ras1^{V12}*-induced cone cell formation. Expression of *UAS-cno^{+}* as driven by *sev-GAL4* in the *cno* wild-type background supports overproduction of cone cells in concert with *sev-Ras1^{V12}*, just as in the case of a reduced level of *cno^{+}* (Fig. 4G). However, the effects on the *sev-Ras1^{V12}* phenotypes of increasing *cno^{+}* expression are not identical to those of decreasing it. For example, the increase in the number of cone cells as a result of *sev-Ras1^{V12}* expression is more extreme in the presence of a decreased rather than an increased level of *cno^{+}* activity. Remarkable differences between the two cases are found in the primary pigment cells, whose fate is determined immediately after cone cell determination. A prominent increase in the number of primary pigment cells is observed when the *cno^{+}* dosage is increased with *UAS-cno^{+}* driven by *sev-GAL4*, while a rather moderate increase is seen following a reduction in the level of *cno^{+}* activity. Furthermore, an unusual 'core' structure is visible in *sev-Ras1^{V12}* ommatidia only when the *cno^{+}* dosage is increased above the wild-type level (Fig. 4G, arrows). The core is composed of several cells stained strongly with cobalt, which presumably represent photoreceptor apical projections attached to the surface of the retina (Fig. 4G, inset).

It is surprising to find that halving of the *cno^{+}* dose enhances the effect of the dominant negative *Ras1^{N17}*, resulting in a further decrease in the number of cone cells (Fig. 4H). There are precedents, however, for mutations that potentiate both constitutively active and dominant negative forms of Ras1, although their effects on cone cell development have not been elucidated (Karim et al., 1996). These findings unambiguously demonstrate that Cno interacts with Ras1 in cells with the potential to develop into cone cells.

The major effector protein for Ras is Raf in many Ras-dependent processes (Han et al., 1993; Zhang et al., 1993). For determination of whether this is also the case in cone cell induction, the pupal eye discs of a hypomorphic *raf* mutant, *raf^{C110}*, were observed. Consistent with the hypothesis that Ras-Raf activation is required for cone cell induction, the *raf^{C110}* mutation results in a marked decrease in the number of cone cells in most ommatidia (Fig. 4I). Furthermore, the *raf^{C110}* mutation completely suppresses the overproduction of cone cells triggered by the *sev-Ras1^{V12}* and *cno^{10B1}* interaction (Fig. 4J).

For confirmation that the extra cells observed on the surface of *sev-Ras1^{V12}/+;cno^{10B1}/+* pupal retinae are cone cells, the discs were subjected to immunohistochemistry with an anti-Cut antibody (Blochlinger et al., 1993) that recognizes differentiated cone cells (Fig. 4K,L). In wild-type pupal retinae, four cone cells were visualized in each ommatidium (Fig. 4K). In contrast, a huge mass of Cut-immunopositive cells are present in *sev-Ras1^{V12}/+;cno^{10B1}/+* pupal retinae (Fig. 4L). This suggests that most of the extra cells produced in *sev-Ras1^{V12}/+;cno^{10B1}/+* retinae are indeed cone cells.

These observations raise a question, namely, from what cells do the extra cone cells originate? We consider two possibilities: (1) they originate from retinal precursors overproduced due to accelerated proliferation (Rogge et al., 1995) and (2) they are generated at the expense of pigment cells that differentiate after the cone cells, and of cells that are fated to die by apoptosis (Wolff and Ready, 1991; Reiter et al, 1996).

Proliferative activity in the third instar larval discs as revealed by BrdU incorporation is indistinguishable between wild-type (Fig. 5A) and *Ras1^{V12}/+;cno^{10B1}/+* discs (Fig. 5B). A striking difference is found between these two genotypes, however, in the pupal retinae treated with acridine orange which stains dead cells: staining is distributed uniformly over the wild-type retinae (Fig. 5C) whereas practically no staining is observed in the *sev-Ras1^{V12}/+;cno^{10B1}/+* retinae (Fig. 5D). These results support the hypothesis that the supernumerary cone cells in the *sev-Ras1^{V12}/+;cno^{10B1}/+* retinae are recruited from a pool of retinal precursors that normally develop into pigment cells and from cells to be eliminated by apoptosis.

Another issue to be addressed is the possible mechanism of action of Cno in modulating Ras1 activity. Recently, we purified, from bovine brain, a novel H-Ras-binding protein (Kuriyama et al., 1996). It was subsequently identified as AF-6, a mammalian homolog of Cno (Kuriyama et al., 1996). We

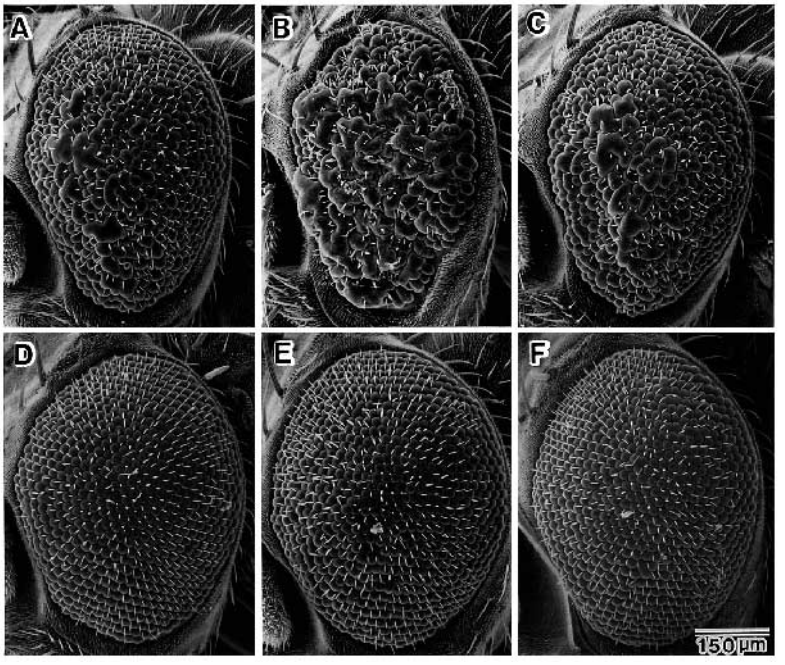

Fig. 2. Scanning electron microscopy of adult compound eyes. (A) *sev-Ras1^{V12}/+*. (B) *sev-Ras1^{V12}/+;cno^{10B1}/+*. (C) *sev-Ras1^{V12}/+;cno^{mis1}/+*. (D) *sev-Ras2^{V14}/+*. (E) *sev-Ras2^{V14}/+;cno^{10B1}/+*. (F) *sev-Ras2^{V14}/+;cno^{mis1}/+*. Anterior is to the left.

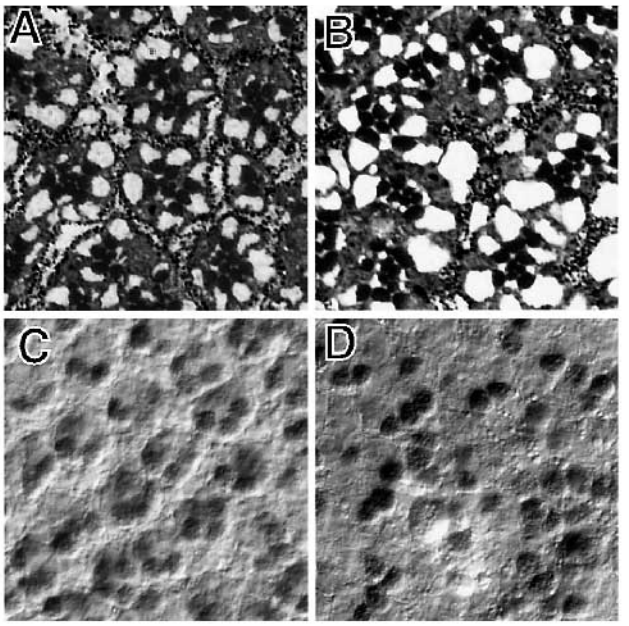

Fig. 3. Overproduction of R7 photoreceptors in *sev-Ras1^{V12}*/+ and *sev-Ras1^{V12}*/+;*cno^{10B1}*/+ flies. (A,B) Tangential sections of adult eyes. (C,D) Pupal retinae at 50 hours APF immunostained against β-galactosidase in an XA12 background. (A,C) *sev-Ras1^{V12}*/+; (B,D) *sev-Ras1^{V12}*/+;*cno^{10B1}*/+. Although the loss of pigment cells is more severe in *sev-Ras1^{V12}*/+;*cno^{10B1}*/+ flies than in *sev-Ras1^{V12}*/+ flies (A,B), the number of extra R7 photoreceptors seems to be the same between the two genotypes (C,D).

further demonstrated that *Drosophila* Cno as well as mammalian AF-6 directly binds to the effector domain of H-Ras in vitro in a GTP-dependent manner (Kuriyama et al., 1996).

The heterologous in vitro experiment suggests that Cno modulates Ras1 activity by its direct binding to the Ras1 effector domain. As shown in Fig. 6, the yeast two-hybrid assay results prove that direct association of Cno with Ras1 occurs. The fact that the plasmid expressing the Cno RA1 and RA2 domains (Ponting and Benjamin, 1996) gave a positive signal whereas that expressing the Cno DHR domain did not (Fig. 6) suggest that Cno binds to Ras1 with its RA1 and RA2 domains.

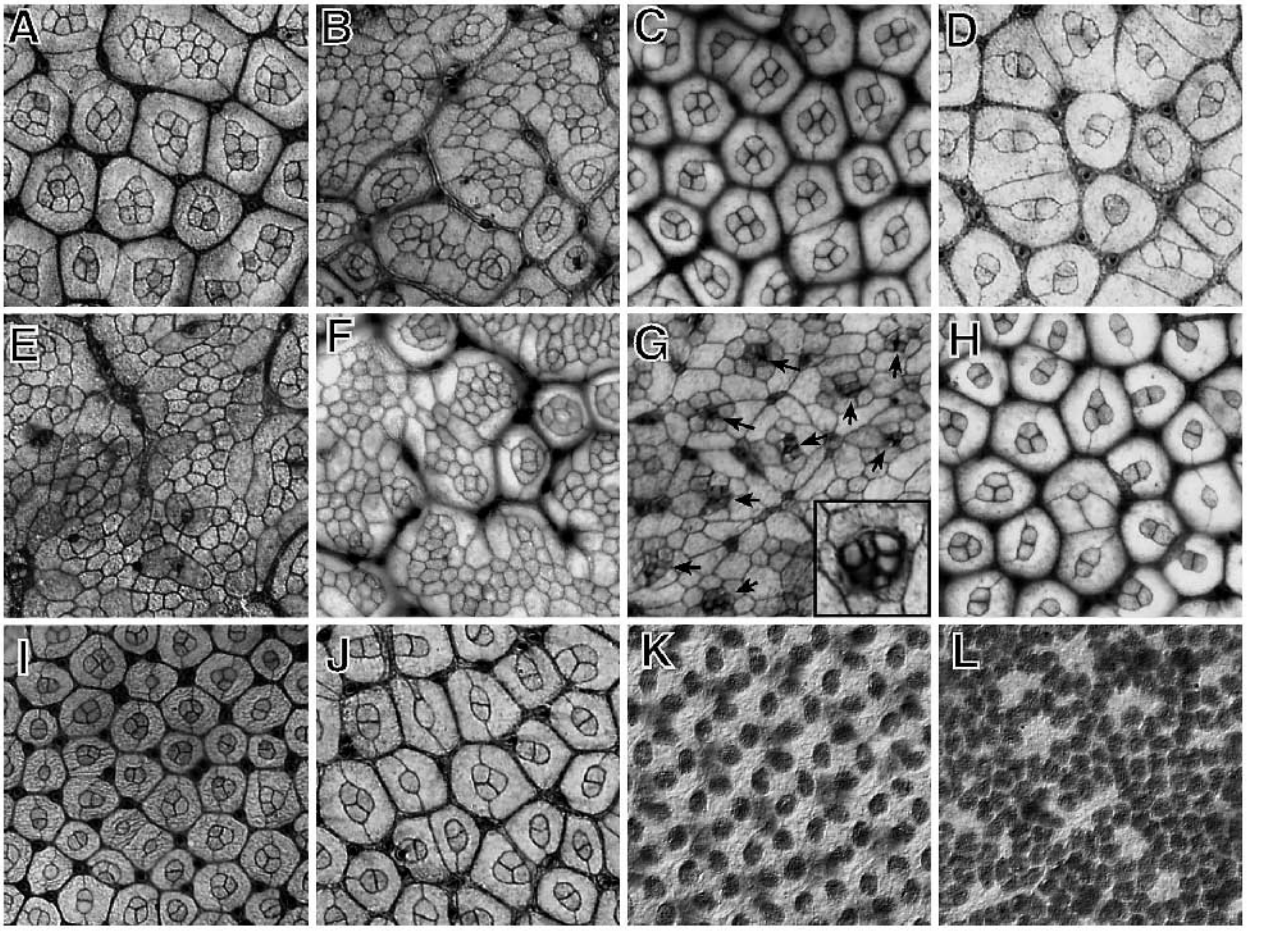

Fig. 4. Interaction between *cno* and *Ras1* in the development of cone cells. (A-J) Cobalt sulfide staining of pupal retinae at 50 hours APF. (K,L) Pupal retinae at 50 hours APF immunostained against Cut (Blochlinger et al., 1993). Cut is specifically expressed in the nuclei of cone cells and bristle cells (out of focal plane in K and L) at this stage. (A) *sev-Ras1^{V12}*/+. (B) *sev-Ras1^{V12}*/*sev-Ras1^{V12}*. (C) *sev-Ras1^{N17}*/+. (D) *sev-Ras1^{N17}*/*sev-Ras1^{N17}*. (E) *sev-Ras1^{V12}*/+;*cno^{10B1}*/+. (F) *sev-Ras1^{V12}*/+;*Df(3R)6-7*/+. (G) *sev-Ras1^{V12}*/*sev-Gal4;UAS-cno^{+}*/+. (H) *sev-Ras1^{N17}*/*cno^{10B1}*. (I) *raf^{C110}*/*Y*. (J) *raf^{C110}*/*Y;sev-Ras1^{V12}*/+;*cno^{10B1}*/+. (K) wild type. (L) *sev-Ras1^{V12}*/+;*cno^{10B1}*/+. See Results for description.

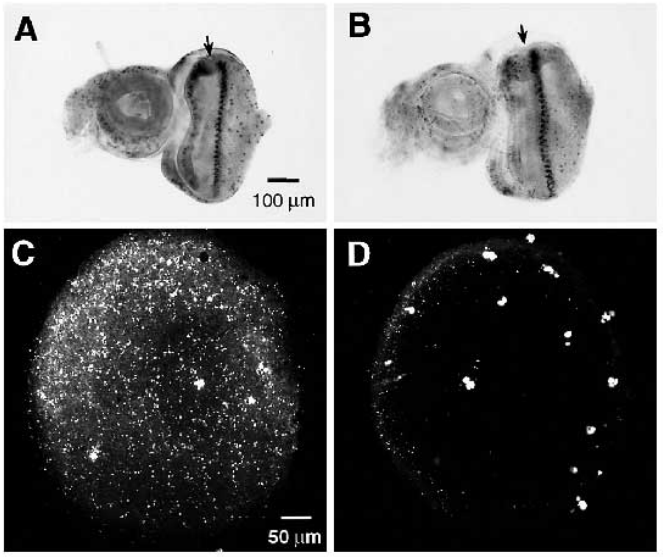

Fig. 5. BrdU incorporation in the eye-antennal discs of third instar larvae (A,B) and acridine orange staining in pupal retinae (C,D). (A,C) Wild-type. (B,D) *sev-Ras1^{V12}/+;cno^{10B1}/+*. The morphogenetic furrows are indicated by arrows in A and B. BrdU is incorporated primarily into cells immediately behind the morphogenetic furrow in both wild-type and *sev-Ras1^{V12}/+;cno^{10B1}/+* eye-antennal discs (A,B). In wild-type retinae, excess precursor cells are eliminated by apoptosis by 50 hours APF at 20°C and dead cells incorporate acridine orange (C). The *sev-Ras1^{V12}/+;cno^{10B1}/+* retina at this stage shows no acridine orange staining (D). Retinae at 40 hours or 60 hours APF show no acridine orange staining (data not shown). Anterior is to the left.

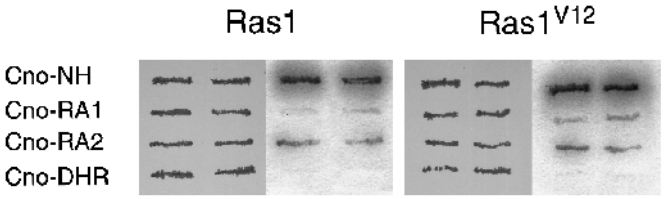

Fig. 6. Interaction of wild-type and constitutively active mutant Ras1 proteins with Cno RA domains in the yeast two-hybrid assay. The *S. cerevisiae* reporter strain Y187 was cotransformed with pairs of genes encoding the indicated proteins fused to GAL4-BD and GAL4-AD. Each patch represents an independent transformant. Cno-NH represents the Cno N-terminal half (1-936 a.a) including the putative Ras-binding domains RA1 and RA2, and the DHR domain. Cno-NH binds to both Ras1 and Ras1^{V12}. Cno-RA1 and Cno-RA2 also weakly bind to Ras1 and Ras1^{V12}, whereas Cno-DHR does not.

DISCUSSION

The *sev-Ras1^{V12}* action is markedly potentiated by halving of the *cno^{+}* gene dosage at the *cno* locus. A similar marked induction of cone cells has been reported to occur when a diffusible form of the EGFR ligand Spi is expressed in developing eye discs (Freeman, 1996). In this respect, Cno seems to counteract the action of Ras1. The mechanism whereby Cno acts in opposition to Ras1 remains to be elucidated. One attractive hypothesis is that direct binding of Cno to Ras1 occludes the effector domain which otherwise provides the binding site for Raf. Since Ras action is mostly mediated by Raf, interference with the binding of Raf to Ras would attenuate the signaling. This hypothesis is, in part, based on the results of the in vitro experiment, which showed that c-Raf-1 competes with AF-6 (a mammalian homolog of Cno) in binding to the H-Ras effector domain (Kuriyama et al., 1996). The direct binding of Cno to *Drosophila* Ras1 was also confirmed to occur in yeast, by the two-hybrid assay results (Fig. 6). It is important to determine whether anti-Cno antibodies can precipitate Ras in *Drosophila* protein extracts.

Negative regulation by Cno of Ras1 activity provides a simple interpretation for the observed synergistic actions of *Ras1^{V12}* and *cno* loss-of-function mutations. However, the hypothesis cannot be used to satisfactorily explain why overexpression of *cno^{+}* results in enhancement, rather than inhibition, of the Ras1^{V12} action in cone cell formation. Another problem with the hypothesis resides in the fact that, in the absence of Ras1^{V12}, a reduction in the Cno^{+} activity causes loss of cone cells whereas overexpression of Cno^{+} promotes formation of extra cone cells. Clearly, the effects of the *cno^{+}* dosage and that of *Ras1^{V12}* are not additive in the flies that bear different combinations of mutations and/or transgenes for *cno* and *Ras1^{V12}*. It is obvious that direct binding of Cno to Ras1 alone cannot explain these observations.

In an attempt to account for such complex interactions between Cno and Ras1, we consider the possibility that there are two counteracting signaling pathways in which Cno participates, one for promoting the induction of the cone cells (via Ras) and the other for repressing this induction (Fig. 7). Here we postulate that Cno acts as a negative regulator for both pathways. Cno predominantly associates with the repressing pathway when Ras activation is minimal, where absence of Cno causes disinhibition of the repression of cone cell formation. With maximal Ras activation (e.g. due to the expression of *sev-Ras1^{V12}*) Ras would titrate Cno. Nevertheless, extra cone cells are formed because the extremely strong activation of Ras1 by *sev-Ras1^{V12}* overrides the inhibition by Cno and the action of the repressing pathway. Under such conditions, a reduction of the Cno level results in disinhibition of the promoting pathway, leading to enhancement of extra cone cell formation. On the contrary, expression of Cno^{+} beyond the normal level by a combination of *sev-GAL4* and *UAS-cno^{+}* would inhibit the repressing pathway, thereby increasing the number of cone cells formed in the presence of *sev-Ras1^{V12}*.

Dominant negative Ras1^{N17} represses cone cell formation and this Ras1^{N17} action is further intensified by a reduction of the *cno^{+}* dosage. The two-pathway model also accounts for this observation. Dominant negative Ras1^{N17} renders endogenous Ras1 inactive by keeping it in the GDP-bound form. Because Cno does not interact with GDP-bound Ras (Kuriyama et al., 1996), the pool of unbound Cno would increase in the cytoplasm. The unbound Cno would be available for inhibition of the repression pathway, thereby diminishing the repressive effect of Ras1^{N17} to some extent. A reduction of the Cno level under such sensitized conditions would diminish the level of Cno-mediated inhibition, resulting in predominance of the repression pathway that blocks cone cell formation in synergy with Ras1^{N17}.

The two-pathway hypothesis postulates another Cno target in the repression pathway. Ponting and Benjamin (1996)

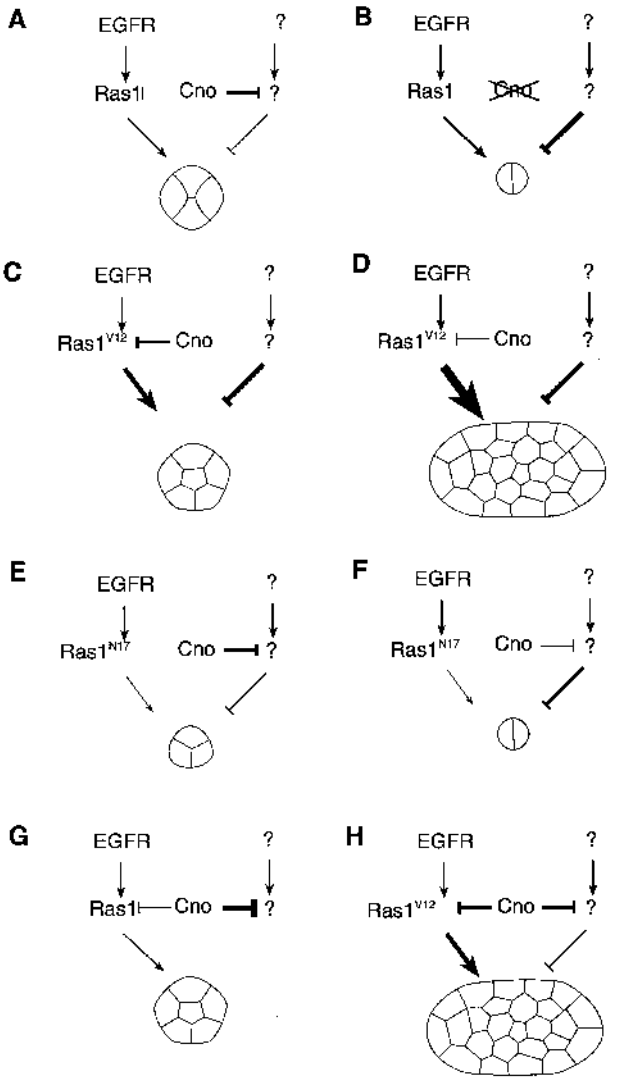

Fig. 7. A model for Ras1-Cno interactions in cone cell formation. The promotion pathway (shown in the left-hand side of each panel) is initiated by the Spi-EGFR interaction, leading to Ras1 activation. Molecular components of the repression pathway (right-hand side) are unidentified. We assume the following order in affinity for Cno protein-binding to small GTP-binding proteins: Ras1(Ras1•GDP) << ?(an unknown molecule in the repression pathway) < $Ras1^{V12}$(Ras1•GTP). The effectiveness of signaling pathways is indicated by thickness of the lines (arrows represent positive signaling and Ts represent negative signaling). (A) Wild type. Cno represses both pathways although it inhibits the repression pathway more efficiently. (B) cno^{mis1}/cno^{10B1}. Loss of Cno (indicated by cross marks on the letter) releases both pathways from inhibition, resulting in repression of cone cell formation. (C) *sev-*$Ras1^{V12}$/+. The constitutively active form of Ras1 ($Ras1^{V12}$) titrates Cno. (D) *sev-*$Ras1^{V12}$/+;cno^{10B1}/+. A reduction in the amount of Cno protein releases a fraction of $Ras1^{V12}$ from inhibition, resulting in further induction of cone cells. (E) *sev-*$Ras1^{N17}$/+. Cno does not bind to $Ras1^{N17}$ (Ras1•GDP) and continues to inhibit the repression pathway. (F) *sev-*$Ras1^{N17}/cno^{10B1}$. The repression pathway is disinhibited by a reduction in the amount of Cno protein, leading to formation of fewer cone cells. (G) *sev-Gal4/+;UAS-*cno^{+}/+. Excess Cno protein strongly inhibits the repression pathway and results in an increase in the number of cone cells. (H) *sev-*$Ras1^{V12}$*/sev-Gal4;UAS-*cno^{+}/+. In contrast to the case of C, $Ras1^{V12}$ is unable to titrate Cno completely because a large amount of Cno is now supplied by the transgene. The fraction of Cno not titrated by $Ras1^{V12}$ acts to inhibit the repression pathway, resulting in an enhancement of formation of extra cone cells.

recently pointed out that the Ras-binding domain of Cno contains a novel motif (RA motif), which is shared by many proteins that bind different small GTP-binding proteins. This raises the intriguing possibility that Cno binds to some small GTP-binding proteins other than Ras1 with different affinities. An in vitro experiment revealed that AF-6 binds to R-Ras, the mammalian counterpart of Ras2, with a lower affinity than to H-Ras (K. Kaibuchi, unpublished observations). The enhancement of the *sev-*$Ras2^{V14}$ rough-eye phenotype by cno^{10B1} implies that Cno-Ras2 association occurs in vivo, although the functional importance of such interactions remains to be addressed. Genetic experiments are currently underway to search for small GTP-binding-protein-encoding genes that interact with *cno*.

In *sev-*$Ras1^{V12}$/+;cno^{10B1}/+ retinae, the extra cone cells seem to be recruited from a pool of undifferentiated cells that normally develop into pigment cells or undergo apoptosis (Wolff and Ready, 1991). Because the expression of $Ras1^{V12}$ is driven by the *sev* enhancer and promoter sequences, these undifferentiated cells should belong to the R7 equivalence group. Alternatively, leaky expression of the $Ras1^{V12}$ transgene outside the R7 equivalence group might be responsible for massive induction of cone cells.

If the excess cone cells do not exclusively originate from the R7 equivalence group cells, then non-cell autonomous effects of $Ras1^{V12}$ need to be postulated to explain the overproduction of cone cells. One might envisage that overproduction of cone cells is, at least in part, a secondary outcome of overproduction of R7 photoreceptors, if extra R7 cells play an inductive role in cone cell formation. This idea does not explain why cno^{10B1} or *sev-GAL4-UAS-*cno^{+} potentiates *sev-*$Ras1^{V12}$ in overproduction of cone cells, even though the number of R7 cells formed in the *sev-*$Ras1^{V12}$ eyes was not increased by cno^{10B1} or *sev-GAL4-UAS-*cno^{+} (see Results). In fact, the action of *cno* in fate determination in eye discs is specific to non-neuronal cells: loss-of-function mutations in the *cno* locus have absolutely no effect on induction of any photoreceptors and overexpression of cno^{+} only marginally interferes with photoreceptor development (Fig. 1). This contrasts with the actions of EGFR and Ras1 that are required for development of all cell types in the compound eye. Thus *cno* may be one of the factors that differentiate the non-neuronal program from the neuronal program in eye development.

A likely signal for initiation of Ras1 activation for cone cell induction is Spi (Rutledge et al., 1992; Tio et al., 1994; Freeman, 1994a), which stimulates EGFR (Freeman, 1996). On the contrary, the *cno* phenotypes in the compound eyes, bristles and wings are enhanced by mutations in the loci associated with the *Notch* (*N*) pathway (Miyamoto et al., 1995). It is worth noting that a reduction of N^{+} in N^{ts} mutants results in either an increase or a decrease in the number of cone cells in ommatidia, depending on the timing of inactivation of N^{+} by a temperature shift (Cagan and Ready, 1989). Thus the N^{+} action is 'bidirectional' in cone cell formation as found for the Cno^{+} action. Involvement of N^{+} in cone cell differentiation is also suggested by the observation that overexpression of activated forms of N under the control of the *sev* promoter-

enhancer interferes with the development of cone cells from R7 equivalence group cells (Fortini et al., 1993). It is therefore possible that Cno action is regulated by the *N* pathway in these cells. In this context, it is of interest to note that some of the components of the EGFR pathway display strong genetic interactions with the *N* group genes in eye, wing and leg morphogenesis (Baker and Rubin, 1992; Rogge et al., 1995). Cno is a reasonable candidate for a molecule that mediates such crosstalk between the *N* pathway and the EGFR pathway.

Our preliminary experiments revealed that Cno is localized to adherens junctions, at which cell-to-cell communications take place. In fact, N, EGFR and some other receptors for inductive signals are known to be enriched in the adherens junctions. Recent studies by other groups suggested that one of the roles for the DHR domain proteins is to assemble composite polypeptides into functional complexes of proteins in the cell membrane. For example, the PSD-95 protein is required for formation of multimeric NMDA receptors (Kim et al., 1996; Kornau et al., 1995; Wyszynski et al., 1997) and of *Shaker* potassium channels (Kim et al., 1995) from several different subunit polypeptides. Two other DHR domain proteins, GRIP and Homer, have been implicated as being involved in the targeting of AMPA receptors and metabotropic glutamate receptors to synaptic junctions, respectively (Dong et al., 1997; Brakeman et al., 1997). Similarly, the association of neuronal NO synthase (nNOS) with the synaptic membrane is mediated by a direct binding of the nNOS DHR domain to the PSD-95 DHR domain (Brenman et al., 1996). These considerations suggest that Cno, as a DHR domain protein, may be involved in physical coupling of membrane proteins, which are otherwise associated with distinct signaling pathways. The incoming signals through these pathways converge onto Cno, which then alters Ras1 activity depending on the developmental (or stimulus) context.

In addition to the DHR motif, Cno has kinesin-like and myosin-V-like domains, the structures that implicate interactions of Cno with cytoskeletal components. If this happens to be the case, Cno may alter the structure of adherens junctions whereby changing the efficacy of signal transmission mediated by multiple pathways in the junctions.

Further studies of the mode of action of Cno should thus provide insights into the mechanism by which multiple signaling cascades are integrated so as to allow the cells to take on the correct fates in a series of complex developmental events.

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