Nitric oxide interacts with the retinoblastoma pathway to control eye development in *Drosophila*

Boris Kuzin*†, Michael Regulski*, Yuri Stasiv*, Vladimir Scheinker*, Tim Tully* and Grigori Enikolopov*

Animal organ development requires that tissue patterning and differentiation is tightly coordinated with cell multiplication and cell cycle progression. Several variations of the cell cycle program are used by *Drosophila* cells at different stages during development [1,2]. In imaginal discs of developing larvae, cell cycle progression is controlled by a modified version of the well-characterized mammalian retinoblastoma (Rb) pathway [3,4], which integrates signals from multiple effectors ranging from growth factors and receptors to small signaling molecules. Nitric oxide (NO), a multifunctional second messenger [5], can reversibly suppress DNA synthesis and cell division [6,7]. In developing flies, the antiproliferative action of NO is essential for regulating the balance between cell proliferation and differentiation and, ultimately, the shape and size of adult structures in the fly [8–10]. The mechanisms of the antiproliferative activity of NO in developing organisms are not known, however. We used transgenic flies expressing the *Drosophila* nitric oxide synthase gene (dNOS1) and/or genes encoding

complex components dE2F and dDP) combined with NOS inhibitors to address this issue. We found that manipulations of endogenous or transgenic NOS activity during imaginal disc development can enhance or suppress the effects of RBF and E2F on development of the eye. Our data suggest a role for NO in the developing imaginal eye disc via interaction with the Rb pathway.

Address: *Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, USA. †Koltzov Institute of Developmental Biology, Russian Academy of Sciences, Moscow 117808, Russia.

Correspondence: Grigori Enikolopov
E-mail: enik@cshl.org

Received: **18 October 1999**
Revised: **7 February 2000**
Accepted: **24 February 2000**

Published: **7 April 2000**

Current Biology 2000, 10:459–462

0960-9822/00/$ – see front matter

Results and discussion

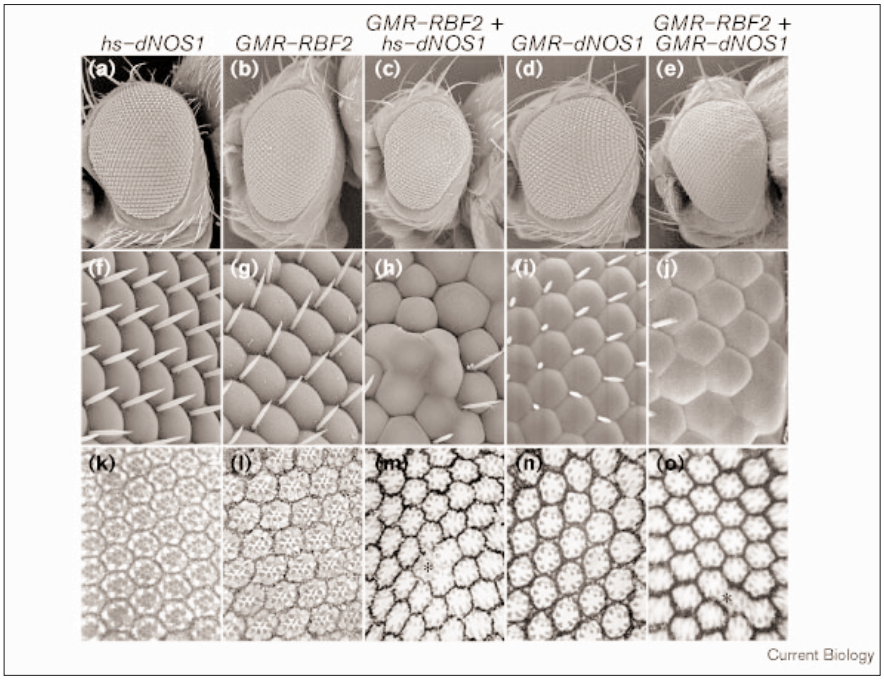
dNOS1 transgenic flies

The correctly formed compound eye of *Drosophila* contains a regular arrangement of ommatidia, each containing an invariant number of cells. Subtle changes in precursor cell proliferation are reflected in changes in the appearance of this structure [11–17]. We have used this developmental context to study the signaling pathways mediating the antiproliferative action of NO. We began by generating transgenic flies that can ectopically express *dNOS1* in a specifically regulated manner. The *dNOS1* gene encodes the major isoform of NOS in developing and adult *Drosophila*, a calcium-dependent enzyme that resembles the mammalian neuronal isoform of NOS [18]. To regulate ectopic production of NO during development, we generated transgenic lines of *Drosophila* in which the expression of *dNOS1* cDNA was controlled either by the heat-shock-inducible *hsp70* promoter (*hs–dNOS1* flies) or by the eye-specific *GMR* promoter, which functions in all cells of the eye imaginal disc in, and posterior to, the morphogenetic furrow [19] (*GMR–dNOS1* flies). *In situ* hybridizations, histochemical analyses, and enzymatic assays of NOS expression in several transformed lines indicated that transcription of *dNOS1* RNA was strongly induced after heat shock in *hs–dNOS1* flies, resulting in an increase both developing head of *GMR–dNOS1* flies (data not shown). Examination of scanning electron micrographs of the eyes of, and thin sections of the retinas of, different transgenic lines did not reveal obvious differences among eyes of wild-type flies, transgenic *hs–dNOS1* flies with or without heat-shock and *GMR–dNOS1* flies (Figure 1a,f,k,d,i,n). This indicates that a moderate increase in NO production on its own does not noticeably affect eye development.

The *hs–dNOS1* or *GMR–dNOS1* transgenes synergize with overexpression of *RBF2*

To investigate the relationship between NOS activity and cell cycle progression, we manipulated NOS activity in transgenic flies ectopically expressing genes of the Rb pathway in the developing eye [12,13]. *Drosophila* RBF is structurally related to the mammalian proteins of the Rb family and, like the Rb proteins, RBF is a negative regulator of cell cycle progression [12]. The *RBF* transgene was placed under control of the *GMR* promoter [12] and flies with either two (*GMR–RBF2*) or four (*GMR–RBF4*) copies of the transgene were used in our experiments. The eyes of adult flies with two copies of the RBF transgenes (*GMR–RBF2*) appeared normal, indicating that at this dosage the RBF transgene did not noticeably disturb cell division in the eye disc (Figure 1b,g,l and [12]). When *GMR–RBF2* flies were crossed to *hs–dNOS1* flies and the

Figure 1



Ectopic expression of dNOS1 affects RBF action in the developing eye. **(a–j)** Scanning electron micrographs of adult eyes. Magnification is 200× in (a–e), and 1,000× in (f–j). **(k–o)** Sections of adult retinas. (a,f,k) *hs–dNOS1*; (b,g,l) *GMR–RBF2*; (c,h,m) *GMR–RBF2* + *hs–dNOS1*; (d,l,n) *GMR–dNOS1*; (e,j,o) *GMR–RBF2* + *GMR–dNOS1*. Anterior is on the left in (a–e). (h,j,m,o) Pigment cells are greatly depleted in *GMR–RBF2* + *hs–dNOS1* and *GMR–RBF2* + *GMR–dNOS1* flies, resulting in several ommatidia fusing together; furthermore, many bristles are missing (the number of photoreceptor cells appears to be normal, however. Note the clusters of four fused ommatidia in the center of (m) and three in the lower right of (o), marked by asterisks.

progeny larvae were treated with heat shock before pupariation, however, the resulting adults had multiple defects in the eyes, including missing bristles and pigment cells. Pigment cells, which comprise the boundaries of each ommatidia, appear as a characteristic honeycomb pattern in thin sections of normal eyes. A lack of the regular number of pigment cells in *GMR–RBF2* + *hs–dNOS1* flies resulted in the appearance of many fused ommatidia and a rough eye phenotype (Figure 1c,h,m). Thus, *hs–dNOS1* and *GMR–RBF2* flies, both of which do not affect the development of eye structure when overexpressed on their own, nevertheless yield eye defects when overexpressed together. This transgenic interaction suggests that *NOS* and *RBF* genes interact synergistically during the development of ommatidia. We observed a similar effect employing a different genetic strategy. This time, the overexpression of *dNOS1* was restricted to the developing eye by crossing *GMR–RBF2* flies with *GMR–dNOS1* flies. These double-transgenic flies also displayed eye defects similar to those of heat-shocked *GMR–RBF2* + *hs–dNOS1* flies — missing pigment and bristle cells and fused ommatidia (Figure 1e,j,o). Thus, regardless of the promoter that drives the expression of the dNOS1 transgene, elevated levels of NO and RBF synergize to limit cell number in the developing eye, supporting the notion of interaction between *dNOS1* and *RBF* genes.

hs–dNOS1 enhances *GMR–RBF4* function

The *GMR–RBF4* transgenic flies contain four copies of the RBF transgene. In the posterior part of the eye of these flies, some of the bristles and pigment cells were missing and some ommatidia were fused (Figure 2a,d,g and [12]), indicating that precursor cell proliferation was suppressed in the eyes of *GMR–RBF4* flies. When these *GMR–RBF4* flies were crossed with *hs–dNOS1* flies and the larvae were subjected to heat shock, the resulting flies had multiple eye defects (Figure 2b,e,h). These defects were similar to, but more extreme than, those of the *GMR–RBF4*, *GMR–RBF2* + *hs–dNOS1*, or *GMR–RBF2* + *GMR–dNOS1* transgenic flies. This increased severity was evident as many more missing pigment cells and extensive fusion of ommatidia throughout the eye, including both the anterior and posterior areas (compare Figure 1c,e and Figure 2a,b). Thus, an increase in dose of RBF in combination with increased synthesis of NO produced a highly abnormal eye phenotype, providing further evidence for genetic interactions between RBF and NOS.

Inhibition of endogenous NOS rescues *GMR–RBF4* function

Both RBF and NOS act to suppress cell division. If indeed NOS acts in concert with RBF during eye development, then inhibition of NOS might suppress RBF function and restore the normal number and shape of ommatidia to *GMR–RBF4* flies. To test this, we blocked endogenous NOS activity in larvae of *GMR–RBF4* flies using a specific NOS inhibitor L-nitroarginine methyl ester, L-NAME (which alone did not affect the eye morphology of the wild-type flies; [8]). Remarkably, the eyes of these drug-exposed transgenic flies had an almost normal phenotype as regards the number of photoreceptor and accessory cells

and the number and shape of the ommatidia (Figure 2c,f,i); only a few bristles were still missing. The antiproliferative activity of NO results from its ability to suppress DNA synthesis, as BrdU labeling of the eye imaginal discs showed that the number of cells in S phase was decreased after heat shock in flies carrying the *hs–dNOS1* transgene and was increased upon inhibition of NOS activity in *GMR–RBF4* flies (see Supplementary material). Thus, the inhibitory effect of RBF overexpression on cell proliferation was almost completely rescued when endogenous NOS activity was inhibited in the developing larvae.

The *hs–dNOS1* transgene rescues the effect of E2F overexpression

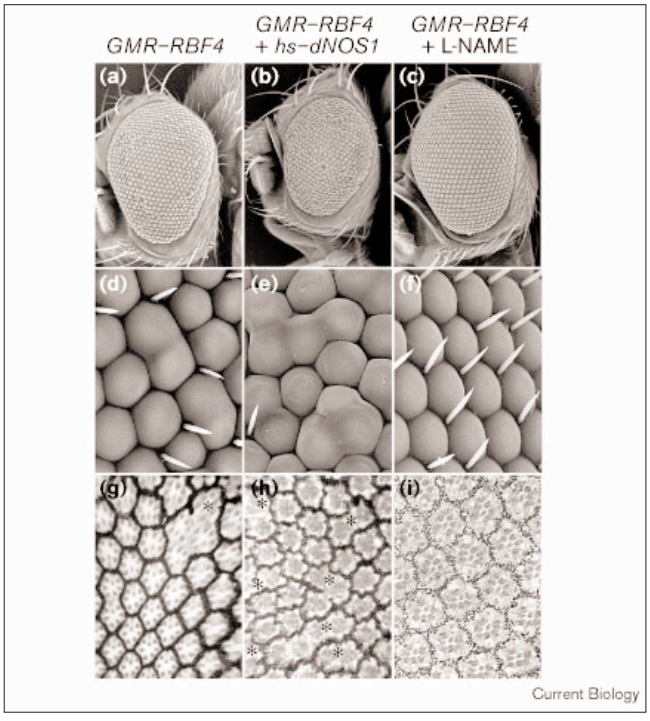
In mammalian cells, Rb and Rb-related proteins bind to transcription factors of the E2F family and inhibit E2F-dependent transcription [3,4]. When phosphorylated by cyclin-dependent kinases, Rb does not bind E2F and E2F-dependent transcription of several genes required for the synthesis of DNA and entry into S phase of the cell cycle is induced. Ectopic overexpression of E2F overcomes the Rb-mediated repression and induces quiescent cells to enter S phase. Similarly, in *Drosophila* cells, RBF is associated with the E2F transcription factor complex [12]. In transgenic flies overexpressing dE2F and dDP under control of the *GMR* promoter (*GMR–dE2FdDP* flies), ommatidia formed irregular rows and lacked their regular hexagonal shape; in addition, many eye bristles were duplicated (Figure 3a,g) [12–14]. This observation indicates that overexpression of RBF and of E2F have reciprocal effects on cell proliferation in the developing eye.

To determine whether the antiproliferative activity of NO can counteract excessive precursor cell proliferation caused by E2F overexpression, we crossed *GMR–dE2FdDP* flies with *hs–dNOS1* flies. When progeny larvae were treated with heat shock, a normalized adult eye developed; in some cases a revertant (wild-type) pattern of ommatidial rows, regularly shaped ommatidia, and the usual number of bristles was seen (Figure 3b,h). Similarly, progeny of a cross between *GMR–dE2FdDP* flies and *GMR–dNOS1* flies developed more normal eyes (Figure 3c,i), corroborating a specific dNOS1-E2F interaction. Thus, in contrast to *GMR–RBF4* flies, with which inhibition of NOS was needed to rescue the mutant phenotype (underproliferation of precursor cells), overexpression of *dNOS1* was needed to rescue the phenotype of *GMR–dE2FdDP* flies (overproliferation of precursor cells). This reciprocal effect of NO levels on RBF and E2F function in cell cycle control adds considerable genetic strength to the idea that NO acts in concert with the Rb pathway to suppress cell division during eye development.

Inhibition of endogenous NOS blocks *GMR–RBF2*-dependent rescue of the *GMR–E2F* eye defect

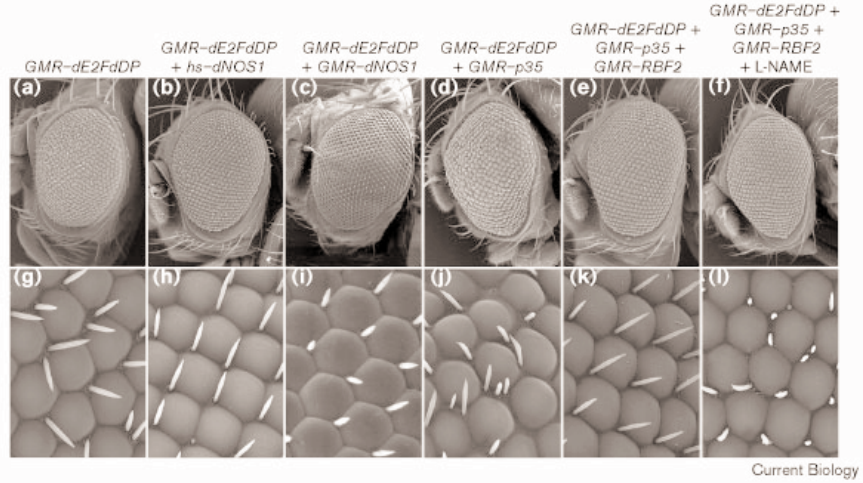
RBF blocks E2F-dependent transcription in cotransfection assays, in accordance with its ability to sequester E2F proteins [12]. When expressed in the eye, GMR-RBF suppresses the rough-eye phenotype of the *GMR–dE2FdDP* transgenic flies [12]. Thus, overexpression of RBF and E2F have opposing effects on the decision of precursor cells to enter the cell cycle. We tested whether NO modulates the effects of *GMR–RBF2* on E2F function by inhibiting NOS activity in *GMR–RBF2* + *GMR–dE2FdDP* flies. Ectopic expression of E2F in the developing eye increases both cell proliferation and programmed cell death [13–15]; the net effect is the appearance of more cells in the eye, however. To minimize the E2F-induced augmentation of cell death, we used an effective inhibitor of apoptosis, the baculoviral *p35* gene, under control of the *GMR* promoter [19]. Whereas the combination of *GMR–dE2FdDP* and *GMR–p35* transgenes produced an even more severe phenotype than the *GMR–dE2FdDP* transgene alone (Figure 3d,j and 3a,g), the *GMR–RBF2* + *GMR–dE2FdDP* + *GMR–p35* flies had a normal eye phenotype, confirming that, in the absence of programmed

Figure 2



Inhibition of NOS activity affects RBF action in the developing eye. **(a–f)** Scanning electron micrographs of adult eyes. Magnification is 200× in (a–c), and 1,000× in (d–f). **(g–i)** Sections of adult retinas. (a,d,g) *GMR–RBF4*; (b,e,h) *GMR–RBF4* + *hs–dNOS1*; (c,f,i) *GMR–RBF4* with L-NAME. Anterior is on the left in (a–c). The rough eye phenotype is much more pronounced when RBF4 is combined with dNOS1 – note (b) gross defects throughout the eye, and (e,h) profoundly fused ommatidia (fused ommatidia are marked by asterisks in (g,h)). (c,f,i) The *GMR–RBF4* phenotype of the adult eye reverts to normal when NOS activity is suppressed in the larvae, however. Note that heat shock treatment did not affect the phenotype of *GMR–RBF4* flies (data not shown).

Figure 3



Ectopic expression of dNOS and inhibition of NOS activity affect E2F action in the developing eye. **(a–l)** Scanning electron micrographs of adult eyes of the indicated genotypes. In (f,l), L-NAME was added. Magnification is 200× in (a–f), and 1,000× in (g–l). Anterior is on the left in (a–f). (a,g) Ectopic expression of dE2F + dDP results in extra cells throughout the eye (note multiple extra bristles), whereas introduction of either (b,h) *hs–dNOS1* or (c,i) *GMR–dNOS1* transgene reverts the eye phenotype to normal. (e,k) RBF expression can also suppress the phenotype of *GMR–dE2FdDP* flies; (f,l) inhibition of NOS activity in the larvae prevents this action of RBF, however (note that the rows of ommatidia are uneven and deformed in (f), and extra bristles are still present in (l)).

cell death, RBF suppresses the consequences of E2F overexpression and rescues the E2F phenotype (Figure 3e,k). In contrast, inhibition of NOS activity in these *GMR–RBF2* + *GMR–dE2FdDP* + *GMR–p35* larvae prevented RBF from rescuing the E2F phenotype (Figure 3f,l). In particular, when endogenous NO production was suppressed, the arrangement of ommatidia was still abnormal, and many additional bristles and pigment cells still were observed. This suggests that the RBF–E2F interaction involves NOS and that RBF requires NO to antagonize the E2F activity.

Our study of the developing *Drosophila* eye presents a series of reciprocal genetic interactions that consistently suggest that NO modulates a signaling pathway involved with cell cycle control. Specifically, increased production of NO in the developing eye acts as an antiproliferative signal, whereas inhibition of NOS activity promotes additional rounds of cell division. We consider that the reciprocal effects of E2F and NOS and complementary effects of Rb and NOS are best explained by our hypothesis that NO affects the Rb signaling pathway, thereby regulating entry into the S phase of the cell cycle.

Supplementary material

Supplementary material including data on BrdU labeling of eye imaginal discs, discussion of how NO could interact with the Rb pathway and additional methodological details is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

We thank Nick Dyson for his gift of *GMR–RBF* and *GMR–dE2FdDP* flies and fruitful discussions; Iswar Hariharan and Bruce Hay for *GMR–dacapo* and *GMR–p35* flies; Gregory Rudomen for help with scanning electron microscopy; Bruce Futcher, Ueli Grossniklaus, Mike Packer, Natasha Peunova, and Jonathan Wallach for critical comments on the manuscript; and Julian Banerji for his invaluable advice and comments on the manuscript. This work was supported by the grants from NIH (to G.E and T.T.), from March of Dimes Foundation, Charles Henry Leach Foundation, and Seraph Foundation (to G.E.), and from Russian Foundation for Basic Research (to B.K.).

References

1. Edgar BA, Lehner CF: **Developmental control of cell cycle regulation: a fly's perspective.** *Science* 1996, **274:**1646-1652.
2. Follette PJ, O'Farrell PH: **Connecting cell behavior to patterning: lessons from the cell cycle.** *Cell* 1997, **88:**309-314.
3. Sherr C: **Cancer cell cycles.** *Science* 1996, **274:**1672-1677.
4. Dyson N: **The regulation of E2F by pRB-family proteins.** *Genes Dev* 1998, **12:**2245-2262.
5. Bredt DS, Snyder SH: **Nitric oxide: a physiologic messenger molecule.** *Annu Rev Biochem* 1994, **63:**175-195.
6. Lepoivre M, Chenais B, Yapo A, Lemaire G, Thelander L, Tenu JP: **Alterations of ribonucleotide reductase activity following induction of the nitrite-generating pathway in adenocarcinoma cells.** *J Biol Chem* 1990, **265:**14143-14149.
7. Kwon NS, Stuehr DJ, Nathan CF: **Inhibition of tumor cell ribonucleotide reductase by macrophage-derived nitric oxide.** *J Exp Med* 1991, **174:**761-767.
8. Kuzin B, Roberts I, Peunova N, Enikolopov G: **Nitric oxide regulates cell proliferation during *Drosophila* development.** *Cell* 1996, **87:**639-649.
9. Enikolopov G, Banerji J, Kuzin B: **Nitric oxide and *Drosophila* development.** *Cell Death Differ* 1999, **6:**956-963.
10. Wingrove JA, O'Farrell PH: **Nitric oxide contributes to behavioral, cellular, and developmental responses to low oxygen in *Drosophila.*** *Cell* 1999, **98:**105-114.
11. Zipursky SL, Rubin GM: **Determination of neuronal cell fate: lessons from the R7 neuron of *Drosophila.*** *Annu Rev Neurosci* 1994, **17:**373-397.
12. Du W, Vidal M, Xie JE, Dyson N: **RBF, a novel RB-related gene that regulates E2F activity and interacts with cyclin E in *Drosophila.*** *Genes Dev* 1996, **10:**1206-1218.
13. Du W, Xie JE, Dyson N: **Ectopic expression of dE2F and dDP induces cell proliferation and death in the *Drosophila* eye.** *EMBO J* 1996, **15:**3684-3692.
14. Brook A, Xie JE, Du W, Dyson N: **Requirements for dE2F function in proliferating cells and in post-mitotic differentiating cells.** *EMBO J* 1996, **15:**3676-3683.
15. Asano M, Nevins JR, Wharton RP: **Ectopic E2F expression induces S phase and apoptosis in *Drosophila* imaginal discs.** *Genes Dev* 1996, **10:**1422-1432.
16. de Nooij JC, Letendre MA, Hariharan IK: **A cyclin-dependent kinase inhibitor, Dacapo, is necessary for timely exit from the cell cycle during *Drosophila* embryogenesis.** *Cell* 1996, **87:**1237-1247.
17. Lane ME, Sauer K, Wallace K, Jan Y-N, Lehner CF, Vaessin H: **Dacapo, a cyclin-dependent kinase inhibitor, stops cell proliferation during *Drosophila* development.** *Cell* 1996, **87:**1225-1235.
18. Regulski M, Tully T: **Molecular and biochemical characterization of dNOS: a *Drosophila* Ca2+/calmodulin-dependent nitric oxide synthase.** *Proc Natl Acad Sci USA* 1995, **92:**9072-9076.
19. Hay BA, Wolff T, Rubin GM: **Expression of baculovirus P35 prevents cell death in *Drosophila.*** *Development* 1994, **120:**2121-2129.