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Report

Capicua Regulates Cell Proliferation Downstream of the Receptor Tyrosine Kinase/Ras Signaling Pathway

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Summary

Signaling via the receptor tyrosine kinase (RTK)/Ras pathway promotes tissue growth during organismal development and is increased in many cancers [1]. It is still not understood precisely how this pathway promotes cell growth (mass accumulation). In addition, the RTK/Ras pathway also functions in cell survival, cell-fate specification, terminal differentiation, and progression through mitosis [2–7]. An important question is how the same canonical pathway can elicit strikingly different responses in different cell types. Here, we show that the HMG-box protein Capicua (Cic) restricts cell growth in *Drosophila* imaginal discs, and its levels are, in turn, downregulated by Ras signaling. Moreover, unlike normal cells, the growth of *cic* mutant cells is undiminished in the complete absence of a Ras signal. In addition to a general role in growth regulation, the importance of *cic* in regulating cell-fate determination downstream of Ras appears to vary from tissue to tissue. In the developing eye, the analysis of *cic* mutants shows that the functions of Ras in regulating growth and cell-fate determination are separable. Thus, the DNA-binding protein Cic is a key downstream component in the pathway by which Ras regulates growth in imaginal discs.

Results and Discussion

Inactivation of *capicua* Results in Increased Growth but Does Not Affect Cell-Fate Determination in the Developing Eye

We performed a genetic screen, by using mitotic recombination in the developing eye, for mutations that allow homozygous mutant cells to outgrow their wild-type neighbors [8]. In addition to mutations in genes, such as *Tsc1*, *Tsc2*, *Pten*, *salvador, warts* and *hippo*, that encode negative regulators of growth (reviewed in [9]) and result in grossly enlarged eyes, we identified mutations where the only observable abnormality was an overrepresentation of mutant over wild-type tissue. Four such mutations belonged to a single lethal complementation group. Eyes containing mutant clones showed an increased relative representation of mutant tissue over wild-type tissue (Figures 1C–1E) when compared to the parent chromosome used in the screen (Figure 1B). Eyes containing mutant clones also consistently contained more ommatidia (mean = 763 ommatidia; n = 6) and were thus slightly larger than eyes containing clones that were homozygous for the parent chromosome (mean = 703 ommatidia; n = 6, p = 0.0037). Otherwise, the eyes were normal in appearance.

All four alleles failed to complement the lethality of cic^{fetU6} and cic^{fetE11}, which are alleles of *capicua* (*cic*) [10]. Mutations in the *cic* locus (also known as *fettucine* and *bullwinkle*) have previously been isolated in screens for mutations that disrupt either embryonic patterning or patterning of the eggshell [10–12], but the role of *cic* as a negative regulator of growth has not been described previously. *cic* encodes a protein with a single high-mobility group (HMG)-box that localizes to the nucleus and that is likely to bind DNA via its HMG-box motif. Each of the four mutant chromosomes isolated in our screen has a mutation in the coding region of the *cic* gene (Figure 1A).

An antibody that recognizes the C-terminal portion of Cic stains nuclei throughout the eye imaginal disc. There is a stripe of increased expression immediately anterior to the morphogenetic furrow and reduced expression in the morphogenetic furrow itself (Figure 1I). Staining is not detected in clones of cic^{Q474X} cells (Figure S1 in the Supplemental Data online), thus confirming that the antibody recognizes the C-terminal portion of the Cic protein.

In the eye imaginal disc, loss-of-function mutations in *cic* appear to increase tissue growth but do not seem to perturb cell-fate specification or differentiation. *cic* mutant ommatidia were indistinguishable from wild-type ommatidia in terms of the size, number, and arrangement of photoreceptor cells in the adult retina (Figure 1F) and appear to develop normally at earlier stages (Figure 1G). Discs containing *cic* clones also showed normal patterns of BrdU incorporation throughout the eye imaginal disc (Figure 1H). However, *cic* clones anterior to the morphogenetic furrow contained a 2- to 3-fold higher density of cyclin-E-positive cells per unit of pixel area than wild-type clones (n = 15, p < 0.0001) (Figures 2A and 2B), consistent with the increased rate of cell proliferation in mutant clones (see below). As in wild-type discs, no BrdU incorporation was observed in *cic* mutant discs posterior to the second mitotic wave (Figure 1H), and ectopic cyclin E protein was not observed in *cic* clones posterior to the second mitotic wave (data not shown). The patterns of mitosis as assessed by staining with anti-phospho-histone H3 [13] were also unchanged (data not shown). Thus, *cic* cells maintain a relatively normal pattern of S phases and mitoses in the eye disc and are still able to exit from the cell cycle in a timely manner. In mature pupal eye discs, occasional

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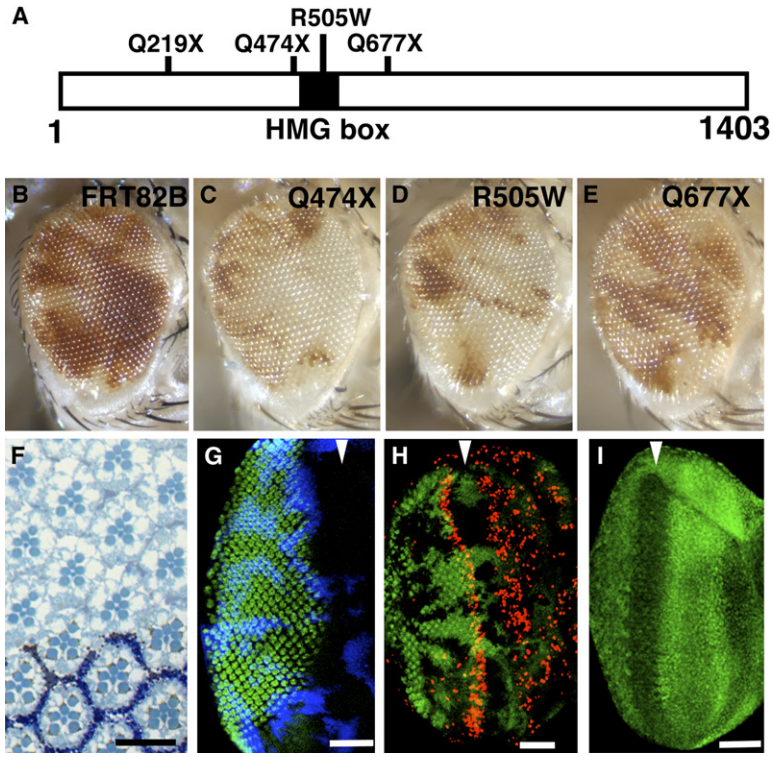

Figure 1. *cic* Mutant Cells Have a Proliferative Advantage over Wild-Type Cells but Undergo Normal Cell-Fate Determination

(A) Cic protein showing the location of the HMG box (shown in black) and the positions of the mutations. In three instances (cic^{Q219X}, cic^{Q474X}, and cic^{Q677X}) the mutations change a CAG (Gln) codon to a TAG (stop), resulting in the generation of a truncated protein. The fourth allele, cic^{R505W}, changes an arginine residue within the HMG box to a tryptophan.

(B–E) Adult mosaic eyes containing tissue homozygous for the parent chromosome bearing the *FRT82B* element (B), the cic^{Q474X} (C), the cic^{R505W} (D), and the cic^{Q677X} (E) alleles. Mutant tissue is shown in white, and wild-type twin-spot tissue is shown in red. The cic^{Q677X} mutation, which is predicted to make a truncated protein that still has an intact HMG-box, has a slightly weaker phenotype than the other alleles (E), indicating that the Cic^{Q677X} protein may retain some of its function.

(F) Retinal section of an adult eye containing cic^{Q474X} mutant clones. Mutant ommatidia do not have the *white* pigment but are otherwise indistinguishable from wild-type ommatidia.

(G) Larval eye imaginal disc showing normal photoreceptor differentiation marked by anti-Elav staining (shown in green) of *cic* mutant tissue as compared to wild-type tissue. Mutant clones fail to stain with anti-β-galactosidase (β-gal) (shown in blue). The approximate position of the morphogenetic furrow is indicated by the arrowhead.

(H) BrdU incorporation (shown in red) showing S phases in the larval third instar eye disc. Mutant clones fail to express GFP (shown in green).

(I) Expression of the Cic protein in the third instar eye disc as shown by anti-Cic staining (green). The anterior is to the right in all panels. Scale bars represent the following for individual panels: (F), 10 μm; and (G)–(I), 50 μm. (For details of methods, see Supplemental Experimental Procedures.)

extra interommatidial cells are observed in mutant clones, suggesting that *cic* cells may have a subtle defect in developmental apoptosis (data not shown).

To examine the growth characteristics of *cic* cells at greater resolution, we dissociated and analyzed cells from the eye and wing discs of early third instar larvae (120 hr AED) by flow cytometry. The distribution of mutant cells in the different phases of the cell cycle as assessed by their DNA content was very similar to that of wild-type cells, as was cell size as assessed by forward scatter in cells of the eye disc (data not shown) or the wing disc (Figures 2C and 2D). As in the adult eye and the eye imaginal disc, the area occupied by mutant clones in the wing disc was larger than the corresponding wild-type twin spots, suggesting that the mutant cells collectively grow (accumulate mass) more quickly than their wild-type neighbors (Figure 2E). Also, mutant clones typically contained more cells than their wild-type twin spots (Figure 2F). The inferred population doubling time calculated from the median clone size was 10.3 hr in mutant clones compared to 12.3 hr in the wild-type twin spots. The simplest interpretation of all of these observations is that *cic* cells have an increased rate of growth (mass accumulation) compared to wild-type cells but maintain a normal size because of a commensurate acceleration of the cell cycle. These findings indicate that a normal function of *cic* is to restrict cell growth in both the eye and wing imaginal discs.

Cic Levels Are Regulated by Ras Signaling in the Eye Disc

Previous work has shown that the levels of Cic protein are responsive to the level of signaling via RTKs and Ras. In the embryo, the level of Cic protein in the terminal regions is decreased upon signaling via the Tor RTK [12]. Activation of Ras in the cells of the wing imaginal disc also reduces Cic levels in those cells [14]. In eye discs, loss-of-function clones of *Egfr* (Figures 3A–3C) or *Ras* (Figures 3D–3I), although small, had clearly elevated levels of Cic protein. Conversely, clones of cells expressing the activated form of Ras, Ras (Val12), had reduced levels of Cic (Figure 3J–3L). Thus, as in other tissues, increased signaling via the Egfr/Ras pathway reduces Cic protein levels in the eye disc. Furthermore, studies with mutations in the effector domain of Ras suggest that Ras regulates Cic primarily via the Raf/MAPK pathway (Figure S2). This is consistent with a recent study that has shown a direct interaction between Cic and MAPK [15].

Inactivation of *cic* Enables Cells to Grow without *Ras* Function

In the eye imaginal disc, clones of $Ras^{\Delta C40b}$ [16], a null allele of *Ras*, were much smaller than their wild-type twin spots. Strikingly, clones of cells that were mutant for both *cic* and $Ras^{\Delta C40b}$ were indistinguishable from *cic* clones in that they were typically larger than their twin spots (Figures 4A–4D and Figure S3). Thus, the loss of