Citron Kinase is an essential effector of the Pbl-activated Rho signalling pathway in *Drosophila melanogaster*

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Summary

Pebble (Pbl)-activated RhoA signalling is essential for cytokinesis in *Drosophila melanogaster*. Here we report that the *Drosophila citron* gene encodes an essential effector kinase of Pbl-RhoA signalling in vivo. *Drosophila citron* is expressed in proliferating tissues but is downregulated in differentiating tissues. We find that Citron can bind RhoA and that localisation of Citron to the contractile ring is dependent on the cytokinesis-specific Pbl-RhoA signalling. Phenotypic analysis of mutants showed that *citron* is required for cytokinesis in every tissue examined, with mutant cells exhibiting multinucleate and hyperploid phenotypes. Strong genetic interactions were observed between *citron* and *pbl* alleles and constructs. Vertebrate studies implicate at least two Rho effector kinases, Citron and Rok, in cytokinesis. By contrast, we failed to find evidence for a role for the *Drosophila* ortholog of Rok in cell division. We conclude that Citron plays an essential, non-redundant role in the Rho signalling pathway during *Drosophila* cytokinesis.

Key words: Cytokinesis, Citron Kinase, Rho Kinase, *Drosophila*, Rho GTPase signalling, Pebble, Rho, GEF

Introduction

Cytokinesis is the final step in the cell division cycle when two prospective daughter cells are separated by ingression of the plasma membrane between separating chromosomes. Although still poorly understood, the strict spatial and temporal coordination of cytokinesis with the other events of mitosis appears to be mediated by a number of proteins that form a complex regulatory network (for a review, see Guertin et al., 2002). A major component of this network is the Rho small GTPase, which serves as a switch in a wide variety of signal transduction pathways that regulate cytoskeletal dynamics in cellular processes such as cell migration, adhesion, morphogenesis, axon guidance and cytokinesis (Hall, 1998). Guanine nucleotide exchange factors (GEFs) catalyse the formation of the GTP-bound active form of Rho GTPases, which can bind and activate downstream effectors (Hart et al., 1998). It has been hypothesised that the activation of Rho GTPases by particular GEFs specifies the downstream Rho effector proteins and, therefore, the pathway that is activated (for reviews, see Miki et al., 1993; Schmidt and Hall, 2002; Takai et al., 1998). *Drosophila pebble* (*pbl*) and its mouse and human homologues, named *Ect2*, encode GEFs that specifically activate RhoA signalling during cytokinesis (Prokopenko et al., 1999; Tatsumoto et al., 1999). Downstream effectors of Rho small GTPases in different cellular contexts are starting to be defined. With respect to cytokinesis, the vertebrate Citron Kinase (Citron) is postulated to be an in vivo target of RhoA during cytokinesis. Citron was isolated in a yeast two-hybrid screen as a protein capable of binding preferentially to GTP-bound RhoA (Madaule et al., 1995). It is a member of a conserved family of serine/threonine kinases described in mouse, rat, human and fly (Di Cunto et al., 1998; Eda et al., 2001; Kimura et al., 2000; Liu et al., 2003; Madaule et al., 1995; Madaule et al., 2000; Sarkisian et al., 2002) (Fig. 1). Consistent with a role in activating myosin II during cytokinesis, Citron can phosphorylate the myosin regulatory light chain (MRLC) in vitro (Yamashiro et al., 2003). Citron and the GTP-bound form of Rho localise to the cleavage furrow and midbody during cell division in mouse, rat and human cells (Di Cunto et al., 1998; Eda et al., 2001). Furthermore, inhibition of Rho in HeLa cells by botulinum C3 exoenzyme abolishes Citron transfer from the cytoplasm to the cleavage furrow (Eda et al., 2001; Sarkisian et al., 2002), suggesting strongly that Rho and Citron interact during cytokinesis. Overexpression of truncated Citron in cell culture blocks cytokinesis, though this phenotype is not induced by full-length or kinase dead protein (Madaule et al., 1998). Embryos homozygous for a mutation in the mouse *Citron* gene have multinucleate testis and brain cells, indicating a role in cytokinesis (Di Cunto et al., 2002). However, proliferation is not blocked in most tissues, suggesting that other factors may compensate for the loss of Citron. One such candidate is another Rho effector kinase, Rok. The overall domain structure of Rok resembles that of Citron (Fig. 1). Rok has recently been implicated in the control of cytokinesis (Chevrier et al., 2002; Kosako et al., 2000) as inhibition of human Rok was found to

delay completion of cytokinesis. Consistent with a level of functional redundancy in their involvement in cytokinesis, both Citron and Rok localise to the cleavage furrow during cytokinesis (Madaule et al., 2000), they are both capable of binding to the same region of Rho (Fujisawa et al., 1998; Yamashiro et al., 2003) and they both phosphorylate the regulatory subunit of myosin II in vitro (Ueda et al., 2002). The vertebrate studies are consistent with Citron being a Rho effector during cytokinesis, but the tissue-restricted mutant phenotypes observed in vivo suggest that Citron is not an essential Rho effector for cytokinesis. With the possibility that phenotypes are being masked by redundancy with Rok, it seemed desirable to analyse the role of Citron in *Drosophila*, which often shows less genetic redundancy than in vertebrates and is more readily amenable to genetic analysis. Here we analyse, in vivo, the properties and functions of the *Drosophila* homologue of Citron. We demonstrate that *citron* is expressed specifically in proliferating tissues and is downregulated in differentiating tissues. We find that *citron* plays a non-redundant role in cytokinesis and, unlike *rok*, exhibits strong genetic interactions with *pbl*, consistent with a role as a downstream target of the Pebble (Pbl)-activated Rho intracellular signalling pathway during cytokinesis.

Materials and methods

Constructs used for the generation of transgenic flies

UAS-citronGFP was made by linker ligation and subcloning from the *citron* EST clone RE26327 into pBD1010 (kindly provided by B. J. Dickson). This construct expresses a fusion of GFP to the C terminus of Citron, and includes all of the Citron open reading frame (ORF) except the last amino acid, with an additional four amino acids (DRTK) between the Citron ORF and GFP. Expression of this construct with the *da-GAL4* driver does not rescue the lethality of *citron* transheterozygotes. The GenBank Accession Number for *citron* is AE003541, locus CG10522. *UAS-pbl*^{RNAi4A} (hereafter *UAS-pbl*^{RNAi}) was generated by PCR amplification of two fragments, 1184-2916 bp and 2182-2916 bp of the *pbl* EST SD01796, and cloning as an inverted repeat with a 298 bp loop into pUAST via a pBluescriptKS+ intermediate.

Fly stocks

The two *citron* (*cit*) alleles used in this study, *KG01697* (a P{SUPor-P} insertion at position +2 of the 5′UTR) and *GS9053* (a UAS-carrying pGSV6 insertion at position +53 of the 5′ UTR of CG10522, kindly provided by Toshiro Aigaki, Tokyo Metropolitan University, Japan), are renamed *cit*^{1} and *cit*^{2} respectively. *Df(3)iro-2* is the smallest available deficiency encompassing the *citron* locus. To confirm that the insertions in these lines gave the phenotypes observed, they were removed by crossing to Δ2-3 transposase and testing the white-eyed progeny for lethality over *Df(3)iro-2*. Both alleles readily reverted with respect to both the white marker and lethality over the deficiency. We found that *cit*^{2} carried a second site lethal on 3R, which we removed by recombination. Ubiquitous expression of *citron* from the UAS in *cit*^{2} was driven by *daughterless-Gal4* (Wodarz et al., 1995). For targeted expression of genes of interest, the yeast Gal4-UAS system was used (Brand and Perrimon, 1993). *UAS-citronGFP* and *UAS-pbl*^{RNAi} constructs were placed under the control of several Gal4-expressing constructs: *Kr-Gal4* (which expresses GAL4 in T2-A4), *prd-GAL4* (drives expression in each alternative segment of the embryo), *en-GAL4* (directs expression in the posterior half of each embryonic segment and posterior domain of the larval wing) and *GAL4l(3)*$^{31\text{-}1\text{-}31\text{-}1RA}$ (hereafter *GAL*$^{431\text{-}1}$; a neural driver). Unless specified, fly stocks, such as UAS-p35 and UAS-CD8GFP, were obtained from the Bloomington *Drosophila* Stock Center (Indiana University, IN, USA).

Histology and cytology

Antibodies used were: rabbit anti-phosphorylated histone H3 (Upstate Biotechnology, NY, USA), rabbit anti-β-galactosidase (Rockland Immunochemicals, PA, USA), mAbs 22C10 (anti-Futsch), 3A9 (anti-α Spectrin), ADL101 (anti-Lamin) (Developmental Studies Hybridoma Bank, IA, USA), mAb anti-α-tubulin (Sigma-Aldrich, Israel), anti-IgG secondary antibodies conjugated with AP, HRP, Cy3, Lissamine-Rhodamine (Jackson ImmunoResearch Laboratories, PA, USA), or Alexa488, Zenon Tricolor labelling kit #1 (for mouse antibodies) and rabbit anti-GFP and Phalloidin-Rhodamine (Molecular probes, OR, USA). For tubulin staining, 3-6 hour embryos were fixed for 2 minutes in 33% formaldehyde, 0.3 M PIPES pH 6.8, 1 mM EGTA, 1 mM $MgSO_4$ before devitellinising in 80% EtOH and staining according to standard protocols. For karyotyping, third larval instar brains were dissected, squashed and Giemsa stained according to the protocol of Pimpinelli et al. (Pimpinelli et al., 2000). TUNEL assay (Roche Diagnostics, IN, USA) and Acridine Orange staining was used to detect cell death (Chen et al., 1996; Wolff and Ready, 1991). For dissociation, third larval instar brains were dissected in 1 × PBS and incubated in 1 × PBS containing 2.5% trypsin and Hoechst 33258 (10 μg/ml) for 3 hours at room temperature with mild agitation. Dissociated live cells were subsequently analysed by Phase-contrast imaging. In situ hybridisation was performed according to Tautz and Pfeifle (Tautz and Pfeifle, 1989). For detection of DIG-labelled riboprobes we used anti-DIG-AP Fab fragment antibodies (Roche Diagnostics, IN, USA). Tissues were viewed with a Zeiss Axiophot, for transmitted light, or for fluorescence, viewed with an Olympus Provis AX70 epifluorescence microscope or a confocal microscope equipped with a BioRad MRC1000 scanhead and a krypton/argon laser.

Yeast two-hybrid analysis of protein interactions

The yeast two-hybrid plasmid vectors pVP16 and pLexA-NLS (pNLX) (Hollenberg et al., 1995), and the yeast strains L40 (*MAT*a *his3-200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop) –HIS3 URA3::(lexAop)8-lacZ GAL4 gal80*) and AMR70 (same genotype as L40 except *MAT*α), were kindly provided by Dr S. Parkhurst (Fred Hutchinson Cancer Research Center, WA, USA). N-terminal VP16-Rho fusion coding sequences were generated with the complete RhoA coding sequence, a dominant negative RhoA-N19 and a constitutively active RhoA-G14V (Prokopenko et al., 2000). Four pNLX-*citron* clones (Citron 1-4), corresponding to amino acids 14 to 846, 160 to 942, 887 to 1599 and 1439 to 1854 respectively, were generated. L40 strains harbouring VP16-RhoA expressing plasmids were mated with AMR70 strains containing lexA-Citron-expressing plasmids. Interactions were tested on X-gal (blue) indicator plates (Sambrook et al., 1989).

RNA interference in S2 cells

Double stranded RNA to *citron* was made by amplifying the EST clone RE26327 with the following primers: TAATACGACTCACTATAGGTACTGTTCGCCGTTCTGGA and TAATACGACTCACTATAGGTACTACGTGGCCGCAATAG, then transcribing with T7 polymerase (Megascript kit, Ambion) and annealing overnight. S2 cells were grown and RNA interference (RNAi) performed according to Clemens et al. (Clemens et al., 2000), using 5 or 15 μg dsRNA.

Statistical and sequence analyses

Wing hair analysis was conducted by scoring the number of multi-haired versus single-haired cells in 14 identical frames for each genotype. A sample of each genotype was tested for homogeneity using a χ^2 2×2 test. The significance of differences between two genotypes in proportions of multihaired cells was evaluated using a χ^2 2×2 contingency test (Mather, 1951). Conserved protein domains were identified by pFam (Bateman et al., 2002) and SMART (Letunic

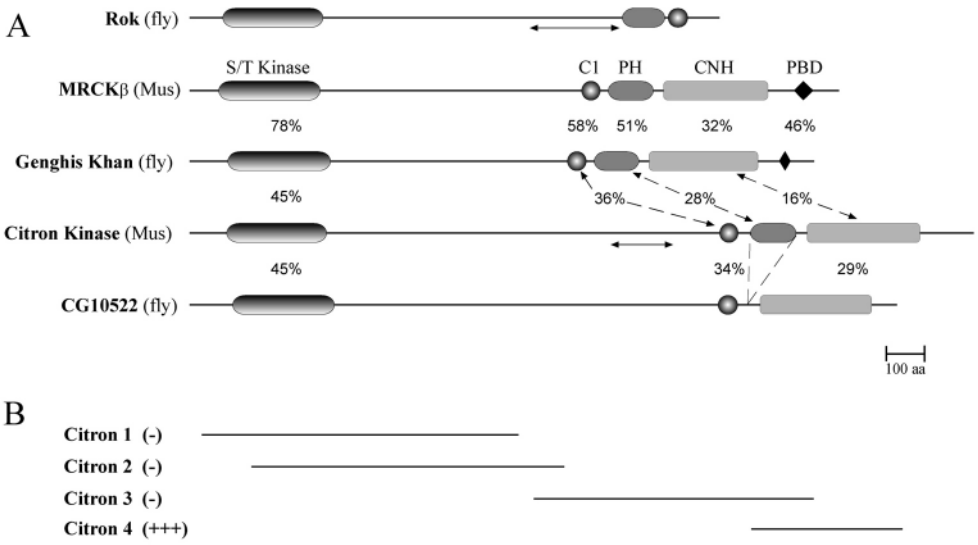

Fig. 1. Evolutionary conservation of the Citron Kinase family of proteins. (A) The family of *Drosophila* proteins containing the same conserved domains as vertebrate Citron. Each line is a scale representation of the protein sequence (scale bar represents 100 amino acids), with each conserved domain marked as a different shape. S/T Kinase, protein kinase C-class Serine/Threonine kinase domain; C1, protein kinase C-type diacylglycerol binding domain; PH, Pleckstrin homology phospholipid binding domain; CNH, Citron homology domain of unknown function; PBD, p21-like Cdc42 binding domain. Numbers between the conserved domains indicate the percentage of amino acid identity between the corresponding domains. Arrowed lines indicate the regions known to be required for binding to Rho. (B) A C-terminal fragment of Citron (Citron 4, amino acids 1439 to 1854) interacts specifically with constitutively active RhoA-G14V. LexA-Citron fusion proteins 1 to 4, represented by the lines below the domain structure of Citron in A, were assayed for interaction with VP16-RhoA-G14V fusion protein by activation of a *lacZ* reporter gene in a yeast two-hybrid assay. The strength of interaction is indicated to the right of each Citron fragment, with (–) indicating no interaction and (+++) indicating a strong interaction.

et al., 2002), and alignments were carried out in Clone Manager (Scientific and Educational Software) using FastScan. Sequences used were from the sptrembl database: CG10522: Q9VTY8; Citron (Mus): O88938; Gek: Q9W1B0; MRCKβ: O54875; Rok: Q9VXE3.

Results

Evolutionary conservation of Citron

The *Drosophila* genome encodes a set of Serine/Threonine kinases related to Citron (Madaule et al., 1998) that show a similar domain structure: an N-terminal PKC-type kinase domain, a coiled-coil region, a C1 (lipid binding) domain, PH (pleckstrin homology) domain and finally a CNH (Citron homology) domain (Fig. 1A) (Madaule et al., 2000). CG10522 and *Genghis khan* (*gek*) are the only *Drosophila* genes encoding this domain order. By sequence arrangement and function, Gek is more closely related to the Cdc42 effector, MRCK (Fig. 1A) (Luo et al., 1997). CG10522 has no PH domain, although the flanking sequences are conserved. The PH domain is present in Gek and the vertebrate homologues, suggesting that the common ancestor of these kinases had a PH domain that was lost in CG10522. The related Rho effector kinase, Rok, lacks a CNH domain and has a different domain order (Fig. 1A). The domain organisation, size, and levels of sequence identity all point to CG10522 encoding the fly homologue of Citron, so hereafter we refer to CG10522 as *citron* (*cit*). We tested whether *Drosophila* Citron, like the mammalian Citron, binds active Rho. In a yeast two-hybrid assay, we found that the C-terminal-most 416 amino acids of *Drosophila* Citron interact specifically with constitutively active RhoA (Fig. 1B and data not shown). Surprisingly, this interaction region, which encompasses the CNH, is distinct from the Rho interaction domain of mammalian Citron that lies N-terminal to the CNH and C1 domains, within a coiled-coil region (Fig. 1A) (Madaule et al., 1998). Thus, although the interaction between Citron and active RhoA is functionally conserved, the interaction domain appears to be different.

Drosophila citron is expressed in proliferative tissues and downregulated in differentiating cells

Patterns of gene expression can provide clues to gene function. In situ hybridisation showed that *Drosophila citron* transcripts occur in nurse cells during oogenesis and ubiquitously in early blastoderm embryos (Fig. 2A,B). This indicates the presence of a significant maternal store of transcript. The maternal transcripts persist only until germ band extension, as transcripts are not seen after this stage in embryos lacking zygotic *citron* (compare Fig. 2C and 2D). With the progression of embryogenesis, *citron* transcripts are restricted to cells in the central and peripheral nervous systems (CNS and PNS respectively, Fig. 2E-H). *citron* expression is gradually lost from the CNS and PNS, correlating with differentiation within these tissues (Jan and Jan, 1993). In third instar larvae, *citron* transcripts were uniformly distributed in all imaginal discs, with the exception of the posterior, differentiating region of the eye disc (data not shown). This pattern of expression, which is initially ubiquitous then restricted to the nervous system (Foe et al., 1993) is very similar to that observed for known cell cycle genes [e.g. *three rows*, D'Andrea et al. (D'Andrea et al., 1993)], consistent with a role for *citron* in proliferation. The association of *citron* expression with proliferating tissues contrasts with the widespread expression of another Rho effector kinase *rok*, and of *RhoA*, the putative upstream activator of Citron, consistent with Rok and RhoA having roles beyond proliferation (Hariharan et al., 1995).

Citron localisation to the cleavage furrow requires activation of RhoA

To explore the intracellular localization of Citron, transgenic *UAS-citron-GFP* flies were generated to produce Citron fused

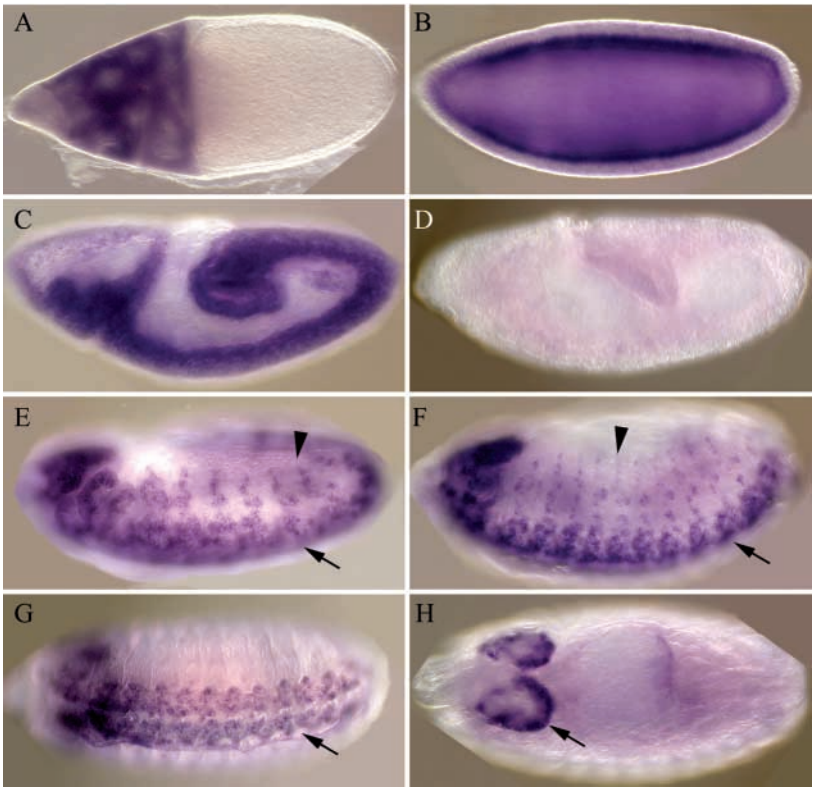

Fig. 2. *citron* is expressed maternally and in proliferating tissues during *Drosophila* development. Whole-mount *in situ* hybridisation with a *Drosophila citron* DIG-labelled RNA probe. (A) A stage 10A oocyte showing *citron* transcripts in the nurse cells. (B) Uniform distribution of *citron* transcripts in a blastoderm embryo. Maternally provided *citron* mRNA has degraded by stage 9, compare wild-type (C) and homozygous *Df(3)iro-2* mutant (D) embryos. (E-H) Zygotic tissue-specific expression of *citron* in the proliferating CNS (arrow) and PNS (arrowhead) starts during germ-band retraction. Anterior is to the left, dorsal to the top.

at the C terminus to Green Fluorescent Protein (GFP). The Citron-GFP fusion protein expressed in embryos and larval brains was scattered throughout the cytoplasm during interphase (data not shown). In dividing cells, Citron-GFP was observed to accumulate at the constricting membrane following anaphase and persisted in the midbody between divided daughter cells upon completion of cytokinesis (Fig. 3A-D, see Movie 1 in supplementary material). The cleavage furrow is enriched in many proteins that are required for the progression of cell division, including RhoA, MRLC and myosin II (for a review, see Guertin et al., 2002). We confirmed that Citron-GFP localises to this contractile ring by showing an overlap of the GFP signal with a stain for RacGAP50C (data not shown), a known cytokinesis regulator that associates with the contractile ring (Somers and Saint, 2003), and by live imaging of contraction (see Movie 1 in supplementary material). The striking similarity in intracellular localisation of fly, mouse, rat and human homologues of Citron suggests that the function of these proteins during mitosis is conserved (this study) (Di Cunto et al., 1998). Localisation of mammalian Citron to the cleavage furrow during cytokinesis is blocked by treatment with a Rho GTPase inhibitor (Eda et al., 2001). The Rho-GEF, Pbl, targets RhoA during cytokinesis (O'Keefe et al., 2001; Prokopenko et al., 1999). By examining Citron-GFP localisation in a *pbl* mutant embryo, we would avoid the drastic disruption that could occur if Rho signalling was blocked indiscriminately (Crawford et al., 1998). In the absence of Pbl, Citron-GFP fusion protein did not accumulate at the cleavage furrow at telophase (Fig. 3E-H). These data indicate that Citron accumulates at the contractile ring in response to activation of Rho by the Rho-GEF Pbl.

Loss of citron function leads to multinucleate embryonic PNS cells

To test *citron* function, we initially inhibited Citron expression in *Drosophila* Schneider line 2 (S2) cultured cells using RNAi. Many *citron* dsRNA-treated cells (~30%, compared with <2% in controls) became multinucleate (Fig. 3I, comparable to *pbl* dsRNA treatment in Fig. 3J), indicating that mitosis was not accompanied by cell division. This result is in line with the identification of *citron* by cell culture-based RNAi screening with a multinucleate phenotype (Kiger et al., 2003; Rogers et al., 2003). To assess whether Citron is required for cytokinesis in vivo, we made use of the available $cit^{KG01697}$ and cit^{GS9053} alleles, which have P-element transposon insertions in the 5′UTR of the *Drosophila citron* locus. We subsequently refer to these alleles as cit^{1} and cit^{2} respectively. To avoid the possibility of homozygosing second site mutations, all analyses used transheterozygotes between cit^{1}, cit^{2} or *Df(3)iro-2*, a small deficiency spanning the *citron* locus. All homozygous and transheterozygous genotypes were lethal, showing that *citron* is essential for *Drosophila* development. A considerable number of *citron* transheterozygotes died during larval development, and the remainder died before eclosion. Transheterozygotes between *citron* alleles and the deficiency did not show an earlier lethal phase or stronger phenotypes in our assays than *citron* mutant homozygotes or transheterozygotes, indicating that cit^{1} and cit^{2} are strong alleles if not genetically null. The insertions were responsible for the lethality observed, as both alleles could be reverted to viability by the expression of P transposase (see Materials and methods). Furthermore, since the cit^{2} insertion carries a UAS element, we were able to show that ubiquitous *citron* gene expression rescued cit^{2}/*Df(3)iro-2* transheterozygotes to viability, confirming that loss of the *citron* transcript is responsible for the phenotypes we describe. Depletion of maternal deposition of *citron* using the FLP/FRT ovoD1 system (Chou and Perrimon, 1996) led to female sterility, indicating that Citron is required during oogenesis. To characterize the *citron* mutants, we initially looked for disruption to neural cell development. As previously shown (Fig. 2), maternal *citron* seems to be depleted by stage 10, the stage at which embryonic sensory organ precursors commence a wave of cell divisions (Campos-Ortega and Hartenstein, 1997). We reasoned that loss of zygotic *citron* might have some effect on these PNS cell divisions and on neural divisions in

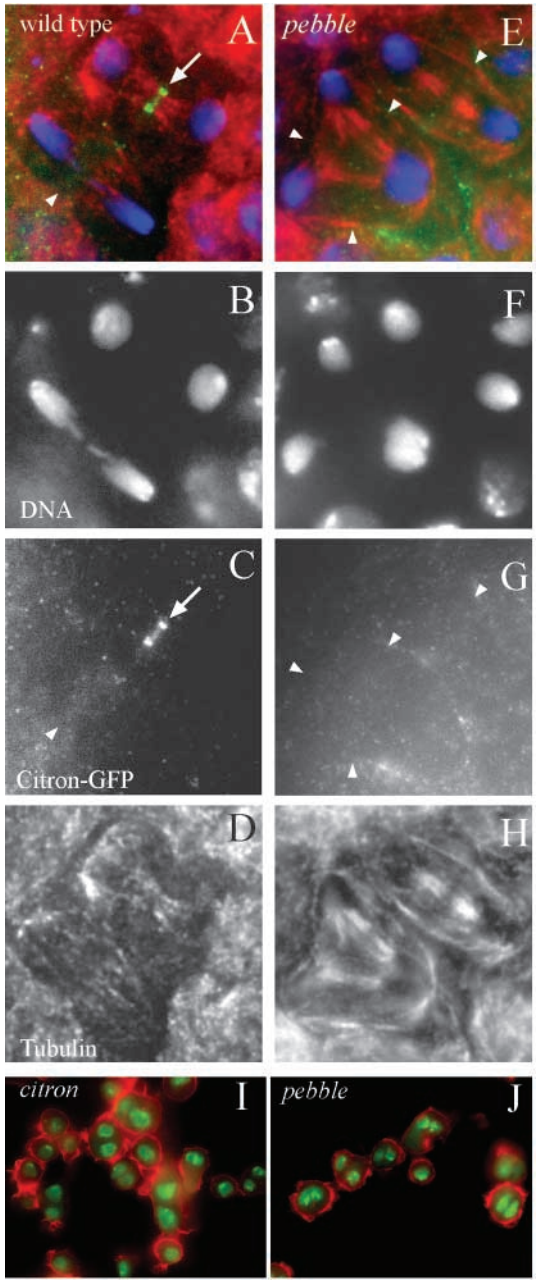

Fig. 3. Subcellular localisation of Citron-GFP to the contractile ring during cytokinesis depends on the normal activation of Rho signalling. (A-H) Citron-GFP was expressed in the 3-6 hour embryonic epidermis using *paired-Gal4*. DNA is stained with Hoechst 33258 (B,F), Citron-GFP is stained with anti-GFP (C,G), and microtubules are stained with anti-α-tubulin antibodies (D,H). (A,E) Merged images with Citron-GFP stained green, DNA stained blue and microtubules stained red. (A-D) A wild-type embryo showing Citron-GFP in the contractile ring (arrow) as it constricts around the central spindle microtubules. Citron-GFP is not localised in the adjacent anaphase cell that is yet to constrict (arrowhead). (E-G) An embryo mutant for the Rho activator *pebble* showing typical tetranucleate and binucleate cells and bipolar and tripolar spindles of cells failing cytokinesis. Citron-GFP shows no localisation to the contractile ring in *pebble* mutant telophase cells. It is found diffusely through the cytoplasm of telophase cells and not at the positions where contractile rings would normally form (arrowheads). (I-J) *Drosophila* Schneider line 2 cultured cells stained for DNA (green) and actin (red). Incubation with *citron* dsRNA (I) or dsRNA corresponding to the Rho activator *pebble* (J) causes the formation of multinucleate cells.

the larva. In wild-type embryos the PNS is organised in distinct, highly stereotyped clusters within each segment (Campos-Ortega and Hartenstein, 1997; Hummel et al., 2000). For instance, the dorsal external sensory (DES) cluster forms a grape-like structure with cells closely linked to each other (Fig. 4A). The lateral chordotonal organ (CH) cluster typically contains five oval-shaped cells of similar size extending parallel processes dorsally (Fig. 4A′). Examination of the PNS in cit^{1}/*Df(3)iro-2* and cit^{2}/*Df(3)iro-2* transheterozygous embryos using the 22C10 monoclonal antibody (Zipursky et al., 1984) revealed a modest disruption to nervous system organization (Fig. 4B,B′) with some disorganisation of clusters and defects in axonal projections, suggestive of a role in neuronal morphology and axonogenesis. Similar phenotypes have previously been shown for hypomorphic alleles of *pbl*, which encodes an upstream activator of the Rho GTPase (Salzberg et al., 1994; Salzberg et al., 1997). Importantly, there was a loss of neurons and concomitant appearance of binucleate neurons in approximately 15% of *citron* mutant PNS cells (Fig. 4C,C′, arrows, E), consistent with the failure of cytokinesis.

Citron mutant larval brains contain polyploid and multinucleate cells

The presence of maternal *citron* transcripts and the late embryonic PNS phenotype suggested that maternally-derived Citron permits early proliferation, but is lost later in embryogenesis. After embryogenesis there is little proliferation in the nervous system until the CNS begins to divide rapidly at the end of the second larval instar (Truman et al., 1993), by which stage the zygotic mutant phenotype should be evident. *citron* mutant larval brains contained massively enlarged nuclei (Fig. 5A,B), DNA stains showing large diffuse aggregates of apparently hyperploid cells. In addition, dissociated mutant larval brain cells from any allelic combination were binucleate at a far higher rate (26/103) than normal (1/100; Fig. 5C). Chromosome preparations from mutant brains (Fig. 5D-F) showed tetraploid metaphase figures (Fig. 5D), confirming that *citron* mutant brain cells can complete S phase and enter mitosis following a previously failed mitosis or cytokinesis. We also observed hyperploid cells undergoing anaphase (Fig. 5E), suggesting that a failure of chromosome disjunction was not the primary defect. Furthermore, the frequency of diploid anaphases and telophases was similar in all *citron* allelic combinations (approximately 37% of mitotic cells) and did not significantly differ from wild-type control (39%). Consistent with this, immunohistochemical analysis showed that centrosomes separate normally in the polyploid cells, since we observed an assembly of microtubule spindles apparently emanating from multiple centrosomes (Fig. 5G-G″,H-H″). Some mutant brain cells were massively hyperploid (Fig. 5F,H′) showing that in the absence of Citron, brain cells can undergo multiple mitotic cycles without dividing. However, with increasing cell ploidy, microtubules form abnormal spindles (Fig. 5G,H) and chromosomes fail to assemble at the metaphase equator (Fig.

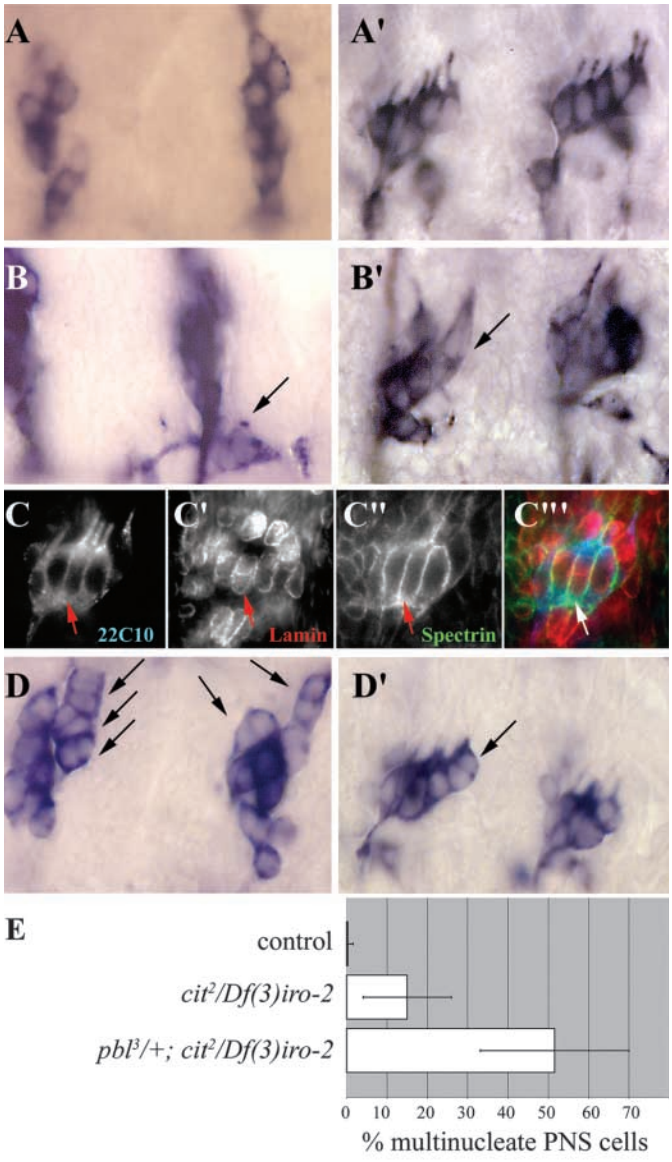

Fig. 4. Citron function during cytokinesis in the *Drosophila* embryonic PNS depends on Rho activation. Flat preparations of the PNS from abdominal segments of stage 16 embryos stained for the neuronal specific antibody 22C10 (A,A′,B,B′,D,D′). (C-C‴) Embryonic PNS cells stained with: 22C10 (C, blue in C‴), nuclear envelope marker anti-Lamin (C′, red in C‴) and plasma membrane marker anti-α Spectrin (C″, green in C‴). Anterior is to the left and dorsal up. (A,B,D) Dorsal external sensory (DES) cluster area. (A′,B′,C-C‴,D′) Lateral chordotonal organ (CH) cluster area. (A,A′) w^{1118} (control) embryos showing typical organization of the DES and CH cell clusters. (B,B′,C-C‴) cit^2/*Df(3)iro-2* mutant embryos exhibit variable abnormalities, such as absence of cells and multinucleate cells (arrows). The number of nuclei in these cells was estimated by presence of Lamin-positive nuclear envelopes within α-Spectrin delineated cellular membrane. A general disorganisation of both clusters and axonal misrouting was also observed. (D,D′) cit^2/*Df(3)iro-2* mutant embryos that are also heterozygous for pbl^3, showing an increase in the number of multinucleate cells (arrow). (E) To quantitate the effect of loss of one copy of the *pbl* gene on the *cit* phenotype, the number of mono- and multinucleate PNS cells in a defined region was scored for each genotype (cells were scored in 30 identical frames). Bars represent the percentage of multinucleate cells in each genotype. Error bars represent one standard deviation.

5G′,H′). These defects are clearly the later consequences of an earlier cell division failure, so we conclude that the primary *citron* larval brain phenotype is a failure of cell division.

Imaginal disc cells mutant for *citron* exhibit cell division defects accompanied by high levels of apoptosis

Examination of *citron* mutant larvae revealed a variable but marked reduction in the size of their imaginal discs (Fig. 6A,B). In line with these observations, we could only generate one or two cell somatic clones of homozygous mutant cells in the wing (data not shown). Cell sizes were not obviously different in the mutants, but wing and leg discs stained with anti-phospho Histone H3 (anti-PH3) showed a mild reduction in the number of mitotic cells relative to wild-type (down to 60% of wild-type for cit^1 and 77% for cit^2, Fig. 6C,D and data not shown). Since this level of perturbation might not be expected to produce such small discs, we examined the possibility that a stronger cell division phenotype could have been masked by the rapid clearance of mutant cells by apoptosis. The TUNEL assay for apoptotic cells and staining with the cell death marker Acridine Orange, revealed an increased level of apoptosis in mutant imaginal discs (Fig. 6E-H). Confirmation that apoptosis was clearing cells with a cytokinesis defect came from expression of the apoptosis inhibitor, p35 (Dorstyn and Kumar, 1997), in *citron* mutant imaginal discs, which led to the accumulation of multinucleate cells (see Fig. S1 in supplementary material).

Genetic interactions confirm Citron as a positive factor in Rho signalling

As discussed above, Citron has been proposed to act downstream of Rho in the regulation of cytokinesis. However, little in vivo evidence has been found to support this proposition. To test whether Citron participates in Rho signalling we examined genetic interactions between *citron* and a known regulator of the Rho pathway, the Rho-GEF-encoding gene, *pebble*. The first assay chosen was the ability to modify the moderate *citron* embryonic PNS phenotype (Fig. 4B,B′,C-C‴,E). We chose *pbl* mutants rather than Rho mutants because Pbl appears to be a specific Rho activator for cytokinesis (Prokopenko et al., 1999), whereas loss of Rho also affects many other processes (Crawford et al., 1998). We found that removing one copy of *pbl* in *cit* mutants resulted in a significant reduction in the overall number of cells in the PNS, while most of the remaining cells (52%) appeared to be multinucleate (Fig. 4D,D′,E). Therefore, a mild reduction in Pbl-mediated Rho activation during cytokinesis resulted in a significant enhancement of the *cit* mutant embryonic PNS defects. A complementary approach was to monitor whether under- or overexpression of *citron* could modify a loss-of-Pbl phenotype. Since strong *pbl* phenotypes arise too early and are too drastic to be of use, we generated an RNAi construct to inhibit Pbl synthesis later in development. Expression of this pbl^{RNAi} construct in the posterior half of the wing resulted in a decrease in the size of the corresponding region (Fig. 7C compared with Fig. 7A). Analysis of the affected area revealed

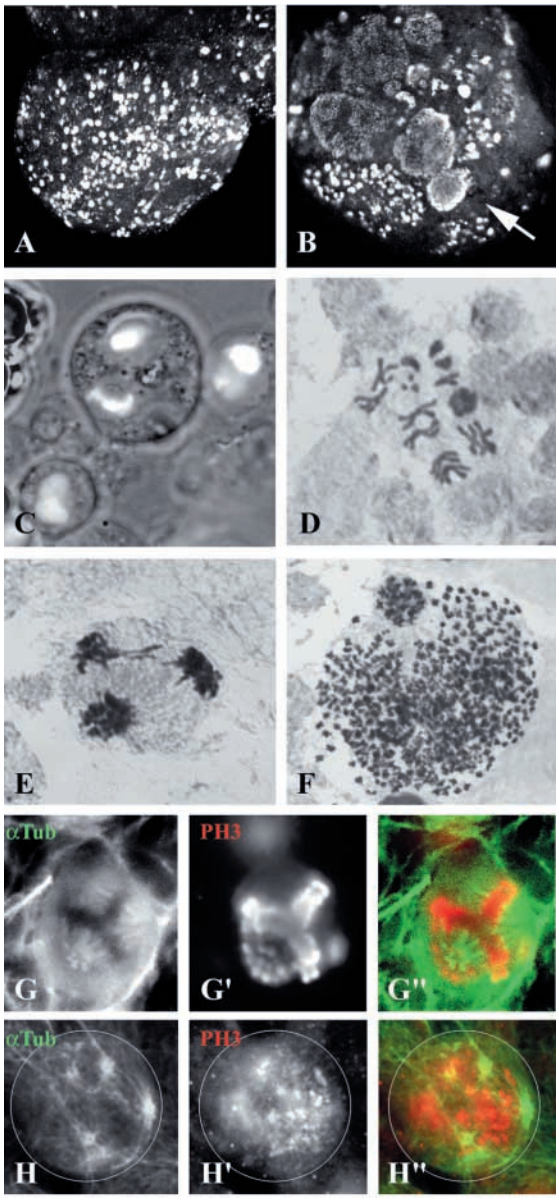

Fig. 5. Loss of *Drosophila citron* leads to failure of cytokinesis and hyperploidy in the larval brain. Wild-type (A) and cit^2/*Df(3)iro-2* mutant (B-H″) brain lobe tissues from third instar larvae. (A,B) Immunochemical staining of whole mount brain lobes with anti-phospho Histone H3 (PH3), to detect cells in mitosis, showing large nuclei (arrow) in cit^2/*Df(3)iro-2* cells (B) compared to wild-type (A). (C) Binucleate cells dissociated from cit^2/*Df(3)iro-2* mutant brain lobes stained with Hoechst 33258 to detect DNA (white) overlaid on the phase-contrast image of the cells. (D-F) Mitotic figures from cit^2/*Df(3)iro-2* mutant cells, which were squashed and stained with Giemsa dye to detect chromosomes. (D) Tetraploid cell at metaphase. (E) Hyperploid cell showing effective chromosome separation at anaphase. (F) Very highly hyperploid cell. (G-G″, H-H″) High magnification view of individual polyploid cells from mutant brain lobes stained with anti-α-Tubulin (G,H) and anti-PH3 (G′,H′). (G″,H″) Merged images. (G-G″) Abnormal metaphase. Three spindle poles are detected by anti-α-Tubulin staining (cf *pebble* mutants in Fig. 3E). (H-H″) The cell exhibits a highly polyploid content of condensed DNA and there are eight spindle poles (four in the field of view) associated with defective microtubule spindles that fail to assemble chromosomes at the cell equator (H′).

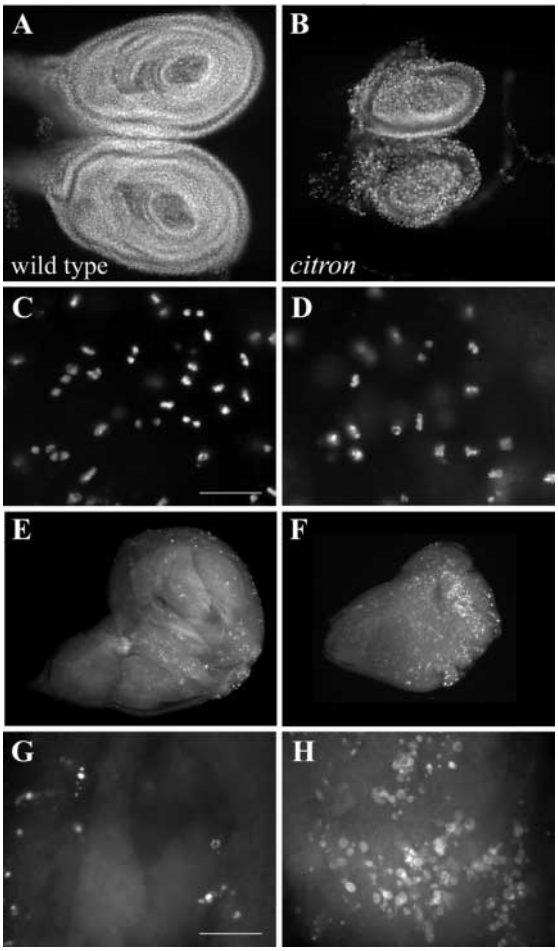

Fig. 6. Loss of *Drosophila citron* gene function results in a dramatic reduction in imaginal disc size. Imaginal discs from wild-type (A,C,E,G) and cit^2/*Df(3)iro-2* mutant (B,D,F,H) third instar larvae labelled with Hoechst 33258 to detect DNA (A,B) or with anti-PH3 to detect cells in mitosis (C,D, shown at high magnification) or labelled using TUNEL to detect cells undergoing apoptosis (E,F and high magnification in G,H). Transheterozygous mutant imaginal discs exhibit a dramatic reduction in size (e.g. leg discs shown in B, compare with wild type in A), contain fewer cells progressing through mitosis (compare D with C) and many more cells undergoing apoptosis (compare mutant wing disc in F and H with wild type in E and G). Scale bars: 25 μm.

that more than 67% of cells produce multiple hairs in contrast to the invariably single-haired cells in wild-type (Fig. 7C′,D′ compared with 7A′,B′), a phenotype observed when cytokinesis is blocked, for example by inhibition of *RacGAP50C* (Somers and Saint, 2003). As expected, co-staining of pupal wings with phalloidin and the DNA stain Hoechst 33258 revealed that the pbl^{RNAi}-expressing cells were abnormally large and typically multinucleate (Fig. 7D), resembling the embryonic phenotype of *pbl* mutants (Hime and Saint, 1992). The intermediate nature of the *en-GAL4>UAS-*pbl^{RNAi} wing size and multiple hair phenotypes allowed detection of enhancement and suppression by prospective interactors. Firstly, to test the specificity of this assay system we examined the pbl^{RNAi} phenotype in a *RhoA*/+ background. Significant diminution of the *pbl*-depleted region of the wing

(Fig. 7E) showed that the *pbl*^{RNAi} phenotype was enhanced by removal of one copy of wild-type *RhoA*, as seen in other genetic assays for *pbl* function (O'Keefe et al., 2001). We quantified the multiple hair phenotype in a defined wing region posterior to vein L5 (Fig. 7A, framed area). A significant increase in the proportion of multihaired cells from 67% to 84% upon loss of one copy of *RhoA* (Fig. 7C′,E′, Fig. 8E) showed that this assay could detect reductions in the dose of cytokinesis effector genes. Removal of one copy of wild-type *citron* also reduced the size of the posterior half of the wing in *en-GAL4>UAS-pbl*^{RNAi} flies (Fig. 8A,B) and enhanced the multiple hair phenotype (up to 86%; Fig. 8E). Identical effects were observed in *Df(3)iro-2* heterozygous mutants (Fig. 8E and data not shown). The genetic interactions between loss of function *citron* and *pbl* phenotypes support the role of Citron as a Rho effector in cytokinesis. Ectopic expression of *citron* in various *Drosophila* tissues generated no dramatic phenotype in wild-type or *pbl*^{RNAi} backgrounds (Fig. 8C,E) (T.S., unpublished), suggesting that the activity of Rho is rate limiting for Citron function.

Drosophila rok alleles do not interact genetically with the cytokinesis Rho pathway

It has been suggested that the human Rho effector kinase Rok plays some role in cytokinesis since inhibition of Rok function by Y-27632 led to significantly prolonged ingression of the cleavage furrow, although cytokinesis was eventually completed (Kosako et al., 2000). *Drosophila* Rok is not required for cytokinesis in wing cells, as somatic mutant *rok* clones do not display any reduction of size (Winter et al., 2001). However this test does not address the issues of delayed completion of cytokinesis or redundancy between Rok and Citron. *Drosophila rok* is uniformly expressed throughout development (Mizuno et al., 1999) and so is present in cells expressing *citron*, making redundant function possible. If the cytokinetic functions of Rok and Citron were redundant, we would expect that halving the dose of one would enhance the cytokinetic mutant phenotype of the other. Since *rok* mutants have no described cytokinetic phenotype we tested for genetic interactions between *rok*^{2}, a strong loss-of-function allele (Winter et al., 2001), and Rho signalling in cytokinesis by introducing the *rok*^{2} allele into our sensitised *pbl* RNAi wing assay. In contrast to the enhancement observed by removing one copy of *citron* (Fig. 8B,E), removal of one copy of *rok* made no significant difference to the *pbl*^{RNAi} mutant phenotype (67% and 65% respectively, Fig. 8D,E). We therefore failed to find evidence in support of a key role for Rok in Pbl-Rho signalling during cytokinesis.

Discussion

A conserved requirement for Citron in animal cytokinesis

We show here that the *Drosophila melanogaster citron* gene is essential for normal cell division in all of the tissues we examined, including the central and peripheral nervous systems, larval brain cells and larval imaginal tissues. Consistent with a role specifically in cell division, *citron* transcripts were detected in proliferating tissues, and *citron* expression was downregulated in post-proliferative cells. This contrasts with the ubiquitous expression of Rho and another

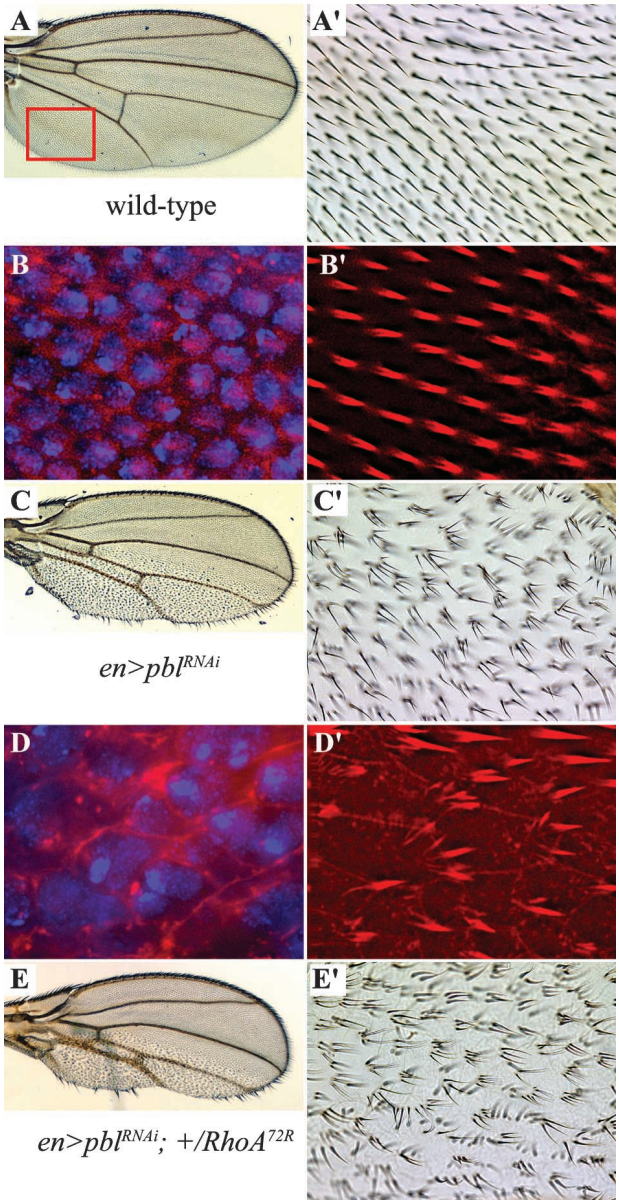

Fig. 7. Reduction of Pebble function in wings by RNA interference causes cytokinetic failure. (A,C,E) Low magnification images of whole adult wings. (A′,C′,E′) Corresponding high magnification images of the posterior region of the wing (framed in A) that was used to evaluate phenotypes (see Fig. 8E). (A,A′) A wild-type wing showing normal size and shape of the wing (A) and ordered arrangement of hairs (A′). (B,B′) A high magnification of a wild-type pupal wing stained with Phalloidin to detect F-actin (red) and Hoechst 33258 for DNA (blue). Each individual wild-type cell has one nucleus (B) and one prehair F-actin bundle (B′). (C,C′) An *en-GAL4>UAS-pbl*^{RNAi} wing showing diminution of Engrailed-domain of the wing (C) and wing hair phenotype (C′) resulting from reduced *pbl* activity. Many *en-GAL4>UAS-pbl*^{RNAi} adult wing cells have more that one hair. (D,D′) A high magnification view of an *en-GAL4>UAS-pbl*^{RNAi} mutant pupal wing stained with Phalloidin for F-actin (red) and Hoechst 33258 for DNA (blue). Mutant cells show more than one nucleus (D) and prehair F-actin bundles (D′). (E,E′) Loss of one copy of *RhoA*^{72R} further reduces the Engrailed-specific region of the *en-GAL4>UAS-pbl*^{RNAi} wing (E) and increases the number of multihaired cells (E′). The multiple haired cell phenotype is quantified in Fig. 8E.

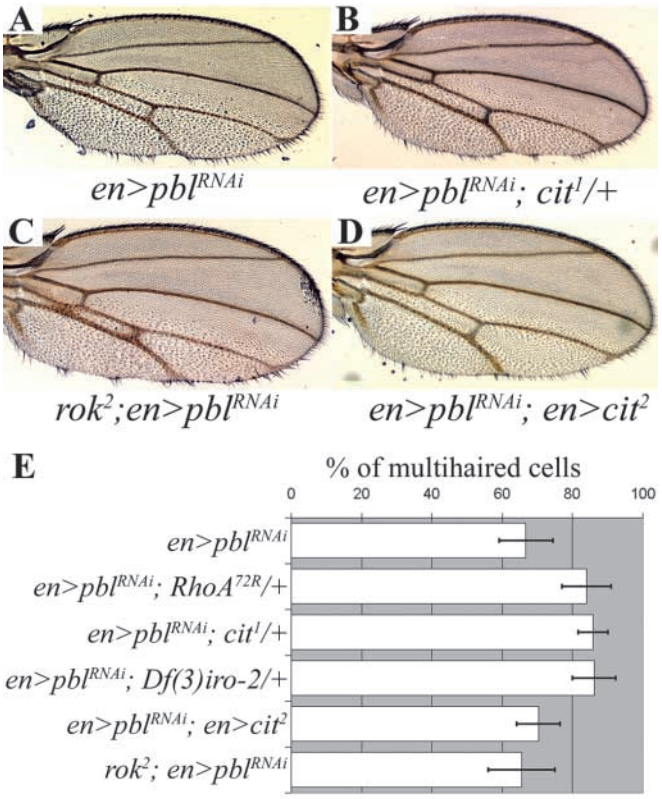

Fig. 8. Citron acts as a positive factor in the Pebble-Rho signalling pathway. Images of whole adult wings. (A) An *en-GAL4>UAS-pbl*^{RNAi} wing in a wild-type background. (B) An *en- GAL4>UAS-pbl*^{RNAi} wing heterozygous for *cit*^{1} shows significant enhancement of the *pbl*^{RNAi} phenotype. (C) An *en-GAL4>UAS-pbl*^{RNAi} wing heterozygous for *rok*^{2} does not appear be significantly modified. (D) Co-expression of a gain-of-function allele of *citron* (*UAS-cit*^{2}) and *en-GAL4>UAS-pbl*^{RNAi} slightly rescues the *pbl*^{RNAi} phenotype. (E) The number of multiple haired wing cells in a defined region (posterior to L5, framed in Fig. 7A) was scored for each genotype shown in Fig. 7 and Fig. 8 in order to quantitate the effect. Multiple haired cells were scored in 14 adult wings for each genotype. Bars represent the percentage of multihaired cells in each genotype. Error bars represent one standard deviation. Statistical significance of the difference between two pairs of genotypes was determined using a χ^2 2×2 contingency test (P<0.001). Significant enhancement of the *en-GAL4>UAS-pbl*^{RNAi} phenotype was seen when flies were heterozygous for *RhoA*^{72R}, *cit*^{1} or *Df(3)iro*-2 alleles.

Rho effector kinase, Rok, both of which play roles in non-proliferating cells during *Drosophila* development (Di Cunto et al., 2000; Hariharan et al., 1995; Mizuno et al., 1999). Our analysis of the *Drosophila citron* mutant phenotype revealed a widespread function in proliferation. In S2 cultured cells treated with *citron* dsRNA, binucleate cells were observed. Furthermore, the PNS of transheterozygous *citron* mutant embryos exhibited a loss of neurons and concomitant appearance of multinucleate neurons, similar to the phenotype seen in hypomorphic *pbl* and other cell cycle control mutants (Hartenstein and Posakony, 1990; Salzberg et al., 1994). *citron* mutant larval brain cells also showed a significant number of binucleate cells. These binucleate cells presumably occurred due to the failure of a single round of cell division. Some of the brain cells had undergone multiple rounds of mitosis without cell division, as evidenced by multiple microtubule spindles, an increase in the number of chromosome complements and polyploid anaphase figures, indicating assembly of an effective mitotic spindle. However, with further increases in cell ploidy and centrosome numbers, the spindle did not form properly and chromosomes were lost at metaphase, but the cells evidently continued to cycle, eventually producing giant cells filled with chromosomes. This brain phenotype is similar to *spaghetti squash* (myosin regulatory light chain) and *diaphanous* mutant phenotypes (Castrillon and Wasserman, 1994; Karess et al., 1991), but is different to phenotypes of mutants such as *makós* or *aurora* that arrest in metaphase (Deák et al., 2003; Glover et al., 1995). We conclude, therefore, that *Drosophila* Citron has an important and conserved function during cytokinesis. In contrast to larval brain cells and embryonic PNS cells, polyploidy and binucleate cells were not observed in larval imaginal tissues. Rather, the discs were dramatically reduced in size and exhibited high levels of apoptosis. The analysis of other tissues shows that *citron* is not generally required for cell survival and we found that inhibition of apoptosis in *citron* mutant discs resulted in the accumulation of multinucleate cells. Therefore, we reason that imaginal cells differ from their brain counterparts by possessing a checkpoint control mechanism that triggers apoptosis following the failure of cell division. The key cytokinetic mutant *diaphanous* (*dia*) exhibits similar hyperploid neuroblasts and loss of larval imaginal tissue (Castrillon and Wasserman, 1994).

Citron is a critical Rho effector in the regulation of cytokinesis in vivo

We have shown that *Drosophila* Citron, like its mammalian counterparts, is localised to the cleavage furrow during cytokinesis. Many of the important regulators of cytokinesis such as the RhoA GTPase and its activators, and structural components such as myosin and actin, are concentrated in this structure (for a review, see Guertin et al., 2002). Importantly, *Drosophila* Citron localisation to the cleavage furrow depends on proper activation of the RhoA cytokinesis signalling pathway. In *pbl* mutant embryos lacking the Pbl Rho-GEF, which activates RhoA during cytokinesis, accumulation of Citron-GFP into the contractile ring does not occur. In vivo confirmation of the importance of Citron function in the Rho cytokinesis signalling pathway came from strong genetic interactions between *cit* and *pbl* mutants using two independent genetic assays, one based on the appearance of multinucleate cells in the PNS and one based on a developing wing phenotype. In both cases, loss of function of one of the genes strongly enhanced the phenotype of the other. The *citron* mutant cytokinesis phenotypes and the enhancement of *cit* and *pbl* phenotypes by a reciprocal reduction in their gene activity provides, for the first time, clear genetic evidence for the involvement of Citron in the Rho cytokinesis signalling pathway. The interaction between active Rho and Citron observed in yeast two-hybrid assays (Madaule et al., 1995) (this study) and the loss of Citron localisation to the cleavage furrow in *pbl* mutant embryos (this study) and in response to Rho inhibitors (Eda et al., 2001) demonstrate that the role of Citron in this pathway is as a downstream effector of Pbl-activated Rho.

Distinct roles in *Drosophila* for the two Rho effector kinases, Citron and Rok

The relationship between the two Rho effector kinases, Citron and Rok, is the subject of ongoing discussion (see Li and Minden, 2003). We have demonstrated a non-redundant essential role for Citron in cell division in all *Drosophila* tissues examined in our study. *Drosophila* Rok plays an important role in planar cell polarity (PCP) via regulatory phosphorylation of myosin light chain (MLC) and subsequent activation of non-muscle myosin II (Winter et al., 2001). We could not detect any PCP specific phenotype in *citron* mutants. Moreover, overexpression or loss of one copy of *citron* had no effect on a PCP-specific *dishevelled*^{1} mutant phenotype (S.L.G., unpublished) arguing against any involvement of Citron in the control of planar polarity by Rho. Conversely, we could find no evidence for a role for Rok in cell division in *Drosophila*. Evidence exists for such a role for human Rok, with depletion of its activity in HeLa cells by chemical inhibitors leading to a substantial delay in the completion of cytokinesis (Kosako et al., 2000). However, other studies using the same cells and Rok inhibitor saw no effect or early completion of cytokinesis (Chevrier et al., 2002; Madaule et al., 1998). Higher doses of Rok inhibitor could effectively prevent cytokinesis, but these concentrations also inhibit Citron (Ishizaki et al., 2000). In *Drosophila*, homozygous *rok*^{2} eye clones do not differ in size from their homozygous wild-type twin spots (Winter et al., 2001). Our analysis of genetic interactions also failed to produce any evidence of a role for Rok in Rho cytokinesis signalling.

In summary, our genetic analyses have demonstrated an essential role for *Drosophila* Citron in cell division and provided clear genetic evidence for its function, in vivo, as an effector in Pbl-activated Rho signalling during cytokinesis. Our analysis has also established a set of genetic tools that will allow a detailed dissection of the roles of Citron within the context of a developing organism.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/131/20/5053/DC1

Note added in proof

After acceptance of this manuscript, the *citron* gene described here was reported as the gene *sticky* by D'Avino et al. (D'Avino et al., 2004).

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