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12. PVC tubing or microtiter plates were coated with antibodies to HA (BAbCO, Richmond, CA) [10 μg/ml in 20 mM $NaHCO_3$ (pH 9.6)] at 4°C overnight. Plates or tubing were washed with Pipes saline [50 mM Pipes (pH 7.0), 150 mM NaCl] and then incubated with 3% (w/v) bovine serum albumin (BSA) (ELISA grade, Sigma) in Pipes saline for 1 hour at room temperature.
13. COS-7 cell extracts were diluted 1:40 with Pipes saline containing 0.1% BSA, 10% (v/v) glycerol, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, leupeptin (10 μg/ml), and pepstatin (10 μg/ml) (Sigma) and then incubated in wells or tubing for 2 to 3 hours at 4°C. This procedure allowed approximately 10 to 15% of the kinase present in the extract to bind to the plastic, saturating the coated antibody. The plastic was washed three times with Pipes saline containing 0.1% BSA and 0.1% Tween-20 and once with the same solution without Tween-20 and was then maintained on ice in Pipes saline containing 0.1% BSA until experimental treatment.
14. Kinase activity was measured in 96-well microtiter plates in 50 mM Pipes (pH 7.0), 10 mM $MgCl_2$, 0.01% BSA, 15 μM AC-3, 25 μM [γ-^{32}P]ATP (1 Ci/mmol), and either 500 μM EGTA (Ca^{2+}-independent activity) or 500 μM $CaCl_2$ and 2 μM calmodulin (Ocean Biologicals, Edmonds, WA) (maximal Ca^{2+}-stimulated activity) in a total volume of 30 μl at 30°C. After 60 s, assays were terminated by pipetting and spotting assay mix on P81 phosphocellulose paper (*27*). The ability of the immobilized kinase to undergo autophosphorylation was examined by incubation for 15 s with stimulus buffer [50 mM Pipes (pH 7.0), 10 mM $MgCl_2$, 0.01% BSA, 250 μM ATP, 500 μM $CaCl_2$, 1 μM calmodulin], after which the solution was replaced with an assay mix containing 500 μM EGTA and the effect of autophosphorylation measured as Ca^{2+}-independent activity (autonomous activity), expressed as a percentage of the maximal Ca^{2+}-stimulated activity. Incubation of autophosphorylated α–CaM kinase II for up to 2 min did not result in a decrease in autonomous activity, suggesting that little or no phosphatase activity was present together with the immobilized enzyme.
15. The flow rate was 5 ml/s, assuring virtually complete (>95%) exchange of solutions inside a tubing (internal diameter, 1.58 mm) 2 cm in length with a 20-ms valve opening or, for most experiments, inside a tubing 4 cm in length with a 50-ms open time (>200 μl of flow through a 15-μl dead volume inside the manifold plus a volume of 80 μl inside the tubing).
16. After 100 pulses (50 ms each, 1 Hz) of perfusion solution (30°C), kinase activity in the tubing was 94 ± 5% (n = 6) of that in untreated tubing.
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19. The phosphorylation of Thr^{286} in one subunit facilitates that of Thr^{286} in a second subunit, resulting in functional cooperativity because a Thr^{286}-phosphorylated subunit can phosphorylate a neighbor without having to bind calmodulin (*7, 8*).
20. α–CaM kinase II was prephosphorylated by a 300-ms pulse of Ca^{2+}, calmodulin (1 μM), and ATP (from the third valve), which was followed by a 400-ms wash and 1-s delay prior to stimulation at 1 Hz. An initial control pulse, with Ca^{2+} and calmodulin but not ATP, generated no autonomy, and the subsequent response to 1-Hz stimulation did not differ from that obtained without a control pulse (Fig. 3B).
21. We confirmed co-assembly of both CaM kinase II isoforms by immunoprecipitation with an antibody to β–CaM kinase II (CB-β-1) [C. Baitinger, J. Alderton, M. Poenie, H. Schulman, R. A. Steinhardt, *J. Cell Biol.* **111**, 1763 (1990)] from a lysate of COS-7 cells transfected with equal amounts of cDNAs encoding HA-tagged α– and β–CaM kinase. Precipitated proteins were separated by SDS–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and subjected to calmodulin overlay blot analysis (*8*). Immunoprecipitated α– and β–CaM kinase II heteromers contained approximately equal amounts of each subunit.
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29. This exponential function is used to illustrate the relation between frequency of stimulation and CaM kinase II autonomy without any implied model. Data were fitted with PSI-PLOT software; the correlations for the fits in Fig. 4 were all ≥0.95, with SSD values of <0.35. The significance of differences between curves was determined with an F test comparing the variance of the pooled data set with the sum of the variance of the individual data sets.
30. We thank D. Profitt and R. Schneeveis for building the pulse-flow device; S. Sather for help with site-directed mutagenesis and early studies on immobilized kinase; A. Braun, P. Hanson, T. Meyer, A. Naini, and R. Y. Tsien for helpful discussions; and J. Ferrell and L. Stryer for their comments on the manuscript. Supported by NIH grants GM40600 and GM30179 and by a Human Frontier Science Program Organization fellowship to P.D.K.

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Requirement for DCP-1 Caspase During *Drosophila* Oogenesis

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Caspases, a class of cysteine proteases, are an essential component of the apoptotic cell death program. During *Drosophila* oogenesis, nurse cells transfer their cytoplasmic contents to developing oocytes and then die. Loss of function for the *dcp-1* gene, which encodes a caspase, caused female sterility by inhibiting this transfer. *dcp-1*$^-$ nurse cells were defective in the cytoskeletal reorganization and nuclear breakdown that normally accompany this process. The *dcp-1*$^-$ phenotype suggests that the cytoskeletal and nuclear events in the nurse cells make use of the machinery normally associated with apoptosis and that apoptosis of the nurse cells is a necessary event for oocyte development.

Apoptosis, a form of programmed cell death, is a mechanism used by organisms to remove cells that are superfluous, abnormal, or no longer needed (*1*, *2*). The failure of cells to undergo apoptosis at the appropriate time during development can lead to abnormal differentiation of tissues and the death of the organism (*3*). Large numbers of cells of the developing germ line of vertebrates and invertebrates are lost as a result of apoptosis (*4*); however, it is not clear if apoptosis is a required event in the germ line of some organisms.

The *Drosophila* ovary consists of individual egg chambers, each of which contains 16 sister germline cells that remain interconnected because of incomplete cytokinesis (*5*, *6*). One germ cell becomes an oocyte, and the rest develop into nurse cells that are linked to the oocyte by cytoplasmic bridges (ring canals). The nurse cells become polyploid and synthesize large amounts of RNA and protein, which are

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rapidly transferred to the oocyte through the ring canals as a result of actin- and myosin-based contraction of the nurse cells late in oogenesis (*7*, *8*). Several hours later, the nurse cell nuclei degenerate, and the oocyte proceeds through the final stages of oogenesis. Mammalian ovarian development also includes the formation of intercellular bridges between germ cells at a period of massive germline cell death (*9*).

Caspases, a class of cysteine proteases, are an essential component of the apoptotic machinery (*10*). Upon activation, caspases cleave a variety of substrates including structural and regulatory proteins (*10*, *11*). Three *Drosophila* caspases have been identified (*12*, *13*), with mutations isolated in one of them, DCP-1. Homozygous *dcp-1* mutants die as larvae (*12*), yet homozygous embryos do not show substantial defects in cell death. However, a role for *dcp-1* in early embryonic cell death cannot be ruled out because *dcp-1* mRNA is synthesized in the ovary and maternally supplied to early embryos (*12*).

To generate a complete loss of function for *dcp-1*, we removed the maternal component by generating homozygous *dcp-1*$^-$ clones in the female germ line (*14*). Females carrying the *dcp-1*$^-$ germline clones (GLCs) were sterile. Although the *dcp-1* GLC females could lay a few eggs, these embryos failed to develop properly, and most embryos were arrested within the first few mitotic divisions. The early arrest of *dcp-1* GLC embryos indicated that oogenesis was not proceeding normally. Indeed, dissected ovaries from *dcp-1* GLC females contained many abnormal late-stage egg chambers (Fig. 1 and Table 1). These egg chambers showed a "dumpless" phenotype (*6*) in which nurse cell contents were not transferred into the oocyte. Nurse cells die by apoptosis late in oogenesis (*15*), because they stain positively for TUNEL (Tdt-mediated deoxyuridine triphosphate nick end labeling) and acridine orange (AO), two markers for apoptotic cells (*16*). Using these methods, we examined whether apoptosis occurred in the *dcp-1* GLC nurse cells (Fig. 1). In control egg chambers, the nurse cell nuclei stained positively for TUNEL and AO during stages 12 and 13 of oogenesis (*15*) [stages according to (*5*)]. In contrast, in *dcp-1* GLC egg chambers, TUNEL staining was frequently delayed and was found in stage 14 egg chambers. Therefore, the loss of *dcp-1* function did not completely inhibit nurse cell apoptosis.

To further analyze the nurse cell phenotype in the *dcp-1* GLC egg chambers, we used a nuclear β-galactosidase (β-Gal) marker carried on the *dcp-1* mutant chromosome (*17*). Ovaries from *dcp-1* heterozygotes and *dcp-1* GLC females were stained with the use of X-gal as a substrate for

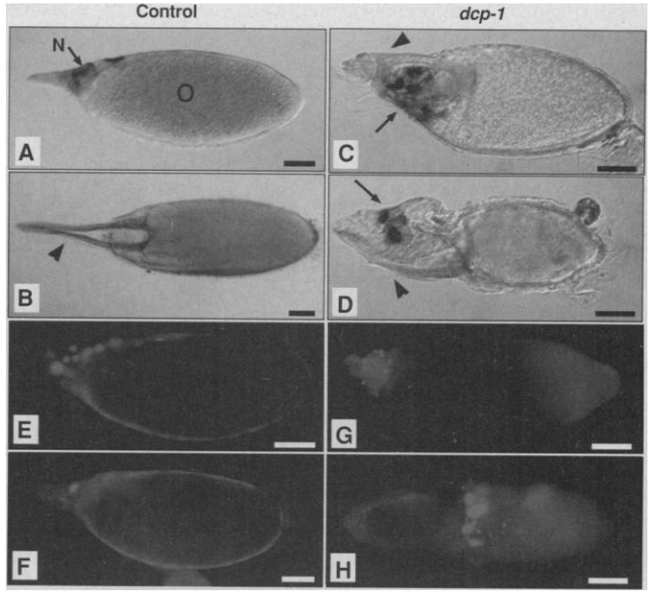

Fig. 1. Dumpless egg chambers from *dcp-1* GLC females with a delay in apoptosis. Egg chambers are labeled with TUNEL (A to D) or AO (E to H) (*16*). All panels are oriented with anterior to the left. (**A**) Stage 13 control egg chamber shows TUNEL-positive staining in the nurse cell cluster. N, nurse cell cluster; O, oocyte. (**B**) By stage 14, the TUNEL-positive material has been cleared from the egg chamber. Arrowheads indicate dorsal appendages that are fully developed at stage 14. (**C** and **D**) Stage 14 *dcp-1* GLC egg chambers have TUNEL-positive nurse cell nuclei. The morphology of these egg chambers is abnormal because a substantial amount of nurse cell cytoplasm (arrows) has failed to be transferred to the oocyte. (**E**) Stage 12 control egg chamber shows AO-positive nurse cell nuclei. (**F**) Stage 13 control egg chamber with one AO-positive nucleus. (**G**) Stage 14 *dcp-1* GLC egg chamber has a substantial amount of AO-positive staining. (**H**) Another stage 14 *dcp-1* GLC egg chamber with a severe dumpless phenotype stains positively for AO. Scale bars, 50 μm.

Table 1. Expressivity of the *dcp-1* phenotype. The severity of the *dcp-1* phenotype is likely to be underestimated because of a low frequency of recombination that has been observed between the *dcp-1* mutant chromosome and the CyO balancer and because of incomplete penetrance of the Cy phenotype. Variability of the *dcp-1* phenotype may be due to the perdurance of *dcp-1* mRNA or protein or the activity of a related caspase, drICE, which is present during oogenesis (*13*). Frequency and strength of the stage 14 dumpless phenotype are given. Egg chambers were scored as strong (for example, Fig. 1D), weak (for example, Fig. 1C), or normal. *chic*, a dumpless mutant (*8*), was used as a control.

Genotype	Strong phenotype (%)	Weak phenotype (%)	Normal phenotype* (%)	Total number
dcp-1^{2132}/+	0	1	99	207
dcp-1^{1862}/+	1	2	98	265
chic^{9951}	12	78	10	244
dcp-1^{2132} GLC	27	28	45	97
dcp-1^{1862} GLC	33	26	40	121

*Eggs appear normal overall but do not develop properly in *dcp-1* and *chic* mutants.

Table 2. Frequency of egg chambers with discrete nuclear β-Gal staining.

Genotype	Stage 10 (%)	Stages 11 and 12 (%)	Stage 13 (%)	Stage 14 (%)
dcp-1^{2132}/+	100 (n = 61)	44 (n = 9)	0 (n = 25)	0 (n = 207)
dcp-1^{1862}/+	100 (n = 116)	48 (n = 21)	0 (n = 61)	0 (n = 265)
dcp-1^{2132} GLC	100 (n = 53)	94 (n = 34)	55 (n = 20)	27 (n = 89)
dcp-1^{1862} GLC	100 (n = 66)	93 (n = 27)	72 (n = 25)	33 (n = 111)

Table 3. Frequency of egg chambers with cytoplasmic actin bundles.

Genotype	Stage 10 (%)	Stages 11 and 12 (%)	Stage 13 (%)	Stage 14* dumpless (%)
Control	29 (n = 77)	100 (n = 16)	100 (n = 19)	–
dcp-1^{1862} GLC	3 (n = 64)	24 (n = 37)	82 (n = 17)	7 (n = 27)

*Phenotype scored only in strong dumpless egg chambers.

β-Gal (Fig. 2). Heterozygous females displayed wild-type egg chambers with β-Gal activity that was tightly localized to nurse cell nuclei at early stages. Beginning in stage 10B, nurse cell nuclei became permeable, and β-Gal diffused into the cytoplasm. β-Galactosidase was then transferred to the oocyte during the dumping stage. The *dcp-1* GLC ovaries had normal β-Gal expression in early egg chambers. However, the *dcp-1* GLC nurse cell nuclei were not permeable during stage 10B, and β-Gal remained nuclear even in some late stage 14 egg chambers (Table 2). Additionally, β-Gal was frequently not transferred to the oocyte. In this assay, β-Gal serves as a marker for the likely fate of other nurse cell nuclear proteins. A failure to transfer critical nuclear proteins may explain the early mitotic arrest of the *dcp-1* GLC embryos.

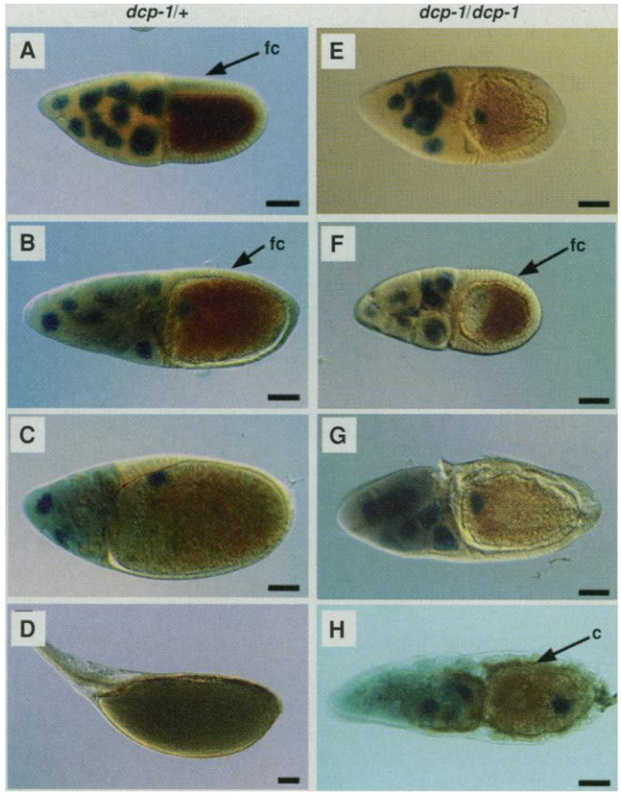

Fig. 2. Nurse cell nuclear permeability blocked in *dcp-1*$^-$ egg chambers. Heterozygous *dcp-1*/+ (A to D) and *dcp-1* GLC (E to H) egg chambers were stained with X-gal to detect β-Gal activity (*17*). Heterozygotes had egg chambers that appeared wild type. (**A**) Stage 10A egg chamber displays nuclear β-Gal staining in the nurse cells and oocyte. Follicle cells (fc) have a columnar shape. (**B**) Stage 10B egg chamber. β-Galactosidase has diffused out of nurse cell nuclei that are most proximal to the oocyte. Follicle cells (fc) have flattened over the oocyte. (**C**) Late stage 11 egg chamber. β-Galactosidase has diffused out of all nurse cell nuclei, except the most distal ones, and has begun to be transferred to the oocyte. (**D**) Stage 14 egg chamber. All β-Gal has been transferred to the oocyte. (**E**) Stage 10A *dcp-1* GLC egg chamber. (**F** and **G**) Stage 10B (F) and 12 (G) *dcp-1* GLC egg chamber. β-Galactosidase remains nuclear and the follicle cells fail to flatten. The size of the mutant egg chambers at late stages was generally smaller than that of the wild-type egg chambers. (**H**) Stage 14 *dcp-1* GLC egg chamber, with chorion (c). Scale bars, 50 μm.

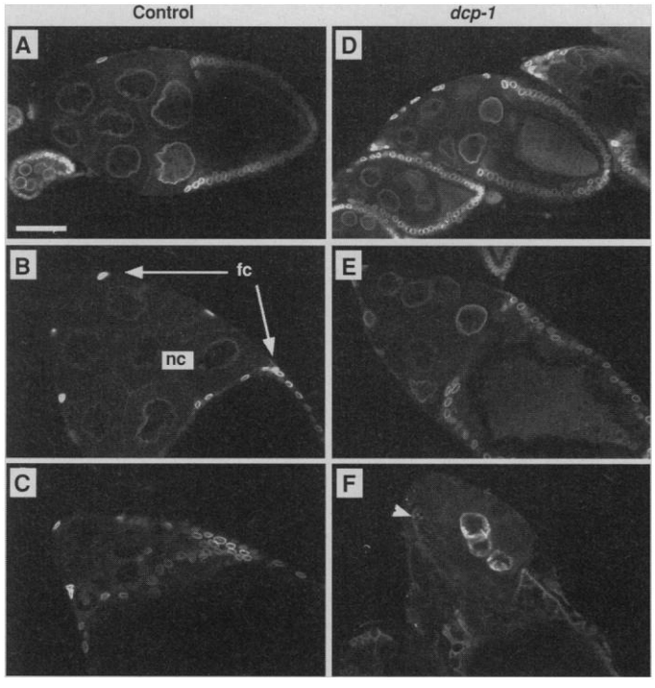

Fig. 3. Inhibition of nuclear lamin breakdown in *dcp-1*$^-$ nurse cell nuclei. Confocal images of control (A to C) and *dcp-1* GLC (D to F) egg chambers labeled with mAb 101, which detects nuclear lamin Dm_0 (*21*). (**A** and **D**) In early stage 10A, mutant and wild-type egg chambers are indistinguishable. (**B** and **C**) Nuclear lamin staining becomes diffuse in stage 11 (B) and 12 (C) control egg chambers. Note the difference in intensity between nurse cells (nc) and follicle cells (fc). (**E** and **F**) *dcp-1* GLC egg chambers continue to show distinct nuclear staining at stages 11 (E) and 14 (F). Arrowhead indicates dorsal appendage. Scale bar, 50 μm.

Breakdown of the nuclear envelope is a central event during apoptosis and is accompanied by caspase-mediated cleavage of nuclear lamins (*18*, *19*). Nuclear lamins are intermediate filament proteins that assemble next to the inner nuclear membrane to form the nuclear lamina (*20*). To address whether nuclear lamins were degraded as the nurse cell nuclei became permeable, we examined egg chambers for the distribution of lamin Dm_0, a *Drosophila* homolog of mammalian lamin B (*21*) (Fig. 3). A monoclonal antibody (mAb) to Dm_0 revealed the loss of lamin Dm_0 signal in control nurse cells. Whereas early egg chambers showed sharp nuclear envelope staining of nurse cells, by stage 11, lamin staining was a diffuse cytoplasmic cloud around the nuclei. In contrast to the control, *dcp-1* GLC nurse cells continued to show distinct nuclear envelope staining even as late as stage 14. Thus, *dcp-1* mutants were defective in the cleavage or disassociation, or both, of nuclear lamins. This failure in lamin breakdown is a likely cause of the defect in nuclear permeability revealed by the β-Gal marker. Lamin breakdown may be directly due to DCP-1 protease activity because purified DCP-1 protein is capable of cleaving lamin Dm_0 in vitro (*22*). In mammalian tissue culture cells, mutation of the caspase cleavage site in nuclear lamin A causes abnormal morphology and a delay in the nuclear dissolution of cells undergoing apoptosis (*19*). Although this effect on cultured cells is transient because the cells still die, in a developing tissue such as the *Drosophila* egg chamber precise timing may be critical. The failure to cleave correct substrates at the appropriate time in development may lead to severe abnormalities.

The process of nurse cell dumping is accompanied by alterations in the actin cytoskeleton (*7*, *8*, *23*). To examine whether *dcp-1* mutants were defective in the cytoskeletal reorganization in nurse cells, we stained egg chambers with rhodamine-phalloidin, which binds to filamentous actin (Fig. 4) (*24*). Actin was localized to the plasma membrane during early stages in control and *dcp-1* GLC egg chambers. During stage 10B in control egg chambers, actin bundles formed throughout the cytoplasm,

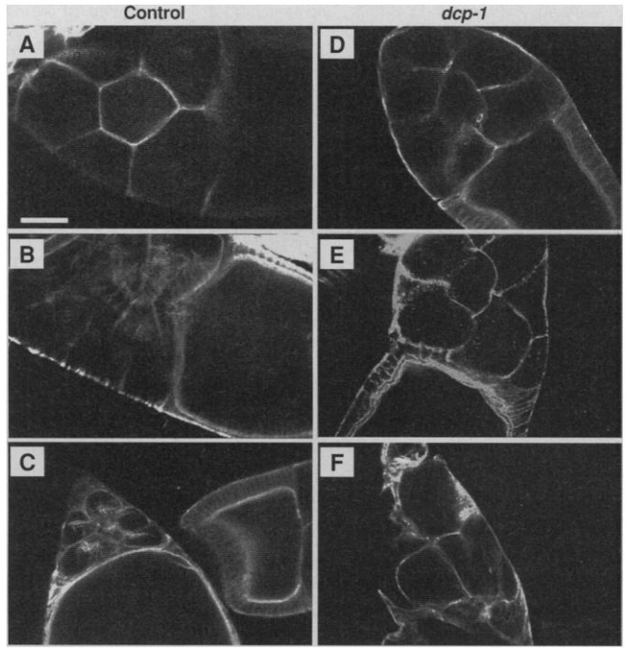

Fig. 4. Failure to reorganize filamentous actin in *dcp-1*$^{-}$ egg chambers. Confocal images of control (A to C) and *dcp-1* GLC (D to F) egg chambers labeled with rhodamine-phalloidin (*24*). (**A** and **D**) Stage 10A egg chambers show subcortical actin staining. (**B** and **C**) Cytoplasmic actin bundles have formed in stage 10B (B) and 12 (C) egg chambers. (**E** and **F**) Actin bundles fail to form in *dcp-1* GLC egg chambers at stages 11 (E) and 14 (F). Scale bar, 50 μm.

connecting the nuclei and plasma membrane. In contrast, actin in many *dcp-1* GLC egg chambers remained associated with the plasma membrane, even in stage 14 egg chambers (Table 3). Therefore, *dcp-1* activity is required for the proper formation of cytoplasmic actin bundles in nurse cells.

Mutations in certain genes, such as *chickadee* and *singed*, lead to dumpless phenotypes (*8*, *25*) similar to *dcp-1* GLCs. Molecular analysis of these genes revealed that they encode *Drosophila* homologs of the actin-binding proteins profilin and fascin, respectively. Mutants lacking these genes showed a disruption of the process of actin polymerization and bundling and are thought to display dumpless phenotypes because the nurse cell nuclei become lodged within the ring canals during nurse cell dumping. The nuclei within the ring canals then obstructed the flow of cytoplasm to the oocyte. Nurse cell nuclei within the *dcp-1* GLC egg chambers remained well spaced and did not appear to block the ring canals, suggesting that *dcp-1* activity may be required for the actin- and myosin-based contraction that drives the dumping process.

These cytoskeletal changes seen in the nurse cells are similar to events that occur in cells undergoing classical apoptosis (*1*, *26*, *27*). In cultured cells, cytoskeletal alterations lead to the formation of blebs and apoptotic bodies. Cytoskeleton-associated proteins such as fodrin, Gas2, and PAK2 may play critical roles in cytoskeletal reorganization because these proteins are cleaved during apoptosis or in vitro by caspases (*11*, *28*). Disruption of actin polymerization or PAK2 function blocks the formation of apoptotic bodies (*11*, *27*).

In the *Drosophila* ovary, the process of nurse cell cytoplasmic dumping and degeneration occurs in a highly ordered and reproducible manner. Our results show that a caspase is required for multiple events during this process. The *dcp-1* GLC nurse cells were defective in nuclear breakdown, cytoskeletal reorganization, and membrane contraction. Although these morphological changes are characteristic of cells undergoing apoptosis, there are several unusual aspects to this nurse cell death. DNA fragmentation in the nurse cells occurs after the majority of cytoplasm has been lost. It is likely that apoptotic effectors, such as active DCP-1 protein, are transferred to the oocyte during the dumping process. However, the oocyte escapes damage, suggesting the existence of a protective mechanism. Whereas current views of apoptosis invoke extensive degradation of cellular proteins and organelles (*10*, *29*), nurse cells contribute functional proteins and mitochondria to the oocyte (*30*). Our findings imply that the activity of caspases in this system is inhibited or restricted, perhaps by cellular compartment or substrate availability. In this way, precise surgical cuts are made of only the appropriate targets. Finally, this process is a clear example of how a single cell, the oocyte, uses the death of its sister cells to develop properly.

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14. Germline clones were generated with the FLP recombinase–dominant female sterile technique [T. Chou and N. Perrimon, *Genetics* **144**, 1673 (1996)]. This technique permits the efficient recovery of clones in over 90% of females in the background of a dominant female sterile mutation such that the only functional germ line is derived from the desired homozygous mutant. Germline clones were generated for two P element alleles of *dcp-1* (*dcp-1*^{1862} and *dcp-1*^{2132}). Females of the genotype *y w*; *P[FRT]*$^{2R\text{-}G13}$ *dcp-1/CyO* were crossed to *y P[HSflp]*^{122}; *P[FRT]*$^{2R\text{-}G13}$ *P[ovoD1]*^{32x9}*/CyO* males and allowed to lay eggs for 1 day. Larval progeny were heat shocked on days 4 and 5 for 1 hour at 37°C to induce mitotic clones in the developing ovary. Progeny females carrying the clones were collected after emergence (genotype *y P[HSflp]/y w*; *P[FRT] P[ovoD1]/P[FRT] dcp-1*) and mated to either *y w* males or their brothers. Newly emerged flies were fed on wet yeast paste for 2 to 5 days before analysis. Seven independent recombinant lines (*P[FRT] dcp-1*) were analyzed initially and found to be sterile. For further experiments, one recombinant line from each *dcp-1* allele was examined; the phenotypes were similar for both alleles (Table 1). As a control, females of the genotype *y w*; *P[FRT]*$^{2R\text{-}G13}$ were carried through the same procedures.
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16. The TUNEL method [Y. Gavrieli, Y. Sherman, S. A. Ben-Sasson, *J. Cell Biol.* **119**, 493 (1992)] detects fragmented DNA in apoptotic cells. We used a protocol modified from K. White, E. Tahaoglu, and H. Steller [*Science* **271**, 805 (1996)]. Briefly, dissected ovarioles were fixed in 4% paraformaldehyde in phosphate buffered saline plus heptane for 30 min, washed several times in PBT (phosphate-buffered saline plus 0.1% Tween 20), treated with proteinase K (10 μg/ml) for 5 min, washed in PBT, and treated as described with reagents from the Apoptag Plus kit (Oncorr, Gaithersburg, MD). For detection, an alkaline phosphatase–conjugated antibody to digoxigenin and reagents from the Genius kit were used (both from Boehringer Mannheim). The AO method also detects apoptotic cells [J. M. Abrams, K. White, L. I. Fessler, H. Steller, *Development* **117**, 29 (1993)]. We used a protocol from (*15*). The dumpless phenotype was variable: In one

experiment, 35% of *dcp-1* GLC stage 14 egg chambers had a severe dumpless phenotype, and 63% of these were TUNEL-positive (also see Table 1). With either TUNEL or AO, a delay in apoptosis was observed in the *dcp-1* GLCs. This delay, however, may not be directly due to loss of *dcp-1* function because a delay in apoptosis is observed in other dumpless mutants (*15*). These authors have suggested that an inhibitor of apoptosis may be present in the nurse cells and that removal of the inhibitor by dumping is required for the completion of apoptosis.

17. Ultrastructural analysis has revealed large (0.5 μm) gaps in the nuclear envelope of nurse cells before the stage of nurse cell dumping [E. Okada and C. H. Waddington, *J. Embryol. Exp. Morphol.* **7**, 583 (1959); A. P. Mahowald and M. P. Kambysellis, in *The Genetics and Biology of* Drosophila, M. Ashburner and T. R. F. Wright, Eds. (Academic Press, New York, 1980), vol. 2d, pp. 141–224]. With a nuclear β-Gal marker, wild-type nurse cell nuclei become permeable during stage 10B, with β-Gal leakage into the cytoplasm (*8*). Both *dcp-1* alleles are caused by insertion of P elements that carry a nuclear β-Gal marker (*12*). We examined either heterozygous *dcp-1/CyO* or *dcp-1* GLC ovaries for β-Gal activity using X-gal as a substrate. We followed the protocol in (*31*), except that ovaries were fixed in 1% glutaraldehyde [in 0.1 M phosphate buffer (pH 6.9)] for 7 min.
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21. Nuclear lamin Dm_0 is described by D. E. Smith, Y. Gruenbaum, M. Berrios, and P. A. Fisher [*J. Cell Biol.* **105**, 771 (1987)] and D. Riemer *et al.* [*J. Cell Sci.* **108**, 3189 (1995)]. For detection of nuclear lamin Dm_0, we used mAb 101, which recognizes an epitope in the coil region. Ovaries were processed for antibody staining as described (*31*). The mAb supernatant was diluted 1:1 in PBT, and for detection a fluorescein isothiocyanate–conjugated goat antibody to mouse immunoglobulin G (Jackson ImmunoResearch) was used at 1:200 dilution. Stained egg chambers were examined on a Bio-Rad confocal microscope, and they had a staining pattern similar to that seen with polyclonal serum to lamin Dm_0 [D. E. Smith and P. A. Fisher, *J. Cell Biol.* **108**, 255 (1989)].
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24. Rhodamine-phalloidin (Molecular Probes) staining was performed essentially as in (*31*). Images were visualized on a Bio-Rad confocal microscope. Because of variability in the phenotype, cytoplasmic actin bundles were found in some *dcp-1* GLC egg chambers (Table 3). However, only 7% of the late-stage severely dumpless egg chambers showed actin bundles.
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Activation of the Cardiac Calcium Release Channel (Ryanodine Receptor) by Poly-S-Nitrosylation

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Several ion channels are reportedly redox responsive, but the molecular basis for the changes in activity is not known. The mechanism of nitric oxide action on the cardiac calcium release channel (ryanodine receptor) (CRC) in canines was explored. This tetrameric channel contains ~84 free thiols and is S-nitrosylated in vivo. S-Nitrosylation of up to 12 sites (3 per CRC subunit) led to progressive channel activation that was reversed by denitrosylation. In contrast, oxidation of 20 to 24 thiols per CRC (5 or 6 per subunit) had no effect on channel function. Oxidation of additional thiols (or of another class of thiols) produced irreversible activation. The CRC thus appears to be regulated by poly-S-nitrosylation (multiple covalent attachments), whereas oxidation can lead to loss of control. These results reveal that ion channels can differentiate nitrosative from oxidative signals and indicate that the CRC is regulated by posttranslational chemical modification(s) of sulfurs.

Mammalian tissues express three major isoforms of nitric oxide synthase (NOS) (*1*). All three NOSs have been identified in cardiac or skeletal muscle in close association with the sarcolemma and have been implicated in the regulation of force production (*1–3*). Cytosolic Ca^{2+}, the primary determinant of force, is released from the sarcoplasmic reticulum (SR) by a ryanodine-sensitive CRC in response to a muscle action potential. In cardiac muscle, the CRC is opened by Ca^{2+} ions that enter the myocyte through a voltage-sensitive dihydropyridine receptor or L-type Ca^{2+} channel in a process known as Ca^{2+}-induced Ca^{2+} release (*4*). Ca^{2+} ions can also increase the activity of NOS 1 and NOS 3 (*1*, *3*). Nitric oxide (NO) then exerts its effects by covalently modifying or oxidizing critical thiols or transition metals in proteins (*5*).

NO may be a physiological modulator of excitation-contraction (E-C) coupling. It is produced at the sarcolemma, it cycles in the beating heart on millisecond time scales, and it modulates contractility (*3*). Both the L-type Ca^{2+} channel (*6*, *7*) and CRC (*8*) are potential targets of NO or related molecules because they contain sulfhydryls whose oxidation modulates their function and influences E-C coupling (*9*). Indeed, intramolecular disulfide formation is thought to be the molecular correlate of NO-mediated changes in channel activity (*7*, *10*). In this model, channels cannot distinguish NO signals from other redox active species—that is, they are thought to sense changes in oxidation state rather than the individual species responsible for such change. Our results indicate that poly-S-nitrosylation reversibly activates the CRC, whereas comparable degrees of thiol oxidation do not. We further identify at least one other redox site whose oxidation irreversibly activates the release channel and could impair muscle function.

CRC purified from canine hearts contained *S*-nitrosothiol (SNO) groups (74 ± 35 pmol of SNO per milligram of CRC; $n = 6$) if dithiothreitol (DTT) was eliminated from the standard buffers used in purification (*11*, *12*), whereas inclusion of DTT resulted in almost complete loss of SNO (0.5 pmol of SNO per milligram of CRC; $n = 2$). In other words, the CRC is endogenously S-nitrosylated and this posttranslational modification is reversible. Low-mass SNOs are one class of endogenous compounds capable of protein S-nitrosylation (*13*). To examine their effects on single cardiac CRCs, we incorporated proteoliposomes containing purified channels into planar lipid bilayers (*11*, *14*). Channel activity can be reliably monitored in a medium containing monovalent cations because the cardiac CRC does not conduct

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