Involvement of caveolin-1 in meiotic cell-cycle progression in *Caenorhabditis elegans*

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Small invaginations called caveolae are present on the surface of many cells and are a form of glycosphingolipid- and cholesterol-enriched microdomains or rafts in the plasma membrane[1,2]. The main component of the caveolar coat is caveolin-1 (refs 3,4), a membrane protein of relative molecular mass (M_r) 21,000 (21K), which binds cholesterol[5] and can form high-molecular-mass homo-oligomers resistant to sodium dodecyl sulphate (SDS)[6,7]. Caveolin-1 has been implicated in signal transduction, but its function remains unclear; whereas overactivation of the p42/44 MAP-kinase cascade was observed after downregulation of caveolin-1 (ref. 8), integrin-mediated activation of the Ras/extracellular-signal-regulated kinase (ERK) pathway[9] or conversion of prostate cancer cells to an androgen-insensitive phenotype[10] required expression of caveolin-1. To resolve the function of caveolin-1 in intact animals, we analysed caveolin-1 and glycosphingolipid/cholesterol-enriched rafts during the development of the nematode *Caenorhabditis elegans*. We show that *C. elegans* caveolin-1 (CAV-1) is expressed in the adult germ line and during embryonic development, and that CAV-1 is essential for Ras/MAP-kinase-dependent progression through the meiotic cell cycle. The function of CAV-1 is dependent on its association with cholesterol-rich membrane microdomains, providing a link between the membrane composition of germ cells and meiotic progression. Our results demonstrate that caveolin-1 and cholesterol-rich microdomains have an essential role in signal transduction *in vivo* and suggest a model for meiotic progression in the *C. elegans* germ line.

C. elegans has two caveolin-like genes, encoding CAV-1 and CAV-2 (ref. 11, and J.S. and T.V.K., unpublished). CAV-1 is about 67% similar and 37% identical to the mammalian caveolins-1 and -3 respectively over their entire length, whereas CAV-2 is a more distantly related member of the caveolin family[11]. Polyclonal antibodies produced against the carboxy-terminal peptide of CAV-1 recognize a single band of about 31K in western blots of *C. elegans* lysates (Fig. 1a). CAV-1 is expressed at low levels in adults (Fig. 1b), where it is found in germ cells (Fig. 1d). The protein is strongly expressed in most, if not all, cells throughout embryonic development (Fig. 1b,e,f). In late embryos and L1 larvae, *cav-1* expression becomes restricted to the nervous system and diminishes during larval development (data not shown).

The function of CAV-1 in *C. elegans* was studied by RNA interference (RNAi)[12], a powerful method of obtaining phenocopies of *C. elegans* mutants by RNA injection into hermaphrodites. Treatment with RNAi for *cav-1*, after which CAV-1 is no longer detectable (data not shown), results in a burst of egg laying by the injected hermaphrodite. One hundred and twelve eggs (s.d. = 3) were laid by injected hermaphrodites (n = 30) within 36 h after injection of late L4 larvae, compared with 46 eggs laid (s.d. = 2) by control animals (n = 30). Behaviour, morphology and lifespan of injected hermaphrodites are not affected. Embryonic or early larval lethality is observed in a small fraction of the progeny of the

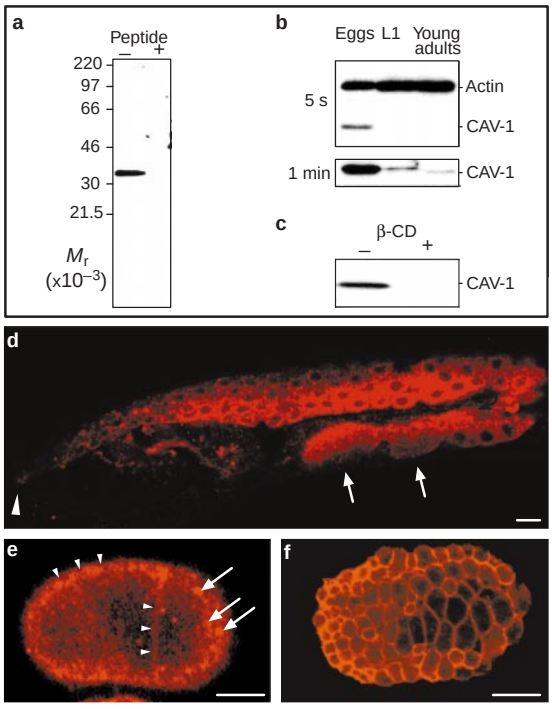

Figure 1 Expression of CAV-1 in *C. elegans*. **a**, Rabbit polyclonal antibodies recognize a protein of about 31K on western blots, consistent with the predicted molecular mass of CAV-1 (ref. 11). This band is abolished after competition by the antigenic peptide, confirming the specificity of the antibody. **b**, Biochemical analysis of the expression of CAV-1 reveals that the protein is expressed at high levels in eggs (top panel, exposure time 5 s; see Methods). At longer exposure times (1 min, bottom panel) expression in L1 larvae is detected. CAV-1 is not detected in L2–L3 stages (data not shown) but appears again in adults. Staining with anti-actin antibodies and Ponceau S staining were used as loading controls. **c**, Extracts in cold Triton X-114, prepared from untreated (–) or β-cyclodextrin (β-CD)-treated (+) *C. elegans* hermaphrodites, were analysed by sucrose gradient centrifugation followed by western blotting of floating gradient fractions. Cholesterol depletion by β-CD treatment abolishes the flotation of CAV-1 with cholesterol-rich microdomains. **d–f**, Immunofluorescence micrographs of *C. elegans* stained with anti-CAV-1 antibodies. **d**, CAV-1 is expressed in the entire germ line of adult hermaphrodites from the distal end (arrowhead) to the developing oocytes (arrows). For a schematic representation of the adult hermaphrodite germ line, see Fig. 2. **e**, CAV-1 is expressed in early embryos. An optical section through a two-cell embryo reveals localization of CAV-1 to intracellular membranes (arrows; a fraction of these granules co-localizes with P-granules), and to the plasma membrane (arrowheads). **f**, CAV-1 is strongly expressed during embryogenesis. An optical section through an embryo at about 4 h of development shows localization of CAV-1 to plasma membranes of all cells. Scale bars: **d**, 25 μm; **e**, **f**, 12 μm.

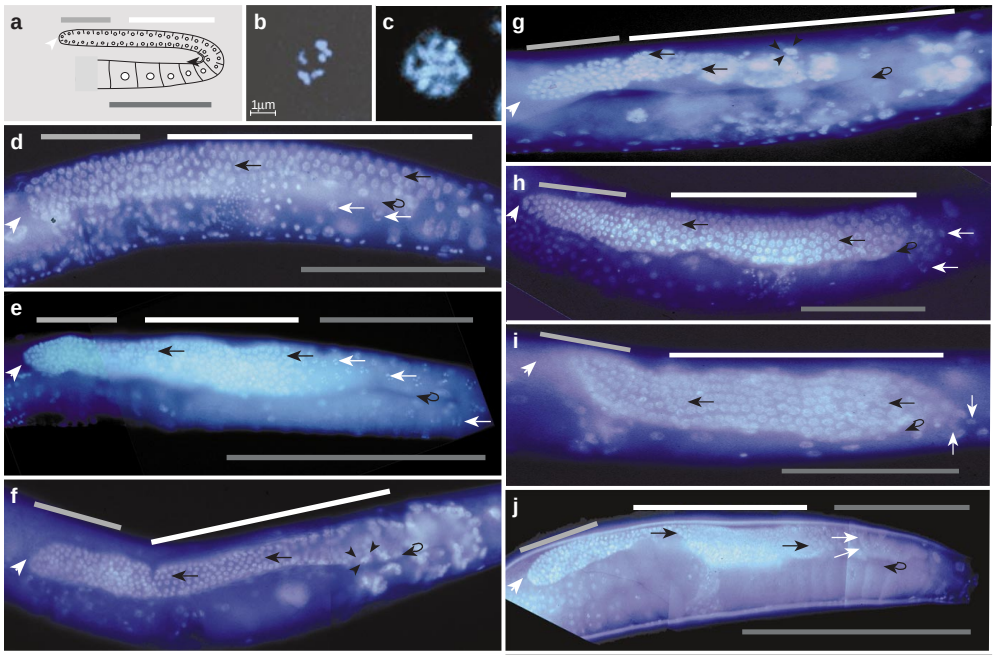

Figure 2 CAV-1 regulates meiotic progression in *C. elegans*. **a**, A gonad arm of adult *C. elegans* hermaphrodite. In this and the following photographs, the distal tip of the gonadal arm is indicated by a white arrowhead, and the inner side of the bend between the distal and the proximal arm of the gonad by a curved arrow. Regions of mitotic germ-cell nuclei are indicated by a mid-grey line, pachytene nuclei by a white bar, and nuclei in diakinesis by a dark-grey bar. **b**,**c**, Typical chromosome morphology of pachytene nuclei (**b**) or nuclei in diakinesis (**c**) in the adult hermaphrodite gonad at high magnification. **d–j**, Fluorescence micrographs of adult *C. elegans* hermaphrodites stained with DAPI. Typical pachytene nuclei are indicated by black arrows, diakinesis nuclei by white arrows. **d**, Wild-type hermaphrodite. A large region of pachytene nuclei (white bar) in the distal arm of the gonad is followed by a region of diakinetic nuclei (dark-grey bar) proximal to the bend of the gonad arm. **e**, Wild-type hermaphrodite after *cav-1* RNAi. The region in diakinesis and developing oocytes are present in the distal arm of the gonad. **f**, *mpk-1(oz140)* hermaphrodite. Meiotic progression is blocked in pachytene. Proximal to a region of normal pachytene nuclei, clumps of pachytene-like nuclei are present in the germ line[13] (black arrowheads). **g**, *mpk-1(oz140)* hermaphrodite after *cav-1* RNAi. *mpk-1(oz140)* mutations suppress the effect of *cav-1* RNAi. **h**, *let-60(n1046gf)* hermaphrodite. Meiotic progression is normal in the presence of a constitutively active form of Ras in *let-60(n1046gf)* mutants (see wild type in Fig. 1d for comparison). **i**, *let-60(n1046gf)* hermaphrodite after *cav-1* RNAi. *let-60(n1046gf)* mutations suppress the effect of cav-1 RNAi, resulting in normal meiotic progression. **j**, Wild-type hermaphrodite treated with 5 mM β-cyclodextrin for 1 h. Depletion of adult hermaphrodites of cholesterol by this treatment results in a phenotype identical to that produced by *cav-1* RNAi, with a reduced zone of pachytenic nuclei and the presence of diakinetic nuclei in the distal arm of the gonad.

injected hermaphrodite 1 to 5 days after injection.

To understand the egg-laying phenotype in more detail, we analysed germline nuclei of injected hermaphrodites. The germ line of adult *C. elegans* hermaphrodites is contained in a U-shaped tubular gonad (Fig. 2a) in which the distal-most cells are in a mitotic cell cycle. Germ cells leave the mitotic cycle and enter meiosis; most meiotic nuclei are found in pachytene, a stage in the prophase of the first meiotic division (Fig. 2a,b), before proceeding to the following meiotic stage, diakinesis (Fig. 2a,c). RNAi of *cav-1* leads to a reduction of the region of germ-cell nuclei in pachytene by up to 50%, resulting in diakinetic nuclei being found more distally compared with control animals (Fig. 2d,e). These results show that interference with *cav-1* function specifically advances meiotic progression.

Meiotic progression is regulated by the Ras/MAP-kinase pathway in *C. elegans*[13]. Loss-of-function (*lf*) mutations in Ras-pathway genes result in an arrest of meiotic nuclei in pachytene (pachytene arrest[13]), a phenotype opposite to that produced by *cav-1* RNAi. *cav-1* RNAi in MAP-kinase mutants (*mpk-1(oz140)*) results in a pachytene-arrest phenotype indistinguishable from that of uninjected *mpk-1* mutant animals (Fig. 2f,g). Only rare escapes from pachytene arrest were observed after *cav-1* RNA injection; these nuclei seem to be arrested in a post-pachytene stage (data not shown). Similar results were obtained after *cav-1* RNAi in Ras mutants (*let-60(s1124)*) or in MAP kinase kinase mutants (*mek-2(q425)*) (data not shown). In contrast to the *lf* alleles of Ras-pathway genes, a gain-of-function (*gf*) allele of Ras, *let-60(n1046)*, encoding a constitutively active Ras protein, does not affect meiotic progression[13] (Fig. 2h). *Let-60(n1046)* completely suppresses the effect of *cav-1* RNAi, resulting in normal meiotic progression in injected *let-60(n1046)* mutants (Fig. 2i). Thus, *cav-1* and the Ras pathway interact to regulate meiotic progression in *C. elegans*.

Cholesterol depletion of *C. elegans* membranes by treatment of late L4 or adult hermaphrodites with β-cyclodextrin (β-CD) disrupts the association of CAV-1 with glycosphingolipid-rich rafts (Fig. 1c). Cholesterol depletion of hermaphrodites also results in a distal shift of diakinetic nuclei. This is indistinguishable from the effect of *cav-1* RNAi (Fig. 2j), suggesting that clustering of CAV-1 in cholesterol-rich rafts is essential for its function in signal transduction *in vivo*. *mpk-1(oz140)* mutations suppress the effect of β-CD (data not shown). This shows that the effect of β-CD is highly specific, and that the cholesterol content of membranes regulates the function of CAV-1 in meiotic progression.

Because *cav-1* RNAi does not affect the initial phase of pachytene, pachytene in the hermaphrodite germ line seems subdivided into two phases. This subdivision is supported by the phenotypes of loss-of-function alleles of Ras-pathway mutants[13] and apoptosis in the germ line[14]. Whereas Ras, CAV-1 or apoptosis seem not to have any role in the first phase of pachytene (P1), progression to the second phase of pachytene (P2) requires the Ras pathway.

Genetic analysis indicates that CAV-1 is not sufficient to initiate Ras signalling, but is required to maintain elevated Ras activity during P2 in a cholesterol-dependent manner. If Ras activity during P2

is maintained constitutively by *let-60(n1046)*, CAV-1 is not required. Maintenance of P2 by CAV-1-dependent Ras activity might be important for germline homeostasis by apoptosis[14]. The direct link between membrane composition and Ras signalling provided by CAV-1 couples meiotic progression to membrane composition during the cellularization of oocytes.

The receptor tyrosine kinases involved in several Ras-dependent signalling processes in *C. elegans* seem to have no role in Ras signalling during meiosis[15]. On the other hand, CAV-1 is not involved in Ras signalling during vulval development or sex myoblast positioning, as it appears not to be expressed in the relevant cells at the correct time. CAV-1 cannot therefore be considered a general regulator of the Ras pathway in *C. elegans*, suggesting that its function *in vivo* can only be studied in the relevant context. □

Methods

Strains.

C. elegans strains were obtained from the Caenorhabditis Genetic Center (St Paul, Minnesota, USA) and maintained under standard conditions. Experiments were performed at 20 °C if not stated otherwise.

Expression analysis.

Rabbit polyclonal antibodies were produced against a carboxy-terminal peptide, (C)NFNIRKYGINQETTA, of CAV-1, and affinity purified. For western blot analysis, worms were washed and dissolved in SDS–PAGE sample buffer. After sonication and boiling, SDS electrophoresis on a 12% gel and subsequent blotting were performed. For detection of bands, the ECL technique was used. In peptide-competition experiments, antibodies were preincubated with 10 μM peptide for 1 h at room temperature.

For immunofluorescence microscopy, embryos, larvae or adult hermaphrodites were fixed in methanol (4 min, –20 °C) after freeze-cracking, and stained with anti-CAV-1 antibodies followed by fluorescently labelled secondary antibodies. Fluorescence micrographs were recorded on a Zeiss Axiophot or on a Leica TCS confocal laser scanning microscope using standard filter sets.

RNAi studies.

Sense, antisense or double-stranded RNA transcribed *in vitro* from the entire complementary DNA of *cav-1*(T13F2.8), or fragments thereof, were injected into the gonads of late 4th larval stage hermaphrodites. Injected animals were analysed at 12-h intervals during the 5 days after injection. Mutants used for injections: *mpk-1 (oz140)*, progeny of *mpk-1(oz140)/ dyp17(e164) unc-79(e1068)* hermaphrodites; *mek-2 (q425) unc-11(e47)*, progeny of *mek-2 (q425) unc-11(e47); sDp2. let-60(s1124) unc-22(s7) unc-31(e169), or let-60(n1046)*, progeny of *let-60(s1124) unc-22(s7) unc-31(e169)/ let-60(n1046)*.

Chromosomal staining.

Worms (treated or untreated) were washed in M9 buffer before fixation in cold methanol/4% formaldehyde for 10 min followed by washes in M9 and staining in 2 μg DAPI per ml M9 for 1 h.

Cholesterol depletion.

For cholesterol-depletion studies, L4 or adult hermaphrodites were incubated with 5 mM β-CD in M9 for 2 h at 20 °C. For morphological analyses, treated hermaphrodites were subsequently washed and incubated in the absence of β-CD for 12 h. To measure cholesterol depletion, animals were incubated in the absence or presence of β-CD, washed, directly resuspended in 100% isopropanol and homogenized using a Potter homogenizator. The extract was incubated at room temperature for 2 h before centrifugation for 10 min at 14,000 r.p.m. (Eppendorf). The cholesterol content of the soluble fraction and the protein content of the insoluble fraction were determined as described[16].

Sucrose gradients.

Hermaphrodites (untreated or treated with β-CD) were collected, washed in M9 and resuspended in 1% Triton X-114 in M9 at 4 °C. After homogenization in a Potter homogenizator, debris was removed by low-speed centrifugation. After adjusting to 1.2 M sucrose, 1.5 ml of the homogenate was placed in the bottom of a 4.5-ml centrifuge tube and overlaid with 2 ml 1.1 M and 0.5 ml 0.2 M sucrose solutions. After centrifugation for 16 h at 33,000 r.p.m. in a SW-60 rotor (Beckman), the interphase between 0.2 M and 1.1 M sucrose was collected and analysed by western blotting.

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