A Secreted Protein Promotes Cleavage Furrow Maturation during Cytokinesis

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Summary

Developmental modifications in cell shape depend on dynamic interactions between the extracellular matrix and cytoskeleton. In contrast, existing models of cytokinesis describe substantial cell surface remodeling that involves many intracellular regulatory and structural proteins but includes no contribution from the extracellular matrix [1–3]. Here, we show that extracellular hemicentins assemble at the cleavage furrow of dividing cells in the *C. elegans* germline and in preimplantation mouse embryos. In the absence of hemicentin, cleavage furrows form but retract prior to completion, resulting in multinucleate cells. In addition to their role in tissue organization, the data indicate that hemicentins are the first secreted proteins required during mammalian development and the only known secreted proteins required for cytokinesis, with an evolutionarily conserved role in stabilizing and preventing retraction of nascent cleavage furrows. Together with studies showing that extracellular polysaccharides are required for cytokinesis in diverse species [4–9], our data suggest that assembly of a cell type-specific extracellular matrix may be a general requirement for cleavage furrow maturation and contractile ring function during cytokinesis.

Results

Hemicentin Is Required for Cleavage Furrow Stability in the *C. elegans* Germline

Approximately 30 distinct *C. elegans him* mutants produce a *h*igh *i*ncidence of *m*ale self-progeny as a result of defects in segregation of the X chromosome in the hermaphrodite germline [10]. All characterized *him* loci encode intracellular proteins associated with the machinery of chromosome segregation, with one exception: *him-4* encodes a large secreted protein with highly conserved vertebrate orthologs, which we have named hemicentin [11]. In addition to a poorly understood defect in germline chromosome segregation, *him-4* mutants exhibit pleiotropic defects in cell adhesion and migration [10,11].

Germ cells in *C. elegans* have incomplete cleavage furrows that connect them to a central cytoplasmic core, allowing them to act as "nurses" while allowing more mature oocytes to fill with bulk cytoplasm [12]. Hemicentin::GFP concentrates in a ring around incomplete cleavage furrows in germ cell plasma membranes (Figure 1). In the absence of hemicentin, germ cell plasma membranes are disorganized and gonads contain multinucleate germ cells [11]. The circumferential distribution of hemicentin::GFP at the periphery of the cleavage furrow and defects in membrane organization observed in the absence of hemicentin suggest

that hemicentin may be required for either the assembly or the stability of nascent membrane structures at the cleavage furrow. To distinguish between these two possibilities, we examined plasma membrane structure in a hemicentin mutant [*him-4 (rh319)*] background, utilizing a membrane-associated RFP-tagged phospholipase C *δ* PH domain (RFP-PH). Temporal studies show that recently synthesized membranes have a relatively normal structure but are unstable and retract with time, resulting in multinucleate germ cells that increase in number and frequency with maternal age (Figure 1; see also Table S1 available online). Wild-type animals of all ages and *him-4(rh319)* mutant animals prior to and early in adulthood have few multinucleate cells. In contrast, older *him-4(rh319)* adults (>24 hr following final molt) have large numbers (~25%) of multinucleate gametocytes (Table S1). Similar nonspecific effects in *him-4(rh319)* and control animals grown in a variety of osmotic conditions (Figure S1) suggest that the role of hemicentin in cytokinesis is distinct from that of several glycosaminoglycan synthesis genes that appear to promote cytokinesis by generating osmotic pressure inside the eggshell [4–7].

Hemicentin is not synthesized by germ cells but is synthesized outside the germline by somatic gonad and body wall muscle cells that secrete hemicentin into the pseudocoelomic fluid [11]. In wild-type animals, secreted hemicentin is targeted to germ cell surfaces by specific sequences within the von Willebrand A domain of hemicentin [13]. Assembly of hemicentin in the gonad is dependent on ECT-2, a highly conserved RhoGEF that activates Rho in a number of eukaryotic species to initiate contractile ring assembly and to promote cleavage furrow stability [14,15]. In an *ect-2(e1778)* mutant background, only a small amount of hemicentin::GFP is recruited to germ cell surfaces inside the gonad, whereas the bulk of the hemicentin:: GFP remains in the pseudocoelomic fluid outside the gonad (Figures 1H–1M). This suggests that ECT-2 is important for the assembly of a structure in the nascent cleavage furrow of dividing germ cells that promotes the recruitment and assembly of hemicentin.

Hemicentin-1 Localizes to the Cleavage Furrow and Is Required for Cytokinesis in Preimplantation Mouse Embryos

To test whether hemicentin's function in cleavage furrow stability is conserved in mammals, we examined the role of hemicentin-1 during mouse development. Two highly conserved hemicentin orthologs, hemicentin-1 (also known as fibulin-6) and hemicentin-2, have a pericellular distribution in a number of mouse tissues including adult epidermis and trophectoderm cell junctions in preimplantation mouse embryos [16]. Examination of hemicentin distribution with an antibody that recognizes hemicentin-1 and -2 prior to trophectoderm formation revealed a diffuse staining pattern that became punctate and concentrated at or near the presumptive cleavage furrow of mitotic one-cell mouse embryos. Hemicentins remained concentrated in the nascent furrow during the division process and formed an open ring structure peripheral to the contractile ring component myosin IIB (Figure 2). This pattern was repeated in successive cell divisions and suggests a potential role for vertebrate hemicentins in mouse blastomere cytokinesis that is analogous to the germline function of *C. elegans* hemicentin (Figure 2).

To determine whether hemicentins have an essential function at this early time during mouse embryonic development, the function of hemicentin-1 was tested by two techniques: double-stranded RNA (dsRNA)-mediated knockdown and targeted inactivation of the hemicentin-1 gene. Injection of hemicentin-1 dsRNA into one-cell wild-type mouse embryos resulted in a large number of embryos (89%) that arrested prior to the four-cell stage, compared to <4% of control embryos that arrested at this stage (Table S2). Arrested embryos had multinucleate cells that were not detected in mock-injected or E-cadherin dsRNA-injected control embryos (Figure 3).

The function of hemicentin-1 in early embryogenesis was also tested by targeted inactivation (Figure S2). Approximately 28% (22 of 80) of embryos obtained by mating heterozygous males and females were homozygous mutant animals that arrested between the one- and four-cell stage, compared to 0% (0 of 76) of animals arrested from matings of wild-type males and females (Figure 4). Homozygous mutant embryos had multinucleate cells and often had internal polar bodies and plasma membranes with multiple invaginations (Figure 4; Figure S3). Time-lapse imaging of negative control and homozygous mutant animals showed that after the initial formation, cleavage furrows failed to ingress and often retracted in the absence of hemicentin-1 (Figure 4; Movie S1; Movie S2). The data suggest that hemicentin is required to stabilize and promote the ingression and/or prevent the retraction of the cleavage furrow in mouse embryos. The presence of internal polar bodies in hemicentin-1-deficient embryos (Figure 4) and the detection of hemicentin on polar bodies at the time of extrusion (data not shown) suggest that hemicentins may have a role in stabilization of the cleavage furrow during polar body extrusion as well as cytokinesis.

Unfertilized eggs obtained from females heterozygous for the null allele of hemicentin-1 were also parthenogenically activated. Approximately 64% of parthenogenically activated eggs from heterozygous mothers arrested before the four-cell stage, as compared to ~25% of parthenogenotes from wild-type mothers (Table S2). It is likely that a fraction of the $hmcn-1^{+/0}$ embryos from wild-type and heterozygous mothers arrest as a result of experimental manipulation or absence of the paternal genome [17,18]. However, a substantial fraction of the arrested embryos that were hemizygous hemicentin-1 null parthenogenotes arrested with multinucleate cells that were never detected in control embryos, and many of the cells in arrested embryos had more than two nuclei (Figure 3).

Discussion

In the *C. elegans* germline, substantial numbers of germ cells with multiple nuclei accumulate in aging hemicentin mutant animals as a result of membrane instability. Distribution of hemicentin peripheral to the incomplete cleavage furrows of syncytial germ cells and retraction of syncytial membranes in the absence of hemicentin suggest that hemicentin has an unprecedented role among secreted proteins in stabilizing the cleavage furrow during cytokinesis in *C. elegans* germ cells. The presence of multinucleate cells in preimplantation mouse embryos lacking hemicentin-1 suggests that this function of hemicentins has been conserved in the vertebrate embryo. Although we suggest that the binucleate cells observed in hemicentin-1-deficient mouse embryos indicate a failure in cytokinesis, a conceivable alternate explanation is that there is a cell-cycle defect or there has been selection for embryos that are fixed just prior to cytokinesis by chance. However, the presence of hemicentin-1 knockdown and knockout embryos with three or more nuclei, including nuclei that have doubled in volume, indicates that the cell cycle remains intact in the absence of hemicentin-1. Moreover, localization of hemicentin in the cleavage furrow of dividing cells and dynamic observations of plasma membrane retraction in hemicentin-1-deficient embryos, together with the fact that multinucleate cells were never detected in control embryos, strongly suggest that the multinucleate phenotype is a result of cytokinesis failure and not a secondary consequence of a cell-cycle defect or an artifact of fixation time.

Although there are striking parallels between hemicentin function in mouse blastomeres and *C. elegans* germ cells, there are also several critical differences. Hemicentin-1 appears to be required for cell proliferation in mouse blastomeres, but hemicentin is not essential for germ cell proliferation in *C. elegans*: hemicentin mutant hermaphrodites produce large numbers of aneuploid gametes [10,11]. This may reflect p53 activity that restricts the proliferation of aneuploid mouse blastomeres but not aneuploid *C. elegans* germ cells [19]. Another critical difference is that the role for hemicentin in cytokinesis appears to be restricted to the

germline in *C. elegans*, because no defects have been detected in *C. elegans* somatic cell divisions in hemicentin mutant animals [11,20]. Instead, hemicentin appears essential for the adhesion and migration of select cells in the nematode soma. It is not known whether hemicentin has a function in cytokinesis or cell adhesion in the post-implantation mouse. *C. elegans* germ cells and mouse blastomeres are uncommitted cell types that divide very slowly, and it is possible that the requirement for hemicentin is limited to cleavage furrows that have structural or dynamic properties not found in rapidly dividing or highly differentiated cells.

Hemicentin-1 is the only known extracellular protein with an evolutionarily conserved role in cytokinesis and may be the first secreted protein required during mammalian development. The ability to form line-shaped elastic fiber-like polymers and transient structures during cell migration [11] suggests that hemicentins may have the properties required to perform structural roles in narrow and highly dynamic groove-shaped structures such as the cleavage furrow. However, not all eukaryotic species have a gene that encodes hemicentin, indicating that hemicentins are not universally required for cytokinesis. Instead, several studies have shown that synthesis of glycosaminoglycans and other extracellular polysaccharides is essential for cleavage furrow stability and maturation in diverse species [4–9]. Together, these studies suggest that extracellular matrices may be required for cytokinesis and that there may be some specificity in the composition and organization of the extracellular matrix required in the cleavage furrow of different cell types.

Experimental Procedures

Targeted Inactivation of the Mouse Hemicentin-1 Gene

Hemicentin-1 clones were identified by hybridization using a PCR-generated probe to screen a mouse 129SvEv genomic bacterial artificial chromosome library (RPCI-22; BAC/PAC Resources, Children's Hospital Oakland) and purchased from Invitrogen. These genomic clones were used to construct a targeting vector designed to replace the first exon of hemicentin-1 with an expression cassette containing GFP and neomycin resistance genes by homologous recombination (Figure S2).

Collection and Culture of Mouse Embryos

All female and male mice used in the experiments were 8–12 and 10–14 weeks of age, respectively, and were maintained in a 12 hr light/12 hr dark cycle. Embryos at different stages were collected from the ampulla and lumen of the uterus depending on experimental requirements. For embryo culture, single-cell oocytes were collected from the uterine ampulla and cultured in potassium-supplemented simplex optimization medium (KSOM) containing 0.5% bovine serum albumin (BSA; Sigma) under embryo test paraffin oil (Sigma) in culture dishes in an atmosphere of 5%CO_2 at 37°C.

Immunocytochemistry

After collection, embryos were washed with KSOM medium, fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS), and permeabilized in PBS containing 0.25% Triton X-100 for 10 min. Immunocytochemical staining was performed by incubating the fixed samples with primary antibodies in PBS/Tween (PBS containing 0.1% Tween 20 and 3% BSA) for 1–2 hr followed by incubation with appropriate secondary antibodies diluted according to the manufacturer's recommendation in PBS/Tween solution for 1–2 hr. Stained samples were analyzed with a Zeiss LSM 510 confocal microscope.

Double-Stranded RNA Preparation and Microinjection

Total RNA was isolated from E12.5 mouse embryos using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcriptions were performed with a SuperTranscript kit (Invitrogen) using random hexanucleotide primers and RNaseH-MMLV reverse transcriptase. Microinjection of dsRNAs was performed as described previously [21,22].

Parthenogenic Activation of MII Phase Oocytes

Murine parthenogenesis of eggs in second meiotic metaphase was performed according to standard protocols [23–25]. Wild-type (*hmcn-1*$^{+/+}$) and heterozygous (*hmcn-1*$^{+/-}$) females 8–12 weeks of age were used as oocyte donors.

Embryonic/Nuclear Volume Measurements

Embryonic and nuclear volumes were calculated as described previously [26] with three distinct measurements of each zona pellucida and nuclear radius using Zeiss LSM 510 image browser software. Volume was calculated as $4/3\pi abc$, and the ratio of nuclear to embryonic volume was used to assess cell-cycle phase. Average values and standard deviations were based on four to six independent measurements of each nucleus.

Nematode Strains

All nematode strains were cultured under standard conditions unless noted otherwise. The *rhIs23* hemicentin:GFP transgene has been described previously [11]. The RFP-phospholipase Cδ1 was obtained from strain DP400 [27]. Histone H2B::GFP was obtained from strain JH1993 [28]. The *ect-2(e1778)* allele was obtained from Caenorhabditis Genetics Center strain CE1857.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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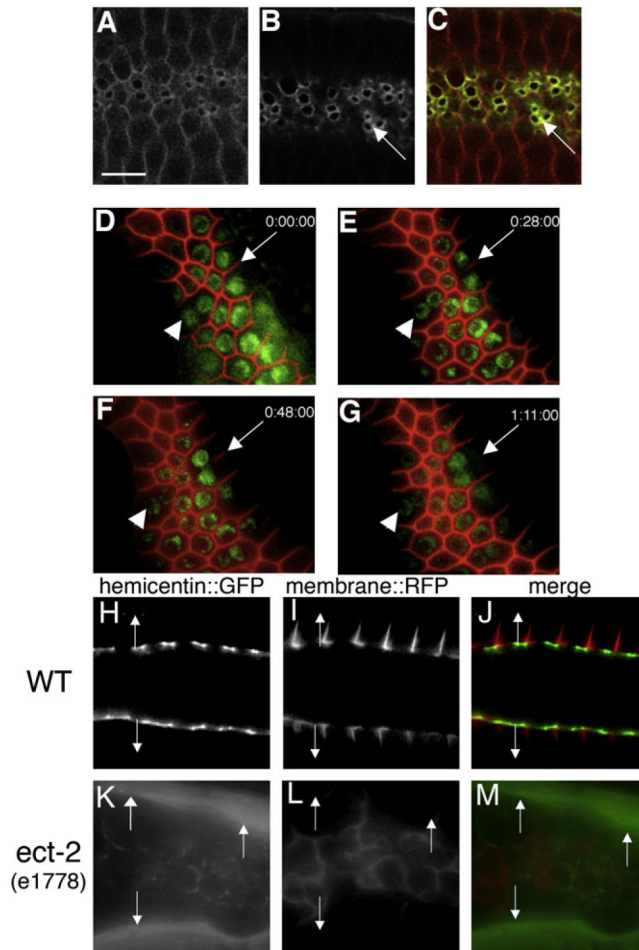
Figure 1. Hemicentin Assembles at the Cleavage Furrow of *C. elegans* Germ Cells and Prevents Membrane Retraction

Germ cells in the hermaphrodite distal gonad have incomplete cleavage furrows.

(A–C) Optical section through the central region of *C. elegans* gonad showing wild-type *C. elegans* hermaphrodite germ cells coexpressing RFP::phospholipase C δ PH domain (A and C) and hemicentin::GFP (B and C). Note that hemicentin::GFP accumulates at the periphery of incomplete cleavage furrows (arrows).

(D–G) Progressive retraction of germ cell membranes in the absence of hemicentin. Images show a time course of a single *him-4(rh319)* hermaphrodite mutant gonad expressing RFP:PH and histone 2B:GFP transgenes. Images were collected at 0 (D), 28 (E), 48 (F), and 71 min (G). Note progressive retraction of the germ cell membrane (arrow). Arrowhead indicates one of several multinucleate germ cells present prior to image collection. This effect does not depend on osmotic pressure (Figure S1). Phenotype quantitation is presented in Table S1.

(H–M) The ECT-2 RhoGEF is required for hemicentin assembly in the gonad. Comparison of membrane RFP:PH and hemicentin:GFP distribution in a wild-type and *ect-2* mutant background is shown. In a wild-type background, hemicentin:GFP assembles peripheral to incomplete cleavage furrows. In an *ect-2(e1778)* mutant background, cleavage furrows do not form properly, and hemicentin does not assemble at membrane surfaces but accumulates outside the gonad (arrows). Scale bar represents 5 μm.
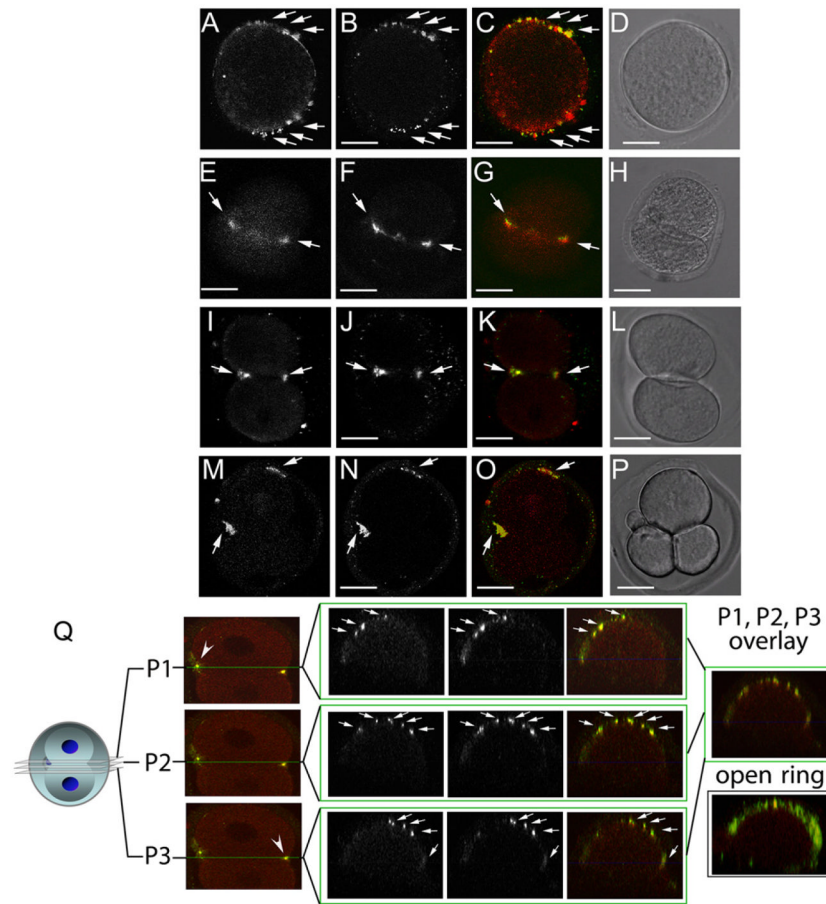
Figure 2. Hemicentin Distribution in Wild-Type Mouse Embryos

(A–P) Immunofluorescence images of dividing one- (A–D), two- (E–L), and three-cell (M–P) wild-type embryos. Embryos were stained at multiple time points with antibodies to hemicentin (A, E, I, and M) and myosin IIB (B, F, J, and N). Merged images (C, G, K, and O) indicate that hemicentin staining accumulates adjacent to myosin II staining in dividing cells. Also shown are corresponding differential interference contrast (DIC) images (D, H, L, and P). Scale bars represent 20 µm.

(Q) Confocal image of three separate planes across the cleavage furrow of a dividing one-cell embryo stained with hemicentin (red) and myosin IIB (green) antibodies. Right: overlay of merged images from two different embryos showing that both proteins form a punctate open ring around the periphery of the cleavage furrow.
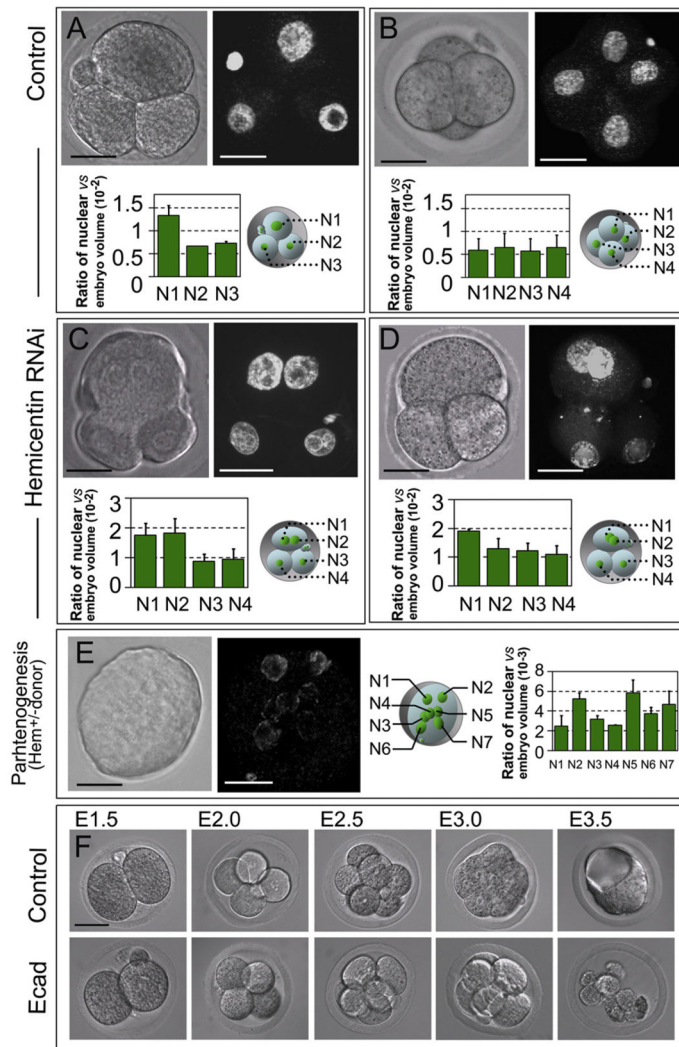
Figure 3. Hemicentin-1 Depletion Results in Embryonic Arrest Prior to Implantation

(A–D) Wild-type embryos were mock injected (A and B) or injected at the one-cell stage with hemicentin-1 dsRNA (C and D). Control embryos progress through the three- and four-cell stage with single nuclei (A and B). In contrast, hemicentin-1 dsRNA-injected embryos arrest prior to the four-cell stage with multinucleate cells (C and D). DIC and corresponding YOYO-1-stained images are shown. Ratio of nuclear volume to embryonic volume ($\times 10^{-2}$) with corresponding schematic diagram is shown below each embryo. Error bars represent standard deviation. Larger nuclear volumes of N1 and N2 in (C) and N1 in (D) suggest that these nuclei are in G2 phase [27].

(E) Parthenogenic activation of eggs obtained from heterozygous hemicentin-1 females results in arrest of embryos with multinucleate cells. DIC and corresponding YOYO-1-stained images of embryos obtained from parthenogenic activation of eggs collected from heterozygous females are shown. Approximately 40% of eggs divide to become mononucleate cells, and ~60% (Table S2) arrest at the one- to three-cell stage, with most cells containing multiple nuclei. At least seven distinct nuclei (N1–N7) are detected within the single arrested blastomere. Ratio of nuclear volume to embryonic volume ($\times 10^{-3}$) with corresponding schematic diagram is shown to the right. Error bars represent standard deviation.

(F) As described above, the majority of mock-injected embryos successfully develop into blastocysts (see Table S2). As a positive control, most E-cadherin dsRNA-injected embryos proliferate normally from the two- to eight-cell stage but fail to form a blastocoele and arrest at the morula stage (Table S2), consistent with published information on the phenotype of E-cadherin knockout mice [29].
Scale bars represent 20 μm.
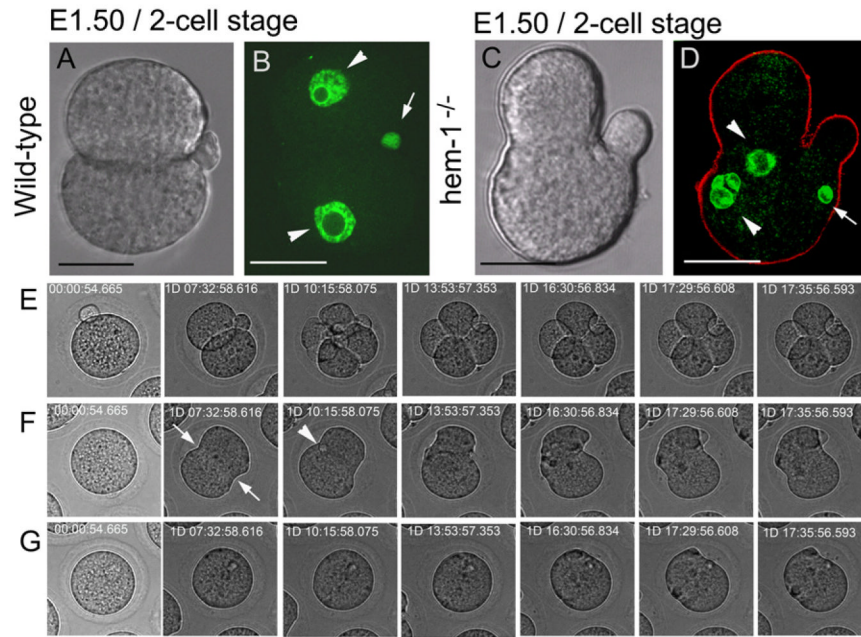
Figure 4. Hemicentin-1-Deficient Embryos Arrest Prior to Implantation with Multiple Nuclei
(A–D) Images of age-matched wild-type (A and B) and hemicentin-1-deficient (C and D) embryos from matings of wild-type and hemicentin-1 heterozygous parents. DIC images are shown with corresponding nuclear staining with YOYO-1 (green) and anti-myosin IIB (red). Scale bars represent 20 μm.
(A and B) Wild-type embryos progress to the two-cell stage with mononucleate cells (arrowheads) and external polar bodies (arrow).
(C and D) In contrast, hemicentin-deficient embryos arrest prior to the four-cell stage. Arrested embryos contain multinucleate cells (arrowheads; Figure S3), multiple invaginations in the plasma membranes, and internal polar bodies (arrow).
(E–G) Time-lapse photography of wild-type and hemicentin-deficient embryos in Movie S1 and Movie S2 during the first 3 days of development. Wild-type embryos have external polar bodies and develop to the four-cell stage (E; Movie S1). In contrast, hemicentin-1-deficient embryos develop membrane invaginations (arrows) and internal polar bodies (arrowhead), but membrane invaginations do not proceed to completion and often retract (F and G; Movie S2).