Transfer of Dorsoventral and Terminal Information from the Ovary to the Embryo by a Common Group of Eggshell Proteins in *Drosophila*

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ABSTRACT The *Drosophila* eggshell is an extracellular matrix that confers protection to the egg and also plays a role in transferring positional information from the ovary to pattern the embryo. Among the constituents of the *Drosophila* eggshell, Nasrat, Polehole, and Closca form a group of proteins related by sequence, secreted by the oocyte, and mutually required for their incorporation into the eggshell. Besides their role in eggshell integrity, Nasrat, Polehole, and Closca are also required for embryonic terminal patterning by anchoring or stabilizing Torso-like at the eggshell. Here, we show that they are also required for dorsoventral patterning, thereby unveiling that the dorsoventral and terminal systems, hitherto considered independent, share a common extracellular step. Furthermore, we show that Nasrat, Polehole, and Closca are required for proper Nudel activity, a protease acting both in embryonic dorsoventral patterning and eggshell integrity, thus providing a means to account for the role of Nasrat, Polehole, and Closca. We propose that a Nasrat/Polehole/Closca complex acts as a multifunctional hub to anchor various proteins synthesized at oogenesis, ensuring their spatial and temporal restricted function.

KEYWORDS *Drosophila*; dorsoventral patterning; terminal patterning; vitelline membrane

EXTRACELLULAR matrices (ECMs) exert a dual role in cell biology. On the one hand, they confer a wide range of specific environments that provide distinct kinds of physical support or protection. On the other hand, they also play a key role in cell signaling as they participate in growth factor storage or modification (Rozario and DeSimone 2010). A particular scenario in which to address the diverse roles of ECMs is that of *Drosophila* oogenesis, a process in which a highly specific ECM, namely the eggshell, is built in parallel with the development of the oocyte.

The *Drosophila* eggshell consists of five morphologically distinct layers. The innermost layer closest to the oocyte is the vitelline membrane, which confers rigidity and protection to the egg but also plays a role in embryonic patterning. During oogenesis, the somatic follicle cells surrounding the oocyte secrete the four major structural components of the vitelline membrane (sV17, sV23, Vm32E, and Vm34C) (Pascucci *et al.* 1996; Waring 2000; Andrenacci *et al.* 2001; Cernilogar *et al.* 2001). The assembly of these proteins in the vitelline membrane requires their stage-specific proteolytic processing and cross-linking among them to render the membrane insoluble. The vitelline membrane also receives contributions from the oocyte. *fs(1)Nasrat* (*fs(1)N*), *fs(1)polehole* (*fs(1)ph*), and *closca* (*clos*) encode a group of extracellular germline proteins that are secreted from the oocyte and are mutually required for their incorporation into the vitelline membrane (Jimenez *et al.* 2002; Ventura *et al.* 2010). Mutant females for null alleles of *fs(1)N*, *fs(1)ph*, or *clos* genes produce fragile eggs that collapse after laying due to defects in vitelline membrane cross-linking (Cernilogar *et al.* 2001; Ventura *et al.* 2010).

At the same time, the ovary provides the oocyte with the basic patterning information that will specify the body plan of the future embryo along the anteroposterior and dorsoventral axes. The anterior and posterior determinants, bicoid and nanos, respectively, are deposited within the oocyte, where they accumulate asymmetrically. In contrast, terminal and dorsoventral systems rely on signals secreted by the embryo

Copyright © 2017 by the Genetics Society of America
doi: https://doi.org/10.1534/genetics.116.197574
Manuscript received November 4, 2016; accepted for publication February 2, 2017; published Early Online February 7, 2017.
Supplemental material is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.197574/-/DC1.
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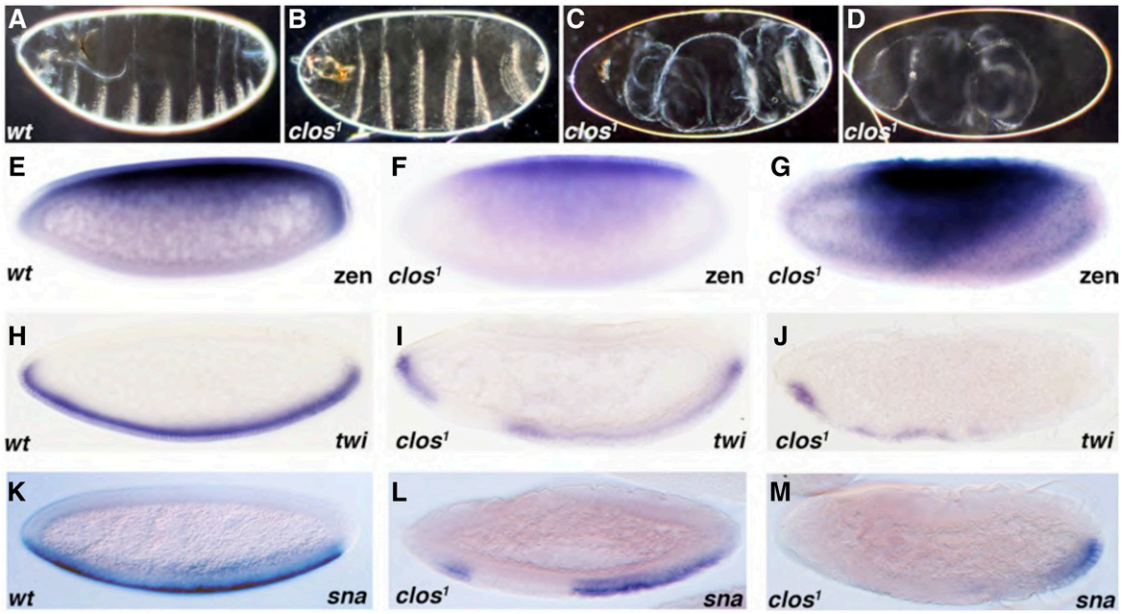

Figure 1 *clos*^{1} mutants show defects in dorsoventral patterning. (A) Cuticle of a wild-type (wt) embryo. (B–D) Cuticles of embryos derived from *clos*^{1} /*clos*^{4152} females raised at 29°. (B) Cuticle of an embryo displaying a terminal phenotype (note the lack of all anterior and posterior terminal structures). (C and D) Cuticles of embryos displaying different degrees of a dorsalization phenotype; note the lack of some ventral denticle belts, only anteriorly for the embryo in (C). (E–G) *In situ* hybridization of *zerknullt* (*zen*) in a wt embryo (E) and in embryos derived from *clos*^{1}/*clos*^{4152} raised at 29° (F and G). In these embryos, *zen* mRNA expression is not sustained at the poles reflecting lack of Tor activation (F and G) and, in some cases, it also expands ventrally reflecting a lack of Toll activation (G). (H–M) *In situ* hybridization of *twist* (*twi*) and *snail* (*sna*) in wt embryos (H and K) and in embryos derived from *clos*^{1} /*clos*^{4152} raised at 29° (I, J, L, and M). In the mutant embryos, *twi* and *sna* mRNA expression is suppressed in some ventral regions consistent with an expansion of dorsal fates. Note that *in situ* whole mounts in embryos from *clos*^{1} females are particularly difficult because of the problems in removing the vitelline membrane in embryos with compromised vitelline membrane integrity [in agreement with the observations of LeMosy and Hashimoto (2000)]; therefore, most fixed embryos do not have a perfect morphology and show a bit of background. mRNA, messenger RNA; wt, wild-type.

that activate the Torso (Tor) and Toll (Tl) receptors in restricted domains of the embryonic membrane [for a review on *Drosophila* early embryonic patterning see St Johnston and Nusslein-Volhard (1992)]. The terminal and dorsoventral systems appear to be tightly coupled to the vitelline membrane (Jimenez *et al.* 2002; Stevens *et al.* 2003; Zhang *et al.* 2009; Ventura *et al.* 2010). Thus, in the terminal system, activation of the Tor receptor at the embryonic poles by its ligand Trunk (Trk) requires fully functional components of the vitelline membrane. In this regard, there are specific mutants for each of the *fs(1)N*, *fs(1)ph*, and *clos* genes that ensure eggshell formation thus allowing embryogenesis to proceed, but impair the activation of the Tor receptor and thus embryos do not develop their terminal regions (Degelmann *et al.* 1990). Likewise, in the dorsoventral system, the Nudel (Ndl) protease is required both for the activation of the Tl receptor in the ventral part of the embryo and for vitelline membrane integrity (LeMosy and Hashimoto 2000). Indeed, *ndl* mutant alleles fall into two phenotypic classes. Eggs laid by females carrying class I alleles [associated with the absence or aberrations of the protein (LeMosy *et al.* 2000)] collapse or arrest in early embryogenesis. In contrast, embryos from females carrying class II alleles [consisting of missense mutations in the protease domain (Hong and Hashimoto 1996)] complete embryogenesis but have defects in vitelline membrane cross-linking and are dorsalized. Therefore, Ndl exerts a proteolytic function involved in embryonic dorsoventral axis specification and vitelline membrane cross-linking.

In the case of terminal signaling, while the complete process that leads to Tor receptor activation remains unclear, we are beginning to unravel how the process is linked to the vitelline membrane. In particular, the work of many laboratories has shown the following: (1) Torso-like (Tsl), a protein secreted by the follicle cells (Savant-Bhonsale and Montell 1993; Martin *et al.* 1994), is required for Trk-mediated activation of the Tor receptor (Casali and Casanova 2001; Furriols and Casanova 2003; Johnson *et al.* 2015); (2) Tsl first accumulates at the vitelline membrane during oogenesis and later translocates to the embryonic plasma membrane, probably at egg activation (Mineo *et al.* 2015); and (3) Nasrat, Polehole, and Closca proteins are required either for the anchoring or stabilization of Tsl at the vitelline membrane (Jimenez *et al.* 2002; Stevens *et al.* 2003; Ventura *et al.* 2010).

Here, we further examined the role of Nasrat, Polehole, and Closca proteins in vitelline membrane integrity and cell signaling. We found that embryos from *clos*^{1} females also show defects in dorsoventral patterning, thereby unveiling that the dorsoventral and terminal systems, hitherto considered independent, share a common step in their extracellular pathways. Furthermore, we show that Nasrat, Polehole, and Closca are required for proper Ndl localization and function, thus providing a link for the functions of these three proteins

Table 1 Percentage of dorsalized phenotypes

	$clos^{1}/clos^{4152}$	$ndl^{-/+}$	$clos^{1}/clos^{4152}$; $ndl^{-}/+$
18°	1% (n = 311)	7% (n = 342)	8% (n = 754)
25°	7% (n = 273)	16% (n = 420)	41% (n = 331)
29°	45% (n = 153)	12% (n = 220)	79% (n = 170)

in vitelline membrane integrity and dorsoventral patterning. We propose that Nasrat, Polehole, and Closca constitute a multifunctional hub for the proper anchorage and activity of extracellular signaling molecules, ensuring their spatial and temporal restricted function to ensure coordinated embryonic axis specification.

Materials and Methods

Fly stocks

We used the following *Drosophila* stocks described in FlyBase: *yw* flies as wild-type, *fs(1)Ph-HA*, *Df(3L)CH12*, $fs(1)ph^{K646}$, $fs(1)N^{14}$, $clos^{1}$, $clos^{4152}$, ndl^{14}, ndl^{111}, $fs(1)N^{A1038}$, $Vm26Ab^{QJ42}$, *Df(2L)BSC183*, and *clos-HA*. In all the experiments, we employed the following transheterozygous combinations: $clos^{1}/clos^{4152}$, $ndl^{111}/Df(3L)CH12$, $ndl^{14}/Df(3L)CH12$, and $Vm26Ab^{QJ42}/Df(2L)$ BSC183 to discard the effect of any putative second site mutation in any chromosome (indeed, the $clos^{1}$ chromosome carries an associated lethal mutation). $clos^{4152}$, ndl^{14}, and $Vm26Ab^{QJ42}$ are null alleles of *clos*, *ndl*, and *Vm26Ab*. *Df(2L)BSC183* and *Df(3L)CH12* uncover the *Vm26Ab* and *ndl* loci, respectively.

Antibodies and immunostaining

For cuticle preparations, embryos were dechorionated with bleach, washed with 0.1% Triton, mounted with Hoyer:lactic (1:1), and incubated at 50–60° overnight. Whole-mount *in situ* hybridizations were performed according to Tautz and Pfeifle (1989) with minor modifications. Embryos were hybridized overnight at 56° with antisense probes labeled with digoxigenin (DIG). Immunostainings were done as described in Furriols and Casanova (2014). Egg chambers for Tsl immunostainings were heat-fixed as described in Mineo *et al.* (2015). The following primary antibodies were used: anti-Tsl 1:50 (Grillo *et al.* 2012), anti-Yl 1:1/500 (Schonbaum *et al.* 2000), anti-HA 1/300 (3F10 Roche), anti-Spectrin 1/5 (3A9, D.S.H.B.), anti-sV23 1/100 (Pascucci *et al.* 1996), anti C-Ndl 1:400 (LeMosy *et al.* 1998), and anti-DIG (1:2000; Roche), and secondary antibodies 1/300 (Jackson ImmunoResearch). Confocal images were obtained with a Leica SPE, analyzed in Fiji, and assembled with Adobe Photoshop.

Western blot analysis

Ovaries were dissected in cold 1 × PBS solution and immediately homogenized. Embryos were collected on apple juice plates and washed extensively with water. Samples were homogenized on ice in 2 × SDS sample buffer containing 6 M urea and 100 mM DTT according to LeMosy *et al.* (1998), boiled for 5 min, and soluble proteins loaded on 10% or 4–20% SDS-polyacrylamide gels (Amersham, Piscataway, NJ). Typically, three ovaries or 50–60 embryos from 0 to 1.5 hr were loaded per lane. Proteins were transferred to PVDF membranes (Millipore, Bedford, MA), blocked with Odissey blocking buffer PBS (Licor), and incubated with primary antibodies overnight. Membranes were analyzed using the Odyssey CLx imaging system (Licor). The following antibodies were used: anti C-Ndl 1:1000 (LeMosy *et al.* 1998), anti-Tubulin 1:5000 (Millipore), and secondary antibodies 1/10,000 (IRDye 800 and 680 from Licor).

Data availability

All data required to confirm the main findings presented in the article are included with the article and in Supplemental Material, Figure S1. Fly strains generated in the laboratory and used for this work are available upon request.

Results

clos mutants show defects in dorsoventral patterning

fs(1)N-, *fs(1)ph*-, and *clos*-null mutations cause eggshell integrity defects that lead to egg collapse and failure to sustain embryonic development. Therefore, it is not possible to assess the requirement of these genes in embryonic patterning from the progeny of these null mutant females. Only females from the hypomorph mutations $fs(1)N^{211}$, $fs(1)ph^{1901}$, and $clos^{1}$ lead to eggs that allow embryonic development and, in these embryos, patterning defects for terminal system patterning have been reported (Degelmann *et al.* 1990; Jimenez *et al.* 2002; Ventura *et al.* 2010). However, we extended the previous analyses on the embryos laid by $clos^{1}$ mutant females at various temperatures (see *Materials and Methods*). When raised at 18°, $clos^{1}$ females lay embryos that display a terminal phenotype (Ventura *et al.* 2010). However, when raised at 29°, 45% of embryos had a dorsalized phenotype displaying a loss of ventral structures at the expense of dorsal ones (Figure 1, C and D), in addition to their terminal defects (Table 1). The dorsalized phenotype was completely rescued by adding a copy of the full *clos* genomic construct (Ventura *et al.* 2010) (100% rescue; n = 390). We confirmed the defects in dorsoventral patterning by examining the expression pattern of *zerknullt* (*zen*), which is detected in a dorsal ectoderm stripe in wild-type stage 4 embryos [Figure 1E and Doyle *et al.* (1989)], and *twist* (*twi*) and *snail* (*sna*) detected in the prospective mesoderm (Figure 1, H and K) (Thisse *et al.* 1987; Leptin and Grunewald 1990). In embryos devoid of terminal activity, *zen* expression is not sustained at the embryonic poles (Helman *et al.* 2012); this was the case for all the embryos laid by $clos^{1}$ females at all the temperatures tested (Figure 1, B and F). However, in some cases, when the females were raised at 29°, we observed a clear ventral expansion of *zen* expression (Figure 1G) and a partial suppression of *twi* (Figure 1, I and J) and *sna* expression (Figure 1, L and M),

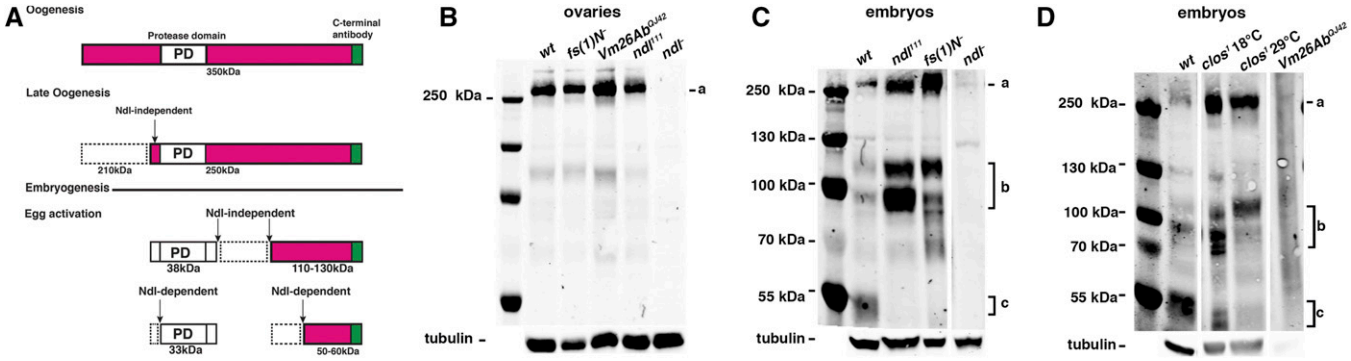

Figure 2 Nasrat, Polehole, and Closca are required for Nudel (Ndl) activity. (A) Schematic diagram of Ndl processing defined by western blots adapted from LeMosy *et al.* (1998). PD indicates the Ndl protease domain. Ndl C-terminal polypeptides are in purple, the region of Ndl recognized by the C-terminal polyclonal antibody is in green. (B) C-terminal Ndl polypeptides in ovaries from wild-type (wt), *fs(1)N*^{14}, *Vm26Ab*^{QJ42}*/Df(2L)BSC183*, *ndl*^{111}*/Df(3L)CH12*, and *ndl*^{14}*/Df(3L)CH12* females. The 350 kDa Ndl is hardly detected with this antibody (LeMosy *et al.* 1998). The 250-kDa Ndl C-terminal polypeptide (a) is detected in all cases except in ovaries derived from *ndl*^{14}*/Df(3L)CH12* females. *ndl*^{14} (lane 4) encodes for a truncated Ndl protein that is not recognized by the antibody (LeMosy *et al.* 2000). (C) C-terminal Ndl polypeptides in embryos from wt, *fs(1)N*^{14}, *ndl*^{111}*/Df(3L)CH12*, and *ndl*^{14}*/Df(3L) CH12* females. Embryos from *fs(1)N*^{14} and *ndl*^{111} females (lanes 2 and 3) completely lack the 50–60-kDa C-terminal polypeptides (c), which require functional Nudel protease activity. Embryos from *fs(1)N*^{14} and *ndl*^{111} females (lanes 2 and 3) show increased levels of two Ndl C-terminal polypeptides of ~100 kDa (b), which are the precursors of the 50–60-kDa polypeptides; compare to wt (lane 1). All lanes come from the same gel and filter. (D) C-terminal Ndl polypeptides from wt females, from *clos*^{1}*/clos*^{4152} females raised at 18° and at 29°, and from *Vm26Ab*^{QJ42}*/Df(2L)* BSC183 females. High levels of the 50–60-kDa processed C-terminal polypeptides (c) are detected in embryos from wt and *clos*^{1}*/clos*^{4152} females raised at 18° (lanes 1 and 2) but are strongly reduced in embryos from *clos*^{1}*/clos*^{4152} females raised at 29° (lane 3). High levels of the Ndl C-terminal polypeptides of ~100 kDa (b) are detected in embryos laid by *clos*^{1}*/clos*^{4152} females (lanes 2 and 3) compared to the wt (lane 1). No Ndl discrete bands could be detected in embryos from *Vm26Ab*^{QJ42}*/Df(2L)* BSC183 (lane 4); the Ndl pattern exhibit a smear suggesting protein degradation. In all cases, an antibody against Tubulin 50 kDa (bottom) was used for a loading control. Note that Tubulin is barely detectable in embryos laid by *Vm26Ab*^{QJ42}*/Df(2L)* BSC183 flies, probably due to protein degradation. All lanes come from the same gel and filter. Some differences between the westerns in (C) and (D) are due to the corresponding gels being of different acrylamide percentages.

revealing an expansion of the dorsal fate, which is in agreement with the cuticle phenotype. In addition, we observed a genetic interaction between *ndl* and *clos* in dorsoventral patterning, as the penetrance of the dorsoventral phenotype in embryos from *clos*^{1} females in the stronger conditions (at 25 and 29°) increased in a *ndl* hemizygous background (Table 1).

Ndl protease activity is impaired in fs(1)N, fs(1)ph, and clos mutants

The eggshell integrity defects of *clos*^{1} mutants (Ventura *et al.* 2010) and their dorsoventral phenotype (Figure 1), and the sensitivity of the latter to the dose of *ndl* (Table 1), prompted us to examine whether Nasrat, Polehole, and Closca are required for Ndl function. As indicated above, Nasrat, Polehole, and Closca are mutually required for their incorporation into the vitelline membrane and thus null alleles for either *fs(1)N*, *fs(1)ph*, or *clos* behave functionally as a triple mutant for all of them (Jimenez *et al.* 2002; Ventura *et al.* 2010). Thus, we have used a null allele for either of them interchangeably in our experiments.

During midoogenesis, Ndl is secreted as a zymogen of 350 kDa, which is cleaved in late oogenesis to generate a C-terminal polypeptide of 250 kDa containing the Ndl protease domain [Figure 2, A–C and LeMosy *et al.* (1998)]. We detected the C-terminus fragment of Ndl both in egg chambers from wild-type and from *fs(1)N*-null mutant females (Figure 2B, a), the latter also lacking Nasrat and Closca accumulation at the vitelline membrane (Jimenez *et al.* 2002; Ventura *et al.* 2010). Later on, in embryogenesis, Ndl is further processed by at least two proteolytic events. The first, occurring at egg activation, is independent of Nudel protease activity, and it generates a 110–130-kDa C-terminal polypeptide from the 250 kDa fragment (LeMosy and Hashimoto 2000). The second one depends on Nudel protease activity itself and gives rise to a 50–60-kDa C-terminal polypeptide from the C-terminal 110–130 kDa polypeptide [Figure 2A and LeMosy *et al.* (1998)].

We could detect the same proteolytic pattern although, in our hands, we observed slightly different molecular weights. Accordingly, western blots from wild-type embryos (Figure 2C, lane 1) showed a C-terminal 50–60-kDa doublet (Figure 2C, c), which is generated by Ndl-dependent protease activity as it is not detected in embryos derived from *ndl*^{111} mutant females with compromised Ndl protease activity (LeMosy *et al.* 2000) [Figure 2C, lane 2 and LeMosy *et al.* (2000)]. Neither was the Ndl C-terminal 50–60-kDa doublet detected in embryos from *fs(1)N*-null mutant females (Figure 2C, lane 3), thereby indicating impaired Ndl protease activity. Of note, in embryos from either *ndl*^{111}- or *fs(1)N*-null mutant females, the impaired Ndl protease activity caused the accumulation of intermediate C-terminal polypeptides (110–130 kDa) (Figure 2C, b) generated in a Ndl-independent way. These results suggest that the failure in Ndl protease activity detected in the embryos laid by *fs(1)N*-null mutant females accounts for the eggshell integrity phenotype observed in *fs(1)N*-, *fs(1)ph*-, and

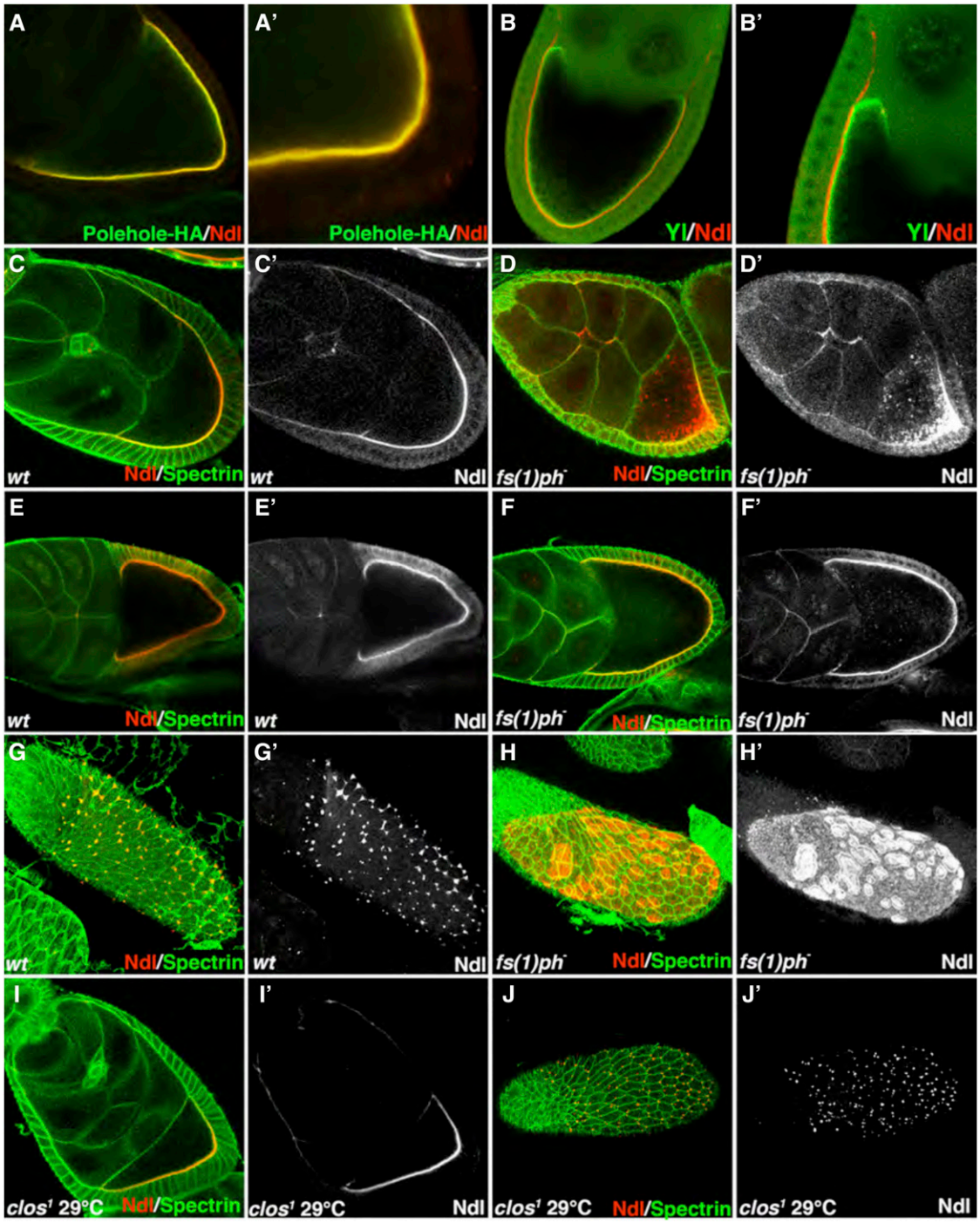

Figure 3 Nudel (Ndl) localize at the vitelline membrane anchored or stabilized by Nasrat, Polehole, and Closca. (A) Confocal section of a stage 9 egg chamber expressing Polehole-HA and immunostained with anti-C-Ndl (in red) and anti-HA(in green) antibodies. (A') A magnification of A showing the colocalization. (B) Confocal section of a stage 9 egg chamber immunostained with anti-C-Ndl (in red) and anti-Yl (in green) antibodies. (B') A magnification of B showing the lack of colocalization. (C–H) Confocal sections of wt (wt) and *fs(1)ph*^{K646} egg chambers at different stages immunostained with anti-C-Ndl (in red) and anti-Spectrin (in green) antibodies. (C and D) stage 9; (E and F) stage 10; and (G and H) stage 12. (C'–H') Same images in the red channel. Note mislocalized Ndl inside the oocyte at stage 9 (D and D') and the Ndl lumpy distribution at stage 12 (H and H') in *fs(1)ph*^{K646} egg chambers. Conversely, Ndl is properly localized at stage 9 (I and I') and at stage 12 egg chambers (J and J') in *clos*^{1} */clos*^{4152} females (raised alt 29°).

clos-null mutant females, although it is probably not its only cause as *ndl* mutants that lack protease activity have weaker eggshell defects (LeMosy *et al.* 1998; LeMosy and Hashimoto 2000) than null mutants for *fs(1)N*, *fs(1)ph*, or *clos*. Furthermore, we found a correlation between the dorsalized phenotype and the eggshell integrity defects in

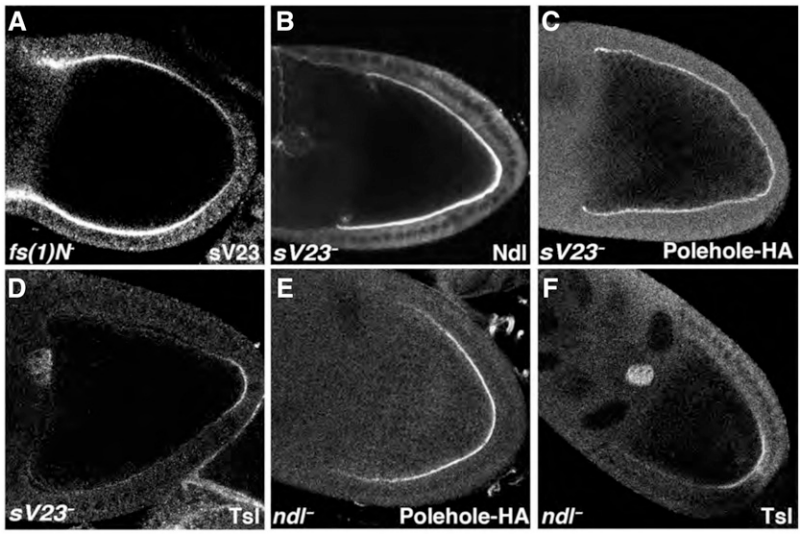

Figure 4 Accumulation of vitelline membrane components in mutant egg chambers. (A) Confocal section of a *fs(1)N*^{14} stage 9 egg chamber showing a wild-type accumulation of sV23 as detected by the anti-sV23 antibody. (B–D) Confocal sections of *Vm26Ab*^{QJ42}*/DefBSC183* stage 9 (B and D) or early stage 10A (C) egg chambers, which lack sV23 protein, immunostained with anti-C-Ndl antibody (B), expressing the Polehole-HA and immunostained with anti-HA antibody (C), and immunostained with anti-Tsl antibody (D). In all three cases, there is a wild-type pattern. (E and F) Confocal sections of *ndl*^{14}*/Df(3L)CH12* stage 9 egg chambers expressing the Polehole-HA and immunostained with anti-HA antibody (E) and immunostained with anti-Tsl antibody (F). In both cases, there is a wild-type pattern.

embryos from *clos*^{1} females and the activity of Ndl protease. Thus, we unveil a lack of proper Ndl protease activity in embryos laid by *clos*^{1} mothers raised at 29° as compared with those laid by *clos*^{1} mothers raised at 18° (Figure 2D, compare lanes 2 and 3). These observations suggest that the dorsalized phenotype and the eggshell integrity defects in embryos laid by *clos*^{1} females (Ventura *et al.* 2010) at 29° are caused by the lack of Ndl proteolytical activity.

Ndl localization is impaired in fs(1)N, fs(1)ph, and clos mutants

As mentioned above, Nasrat, Polehole, and Closca proteins are required either for the anchoring or stabilization of Tsl at the vitelline membrane (Jimenez *et al.* 2002; Stevens *et al.* 2003; Ventura *et al.* 2010). Therefore, we examined whether this could also be the case for Ndl. Indeed, we found that secreted Ndl overlaps with the vitelline membrane component Polehole (Figure 3, A and A') but not with a protein of the oocyte plasma membrane such as the yolk receptor Yolkless (Yl) [Figure 3, B and B' and Schonbaum *et al.* 2000)], thereby indicating that Ndl accumulates at the vitelline membrane during oogenesis once secreted from the follicle cells.

Furthermore, we found that the proper accumulation of Ndl at the vitelline membrane depends on Nasrat, Polehole, and Closca proteins. In particular, Ndl was partially mislocalized inside the oocyte in all stage 9 egg chambers examined from null *fs(1)ph* mutant females, (Figure 3, C–D' and Figure S1), thereby suggesting that the vitelline membrane does not hold or stabilize all the Ndl protein in the absence of Nasrat, Polehole, and Closca. The nonlocalized Ndl protein may be degraded and/or eliminated, as we did not detect it at stage 10 (Figure 3, E–F' and Figure S1, C–D'). However, again, we observed an abnormal pattern at stages 12–14, when in the wild-type egg chambers Ndl is detected between follicle cells in aggregates together with other vitelline membrane proteins (data not shown; Furriols and Casanova 2014); conversely, in ~70% of *fs(1)ph* mutant females, Ndl showed a lumpy distribution between follicle cells and the oocyte plasma membrane (Figure 3, G–H' and Figure S1, E–F'). However, we observed a proper Ndl pattern in *clos*^{1} egg chambers even at 29° (Figure 3, I and J), indicating that a dorsalized phenotype (Figure 1 and Table 1) and gross mislocalization of Ndl are not necessarily always linked.

Since we found that Ndl anchorage or stabilization at the vitelline membrane partially depends on Nasrat, Polehole, and Closca, we questioned whether this was due to a specific role of these proteins on Ndl or whether Ndl might be mislocalized by a general problem in vitelline membrane integrity. To address this point, we first examined whether structural vitelline membrane components were affected in egg chambers from *fs(1)N* mutant mothers and found this not to be the case as sV23 (Figure 4A) and VM32E (data not shown) localized correctly in these chambers. We next examined Ndl localization in egg chambers from *Vm26Ab*^{QJ42} mutant females, which lack the sV23 protein and lay eggs that collapse due to severe defects in vitelline membrane integrity (Savant and Waring 1989). We found that Ndl, as well as Polehole and Tsl, localized correctly at the vitelline membrane (Figure 4, B–D). While Ndl accumulates correctly in *Vm26Ab*^{QJ42} ovaries (Figure 2A and Figure 4B), we could not assess Ndl protease activity in embryos from *Vm26Ab*^{QJ42} females because there appears to be a general problem of protein degradation in these embryos; in fact, we could not detect or detected at very low intensity the band of tubulin used as a control (Figure 2E). Finally, while Nasrat, Polehole, and Closca were found to be required for Ndl localization, the opposite was not true, as Polehole and also Tsl localized correctly in egg chambers from *ndl*-null flies (Figure 4, E and F). All together, these observations suggest that Nasrat, Polehole, and Closca have a specific role in Ndl stabilization or localization at the vitelline membrane, and that the impairment of Ndl function in *fs(1)N*, *fs(1)ph*, or *clos* mutants is not just a consequence of a membrane integrity problem.

Discussion

Terminal and dorsoventral signaling rely on initial spatial cues, which originate in the follicle cells surrounding the oocyte, that induce pattern formation in embryogenesis. Since follicle cells degenerate long before the cues perform their action in embryogenesis, all the information necessary for embryonic patterning has to be retained in the egg. In this scenario, the role of Nasrat, Polehole, and Closca in the localization of Tsl and Ndl suggests that a Nasrat/Polehole/Closca complex acts as a multifunctional hub at the vitelline membrane to anchor various proteins synthesized at oogenesis and with later functions in the eggshell and/or in triggering embryonic patterning.

Although the mechanism responsible for eggshell integrity is not fully understood, Ndl, and in particular its protease activity (LeMosy and Hashimoto 2000), Nasrat, Polehole, and Closca clearly participate in this process (Cernilogar *et al.* 2001). Our results now identify Ndl as an effector of Nasrat, Polehole, and Closca both in eggshell integrity and in their so far unknown role in dorsoventral patterning. In this regard, it is worth mentioning that, in spite of the many analyses of Ndl activity, it remains an open question as to whether its function in dorsoventral axis specification and eggshell integrity are independent of each other. Besides, LeMosy and collaborators have proposed an additional role for the nonprotease regions of Ndl in eggshell integrity (LeMosy *et al.* 1998; LeMosy and Hashimoto 2000).

Likewise, it is difficult to establish whether the diverse roles of Nasrat, Polehole, and Closca imply specific and independent functional protein domains. Although Nasrat, Polehole, and Closca belong to a common group of proteins, they show only moderate similarity, and no functional domains have been identified in any of them (Jimenez *et al.* 2002; Ventura *et al.* 2010). The observation that a point mutation impairs the terminal functions of Clos proteins, as well as dorsoventral patterning and eggshell integrity, suggests a lack of clear independent domains responsible for each individual function. However, $fs(1)N^{211}$ and $fs(1)ph^{1901}$ mutants are thought to specifically impair the terminal function of Nasrat and Polehole proteins, respectively, which suggests that these might be modular proteins with different functional domains. Similarly, the $fs(1)N^{A1038}$ mutation supports the notion of independent functional domains (Degelmann *et al.* 1990). In particular, all eggs from homozygous $fs(1)N^{A1038}$ females collapse due to eggshell integrity defects; the same phenotype is observed in hemizygous $fs(1)N^{A1038}$ females and in transheterozygote females of $fs(1)N^{A1038}$ over a null *fs(1)N* mutant allele (Degelmann *et al.* 1990). However, eggs from transheterozygous females of $fs(1)N^{A1038}$ over the $fs(1)N^{211}$ terminal allele give rise to wild-type larvae and adults [Degelmann *et al.* (1990) and our own observations]. This intra-allelic complementation suggests that separate domains specifically affect the integrity and the terminal functions of the Nasrat protein. To further characterize these putative protein domains, we mapped the molecular lesion in the $fs(1)N^{A1038}$ mutation and found it to correspond to an E to V transition at residue 350 (data not shown). This observation suggests that the domain of the Nasrat protein encompassing this residue is required for eggshell integrity but has no effect on embryonic patterning.

In conclusion, we have found that terminal and dorsoventral signaling, hitherto considered independent in their extracellular pathways, have Nasrat, Polehole, and Closca as common mediators. We propose that a complex of these proteins constitutes a multifunctional hub to ensure the proper temporal localization/stabilization and activity of proteins synthesized at oogenesis and required at egg activation, thus guaranteeing the coordination of the hardening of the eggshell with the trigger of early embryonic patterning.

Acknowledgments

We thank N. Martín and E. Fuentes for technical assistance; E. K. LeMosy, M. Forés, N. Samper, G. Jimenez, G. Gargiulo, A. Ephrussi, and G. Waring for reagents and stocks; and A. Casali, M. Llimargas, and S. Araujo for comments on the manuscript. A.M. was supported by a fellowship from La Caixa Foundation. This work has been supported by the Generalitat de Catalunya, the Spanish Ministerio de Ciencia e Innovacion. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors declare no competing or financial interests.

Author contributions: A.M. and M.F. performed the experiments. A.M., M.F., and J.C. conceived and designed the experiments, analyzed the data, and wrote the paper.

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Communicating editor: N. Perrimon

GENETICS

Supporting Information
www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.197574/-/DC1

Transfer of Dorsoventral and Terminal Information from the Ovary to the Embryo by a Common Group of Eggshell Proteins in *Drosophila*

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DOI: 10.1534/genetics.116.197574