Developmental Biology 453 (2019) 107–109

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Commentary

Cracking the eggshell: A novel link to intracellular signaling

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In sexually reproducing organisms, oocytes arrested in prophase I of meiosis reenter the cell cycle in response to hormonal or developmental stimulation and undergo physiological changes during maturation to become competent for fertilization. After fertilization, oocytes from different species employ conserved as well as organism-specific regulatory strategies that govern a collection of events known as egg activation (Von Stetina and Orr-Weaver, 2011). Egg activation is triggered by sperm entry and involves multiple, concurrent downstream events that include progression through the meiotic cell cycle, rearrangement of the cytoskeleton, generation of a block to polyspermy, and activation and degradation of selected inherited maternal mRNAs and proteins (Horner and Wolfner, 2008). The block to polyspermy partially depends on the extracellular matrix, which has a different composition and organization in different species. The *C. elegans* oocyte is a useful system for studying oocyte maturation and the coordination of the complex events during egg activation.

A crucial function of the egg activation process is securing the block to polyspermy. Prior to fertilization, oocytes arrested prophase of meiosis I are covered by a receptive vitelline layer coating that mediates interactions with sperm. Immediately after fertilization, the vitelline is modified by the release of cargo through cortical granule exocytosis that converts this receptive coat into a block to polyspermy through addition of new protein components and enzymatic modifications (Wessel et al., 2001). Although the cargo delivered to the oocyte surface and the resulting modification to the extracellular matrix differs between organisms, cortical granule exocytosis is a highly conserved process (Liu, 2011). In *C. elegans,* the oocyte is covered by a vitelline layer that becomes the outermost layer of a multilayered eggshell built sequentially after fertilization. The eggshell contains several layers from the outside in: the outer vitelline layer, a chitin layer, the chondroitin proteoglycan layer, a region called the extra-embryonic matrix (referred to as the perivitelline space by Gonzalez et al.), the permeability barrier layer, and the peri-embryonic layer (Stein and Golden, 2015). The only known marker of the vitelline layer is the chitin-binding domain containing protein, CBD-1 (Johnston et al., 2010). The chitin layer is synthesized by the transmembrane chitin synthase, CHS-1, in metaphase I immediately after fertilization (Zhang et al., 2005). Cortical granule exocytosis, which occurs during anaphase I, releases chondroitin proteoglycans CPG-1, which is stably incorporated into the chondroitin proteoglycan layer, and CPG-2, which remains diffusive within the extra-embryonic matrix (Bembenek et al., 2007; Olson et al., 2012). The permeability barrier is made later during meiosis II by a process requiring lipid biosynthesis (Stein and Golden, 2015; Olson et al., 2012; Benenati et al., 2009). Therefore, eggshell formation and cell cycle progression are tightly integrated to ensure a highly coordinated series of developmental events. Inactivation of many genes that disrupt eggshell formation also block polar body extrusion, establishment of embryonic polarity and other actin-dependent processes (Johnston et al., 2006). In contrast, isolated blastomeres stripped of the eggshell, are viable *in vitro* (Edgar and Goldstein, 2012), begging the question as to why eggshell mutants are so severe. Results from the new paper from Gonzàlez et al. suggest that eggshell defects may cause these phenotypes due to an unappreciated function for the eggshell in organizing signaling during the oocyte-to-embryo transition.

CBD-1 is the first characterized component of the vitelline layer and is critical in proper eggshell formation. Prior to fertilization, CBD-1 is found on the surface of oocytes and is required for anchoring the transmembrane EGG-1 and EGG-2 proteins to the oocyte cortex where they promote sperm recognition (Johnston et al., 2010; Kadandale et al., 2005). CBD-1 is also required for normal distribution of CHS-1 in the oocyte plasma membrane so that it can synthesize chitin into the extracellular space (Johnston et al., 2010). Deposition of the chitin layer precedes the delivery of cortical granule cargo proteins, CPG-1 and CPG-2 (Olson et al., 2012). CBD-1 and CHS-1 are required for proper cortical recruitment of a complex containing the kinase, MBK-2 and its regulators EGG-3, -4, and -5 (Johnston et al., 2010; Maruyama et al., 2007; Parry et al., 2009). MBK-2 is a critical regulatory kinase required for oocyte-to-embryo transition (Cheng et al., 2009). After fertilization, the EGG proteins, CHS-1 and MBK-2 are internalized on vesicle like structures during the egg activation process. EGG-1 and EGG-2 are

DOI of original article: https://doi.org/10.1016/j.ydbio.2018.08.005.
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https://doi.org/10.1016/j.ydbio.2019.05.014
Received 29 January 2019; Accepted 28 May 2019
Available online 8 June 2019

endocytosed following formation of the chitin layer (Johnston et al., 2010). Similarly, EGG-3 is internalized and degraded following resumption of the cell cycle (Maruyama et al., 2007). EGG-4 and EGG-5, two redundant pseudo-phosphatases, whose essential role is to sequester and inhibit MBK-2 at the cortex until resumption of meiotic divisions, are also removed from the surface (Cheng et al., 2009). Subsequently, MBK-2 is gradually released into the cytoplasm where it is active and phosphorylates substrates to control the transition into the embryonic divisions (Cheng et al., 2009; Stitzel et al., 2006). These observations, therefore, indicate that CBD-1 is a key upstream organizer of the intracellular signaling molecules that govern the oocyte-to-embryo transition. However, there is no detailed understanding of how CBD-1 functions in the eggshell.

Despite the extensive research into the molecular signaling that drives early *C. elegans* development as well as the eggshell formation process, there is still a dearth of knowledge about the vitelline layer. The components that make up the vitelline layer and how these components assemble on the oocyte surface have not been determined. In their recent article, Gonzàlez et al. (González et al., 2018) identify new molecules that make up the vitelline layer and define a novel function for CBD-1 in the formation of the vitelline layer of the eggshell. This article advances our understanding of the establishment and composition of the vitelline layer by presenting compelling evidence that two proteins, PERM-2 and PERM-4 are critical for vitelline layer formation. Loss of either PERM protein results in increased permeability of the eggshell (Olson et al., 2012). CBD-1 is required for the recruitment of both PERM-2 and -4 on the oocyte surface prior to fertilization but does not depend on PERM proteins for its own proper localization. However, PERM-2 and PERM-4 both depend on each other to properly localize to the vitelline layer. There is also compelling biochemical evidence that CBD-1 and PERM-2/4 associate with each other indicating that they form a novel protein complex critical in forming the vitelline layer. Therefore, CBD-1 not only serves to organize MBK-2 pathway components to ensure proper oocyte-to-embryo transition signaling, but also plays a key role in the structural assembly of the vitelline layer.

Gonzàlez et al. also provide insight into the mechanism by which CBD-1 recruits PERM 2 and 4. Using an in-frame deletion, which eliminates two N-terminal chitin binding domains of CBD-1, they demonstrate that the N-terminus of CBD-1 is required for the recruitment of PERM-2 and 4 and eggshell integrity. Curiously, this interaction occurs prior to fertilization and before the time when chitin is synthesized in the eggshell. This complex may control the organization of chitin as it is extruded into the eggshell. CBD-1's presence at the oocyte cortex prior to fertilization and its chitin binding domains make it an ideal candidate for organizing newly synthesized chitin. It will be important to determine the dynamics of the vitelline complex containing CBD-1 and PERM-2/-4 after synthesis of chitin. It will be interesting to determine whether chitin binding to CBD-1 is competitive with PERM-2/-4 or whether the complex remains associated when chitin is present. Competitive binding between the PERM proteins and chitin may constitute a regulatory scheme that drives molecular rearrangements to convert the oocyte vitelline layer into a multi-layered impenetrable eggshell. Importantly, deletion of the two N-terminal chitin binding domains does not appear to affect the cortical recruitment of either EGG-1 or EGG-3, demonstrating that CBD-1 has two separate functions in organizing the vitelline layer and the intracellular signaling complexes at the plasma membrane. EGG-3 is required for chitin synthesis and is endocytosed upon fertilization and chitin synthesis (Maruyama et al., 2007). Gonzàlez et al.'s observation also raises the question of what drives the internalization of EGG-1 and -3 and how CBD-1's affinity for these proteins changes following fertilization. Furthermore, the recruitment of the EGG and PERM complexes are not interdependent. Depletion of PERM-2 or -4 has little effect on the recruitment of either EGG-1 or EGG-3. Likewise, co-depletion of EGG-1 and -2 does not prevent PERM-2 or 4 recruitment by CBD-1. These data demonstrate that CBD-1 is a critical organizer of both a signaling protein complex required for oocyte-to-embryo transition and vitelline layer formation.

Interestingly, there is evidence that CBD-1 not only anchors EGG-1 and EGG-3 but also might impact the intracellular transport mechanism that delivers these proteins to the cortex. CBD-1 depletion results in EGG-1 being trapped in intracellular vesicles in the cytoplasm rather than localized to the plasma membrane as also observed previously (Johnston et al., 2010). In contrast, EGG-3 fails to evenly distribute as a continuous layer along the cortex. These observations suggest that EGG-1 might be continuously removed from the cell cortex and that CBD-1 tethers EGG-1 resulting in its cortical enrichment. If this is the case, it would also be interesting to investigate if oocyte endocytic mechanisms are required to remove EGG-1 (Fares and Grant, 2002). It is noteworthy that compensatory endocytosis occurs after cortical granule exocytosis in other species and might be involved in internalizing the EGG and MBK-2 complexes (Bement et al., 2000). Alternatively, CBD-1 may be required for cellular signaling that promotes the transport of EGG-1 to the cortex. Future investigation of the trafficking of the different signaling and extracellular coat proteins in the oocyte and activated egg will be necessary to differentiate between these possibilities.

Beyond the mechanism of vitelline layer formation, Gonzàlez et al. provide evidence of a yet unexplored function of this layer as a diffusion barrier for proteins secreted into the extra-embryonic matrix. CPG-1 and CPG-2, both cargos transported by cortical granules, have different fates following their delivery. In the presence of a properly formed vitelline layer, CPG-1 is readily incorporated into the chondroitin proteoglycan layer whereas CPG-2 freely diffuses within the extra-embryonic matrix (Olson et al., 2012). Defects in the vitelline layer introduced by PERM protein loss leads to diffusion of CPG-2 into the uterus, indicating improper integrity of the eggshell. It is also notable that loss of PERM-2/4 leads to embryos that abnormally adhere to each other, suggesting changes to the properties of the outer surface of the egg coating. PERM-2/4 could regulate or be enzymes that modify the eggshell, or they could serve as platforms to recruit uterine factors that might associate with the embryo exterior. It will be interesting to investigate how the loss of PERM-2 and PERM-4 alters the molecular structure of the vitelline layer.

The discoveries presented in this article have the potential to expand our understanding of pathogenic worms. Chitin synthesis is critical for the survival of many parasitic nematodes, but not their hosts, making drugs that target chitin synthesis attractive targets for treating infections (Foster et al., 2005). Development of such anthelmintic drugs against chitin synthase would also reduce the risk of side effects of such drugs. The work presented by Gonzàlez et al. identifies new molecules critical for eggshell formation in *C. elegans* that are required for embryo viability and may also be operative during the growth of parasitic worms (Foster et al., 2005; Wharton, 1980). Protein sequence analysis suggests that CBD-1, PERM-2 and PERM-4 are conserved among nematodes and may be attractive drug targets. This potentially identifies novel drug targets unique to parasitic nematodes that could lead to development of new anthelminthic therapies.

The need for altering the extracellular matrix of a fertilized embryo to prevent polyspermy is a long-established developmental phenomenon in oocytes of many species including mammals (Hoodbhoy and Talbot, 1994). The observation that CDB-1 is critical for recruiting a protein complex controlling oocyte-to-embryo intracellular signaling as well as extracellular components that build the outer most layer of the eggshell is a significant discovery. Modifications of the egg coating have long been thought of as downstream events in the egg activation process, but these results from *C. elegans* indicate that there is a direct link between components of the extracellular coat and intracellular signaling complexes. Cues from the extracellular matrix are known to influence cellular signaling in other contexts (Mammoto et al., 2013), hence, the molecular linkage between structural integrity of the egg coat and organization of intracellular signaling in oocytes might be conserved in other organisms and represents a significant conceptual advance of this study. Future studies into the function of CBD-1 will advance our understanding of how

the oocyte coordinates multiple disparate processes during egg activation.

Acknowledgements

We thank Andy Golden, Andrew Singson and members of the Bembenek laboratory for helpful comments on the manuscript. Funding was provided by NIH R01 GM114471 to JNB.

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