

Expression of a set of glial cell-specific markers in the *Drosophila* embryonic central nervous system

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The types of glia in the central nervous system (CNS) of the *Drosophila* embryo include longitudinal glia (LG), cell body glia (CBG), and peripheral glia (PG). Transcription factors, such as *glial cell missing* and *reverse polarity*, are well-established general glial cell markers. Only a few glial cell-specific markers have been identified in the *Drosophila* embryonic CNS, thus far. In the present study, we employed the glial cell-specific markers for LG (*vir-1*/CG5453 and CG31235), CBG (*fabp*/CG6783 and CG11902), and PG (CG2310 and *moody*/CG4322), and comprehensively analyzed their expression patterns, during the embryonic CNS development. Our study validated the specificity of a set of glial markers, and further revealed their spatio-temporal expression patterns, which will aid in the understanding of the developmental lineage, and investigating their role in the development and homeostasis of the *Drosophila* CNS *in vivo*. [BMB Reports 2014; 47(6): 354-359]

INTRODUCTION

Glia play active roles in axon guidance and the development of neurons, supporting neurotransmission, maintaining ionic balance, storing energy, and insulating axons to speed up electrical communication (1, 2). It is also thought that glia play primary roles in information processing, like neurons, synaptic remodeling, and homeostasis of the CNS, by surveying the brain for an infection and damage, and subsequently engulfing dead cells and foreign antigens (3-5). In order to clearly understand the functional contributions of glia to information processing, synaptic remodeling, and innate immune reactions, it is important to establish an *in vivo* model system. *Drosophila* serves as one of the excellent *in vivo* model systems for study-

ing the diverse functions of glia, using well-established molecular genetic tools, and high-resolution cellular imaging techniques, with molecular markers that yield images at single-cell resolution (6, 7).

The glia in *Drosophila* are evolutionarily conserved, and exhibit many developmental, morphological, and functional similarities to their mammalian counterparts (8, 9). Furthermore, cell-body glia (CBG) in *Drosophila* are similar to astrocytes, being embedded within the cell cortex, in close contact with neurons. The longitudinal glia (LG), belonging to a subclass of *Drosophila* neuropile glia, are functionally similar to vertebrate oligodendrocytes that insulate axons from the environment, and promote neuronal survival, via trophic support mechanisms. The peripheral glia (PG) migrate long distances into the periphery, where they interact closely with axons, to support and ensheath peripheral nerves, similar to the mammalian Schwann cells. In addition, they are required for formation of the blood-brain barrier, which serves as a major entry site for gases and nutrients into the CNS, and are essential for growth cone guidance of motor and sensory neurons.

The master regulatory gene of glial cell development, *glial cells missing*/*glial cell deficient* (*gcm*/*glide*), encodes a novel transcription factor with Gcm-binding motifs, which is transiently expressed in all glia, except the midline glia. *gcm* functions as a binary genetic switch for glial versus neuronal cell fate (10-12). *Gcm* activates downstream target genes, including *reverse polarity* (*repo*) and *pointed* that are required for glial differentiation, as well as *tramtrack*, which represses neuronal fate within the glial cell lineage. Amongst these genes, *Gcm* and *Repo* have been extensively employed as general glial markers for gliogenesis studies.

Thus far, more than 40 novel *Drosophila* glial genes have been identified, using several genomic approaches, in order to gain deeper insights into the development and functions of glia (13-15). They are regulated by the master gene for glial cell fate in *Drosophila*, *gcm*, and are expressed in the glial cell lineage. Although this study provided brief expression data of the selected glial genes that show unique spatio-temporal patterns in the glia sublineage during gliogenesis, this information did not provide enough knowledge that is required for gaining molecular and cellular insights into the function and development of the specific glial cell lineage. In order to promote gaining the

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deeper insights into glial development and function in *Drosophila*, we validated the specificity of the glial markers for LG (*vir-1*/CG5453 and CG31235), CBG (*fabp*/CG6783 and CG11902), and PG (CG2310 and *moody*/CG4322), and comprehensively analyzed their expression patterns during the embryonic CNS development of *Drosophila*, by using the *in situ* hybridization method.

RESULTS

The expression of the LG markers, *vir-1*/CG5453 and CG31235 was examined during the *Drosophila* embryonic development. *vir-1*/CG5453 expression started in the midline cells of the ventral neuroectoderm at stage 10, and became detectable shortly thereafter, in the tracheal precursor cells, in addition to the midline cells at stage 11 (Fig. 1A-D). Its expression in the trachea and 3-4 midline cells became prominent in a segmentally repetitive fashion at stage 12 (Fig. 1E-H).

Subsequently, it was found to be expressed in approximately 4-5 LG of each segment, in addition to tracheal cells and midline cells at stage 14 (Fig. 1M-P). While its expression in the LG and midline cells peaked at stage 16, the first *vir-1*/CG5453-positive oenocytes were detected (Fig. 1U-X). The expression of another LG marker, CG31235 first appeared in the LG at stage 11 (Fig. 1A'-C'). Its expression was repeated in every 2-3 LG per hemisegment, and became detectable shortly thereafter, in the brain at stage 12 (Fig. 1D'-F'). The CG31235-expressing LG were found to be connected along the longitudinal tracts at stage 13 (Fig. 1G'-I'), and the number of CG31235-positive LG increased to approximately 3-4 at stages 14 and 15 (Fig. 1J'-O'). At stages 16 and 17, the number of CG31235-positive LG increased to 5-6 cells per segment, and its expression was seen in a few commissural glia (Fig. 1P'-U').

The expression of the CBG marker, *fabp*/CG6783 was initially detected in the surface-associated glia in each segment at

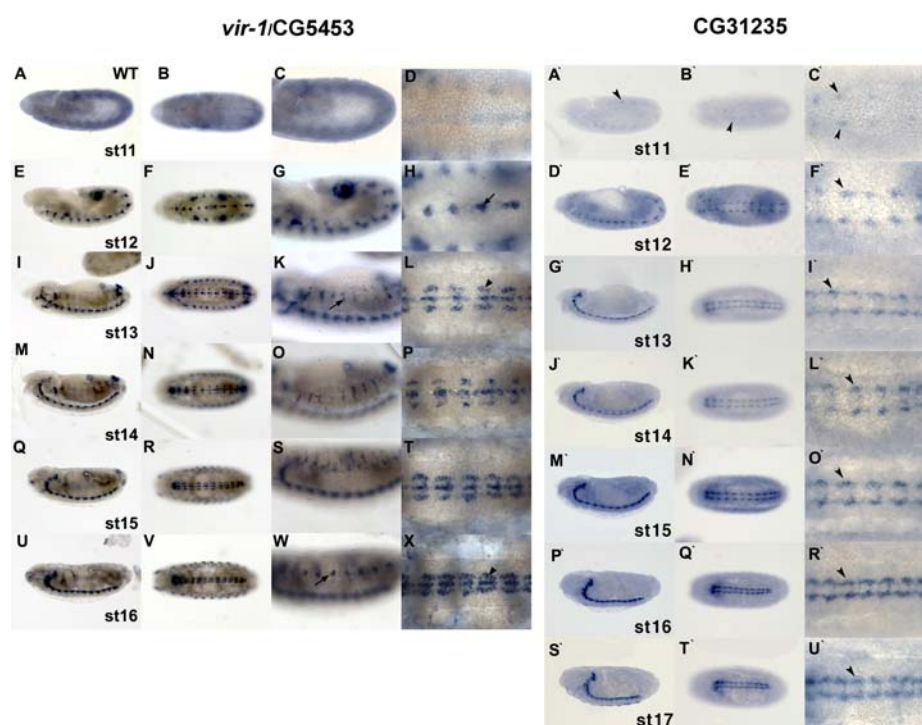


Fig. 1. The expression of the LG-specific markers, *vir-1*/CG5453 and CG31235. (A-D) *vir-1*/CG5453 expression is detected in the midline cells and the tracheal precursor cells, at stage 11. (E-H) Its expression in the trachea and 3-4 midline cells (arrow) becomes prominent in a segmentally repetitive fashion, at stage 12. (I-P) At stages 13 and 14, it is expressed in approximately 4-5 LG (arrowhead) of each segment, in addition to tracheal cells (arrow) and midline cells. (Q-T) Its expression becomes stronger in the LG and midline cells, at stage 15. (U-X) *vir-1*/CG5453 expression reaches the maximum level at stage 16, and the first *vir-1*/CG5453-positive oenocytes (arrow) are detected. (A'-C') It first appears in the LG (arrowheads) at stage 11. (D'-F') Its expression is repeated in every 2-3 LG (arrowhead) per hemisegment, and becomes detectable shortly thereafter, in the brain at stage 12. (G'-I') The CG31235-expressing LG are connected along the longitudinal tracts, at stage 13. (J'-O') The number of CG31235-positive LG increases to approximately 4-5, at stages 14 and 15. (P'-R') The number of CG31235-positive LG increases to 5-6 cells per segment, at stages 16 and 17. (S'-U') Its expression is observed in a few commissural glia, in addition to LG, at stage 17. In all panels, anterior is to the left. In panels A, C, E, G, I, K, M, O, Q, S, U and W, side views are shown. In panels A', D', G', J', M', P' and S', side views are shown. In all the remaining panels, ventral views are shown.

stages 12 (Fig. 2A-C). At stage 13, its expression became prominent in most medial (MM)-CBG, intermediate glia, and lateral glia (Fig. 2D-F). Its expression became stronger in many MM-CBG and lateral glia at stages 15 and 16 (Fig. 2J-O), and peaked at stage 17 (Fig. 2P-R). The expression of the CBG marker, CG11902 appeared in a pair of surface-associated glia (SG) in each segment at stage 10 (Fig. 2A'-C'). At stage 11, it was found to be expressed in the medial CBGs, which were located adjacent to the anterior SG in each segment (Fig. 2D'-F'). Subsequently, it appeared in the intermediate glia that had resulted from the division of SG in each segment, and medial CBG were divided into two medial CBG at stage 12 (Fig. 2G'-I'). Its expression became significantly reduced by stage 14 (Fig. 2M'-O'). However, its expression remained in the medial CBG, intermediate and lateral glia of each segment at stage 15 (Fig. 2P'-R').

The expression of the PG marker, CG2310 was observed in the lateral neuroectoderm of each segment at stages 11 and 12 (Fig. 3A-H). However, it was expressed only in the PG that are located at the boundary between the ventral nerve cord and the lateral epidermis at stage 13 (Fig. 3I-L). CG2310-expressing

PG were found to migrate towards the lateral side at stage 14 (Fig. 3M-P), and continued to migrate further away along the sensory axonal tracts, at stage 15 (Fig. 3Q-T). Notably, 3-4 PG expressed CG2310 along the sensory axonal tracts, at stage 16 (Fig. 3U-X). The expression of the PG marker, *moody*/CG4322 was initially detected in the head mesoderm and the anterior midgut, at stages 9 and 10 (Fig. 4A-F). It was found to be expressed in a pair of LG in each segment at stage 11 (Fig. 4G-I). At stages 12 and 13, it was also expressed in PG, in addition to LG (Fig. 4J-O). *moody*/CG4322-expressing PG were also found to migrate along the sensory axonal tracts in each segment (Fig. 4P-R) at stage 14, and thereafter 3-4 *moody*/CG4322-positive PG were observed in each sensory axonal tract, at stages 15 and 16 (Fig. 4S-X).

DISCUSSION

The present study is the first of its kind to comprehensively analyze the expression patterns of a set of glial-specific markers for CBG, LG and PG, during the embryonic CNS development of *Drosophila*. Based on the expression patterns described

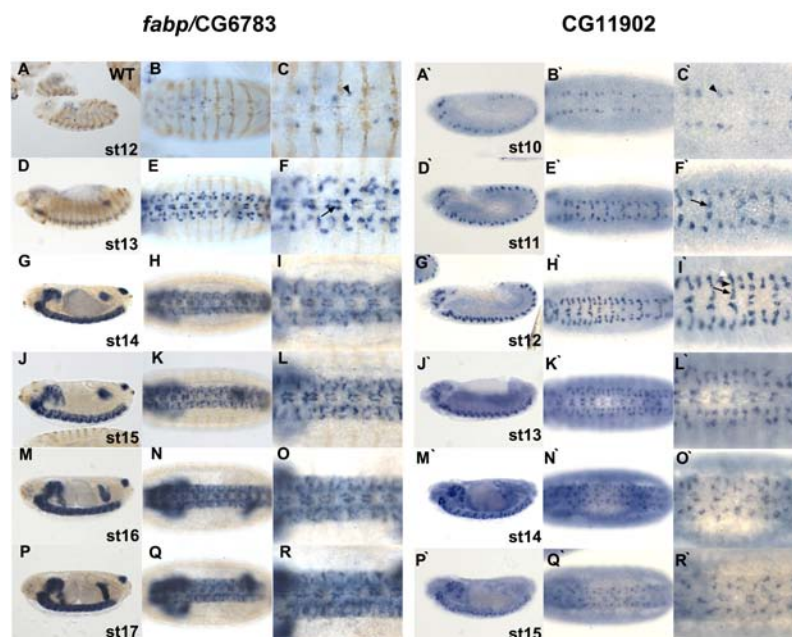


Fig. 2. The expression of the CBG-specific markers, *fabp*/CG6783 and CG11902. (A-C) It is initially detected in the SG (arrowhead) in each segment, at stages 12. (D-F) At stage 13, its expression becomes prominent in MM-CBGs (arrow), intermediate and lateral glia. (G-O) Its expression becomes stronger in many MM-CBG and SG, and is detected in the brain and ventral nerve cord at stage 17. (A'-C') CG11902 expression appears in a pair of SG (arrowhead) in each segment, at stage 10. (D'-F') At stage 11, it is expressed in the medial CBG (arrow), which are located adjacent to the anterior SG in each segment. (G'-I') At stage 12, it is detected in the intermediate glia (arrowhead) that had resulted from the division of SG (white arrow) in each segment, and medial CBGs (arrow) are divided into two medial CBG in the thoracic segments. (J'-L') Its expression becomes weaker at stage 13. (M'-O') Its expression becomes significantly reduced by stage 14. (P'-R') Its expression remains in the medial CBG, intermediate and lateral glia of each segment at stage 15. In all panels, anterior is to the left, and each segment is marked by staining with anti-En antibody (brown). In panels A, A', D, D', G, G', J, J', M, M', P and P', side views are shown. In all the remaining panels, ventral views are shown.

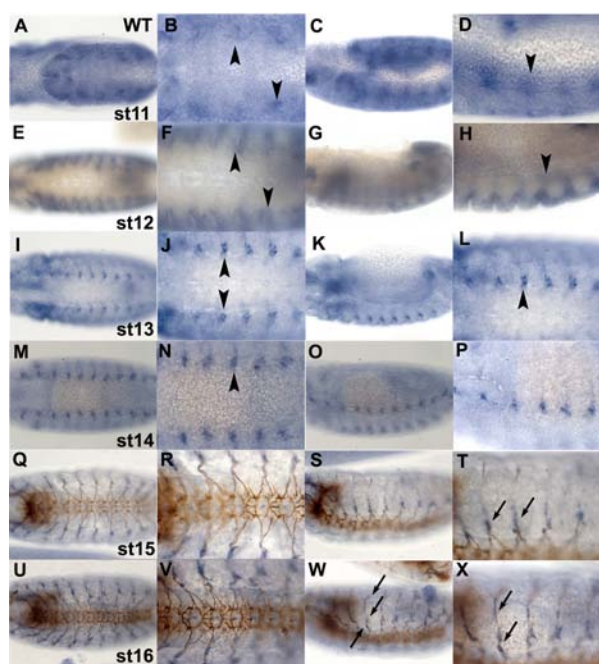


Fig. 3. The expression of the PG marker, CG2310. (A-H) CG2310 expression is observed in the lateral neuroectoderm (arrowheads) of each segment, at stages 11 and 12. (I-L) It is expressed only in the PG that are located at the boundary between the ventral nerve cord and the lateral epidermis (arrowhead), at stage 13. (M-T) CG2310-expressing PG (arrows) begin to migrate towards the lateral side at stage 14, and continue to migrate further away along the sensory axonal tracts, at stage 15. (U-X) It is expressed in 3-4 PG (arrows) along the sensory axonal tracts, at stage 16. In all panels, anterior is to the left. In panels C, D, G, H, K, L, O, P, S, T, W and X, side views are shown. In all the remaining panels, ventral views are shown. In panels Q-X, sensory axonal tracts are stained with 22C10 antibody (brown).

above, the glial markers for LG (*vir-1*/CG5453 and CG31235), CBG (*fabp*/CG6783 and CG11902), and PG (CG2310 and *moody*/CG4322) could be very useful for studying spatial and temporal glial development patterns, including identity determination, neuronal proliferation and survival, axon pathway formation, and migration. Moreover, they could serve as valuable tools in the investigation of glial functions, such as the regulation of synaptic efficacy, buffering of the CNS, the formation of the blood-brain barrier, and the removal of dying cells and invading pathogens.

In the present study, the LG-specific gene, *vir-1*/CG5453 was initially detected in a subset of 5-6 LGs at stage 13, and thereafter reached its peak at approximately stage 16 (Fig. 1). *vir-1*/CG5453 is also known as *virus-induced RNA-1* (*vir-1*), and its function involves host protection against foreign virus attack, by RNA interference mechanism (16). This and other findings suggest that *vir-1*/CG5453 plays a crucial role in destroying foreign viral RNA, by inducing RNA interference in the LG. These data thus suggest that the functional study of

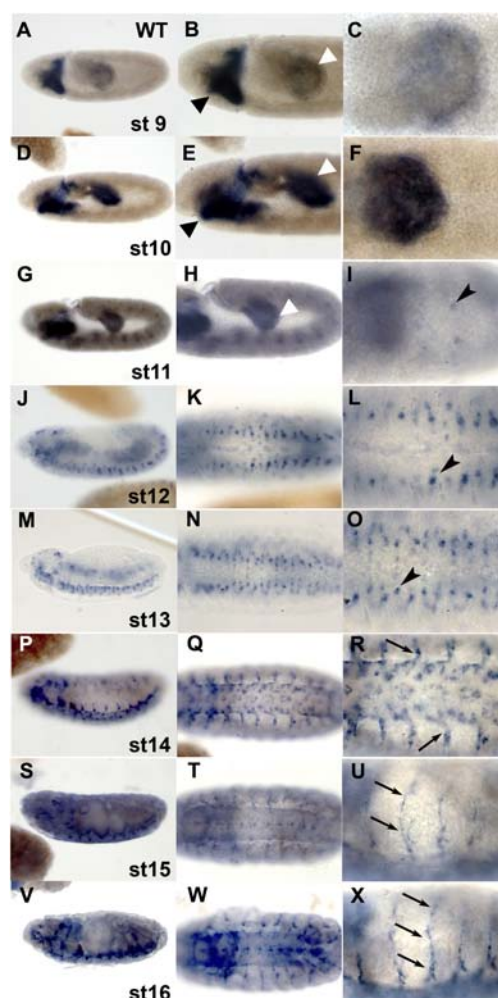


Fig. 4. The expression of the PG marker, *moody*/CG4322. (A-F) It is initially detected in the head mesoderm (arrowhead) and the anterior midgut (white arrowhead), at stages 9 and 10. (G-I) It is expressed in a pair of LG (arrowhead) in each segment, at stage 11. (J-O) At stages 12 and 13, it is also expressed in PG (arrowhead), in addition to LG. (P-R) *moody*/CG4322-expressing PG migrate along the sensory axonal tracts in each segment, at stage 14. (S-X) *moody*/CG4322-positive 3-4 PG are observed in each sensory axonal tract, at stages 15 and 16. In all panels, anterior is to the left. In panels A, B, D, E, G, H, J, M, P, S, U, V and X, side views are shown. In all the remaining panels, ventral views are shown.

vir-1/CG5453 gene may provide insightful knowledge on the role of the defense mechanism of RNA interference in glial cells. The expression of the LG-specific gene, CG31235 became detectable in a subset of 5-6 LG from stage 12, and subsequently reached the maximum level at stage 16 (Fig. 1). Similar to our study, a previous study demonstrated the expression of CG31235 is detected in the 9 LGs in each segment (17). Since this gene is known to encode dehydrogen-

ase/reductase, which is involved in lipid metabolism (14), we hypothesize that CG31235 may be involved in lipid synthesis and degradation, in order to balance the lipid levels in the neurons.

In our study, the CBG-specific gene, *fabp*/CG6783 was found to be expressed from stage 12, with its expression levels reaching the peak at stage 17, in the brain and ventral nerve cord (Fig. 2P-R). *fabp*/CG6783 is known to be *fatty acid-binding protein (fabp)* (Flybase). Since it is involved in lipid metabolism, and showed highest expression in CBG at stage 17, *fabp*/CG6783 may play an important role in lipid metabolism of the CBG, which are the mammalian counterparts of astrocytes. Future studies on the functional role of *fabp*/CG6783 in CBG may provide further insights into the functions of astrocyte-like glia in the mammalian CNS. Our study demonstrated that the expression of the CBG-specific gene, CG11902 peaked at stage 12 (Fig. 2G'-I'), wherein the majority of CBG were found to be positive, although its expression was initially detected in a subset of 1-2 SG at stage 10. Given that CG11902 encoded a C2H2-type zinc finger transcription factor (14), its spatial and temporal expression pattern observed in our study suggests that CG11902 plays an important role in generating glial subtype diversity at a specific time of the CNS development, in conjunction with Gcm expressed in all glia. Furthermore, our data highlight that CG11902 may be useful in investigating the roles of the 3 columnar genes involved in gliogenesis in the ventral nerve cord, since it is expressed in all 3 columns along the dorsoventral axis (Fig. 2I').

Interestingly, our study showed that expression of the PG-specific gene, CG2310 was restricted to the lateral neuroectoderm at stage 12, which later became prominent in the boundary between ventral nerve cord and peripheral CNS (Fig. 3L). Later, at stage 16, 3-4 PG were found to migrate into the sensory axon tracts (Fig. 3X). Stepwise temporal expression of CG2310 in the PG suggests that CG2310 may play an essential role in the proper development and migration of PG along sensory axon tracts in the lateral neuroectoderm. Since CG2310 gene is known to be a novel gene in the development of PG, further studies on the functional role of CG2310 in the PG development and migration are warranted, in order to clearly understand the developmental contribution of PG to the proper formation of the CNS. The PG-specific gene, CG4322 is known as *moody*, and is involved in formation of the blood-brain barrier along the ventral nerve cord and brain that protect the CNS (18, 19). As expected, our findings demonstrated that *moody*/CG4322-positive LG were widely distributed along the ventral nerve cord, and that a strong expression was noted on the surface of the ventral nerve cord and sensory axon tracts at stage 14 (Fig. 4R). Moreover, as *moody*/CG4322 is involved in the formation of septate junctions between SG surrounding the CNS (6), future studies on the functional role of *moody*/CG4322 may provide useful knowledge on how blood-brain barrier forms and functions, to maintain and protect the CNS.

Although glia are the most abundant cell type in the CNS, glial development and functions are not yet well understood. Our pilot data on the analysis of the expression patterns of the glial-specific genes therefore provide an invaluable starting point to delve deeper, in order to understand the myriad functions of glial cells in the CNS development. Despite being a relatively simple and tractable CNS, *Drosophila* embryos promise to provide an insightful perspective on the glial identity determination, generation of diverse subtype specificity, and formation of the blood-brain barrier. In addition, it serves as an attractive model for understanding various aspects of glial functions, including glia-neuron interactions, axon growth, regulation of synaptic efficacy and synaptogenesis, buffering of the CNS, and induction of protective inflammatory response to foreign pathogens and injury.

In conclusion, the present study revealed unique spatio-temporal expression patterns of a set of glial-specific markers in the glia sublineage, during embryonic gliogenesis of *Drosophila*. The observations described in the present study impart a deeper understanding of the glial cell development and function in *Drosophila*, and also provide a basis for defining future studies, to investigate the role of the glial-specific markers in glial development and function *in vivo*. However, further studies that elucidate the molecular and cellular functions of a specific glial cell lineage are warranted.

MATERIALS AND METHODS

Mouse monoclonal antibodies 22C10 (dilution 1:50) and Engrailed (En; 1:20) were obtained from the Developmental Studies Hybridoma Bank. LG markers-*vir-1*/CG5453 and CG31235, PG markers-CG2310 and *moody*/CG4322, and CBG markers-*fabp*/CG6783 and CG11902 cDNAs (12) were obtained from the *Drosophila* Genomics Resource Center, and used for analyzing specific glial cells. Whole-mount *in situ* hybridization using digoxigenin-labeled RNA probes was performed, as described (20). *In situ* hybridization, together with consecutive antibody staining, was carried out, as described (21). Differential interference contrast microscopy was performed on stained embryos, using an Olympus BX51 microscope equipped with Nomarski optics. Images were acquired with Leica DC480 digital camera, and processed using Image J, and Adobe Photoshop.

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