Supplementary Methods:

Sp-C5a antibody generation and validation

Rabbit polyclonal antibodies to C5a (anti-C5a) were produced against recombinant C5a expressed in bacteria. The following N-terminal fragment of C5a was cloned into the pProEx Htb expression vector (Invitrogen) and expressed in BL21 Codon Plus cells: MIIEGNDPLSMTSPHRASEGDIHNDEGFGVQERSSSLEDQTVIEMDSQIDTALSYTDGK TPGELKDGRIGEQEDDPDETEQLLDKREEGDTEEQKSSNTGTKYWATGNFISVVTSQ WLTPLFRAAKKRGLNDDDLYHILPVDSAEKNAKIFAQLWEEEIKHHGGNAVKASLRRVI LR. Recombinant protein was produced and purified at the PEP Core facility of The Scripps Research Institute. Purified protein was run on polyacrylamide gels, and the protein bands were excised and sent to Lampire Biological Laboratories (Pipersville, PA) for antigen preparation, rabbit immunization, and affinity purification. The recombinant protein was used to affinity-purify an aliquot of the C5a-specific IgG from antiserum. This affinity-purified antibody was used for Western blots, while adsorbed whole serum (described below) was used for immunohistochemistry (IHC) (and affinity-purified antibody was tested with IHC as a control).

To determine specificity of anti-C5a, we used Western blots to compare protein expression between embryos expressing endogenous C5a, overexpressing C5a-mCherry, and expressing no C5a (i.e. MASO knockdown) (Fig. S1A). Western blotting was performed with 70 hpf embryos from the same batch, and samples included: (1) C5a-CmCherry overexpressing (0.8 mg/ml), (2) control-injected (0.05 mg/ml histone H2B-CFP), (3) control-uninjected, (4) C5a knockdown with MASO1 (300 µM), and (5)

C5a knockdown with MASO2 (600 µM). Ten embryos were pooled for the C5a-CmCherry over-expressing lane, and 200 embryos were pooled per lane for the control-injected, control-uninjected, MASO1, and MASO2 samples.

Antibody adsorption for immunohistochemistry: Because an 80 kDa band inconsistent with mature C5a appeared strongly on blots of all developmental stages, while the 210 kDa C5a band appeared only after 26 hpf, anti-C5a whole serum was adsorbed to fixed and permeabilized 22 hpf embryos to remove immunoglobulins reacting with non-C5a antigens. Sera (diluted to 1:1500 in 2% BSA-PBS) were adsorbed for 24 hours at 15°C and then collected and stored at 4°C with 5 mM sodium azide prior to immunolocalizion of C5a. Examples of C5a immunolocalizations are shown using pre- and post-adsorbed antibody (Fig. S1B,C). After adsorption, localization was restricted to *gcm*-expressing pigment cells, consistent with the localization of *C5a* transcripts (Fig. 3).

Time-lapse imaging of C5a-knockdown embryos

Embryos were mounted in FSW on protamine sulfate-coated Delta-T dishes (Bioptechs, Butler, PA) and retained in an enclosure of Scotch double-sided tape, with four ~50 µm channels through which FSW could flow (Fig. S3). A coverslip was placed on top of the tape to restrict embryo movement. A chilled stage maintained embryos at 15°C, and images were captured on a Leica Sp8 confocal microscope with a Plan-Apochromat 20X objective (0.7 numerical aperture). In long time-lapses (> 15 hours), embryonic development was slowed to ~0.5-0.75 times the normal rate.

Supplemental Figures:

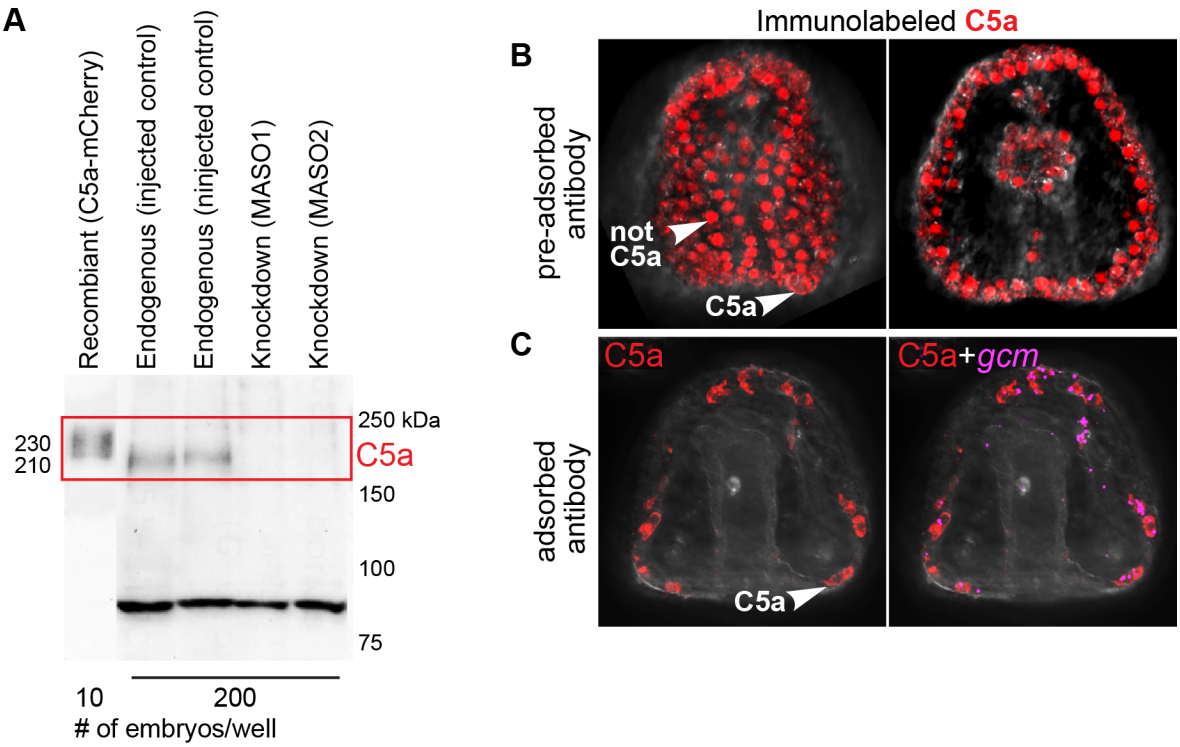


Figure S1. Detecting C5a with affinity-purified and adsorbed anti-C5a, and demonstrating knockdown with two MASOs.

(A) Western blot using lysates from 70 hpf embryos, probed with affinity-purified anti-C5a. C5a-mCherry runs as a ~230 kDa doublet, while endogenous C5a runs at 210 kDa. Ten embryos were lysed and run per lane for the C5a-mCherry sample, while 200 embryos per lane were run for all other samples. Endogenous C5a (210 kDa band) is knocked down by both C5a MASO1 and MASO2, while an ~80 kDa band not corresponding to C5a is unaffected by both MASOs. Lysate from the ten C5a-mCherry overexpressing embryos had insufficient ~80 kDa antigen to be detected in the first lane.

(B,C) Immunolocalization of C5a (red) in prism stage embryos using pre- and post-adsorbed anti-C5a (from whole serum).

(B) Embryo is immunolabeled with pre-adsorbed anti-C5a serum. Two z-sections are shown. Pre-adsorbed anti-C5a recognizes a nuclear antigen (likely corresponding to the 80 kDa band seen in (A)) in most cells. In addition, in only cells with pigment cell-like distribution around the embryo, it recognizes a membrane-localized and intracellular signal corresponding to C5a.

(C) Embryo is immunolabeled with adsorbed anti-C5a serum, and *gcm* transcripts (magenta) are labeled with FISH. Adsorbed anti-C5a does not detect a nuclear antigen, but recognizes membrane-localized and intracellular C5a in *gcm*-expressing pigment cells, which is consistent with the localization of *C5a* transcripts in *gcm*-expressing cells (Fig. 3).

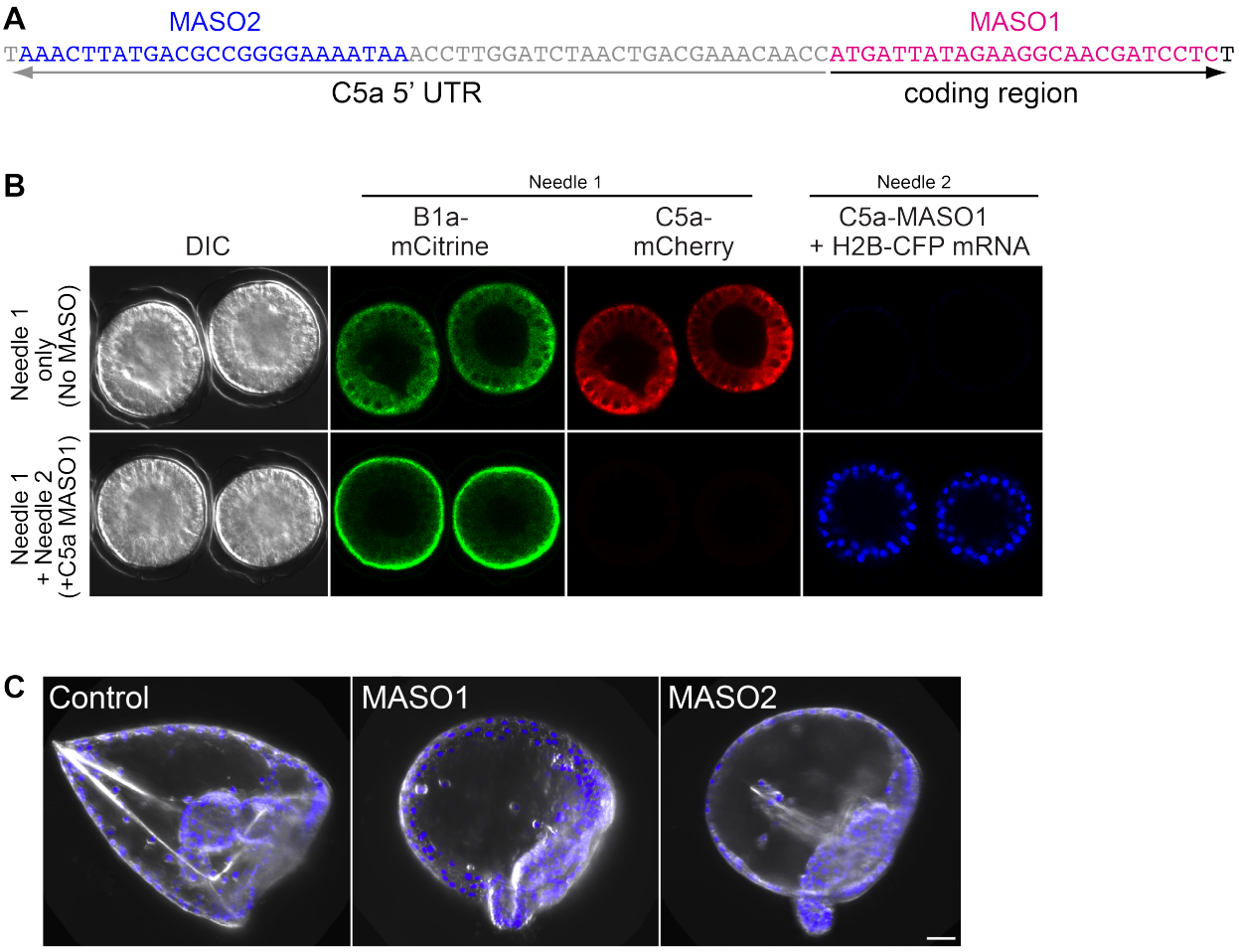


Figure S2. Two MASOs block translation of *C5a* and cause hindgut prolapse.

(A) Two non-overlapping MASOs block translation of *C5a*. MASO1 targets the first 25 bases of the coding region, while MASO2 targets the 5' UTR.

(B) C5a-MASO1 knocks down C5a-mCherry, but not B1a-mCitrine. Fertilized eggs were all injected with Needle 1, containing 1 mg/ml each of B1a-mCitrine and C5a-mCherry mRNA and imaged at the 20 hpf blastula stage. In the top panel (no MASO), both B1a-mCitrine and C5a-mCherry are expressed.

In the bottom panel, samples were injected a second time with Needle 2, containing 0.3 mM C5a-MASO1 and H2B-CFP mRNA as an injection marker. In the presence of MASO1, no C5a-mCherry expression is detected, while B1a-mCitrine is robustly

expressed. B1a-mCitrine localizes to apical membranes, presumably due to a relief on the protein sorting machinery, as C5a-mCherry is no longer present as a membrane-bound protein.

Interestingly, in the absence of MASO, neither protein membrane-localizes, presumably because the trafficking machinery is overwhelmed. Consistent with this hypothesis, B1a trafficking is normal in the presence of C5a-MASO1.

(C) Both MASO1 and MASO2 cause hindgut prolapse as shown in 70 hpf embryos. The phenotypes that result from injection of MASO1 and MASO2 are indistinguishable. Nuclei (blue) are labeled with H2B-CFP. Scale bar is 20 μm.

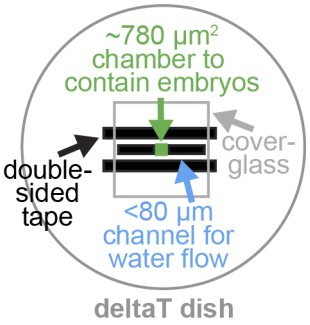
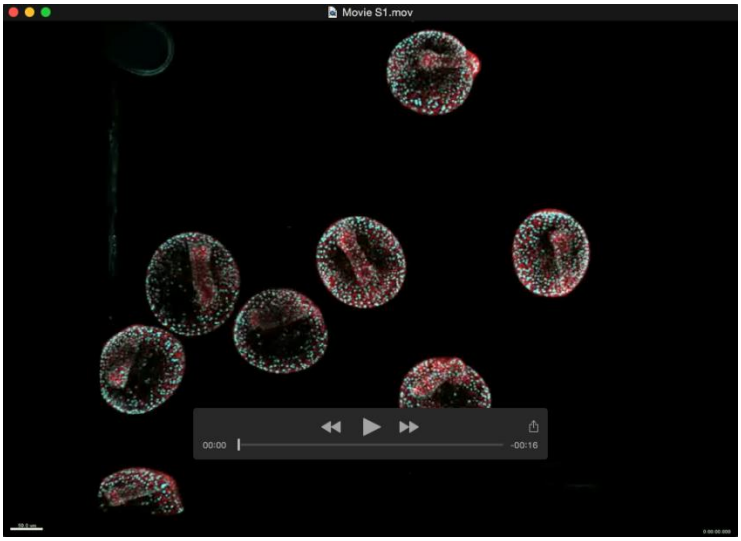
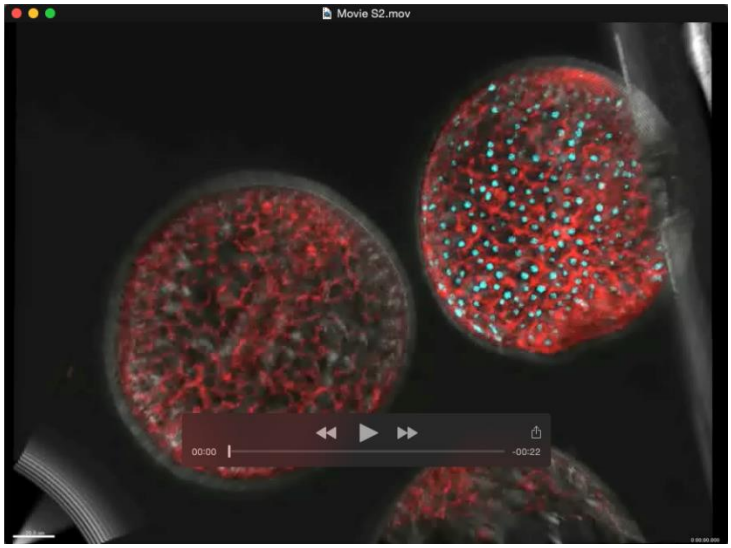


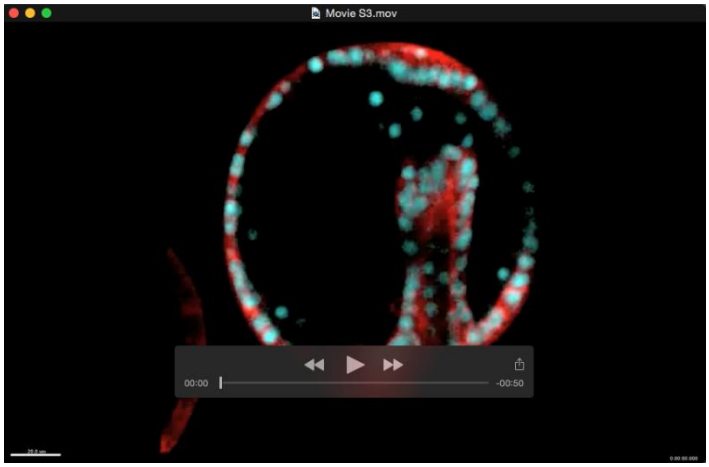
Figure S3. Chambers used to contain swimming embryos for long-term imaging.



Movie 1: Maximum intensity projection (MIP) time-lapse of seven C5a knockdown embryos. Plasma membranes are red (LCK-mCherry), and nuclei are blue (H2B-CFP). 46 hpf embryos were imaged every 10 minutes for 144 frames (~24 h). The video is sped up 5355X. Scale bar (bottom left) is 50 µm.



Movie 2: MIP (fluorescent channels plus brightfield) time-lapse of a control embryo (left, red membrane) and a C5a knockdown embryo (right, red membrane and blue nuclei) shown side by side. 48 hpf embryos were imaged every 2.5 min for 432 frames (~18 h). The video is sped up 3085X. Scale bar (bottom left) is 20 µm.



Movie 3: Cross-section of the C5a knockdown embryo from Movie 2, shown as a partial MIP with raw fluorescence, then repeated with isosurface renderings. Scale bar (bottom left) is 20 μm.