Cofilin-Mediated Actin Stress Response Is Maladaptive in Heat-Stressed Embryos

Graphical Abstract

Highlights
- *Drosophila* embryos mount an actin stress response (ASR) against heat stress
- Cofilin mediates the ASR, affecting actin in both the nucleus and the cytoplasm
- In nuclei, actin rods assemble; in cytoplasm, F-actin structures are destabilized
- F-actin destabilization disrupts morphogenesis, and embryo ASR is overall maladaptive

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In Brief
Figard et al. show that heat stress induces an actin stress response (ASR) in early *Drosophila* embryos. This ASR is mediated by a heat-induced increase in Cofilin activity. Increased Cofilin activity destabilizes F-actin structures required for morphogenesis. In addition, the Cofilin-mediated ASR reduces embryo viability.

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Cofilin-Mediated Actin Stress Response Is Maladaptive in Heat-Stressed Embryos

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INTRODUCTION

Environmental stress significantly challenges developing embryos. In humans, prenatal exposure to hypoxia, drugs, pathogens, or high temperature is associated with increased risk of fetal death or physical malformations and/or defects following birth (Edwards, 2006; Dixon et al., 2011; Hamdoun and Epel, 2007). Environmental stress disrupts cellular function in embryos by generating reactive oxygen species, altering gene expression, inducing apoptosis and heterochronicity, and disrupting signaling networks (Parman et al., 1999; Puscheck et al., 2015; Salilew-Wondim et al., 2014; Crews et al., 2016). Whether these stress-induced disruptions impinge on the actin cytoskeleton, the ultimate architectural driver of embryonic morphogenesis, remains unknown.

Recently, the actin cytoskeleton itself has emerged as a mediator of stress response (Amberg et al., 2012; Baird et al., 2014; Bernstein et al., 2006; Chambers et al., 2015; Higuchi et al., 2013). Many cell types, including neurons, myocytes, and epithelial cells, reorganize their F-actin upon exposure to heat or oxidative stress as part of an inducible actin stress response (ASR; reviewed in Bamburg and Bernstein [2016]; Kanellos and Frame [2016]; Munsie and Truant [2012]). This F-actin reorganization is typified by the assembly of actin "rods" in the nucleus or cytoplasm of affected cells (Ashworth et al., 2003; Iida et al., 1986; Minamide et al., 2000; Ono et al., 1993; Sanger et al., 1980; Vandebrouck et al., 2010). In transiently stressed neuronal cells, rod assembly promotes increased cell survival through an unknown mechanism (Bernstein et al., 2006; Munsie and Epel, 2007). However, the full extent of the ASR’s protective value is still undefined in most cells and is likely to be context dependent.

So far, the ASR has not been described in any embryo. What’s more, like other inducible stress responses in early embryos, it is unclear whether an ASR would be protective or would adversely divert normal developmental programs (Hamdoun and Epel, 2007). Here, we identify a maladaptive ASR in heat-stressed Drosophila embryos. This ASR destabilizes cytoplasmic F-actin structures, compromises cellularization—the first morphogenetic event in fruit fly development—and reduces embryo viability. Intriguingly, reduced viability appears to be more of a consequence of F-actin destabilization than of mild morphogenesis mistakes.

RESULTS

Heat Stress Induces Intra-Nuclear Actin Rod Assembly in Embryos

During cellularization, plasma membrane furrows invaginate synchronously to package ~6,000 nuclei of the syncytial embryo into a sheet of mononucleate epithelial cells that immediately goes on to gastrulate and eventually forms the larva. Furrow invagination depends on assembly of F-actin and Myosin-2 structures at furrow tips (Sokac and Wieschaus, 2008; Figure 1A,
cross section). Considered in three dimensions, these furrow tip structures make a hexagonal network across the embryo surface, with each hexagon encircling one nucleus (Figure 1A, surface view). Mutations in positive regulators of F-actin (e.g., Diaphanous/Formin) reduce F-actin levels at all furrow tips and result in the regression of a fraction of furrows (Padash-Barmchi et al., 2005; Grosshans et al., 2005; Sokac and Wieschaus, 2008; Zheng et al., 2013). Furrow regressions manifest as multinucleate cells. Thus, multinucleation serves as a proxy for compromised F-actin function during cellularization.

We previously found that wild-type embryos are prone to multinucleation when reared under heat stress (Figures 1B and 1C; Zheng et al., 2013), leading us to hypothesize that F-actin is somehow disrupted by high temperature. To test this hypothesis, we first visualized the actin cytoskeleton in live wild-type embryos injected with rhodamine labeled G-actin (G-actinRed). We chose G-actinRed as a probe, because it readily incorporates into furrow tips during cellularization with no known adverse effects on the process (Cao et al., 2008; Xue and Sokac, 2016). Embryos were injected with G-actinRed at 18°C and then imaged at either 18°C or under heat stress at 32°C (Table S1). This is a mild heat stress, given that standard culturing conditions are 18°C–29°C, and heat shock experiments in Drosophila typically use ≥ 37°C (e.g., Bergh and Arking, 1984; Crews et al., 2016). In injected embryos, single plane, confocal surface views captured furrow tip F-actin, as well as cross sections through nuclei. Strikingly, numerous actin rods were seen inside the nuclei of embryos at 32°C (Figures 2A and S1A). These rods resembled actin rods previously associated with the ASR in heat and oxidatively stressed adult cell types (Kanellos and Frame, 2016).

Actin rods in stressed neurons are “stable,” meaning there is no actin subunit turnover along their length (Bernstein et al.,...
To assess subunit turnover in rods in embryos, we measured fluorescence recovery after photobleaching (FRAP) for actin in the central region of rods. Nuclear rods showed no recovery (Figure 2B), whereas F-actin in cytoplasmic furrow tips recovers in tens of seconds (Xue and Sokac, 2016). So, analogous to the ASR in neurons, rods are stable in heat-stressed embryos. Despite this stability, rod assembly is reversible if embryos are shifted back to lower temperature, as previously described for ASR in transiently stressed neurons (Figure S1B; Bamburg and Bernstein, 2016). We wondered whether intra-nuclear rod assembly depends on free nuclear actin concentration. For embryos at 18°C, a few rods formed, but only at ~3-fold higher free nuclear actin level compared to 32°C (Figures 2C and 2D). Thus, rod assembly is concentration dependent at both temperatures, but rods form at lower free actin levels during heat stress. The difference in rod assembly at 18°C versus 32°C was not due to a change in the ratio of free nuclear to free cytoplasmic actin (Figure S1C). Free nuclear and cytoplasmic actin concentrations show the same linear relationship to each other at 18°C and 32°C (Figure S1D), perhaps because embryos are undergoing rapid rounds of mitoses with partial nuclear envelope breakdown. So, regardless of temperature, actin is incorporated into newly reformed nuclei at levels reflecting its free cytoplasmic concentration. Consistent with this, the dependencies between rod assembly and either free nuclear or free cytoplasmic actin concentration were similar (Figures 2C, 2D, S1E, and S1F). Finally, we found a maximum level of free actin in the nucleus that is not exceeded at 32°C, but instead at which additional actin rods are made (Figure S1G). Together, these results support a model where the proportion of free nuclear to free cytoplasmic actin does not change with temperature; however, during heat stress, some additional activity lowers the threshold concentration for actin sequestration in rods. Cofilin Promotes Actin Rod Assembly in Heat-Stressed Embryos

Next, we asked whether Cofilin provides the activity that promotes rod assembly in heat-stressed embryos. In mammalian cell types, ASR is mediated by stress-induced hyper-activation...
of Cofilin, an actin binding protein with diverse functions that influence both cytoplasmic and nuclear actin (Ashworth et al., 2003; Huang et al., 2008; Kim et al., 2009; Minamide et al., 2000; Ohta et al., 1989). Under normal conditions, Cofilin in the cytoplasm binds and severs F-actin to accelerate filament turnover (Andrianantoandro and Pollard, 2006). Cofilin also binds G-actin and works with importins to shuttle free actin into the nucleus (Dopie et al., 2012, 2015). Finally, Cofilin at high concentrations may stabilize F-actin regardless of cellular location (Andrianantoandro and Pollard, 2006; McCullough et al., 2008). During egg laying, Cofilin using a commercially available phospho-specific antibody against Cofilin, an actin binding protein with diverse functions that in-
Figure 3. Cofilin Mediates an ASR that Changes Actin Organization in Nuclei and Cytoplasm

(A) Surface views show G-actinRed in furrow tips in cofilin+/− embryos at the indicated temperatures. Intra-nuclear rods (yellow arrows) are reduced in cofilin+/− embryos at 32°C.

(B) Percentage of embryos with rods in wild-type (WT) and cofilin+/− embryos at the indicated temperatures (n ≥ 14 embryos per condition, with ~500 nuclei analyzed per embryo; mean ± SE).

(C) Representative western blots for Dm-Cofilin, Pi-cofilin from WT embryos at the indicated temperatures. β-actin is used as a loading control. Antibody validation in Figure S2.

(D) Ratio phosphorylated to total Cofilin in WT embryos at the indicated temperatures (n = 4 independent experiments; mean ± SE).

(E) F-actin levels in furrow tips in WT embryos at the indicated temperatures (n ≥ 29 embryos per temperature, with 15 furrows analyzed per embryo; mean ± SE). Normalization to Histone-GFP embryos at 25°C, according to Figure S3.

(F) Cross sections show FRAP of furrow tip F-actin (G-actinRed) in WT embryos at the indicated temperatures. Yellow boxes show bleached furrow tips. Pre, immediate pre-bleach time point; bleach, immediate post-bleach time point; sec, seconds after bleach.

(G) FRAP kinetics for furrow tip F-actin in WT and cofilin+/− embryos at indicated temperatures. Each point represents one embryo (n ≥ 14 embryos per temperature, with 1–3 furrows analyzed per embryo; horizontal lines represent means ± SE).

(H) F-actin levels in furrow tips in WT and cofilin+/− embryos at 32°C (n ≥ 48 embryos per condition, with 15 furrows analyzed per embryo; mean ± SE). Normalization to Histone-GFP embryos at 32°C.

(E) and (H) correspond to scatterplots in Figures S4C and S4D.

Student’s t test used to calculate p values in (B), (E), (G), and (H).
to room temperature until larval hatching was scored (Table S1). For the 32°C embryo group, more cofilin+/− embryos survived and hatched than did wild-type (Figures 4E and S4F), suggesting that the ASR is not only disruptive for cellularization but also can be harmful to embryos overall.

We wondered whether the reduced viability of heat-stressed embryos was a result of cellularization mistakes. However, inspection of cofilin+/− embryos showed a similar likelihood of furrow regressions as seen in wild-type embryos (Figures 4C and 4D). We do not know why overstabilization of F-actin at 32°C in cofilin+/− embryos causes multinucleation, though our combined data suggest that some “precise” level of stability is required for best cellularization outcomes. Nonetheless, cofilin+/− embryos had improved viability compared to wild-type embryos, even though they showed the same extent of multinucleation as wild-type. Thus, embryos must be robust to a small number of furrow regressions. Instead, the reduced viability accompanying ASR seems more closely associated with F-actin destabilization in the cytoplasm, perhaps due to more free actin entering the nucleus.

**DISCUSSION**

Here, we identify an embryonic ASR. While previously ASR was defined in terms of actin rod assembly (e.g., Kanellos and Frame, 2016), our work argues that the actin cytoskeleton is more...
extensively modified throughout cells. We suggest a model whereby increased Cofilin activity during ASR in fly embryos leads to increased F-actin severing in the cytoplasm and destabilization of normal F-actin structures. This F-actin destabilization has two consequences: first, it puts morphogenesis at risk. Specifically, during cellularization, F-actin destabilization at all furrow tips leads to the stochastic regression of a small number of furrows. Second, cytoplasmic F-actin destabilization increases the free actin monomer pool in the cytoplasm and, consequently, the nucleus, to a level that can promote concentration-dependent actin rod assembly in the nucleus. Rod assembly buffers free nuclear actin levels, perhaps in an attempt to sequester subunits from some adverse function in either the nucleus or cytoplasm. While many details of this model remain to be tested, we believe that the ASR should now be viewed in terms of the entire actin cytoskeleton and all its functions, both nuclear and cytoplasmic.

This work adds to a growing list of examples in which Cofilin emerges as a specific target for, and vulnerability to, environmental stress. Beyond the ASR, Cofilin oxidation and responsiveness to heat shock proteins in environmentally stressed cells affects actin-based behaviors, as well as cell survival via mitochondrial health and apoptotic signaling (Klamt et al., 2009; Kiemke et al., 2008; Simard et al., 2011). While Cofilin’s functions will surely differ with context, we suggest it as a key general node in actin-based stress response, acting across diverse cell types and organisms. Our observations in the fly embryo lead us to consider whether stress-induced changes in Cofilin activity could partially explain why high temperature is teratogenic (Auger et al., 2017; Chambers et al., 1998; Dixon et al., 2011; Edwards 2006). Because F-actin remodeling drives morphogenesis (Rodal et al., 2015), we think it possible that Cofilin-mediated stress response could compromise the fidelity of other morphogenetic events, just as it precipitated cellularization failures here. In addition, by changing the levels or organization of nuclear actin, Cofilin-mediated ASR could also alter nuclear events critical to development, including transcription and genome remodeling.

**STAR METHODS**

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Supplemental Information can be found with this article online at https://doi.org/10.1101/j.celrep.2019.02.092.

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**AUTHOR CONTRIBUTIONS**

L.Z. and A.M.S. conceived of the idea. L.F. and L.Z. performed genetics and imaging. Z.X. and N.B. generated the Dm-Cofilin antibody. N.B. performed the biochemistry. L.F. performed the hatching assays. L.Z., L.F., H.S., S.C., I.G., and A.M.S. performed the image and the data analysis. L.Z., L.F., N.B., and S.C. prepared the figures. L.F. and A.M.S. wrote the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| Antibodies           |        |            |
| Anti-β-actin, mouse  | SCBT   | Cat#sc-47778; RRID: AB_626632 |
| Anti-D. melanogaster Cofilin (Dm-Cofilin), rabbit | This study | N/A |
| Anti-PhosphoCofilin (P-Cofilin), rabbit | SCBT | Cat#sc-12912-R; RRID:AB_673572 |
| Anti-Mouse HRP       | Jackson Immuno Research | Cat#115-035-003; RRID:AB_10015289 |
| Anti-Rabbit HRP      | Jackson Immuno Research | Cat#111-035-144; RRID:AB_2307391 |
| Bacterial and Virus Strains | Thermo Scientific | Cat#C600003 |
| Actin protein (rhodamine), human platelet (G-actin<sub>red</sub>) | Cytoskeleton, Inc. | Cat#APHL99-E |
| 6X-His-Dm-Cofilin     | This study | N/A |
| Alexa Fluor 488 phallolidin | Invitrogen-Thermo Scientific | Cat#A12379 |
| Alexa Fluor 546 phallolidin | Invitrogen-Thermo Scientific | Cat#A22283 |
| Hoechst 33342         | Invitrogen/Fisher Scientific | Cat#H3570 |
| Halocarbon 27 oil     | Sigma-Aldrich | Cat#H8773-100ML |
| Halocarbon 700 oil    | Sigma-Aldrich | Cat#H8898-100ML |
| Nickel-NTA Agarose    | QIAGEN  | Cat#30210 |
| Pierce Protease Inhibitor Mini Tablets, EDTA-free | Thermo Scientific | Cat#A32955 |
| Melon Gel IgG Spin Purification Kit | Thermo Scientific | Cat#45206 |
| Lambda phosphatase    | SCBT   | Cat#sc-200312A |
| BCA Protein Assay     | Thermo Scientific | Cat#23225 |
| Pierce Disposable Columns (10 mL) | Thermo Scientific | Cat#29924 |
| Amicon-Ultra 15 mL centrifugal filters, MWCO 30 kDa | Millipore-Sigma | Cat#UFC903008 |
| Experimental Models: Organisms/Strains |        |            |
| Oregon R (OreR)       | DGGR   | Cat#109612; RRID:DGGR_109612 |
| ubi::H2A-GFP (Histone-GFP) | Gift of E. Wieschaus | N/A |
| tsr<sup>1</sup>/CyO (1/2 cofilin) | BDSC | Cat#9107; RRID:BDSC_9107 |
| Df(3R) X3F/TM3, Sb (sry-α --/--) | Merrill et al., 1988 | N/A |
| Oligonucleotides      |        |            |
| Primer: Forward: sry-α-RNAi-F (5'-TAATACGACTCACTATTAGGGTCAGGAGCTAATC-3'); Reverse: sry-α-RNAi-R (5'-TAATCAGACTCTAATAGGCCGCCAGCATGTCCA-3') | Zheng et al., 2013 | N/A |
| Recombinant DNA       |        |            |
| pET45b-tsr(B/X) (6X-His-Dm-Cofilin) | This study | N/A |
| Software and Algorithms |        |            |
| ImageJ/FIJI           | NIH    | https://fiji.sc |
| Adobe Photoshop CC    | Adobe  | https://www.adobe.com/creativecloud.html |
| Adobe Illustrator CC  | Adobe  | https://www.adobe.com/creativecloud.html |
| MATLAB                | MathWorks | https://www.mathworks.com/products/matlab.html |
| Segmentation Algorithm (custom MATLAB code) | Zheng et al., 2013 | N/A |
| Furrow tracking algorithm (custom MATLAB code) | Zheng et al., 2013 | N/A |
| Furrow canal intensity algorithm (custom MATLAB code) | Zheng et al., 2013 | N/A |
Further information and requests for resources and reagents should be directed to the Lead Contact, Anna Marie Sokac (sokac@bcm.edu).

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

*Drosophila melanogaster* stocks were housed at 22°C on standard molasses food. Most analyses focus on embryos in Bownes Stages 4 through 5 (pre-cellularization through the end of cellularization) (Bownes, 1975), unless noted in the Method Details, such as mixed-stage embryo collections for some Western Blotting experiments.

**Fly stocks and genetics**

OreR was used as the wild-type stock. For Histone-GFP, embryos were collected from *ubi::H2A-GFP* (gift of E. Wieschaus). For *cofilin*+/-, adults from stock *tsr1/CyO* (Bloomington Stock Center #9107) were crossed with OreR, and F2 embryos collected from F1 *tsr1/+* females crossed with either sibling *tsr1/+* males (imaging and western blotting experiments) or OreR males (larval hatching assays). For *sry-α−/−*, embryos were collected from *Df(3R)X3F/TM3, Sb* (Merrill et al., 1988). For *sry-α−/−+ cofilin*+/-, adults from stock *tsr1/CyO* were crossed with *Df(3R)X3F/TM3, Sb*, and F2 embryos collected from F1 *tsr1/+; Df(3R)X3F/+* females crossed with sibling *tsr1/+; Df(3R)X3F/+* males.

**METHOD DETAILS**

**Embryo collection, fixation, and staining**

Embryo collection cups were set up on apple juice plates at 18°C, incubated for 24-48 hours, and embryos were hand-selected under a dissecting microscope, transferred to fresh apple juice plates, covered in Halocarbon oil 27 (Sigma), and incubated at 22°C in humidifying chambers (80x15 mm glass Petri dish lined with moist paper towels). Embryos were collected 30 minutes post-egg laying from collection cups at 18°C. Embryos were hand-selected under a dissecting microscope, transferred to fresh apple juice plates, covered in Halocarbon oil 27 (Sigma), and incubated for 24-48 hours. Embryos were mounted in a line on the edge of a glass coverslip with “embryo glue” (a solution made from double-sided tape saturated in n-heptane), dessicated for 7-10 minutes. After 48 hours plates were scored for the percent of hatched larvae.

**Rhodamine G-actin injections and RNAi**

Embryos were collected 30 minutes post-egg laying from collection cups at 18°C. Embryos were mounted in a line on the edge of a glass coverslip with “embryo glue” (a solution made from double-sided tape saturated in n-heptane), dessicated for 7-10 minutes prior to injection, and covered with 0.5% agarose in 1x M9 before imaging. For F-actin staining, mixed-stage embryos were fixed in 8% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4); n-heptane (1:1) and hand-peeled for staining with 5 U ml-1 Alexa Fluor 488 phalloidin or Alexa Fluor 546 phalloidin (Thermo Scientific, Rockford, IL). For nuclear staining, Hoescht 33342 was used at 1.0 μg ml-1 (Invitrogen, Carlsbad, CA).

**Image acquisition**

Images were collected on a Zeiss LSM 710 confocal microscope with a 40X/1.2 numerical aperture water-immersion objective (Carl Zeiss, Inc., Oberkochen, Germany). For presentation, images were cropped, resized, and adjusted for brightness and contrast in ImageJ/FIJI (Schindelin et al., 2012) or Adobe Photoshop CC (Adobe, San Jose, CA). Channels were adjusted separately in multicolor images.

For FRAP, embryos were imaged at 18±2°C or at 32±2°C in a thermal incubator. G-actinRed was bleached to approximately 50% fluorescence intensity using 100% laser power in cross-section within a 2 μm x 1 μm box for furrow canals or in surface views within a 2 μm x 2 μm box for rods. Fluorescence recovery was recorded at 1 or 2 s intervals for ≥ 80 s, which encompassed the time to full recovery. Bleached furrow canals ingress at the same rate and with the same morphology as their unbleached neighbors, suggesting that bleaching does not induce significant phototoxicity (Figure 3F; Xue and Sokac, 2016).
For imaging actin rods, wild-type or Histone-GFP embryos injected with G-ActinRed were imaged at 18 ± 2 °C or at 32 ± 2 °C in a thermal incubator, ~10 μm beneath the embryo’s surface, at the mid-section of the nuclei.

**Western Blotting**

Embryos (200 per condition) in early cellularization were hand-selected under a dissecting microscope and rapidly snap-frozen in liquid nitrogen. Frozen embryos were homogenized on ice in lysis buffer (150 μL 0.05 M Tris pH 8.0, 0.15 M KCl, 0.05% NP-40, 1X protease inhibitor cocktail (Pierce Protease Inhibitor Mini Tablets, EDTA-free, Thermo Scientific)). After spinning to remove yolk and debris, protein concentrations were determined using a BCA Protein Assay (Thermo Scientific). Equal amounts of protein were loaded per well and separated on hand-cast 12% SDS-PAGE gels. Proteins were transferred to 0.2 μm nitrocellulose (Bio-Rad, Hercules, CA) and probed with 1:50 mouse anti-β-actin (sc-47778, Santa Cruz Biotechnology, Dallas, TX), 1:5000 rabbit anti-mouse HRP secondary antibodies (Jackson Immuno Research, West Grove, PA).

**Antibody production and validation**

Recombinant protein 6X-His-Dm-Cofilin (Twinstar) was expressed in E. coli strain BL21 and purified in gravity-flow columns (Thermo Scientific) using Nickel-NTA affinity beads (Qiagen, Hilden, Germany). Antibody was raised in rabbits by Covance (Princeton, NJ), and precipitated from sera using ammonium sulfate (Harlow and Lane, 1988). IgG was further purified using the Melon Gel Purification Kit (Thermo Scientific).

**Phosphatase assay**

Collections of mixed-stage embryos were snap-frozen in microcentrifuge tubes in liquid nitrogen. Frozen embryos were homogenized on ice in EDTA-free lysis buffer (200 μL 0.05 M Tris pH 8.0, 0.15 M KCl, 0.5% NP-40, 1X protease inhibitor cocktail (Pierce Protease Inhibitor Mini Tablets, EDTA-free, Thermo Scientific)). For lysates with phosphatase inhibitor, final concentration of 0.04 M sodium orthovanadate (Santa Cruz Biotechnology) and 0.20 M sodium fluoride (Sigma Aldrich) were included in the lysis buffer. For phosphatase reactions, 80 μg embryo protein (calculated from BCA assay, Thermo Scientific) were incubated with 4000 units of λ phosphatase in λ phosphatase reaction buffer supplemented with MnCl₂ per the manufacturer’s instructions (Santa Cruz Biotechnology) for 20 minutes at 30 °C. Equal concentrations of protein from each reaction were separated on hand-cast 12% SDS-PAGE gels, followed by Western Blotting.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Student’s t tests were performed using GraphPad QuickCalcs (GraphPad, San Diego, CA). Comparisons with p values ≤ 0.05 were considered to be significant. Specific information regarding p values and n values can be found in the figure legends. All graphs were generated in MATLAB (MathWorks, Natick, MA) and edited in Adobe Illustrator CC (Adobe, San Jose, CA). Figures were assembled in Adobe Photoshop CC.

**Multinucleation quantification**

The percent of embryos displaying multinucleation was counted manually using raw, single plane, surface view images collected at the furrow canals, where an entire embryo side was visible (≥ 1000 nuclei assayed per embryo). The ratio of mononucleate cells to nuclei was determined by manually counting in two quadrants from a raw, single plane, surface view image collected at the furrow canals (quadrant size = 2500 μm²); and the mean was calculated per embryo. For presentation of the multinucleation phenotype, images were segmented using our previously described custom MATLAB code (Zheng et al., 2013).

**F-actin levels quantification**

We quantified F-actin levels in furrow tips of embryos with 3-6 μm furrow lengths using custom MATLAB code, as previously described (Zheng et al., 2013), with the following modification: To control for tube-to-tube variation in immunostaining, we used Histone-GFP embryos collected at 25 °C (for temperature comparisons) or 32 °C (for genotype comparisons) as internal controls that were mixed into each tube of experimental wild-type or cofilin⁻/⁻ embryos collected at either 18 °C or 32 °C, following the scheme shown in Table S1. GFP signal distinguished the experimental embryos from Histone-GFP controls. We confirmed that differences between wild-type and Histone-GFP embryos at 25 °C were insignificant, with a ratio of 1.006 ± 0.035 (n = 3 experiments from 37 embryos for each genotype; mean ± SE). Thus, we normalized F-actin levels as follows: The F-actin fluorescence value from each embryo in an experimental group was normalized to the F-actin fluorescence value from each Histone-GFP embryo in the same tube, generating a pairwise series of normalized F-actin levels. To compare between experimental conditions, pairwise normalized F-actin levels were pooled from each experimental condition (temperature or genotype) and used to calculate average and standard error of the mean. An alternative method, in which F-actin levels from each embryo in an experimental group were normalized to the average of the corresponding Histone-GFP embryos, was also performed and gave similar results.
FRAP quantification

Fluorescence intensity was measured using FIJI/ImageJ. Intensity of the bleached furrow canal (IFRAP) was normalized (INORM) by the intensity of an unbleached furrow canal (IREF) and the data fitted using two methods (Hardy, 2012; Phair et al., 2004). In both methods, IREF-PRE and IFRAP-PRE are the pre-bleach fluorescence intensity in the respective region, and I0 is the normalized intensity after bleach. In method 1: INORM(t) = (Ifluor(t)*IREF-PRE)/|IFRAP-PRE*IREF(t)). Using MATLAB, recovery was fit to a single exponential function: INORM(t) = lmax-(lmax-I0)*e(-k*t). In method 2: INORM(t) = Ifluor(t)/IFRAP-PRE. Using MATLAB, recovery was fit to a modified exponential function: INORM(t) = lmax-(lmax-I0)*e(-k*t)-Ka*t, where Ka is the acquisition bleaching rate calculated by the slope of the reference fluorescence intensity. For both methods, the mobile fraction and half-time to recovery were calculated by the following equations: Mobile fraction = (Imax-I0)-(1-I0) and Half time = -ln(0.5)/k. The mobile fraction and half-time values calculated using method 1 are plotted throughout. The same trends were confirmed using method 2.

Actin rod abundance quantification

For rod quantifications, we chose G-actinRed imaging in live embryos to avoid confounding results that were likely to be introduced by poor retention of rods in chemically fixed embryos. (Note that while rods could be minimally preserved by fixation and stained with phalloidin in some embryos, to the eye, rod numbers and size were drastically reduced compared to live embryos). To compare rod formation between temperatures and genotypes, we quantified the percent embryos containing rods within each experiment. However, the same trends were confirmed using two additional measurements of rod abundance: rods per nucleus, as well as percent nuclei containing rods.

For rod abundance quantifications, the total number of nuclei and rods were manually counted and expressed as a ratio (rods / nucleus). Each image was assigned a code so that the experimenter performing the quantifications was blind to the conditions. Free nuclear actin fluorescence and free cytoplasmic actin fluorescence were quantified by averaging fluorescence measurements from 2 μm x 2 μm boxes in three nuclei or three cytoplasmic (non-furrow) regions per embryo. To generate the plots in Figure 2C, 2D, S1E, S1F, and S1G, MATLAB was used to bin data along the x axis with equal numbers of data points in each bin. Binned data were fit to a Hill Function.

Western blot quantification

Films were scanned in black and white at 600 dpi and quantified after applying the “Subtract Background” algorithm in Fiji/ImageJ and then inverting. Per blot (bit depth = 8), integrated intensities for each band were normalized against the value of the 18’C band for that antibody or Cofilin species (i.e., phosphorylated or de-phosphorylated) so that results from different experiments could be related. The average and standard deviation for the normalized values for eight or four experiments for actin or Cofilin, respectively, were calculated. Dm-Cofilin blots were used to calculate the ratio of phosphorylated to total Cofilin. Consistent with the decrease in this ratio, the Pi-Cofilin values consistently decreased in four out of four experiments.

DATA AND SOFTWARE AVAILABILITY

All raw data, image files, and custom MATLAB algorithms are available upon request to the Lead Contact.
Supplemental Information

Cofilin-Mediated Actin Stress Response Is Maladaptive in Heat-Stressed Embryos

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Table S1. **Timeline of heat stress experiments. Related to Figures 1-4.**
Schematic representation of the developmental timing of heat stress treatments for various experiments.
Figure S1. Intra-nuclear actin rods assemble in heat stressed embryos. Related to Figure 2.

(A) Surface views show G-actin\(^{35S}\) (green) in furrow tips encircling nuclei (Histone-GFP, purple) in live wild-type (WT) embryos at 32°C. Rods (yellow arrows) assemble inside nuclei. (B) Rod abundance (rods / nucleus) in a single live embryo after downshift from heat stress at 32°C. (C) Ratio of free nuclear to free cytoplasmic actin fluorescence at indicated temperatures (n=147 embryos, with free actin fluorescence averaged from 3 nuclear or cytoplasmic regions per embryo). Each point represents one embryo. Horizontal lines are means ± SE. (D) Free nuclear versus free cytoplasmic actin fluorescence in live WT embryos at indicated temperatures (n=147 embryos, with free actin fluorescence intensity averaged from 3 nuclear or cytoplasmic regions per embryo). Each gray or black point represents one embryo at 18°C or 32°C, respectively. Gray or black lines are linear fits for corresponding data at 18°C or 32°C, and fits yield slopes that are statistically indistinguishable. (E) and (F) Rod abundance (rods / nucleus) versus free cytoplasmic actin fluorescence in live WT embryos at indicated temperatures (n\(\geq\)31 embryos, with rod abundance counted in \(\geq\)60 nuclei per embryo; free actin fluorescence averaged from 3 cytoplasmic regions per embryo). Related data shown in Figure 2C, 2D. (G) Free nuclear actin fluorescence versus rod abundance (rods / nucleus) in live WT embryos at 32°C (n=147 embryos, with rod abundance counted in \(\geq\)60 nuclei per embryo; free actin fluorescence averaged from 3 nuclei per embryo). Related data shown in Figure 2D. Each gray point represents one embryo, and black points are binned data (mean ± SE) in (E), (F), and (G). Green line is binned data fitted to a Hill Function with Hill Coefficient=4 in (E) and (F), and Hill Coefficient=3.5 in (G). Student’s t-test used to calculate P value in (C).
**Figure S2. Validation of Dm-Cofilin and Pi-Cofilin antibodies. Related to Figure 3.**

(A) Representative full lane Western blots for Dm-Cofilin and Pi-Cofilin antibodies from wild-type embryos at 25°C. (B) Representative Western blots for Dm-Cofilin and Pi-Cofilin antibodies for lysates from wild-type embryos following indicated λ-phosphatase or Phosphatase inhibitor treatments. β-actin used as loading control.
Figure S3. Method used to quantify F-actin levels in embryos reared at different temperatures. Related to Figure 3. (A) Strategy used to quantify F-actin levels in embryos reared at different temperatures. To minimize experimental variability, embryos reared at each temperature were stained in the same tube as and normalized against internal control, Histone-GFP embryos reared at 25°C. (B) Cross sections show furrow tip F-actin (Phalloidin, green) and nuclei (Histone-GFP purple) in Histone-GFP and wild-type (WT) embryos at indicated temperatures. Arrows indicate furrow tip position where quantification was done.
Figure S4. Percent mobile fraction from all FRAP experiments and scatter plots for F-actin quantifications and hatching assays. Related to Figures 3 and 4.

(A) and (B) Percent mobile fraction for furrow tip F-actin in wild-type (WT) or colin^+/- embryos at indicated temperatures (n≥20 embryos per temperature, with 1-3 furrows analyzed per embryo). (C) F-actin levels in furrow tips in WT embryos at indicated temperatures (n≥29 embryos per temperature, with 15 furrows analyzed per embryo). (D) F-actin levels in furrow tips in WT and colin^+/- embryos at 32°C (n≥48 embryos per condition, with 15 furrows analyzed per embryo).

(E) Percent mobile fraction for furrow tip F-actin in indicated genotypes at 32°C (n≥20 embryos per temperature, with 1-3 furrows analyzed per embryo). (F) Larval hatching rates for indicated conditions (n≥5 independent experiments, with ≥9 embryos per experiment).

Each point represents one embryo, and horizontal lines are means ± SE for (A), (B), (E) and (F).
Method shown in Figure S3 for (C) and (D).
Each point represents an individual pairwise comparison between all WT embryos and all Histone-GFP embryos, and horizontal lines are means ± SE for (C) and (D).
Student’s t-test used to calculate P values in (A)-(F).