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Using GAL4-inducible transgenics to modulate Rho GTPase activity in zebrafish

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Abstract

Rho GTPases are Ras-family G-proteins that regulate many critical cellular functions. Due to their requirement during early embryonic development, investigations into the function of Rho GTPases at a tissue-specific level require inducible and spatially-targeted modulation of Rho GTPase activity. Here, we describe the use of ten novel zebrafish transgenics enabling GAL4-specific expression of Rho GTPases to modulate Rho GTPase activity with spatial and temporal control.

Keywords

Cdc42; Rac1; RhoA; Zebrafish; GAL4/UAS; heat shock; transgenics

1. Introduction

Rho GTPases are monomeric G-proteins that function as molecular switches to regulate myriad cellular processes [1], [2]. When bound to GTP, Rho GTPases assume a conformational state enabling the activation of a diverse array of effector proteins. Upon hydrolysis of GTP to GDP, Rho GTPases assume an inactive conformation and their signaling activity is abrogated. The best-studied Rho GTPases are Cdc42, Rac1, and RhoA [3]. Since their discovery, these three proteins have been shown to regulate numerous processes, including cell polarity, cell cycle progression, and actin polymerization [4]–[6].

The majority of studies focusing on Rho GTPases have been conducted in *in vitro* systems. Due to the ubiquitous expression and important functions of Rho GTPases, embryonic knockout models display pleiotropic defects and early embryonic lethality [7], [8]. To circumvent these issues, it is necessary to modulate Rho GTPase activity in a temporally- and spatially-controlled fashion. To this end we have recently introduced ten transgenic zebrafish lines which utilize a GAL4-inducible promoter to express Cdc42, Rac1, and RhoA [9]. In the GAL4/UAS system, transcription of the yeast transcriptional activator GAL4 is driven by a promoter fragment in a particular cell type. Once translated, GAL4 protein then binds an upstream activating sequence (UAS), which activates transgene expression. In these lines a UAS sequence drives expression of wild-type, dominant negative, and constitutively

active isoforms of Cdc42, Rac1, and RhoA, which are placed downstream of a fluorophore and the self-cleaving peptide f2a (Fig. 1). When used in conjunction with tissue-specific GAL4 driver lines, these transgenic lines provide the opportunity to determine novel roles for Rho GTPases in previously inaccessible contexts.

Here we describe five methods for modulating Rho GTPase activity using these lines: (1) global transgene expression using *hsp70l:GAL4*, (2) global transgene expression via the injection of GAL4 mRNA, (3) mosaic induction via cDNA injections, (4) crossing to tissue-specific GAL4 driver lines and (5) spatially and temporally-restricted expression via focal heat shock [10].

2. Materials

Prepare all solutions using ultrapure water, and store all reagents at room temperature unless otherwise noted. Properly dispose of all waste in accordance with institutional standards.

2.1. Maintenance of zebrafish

All zebrafish must be treated in accordance with provisions established by Institutional Animal Care and Use Committees, and maintained at 28.5°C on a 14-h-light/10-h-dark cycle. Embryos must be kept at 28.5°C in the dark. Keep all fish medium used for rinsing at 28.5°C.

2.2. Zebrafish and embryo rearing

1. Rho GTPase toolkit lines (Fig. 1)
 - a. UAS:mCherry-f2a-myc-Cdc42^{XX} lines
 - b. UAS:GFP-f2a-Rac^{XX} lines
 - c. UAS:mCherry-f2a-myc-RhoA^{XX} lines
 - i. Available and from the authors [9]
2. *Hsp70l:GAL4*^{kca4} [11]
 - a. Obtainable from ZIRC
3. Tissue-specific GAL4 driver (e.g. *pou4f3:GAL4* [12])
 - a. *pou4f3:GAL4* available from the authors [12].
4. 28.5° incubator
5. Phenylthiourea (PTU)

2.2. Global Heat Shock

- a. 96-well PCR plate and plate sealing tape
- b. Fish system water or embryo medium
 - i. If using Danieu's medium, prepare according to [13]
 - ii. Fish system parameters are found in The Zebrafish Book [14]

- c. PCR thermocycler (with a block capable of accommodating 96-well plates, e.g. BioRad-PTC-1196)
- d. Petri dishes
- e. Squirt bottle

2.3. Laser-pointer heating apparatus:

For comprehensive materials and guide for apparatus assembly, refer to [10].

- a. Petri dishes
- b. 75mW laser pointer
- c. Variable power source
- d. Breadboard
- e. Focusing lens and lens mount
- f. 50µm fiberoptic fiber
- g. Fiberoptic fiber clamp
- h. Translation stage
- i. Power meter
- j. Micropipette puller (optimized for fiber pulling)
- k. Permanent black ink
- l. Thermocouple attached to thermometer
- m. Micromanipulator
- n. Wooden dowel

2.4. Microinjection (researchers should familiarize themselves with the technique as described elsewhere, such as [15])

- a. 2 mg/mL Pronase dissolved in fish system water or embryo medium
- b. 1% agarose in fish in fish system water or embryo medium
- c. 1% low-melt agarose in fish system water or embryo medium
- d. 3.2% methylcellulose in deionized water
- e. Methylene blue
- f. Petri dishes
- g. Injection molds
- h. Pipette pump
- i. Glass pipettes
- j. Diamond scoring pen

- k. Insect pin
- l. Q-tips
- m. Forceps
- n. Micropipettete needle puller
- o. Micromanipulator
- p. Pressure regulator

2.5. Molecular equipment (for generating/storing mRNA)

- a. mRNA Transcription kit (i.e. Ambion mMessage mMachine)
- b. PCR thermocycler

2.6. Whole-embryo imaging and cryosection analysis equipment:

(for comprehensive methods and materials, see [16])

- a. DMSO
- b. Normal Goat Serum
- c. Paraformaldehyde, 4% dissolved in 1X PBS
- d. Phosphate buffered solution
- e. Primary antibody
- f. Secondary antibody
- g. Sucrose, 25% and 35%, dissolved in 1X PBS
- h. Tween-20
- i. Vectashield mounting medium
- j. Coverslips, No. 1 thickness
- k. Cryostat
- l. Cryomolds
- m. Humid chamber
- n. Microslides, 1mm thick
- o. Hydrophobic pen
- p. Razor blade
- q. Slide jar or Coplin jar
- r. Tissue freezing medium
- s. Epifluorescent-enabled dissecting microscope with GFP and mCherry filter sets

3. Methods

3.1. Ubiquitous transgene expression using *hsp70l*:GAL4

1. Set up timed pairwise crosses using standard zebrafish husbandry techniques [14] to cross *hsp70l*:GAL4 transgenics to the UAS-driven Rho GTPase transgenic of your choice. As soon as possible following fertilization, collect embryos and place them into the embryo incubator (*see* Note 1).
 - a. Depending upon the tissue/process of interest, treatment with phenylthiourea (PTU) may be necessary to inhibit melanin pigmentation to facilitate visualization of internal organs. To accomplish this, we add 0.3 mM PTU (commonly referred to as 1.5× PTU) to embryos beginning ~12 h post fertilization and maintain PTU with daily medium changes throughout development.
2. At the desired developmental time point, carefully dechorionate embryos manually or using freshly-made 2 mg/mL pronase (*see* Note 2).
3. Prepare a 96-well PCR plate for the addition of embryos by dispensing ~50µL of fish medium to each well.
4. Using a diamond pen, cut a glass Pasteur pipette tip so that individual embryos can pass safely through, and attach it to a pipette pump. Using the pipette pump, carefully transfer embryos into PCR tubes, with one embryo per well of the PCR plate. After distributing the desired number of embryos, use the pipette pump to add or remove medium in each tube to ensure the total volume per tube is ~120 µL.
5. Seal the top of the PCR plate using sealing tape, and place into a PCR thermocycler. Run the thermocycler with the following conditions:
 - a. Lid temperature: 28.5°C
 - b. 30 min at 39.5°C
 - c. 28.5°C forever
6. Immediately after the PCR thermocycler returns the temperature to 28.5°C, remove the PCR plate from the machine and remove the sealing tape. Using a squirt bottle, gently squirt fish medium into the wells to wash the embryos out of the plate and into a Petri dish (*see* Note 3).

¹Because these embryos will ultimately be subjected to stresses such as dechoriation and heat shock, we take extra precautions to care for embryos resulting from *hsp70l*:GAL4 crosses. These include daily water changes after harvesting, and treatment with inhibitors of fungal and bacterial growth such as methylene blue. We find that 1.5× PTU effectively inhibits melanization throughout embryonic development. However, PTU has nonspecific effects which can disrupt developmental processes [17]–[19], so proper controls are required when interpreting phenotypes in PTU-treated embryos.

²Our UAS-driven Rho GTPase transgenics include a *cmhc2*:GFP transgenesis marker. In our hands, we are able to reliably see GFP expression in cardiomyocytes as early as 13-somite stage (~14 h post fertilization [20]). Once this occurs, it is possible to separate Rho GTPase transgenics from non-transgenics. However, we recommend keeping the groups intermixed at this stage so that both groups are heat shocked simultaneously and under similar conditions.

³Squirting fish medium may not remove every embryo from 96-well plates. To avoid unnecessary pipetting—and potential injury—of embryos, we recommend squirting an excess of fish medium onto the plate before firmly flicking the plate downward above a

7. Using a fluorescence dissecting scope, select embryos expressing *cmlc2:GFP* and sort them into a separate dish from non-transgenic embryos. Keep *cmlc2:GFP*-negative embryos as controls for later analysis.
8. Using a fluorescence dissecting scope, determine when fluorescent expression initiates in *hsp70l:GAL4;Rho* GTPase double-transgenic embryos (see Note 4).
9. Sort embryos displaying ubiquitous fluorophore expression from nonfluorescent siblings (see Note 5).
10. Analyze transgene-expressing embryos for phenotypes (Fig. 2) (see Note 6).

3.2. Mosaic transgene expression

1. Set up timed pairwise crosses using standard zebrafish husbandry techniques [14] to cross *hsp70l:GAL4* transgenics to the UAS-driven Rho GTPase transgenic of your choice. As soon as possible following fertilization, collect embryos and place them into the embryo incubator (see Note 1).
2. Inject cDNA (e.g. [15]) encoding the transgene for the desired Rho GTPase (i.e. UAS:mCherry-f2a-myc-Cdc42^{WT}), into the nucleus of single-celled embryos (see Note 7). Retain a population of uninjected siblings as controls for later analysis.
 - a. When co-injecting cDNA with Tol2 mRNA, we frequently found that the Tol2 mRNA degraded while assembling the injection mixture. To avoid this issue, we prepared fresh mixtures before each injection as follows: first, we prepared a 2× cDNA/phenol red solution and placed it in a PCR thermocycler for 10 min at 95°C. Then, immediately before injection, we mixed that solution 1:1 to a mixture containing 2× the desired mRNA concentration to produce the final injection mix.
3. Incubate embryos until developmental time points of interest (see Note 8).

partially full petri dish. When done correctly, embryos will float to the top of the wells after addition of fish medium and then be poured into the petri dish.

⁴In our experience, we observed a lag time between heat shock and UAS:fluorophore expression of around 6–8 h. This lag time varies, and this is likely based at least in part upon the precise rearing conditions of embryos. Additionally, due to the bicistronic nature of our transgenes, it may be possible that Rho GTPase expression affects cellular processes before sufficient levels of fluorophore have accumulated to produce a visible signal. For these reasons, we highly recommend careful observation of induced embryos after heat shock, both to determine when fluorophore expression initiates, and to determine if and when a reliable phenotype is discernible.

⁵Despite our efforts to standardize the amount of heat delivered to each individual embryo, we routinely observed a wide range of fluorophore expression from embryo-to-embryo. This range of expression likely stems from unavoidable variability in transgene expression and/or GAL4 induction. We commonly excluded the dimmest- and brightest- expressing embryos in an attempt to analyze embryos at a consistent level of induction from batch-to-batch.

⁶To differentiate heat shock stress from *bona fide* Rho GTPase phenotypes, we carefully compared embryos from induced embryos (*hsp70l:GAL4*⁺; *cmlc2:GFP*⁺) to both *hsp70l:GAL4*[−]; *cmlc2:GFP*⁺ and to *cmlc2:GFP*-negative embryos sorted during Step 7. After carefully analyzing any phenotypes in uninduced embryos, we could determine whether phenotypes observed in induced embryos likely result from Rho GTPase modulation, or whether those phenotypes are produced by heat shock.

⁷We also strongly recommend running the remaining injection mix on a 1% agarose gel after injection to confirm the presence of intact Tol2 mRNA after injections are completed. While the concentration of Tol2 mRNA and cDNA must be optimized by each user for their specific experiment, we had good results injecting 75 pg of mRNA and 50 pg of cDNA.

⁸Due to mosaic construct expression, we regularly observed a range of *cmlc2:GFP* expression, from one or two very dimly-fluorescent cells in the heart to the entire heart being labeled by brightly-fluorescent GFP positive cells. We did not, however, exclude dimly-expressing embryos at this stage, as dim *cmlc2:GFP* expression does not necessarily indicate that transgene expression will be poor in other tissues.

4. As detailed above (Subheading 3.1. steps 3 to 6), prepare a 96-well PCR plate and heat shock injected embryos for 30 minutes at 39.5°C
5. After heat-shocked embryos begin to express transgene proteins, separate fluorophore-expressing embryos from non-expressing embryos and analyze induced embryos for a phenotype (Fig. 3) (*see Note 9*).

3.3. Ubiquitous transgene expression using GAL4 mRNA injections

1. Prior to fish breeding, synthesize GAL4 mRNA and store at either –20° or –80°.
 - a. We use the mMessage mMachine transcription kit (ThermoFisher Scientific) to synthesize GAL4 mRNA. After synthesis, we typically elute mRNA in 20 µL of ultrapure water. After verifying RNA concentration and quality by Nanodrop analysis, we aliquoted the purified mRNA into 5-µL aliquots and stored them at –80°C. Individual aliquots are then thawed as needed, and diluted to 2× the desired injection concentration. Immediately prior to injection, we combined the 2× mRNA mixture with a heat-treated 2× mixture of water and phenol red at a 1:1 ratio.
2. Set up timed pairwise crosses using standard zebrafish husbandry techniques [14] to cross the UAS-driven Rho GTPase transgenic of your choice to wild-type zebrafish. Collect embryos immediately after fertilization.
3. Inject GAL4 mRNA [15] into single-celled embryos.
4. Using a fluorescence microscope, carefully monitor injected embryos for expression (*see Note 10*).
5. Sort for embryos ubiquitously expressing GFP or mCherry (induced). Keep both induced and fluorescence-negative embryos in separate dishes.
6. Analyze induced embryos for phenotypes (*see Note 11*).

3.4. Transgene expression using tissue-specific GAL4 driver lines

1. As described above, set up timed pairwise crosses between the Rho GTPase transgenic of your choice and tissue-specific GAL4 transgenic zebrafish of your choice. As soon as possible following fertilization, collect embryos and place them into the embryo incubator.

⁹Similar to Note 6, carefully compare induced embryos and controls (i.e. uninjected and *hsp70l*:GAL4-negative groups). Extra scrutiny will be required when analyzing injected embryos for phenotypes because of the possibility of nonspecific phenotypes arising from RNA toxicity after cDNA/Tol2 mRNA injections.

¹⁰We commonly observe a lag time of ~4–5 h between mRNA injection and transgene induction. Since there is no requirement to activate GAL4 expression via heat shock, the lag time we see after mRNA injection is shorter compared to the lag time between heat shock and transgene induction (*see Note 4*). Therefore, this technique will enable the global modulation of Rho GTPases at slightly earlier time points than were possible using *hsp70l*:GAL4 transgenes.

¹¹Despite careful controls, we regularly observe nonspecific phenotypes arising from RNA toxicity. Therefore, careful analysis is critical when analyzing induced embryos for phenotypes after GAL4 mRNA injection. Due to these nonspecific phenotypes, we also strongly recommend performing rigorous dose-response experiments to determine the optimal concentration of mRNA in your experiments.

2. Using a fluorescent microscope, screen for embryos expressing *cmlc2:GFP* and sort them into a separate dish. Keep *cmlc2:GFP*-negative embryos as controls for later analysis.
3. Monitor *cmlc2:GFP*-positive embryos for Rho GTPase transgene expression (*see* Note 12).
4. Carefully analyze induced embryos for phenotypes and compare them to uninduced controls (*see* Note 13).

3.5. Local heat shock induction:

1. Prior to breeding, prepare fiberoptic fiber tips for local heat shock by burning away fiber insulation and/or using micropipette puller to reduce tip size. Carefully store the tips after preparation, as they are very fragile.
2. Set up timed pairwise crosses using standard zebrafish husbandry techniques [14] to cross *hsp70l:GAL4* transgenics to the UAS-driven Rho GTPase transgenic of your choice. As soon as possible following fertilization, collect embryos and place them into the embryo incubator (*see* Note 1).
3. Using a fluorescent microscope, screen for embryos expressing *cmlc2:GFP* and sort them into a separate dish. Keep *cmlc2:GFP*-negative embryos as controls for later analysis.
4. At the desired developmental time point, select an appropriate number of both *cmlc2:GFP*-positive and *cmlc2:GFP*-negative embryos for heat shock analysis (*see* Note 12), and carefully dechorionate manually or using freshly-made 2 mg/mL pronase (*see* Note 2).
5. Using a pipet, place beads (~0.5–1 mL) of 1% low melt agarose or 3.2% methylcellulose on the surface of a Petri dish. Using a pipet pump, gently drop embryos on the top surface of the beads and remove as much water as possible from the embryo. Using an insect pin or hair, gently guide embryos into the agarose or methylcellulose and position them as desired (*see* Note 14).
6. Prepare the local heat shock apparatus by mounting the prepared fiberoptic fiber on a wooden dowel and securing it in a micromanipulator. Dip the tip of the fiber in permanent ink, and then turn the power supply on. After confirming proper tip

¹²-Roughly half of *cmlc2:GFP*-positive embryos will contain both the Rho GTPase transgene (*cmlc2:GFP*-positive) and the *GAL4* driver transgene. Additionally, due to mosaic UAS silencing, *GAL4* induction may be reduced in some embryos and completely silenced in others. Therefore, it is likely that <50% of *cmlc2:GFP*-positive embryos will display transgene induction, and within that population there may be varying degrees of expression.

¹³-We strongly recommend careful observation of embryos around the time points *GAL4* expression is known to initiate in your driver line of choice. Additionally, similar to Note 4, due to the bicistronic nature of our transgenes it may be possible that Rho GTPase activity modulation can disrupt normal cellular processes before detectable levels of fluorophore have accumulated. Therefore, it may be beneficial to start analyzing embryos at time points slightly preceding the time points when your driver line of choice is known to initiate expression of *GAL4*.

¹⁴-If using 1% low melt agarose, be sure to allow the agarose to cool sufficiently so as to avoid harming embryos. Methylcellulose and low melt agarose offer distinct advantages and disadvantages: low melt agarose hardens and therefore prevents embryos from drifting out of position once that position is reached. However, repositioning embryos during the procedure, or freeing them from hardened low melt agarose after heat shock, may be problematic. Meanwhile, methylcellulose never fully hardens and therefore allows more flexibility for repositioning and removing embryos, though embryos may drift out of position.

temperature by bringing the tip into contact with a thermocouple submerged under a droplet of water, bring the fiberoptic tip into contact with your tissue/region of interest and apply heat for 50°C for 3 (see Note 15).

7. Free heat shocked embryos from low melt agarose by submerging the immobilized embryos in fresh fish medium. Then, making gentle circular motions with a hair attached to a wooden stick, gently disrupt the low melt agarose immobilizing the embryos until they are able to make unimpeded swimming motions. Transfer embryos to a petri dish containing fresh fish medium for recovery.
8. Compare induced embryos to uninduced controls and carefully analyze them for phenotypes (see Note 13).

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References

- [1]. Etienne-Manneville S and Hall A (2002) Rho GTPases in cell biology. *Nature*, 420, 6916, 629–635, 2002. [PubMed: 12478284]
- [2]. Takai Y, Sasaki T, and Matozaki T (2001) Small GTP-binding proteins, *Physiol Rev*, 81, 1, 153–208, 2001. [PubMed: 11152757]
- [3]. Luo L (2000) Rho GTPases in neuronal morphogenesis. *Nat. Rev. Neurosci*, 1, 3, 173–180. [PubMed: 11257905]
- [4]. Hall A (1998) Rho GTPases and the actin cytoskeleton. *Front Cell Dev Biol*, 9, 7, 2632–2641.
- [5]. Bosco EE, Mulloy JC, and Zheng Y (2009) Rac1 GTPase: A ‘Rac’ of all trades. *Cell. Mol. Life Sci*, 66, 3, 370–374. [PubMed: 19151919]
- [6]. Erickson JW, and Cerione RA, (2001) Multiple roles for Cdc42 in cell regulation. *Curr. Opin. Cell Biol*, 13, 2, 153–157. [PubMed: 11248548]
- [7]. Sugihara K, Nakatsuji N, Nakamura K, Nakao K, Hashimoto R, Otani H, Sakagami H, Kondo H, Nozawa S, Aiba A, Katsuki M (1998) Rac1 is required for the formation of three germ layers during gastrulation. *Oncogene*, 17, 26, 3427–3433. [PubMed: 10030666]
- [8]. Chen F, Ma L, Parrini MC, Mao X, Lopez M, Wu C, Marks PW, Davidson L, Kwiatkowski DJ, Kirchhausen T, Orkin SH, Rosen FS, Mayer MJ, Kirschner MW, Alt FW (2000) Cdc42 is required for PIP(2)-induced actin polymerization and early development but not for cell viability. *Curr. Biol*, 10, 13, 758–65. [PubMed: 10898977]
- [9]. Hanovice NJ, McMains E, and Gross JM, (2016) A GAL4-inducible transgenic tool kit for the in vivo modulation of Rho GTPase activity in zebrafish. *Dev. Dyn*, 245, 8, 844–853. [PubMed: 27105927]
- [10]. Placinta M, Shen M-C, Achermann M, and Karlstrom RO (2009) A laser pointer driven microheater for precise local heating and conditional gene regulation in vivo. *Microheater driven gene regulation in zebrafish. BMC Dev. Biol*, 9, 73. [PubMed: 20042114]

¹⁵ Karlstrom *et al* [10] recommends either 3 min at 50°C or 30 min at 40°C. While we found the best results when we heat shocked embryos at 50°C for 3 min, we strongly encourage the reader to rigorously optimize the precise time and temperature required for their purposes.

- [11]. Scheer N, Groth A, Hans S, and Campos-Ortega JA, (2001) An instructive function for Notch in promoting gliogenesis in the zebrafish retina. *Development*, 128, 1099–1107. [PubMed: 11245575]
- [12]. Xiao T, and Baier H (2007) Lamina-specific axonal projections in the zebrafish tectum require the type IV collagen Dragnet. *Nat. Neurosci*, 10, 12, 1529–1537. [PubMed: 17982451]
- [13]. “Danieau’s solution (30×),” *Cold Spring Harb. Protoc*, vol. 2011, no. 7, p. pdb.rec12467, 7 2011.
- [14]. Westerfield M (2015) *The zebrafish book a guide for the laboratory use of zebrafish Danio (Brachydanio) rerio*. Eugene, OR: ZFIN.
- [15]. Xu Q (1999) Microinjection into zebrafish embryos. *Methods Mol. Biol*, 127, 125–132. [PubMed: 10503230]
- [16]. Uribe RA, and Gross JM (2007) Immunohistochemistry on cryosections from embryonic and adult zebrafish eyes. *CSH Protoc*, 1.
- [17]. Elsalini OA, and Rohr KB, (2003) Phenylthiourea disrupts thyroid function in developing zebrafish. *Dev Genes Evol*, 212, 593–598. [PubMed: 12536323]
- [18]. Li Z, Ptak D, Zhang L, Walls EK, Zhong W, and Leung YF (2012) Phenylthiourea specifically reduces zebrafish eye size. *PLoS One*, 7, 6, 1–14.
- [19]. Tingaud-Sequeira A, Forgue J, Andre M, and Babin PJ, (2006) Epidermal transient down-regulation of retinol-binding protein 4 and mirror expression of apolipoprotein Eb and estrogen receptor 2a during zebrafish fin and scale development. *Dev. Dyn*, 235, 11, 3071–3079. [PubMed: 16929533]
- [20]. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, and Schilling TF (1995) Stages of embryonic development of the zebrafish. *Dev. Dyn* 203, 3, 253–310. [PubMed: 8589427]

Figure 1:

Overview of transgenic constructs. A: 10X*uas* drives bicistronic expression of a fluorophore (mCherry or GFP) and Rho GTPases Rac1, myc-Cdc42, and myc-RhoA. B: Chart detailing each line, including the isoform of Rho GTPase and its predicted activity and species of origin.

Figure 2:
Expression of mCherry and Cdc42^{WT} following global heat shock in *hsp70l:GAL4⁻* (A,B) and *hsp70l:GAL4⁺* (C,D) embryos.

Figure 3:
Mosaic expression of GFP-f2a-Rac^{WT} in the retina at 3dpf. Red = phalloidin, green = GFP.
Scale bar = 50µm.