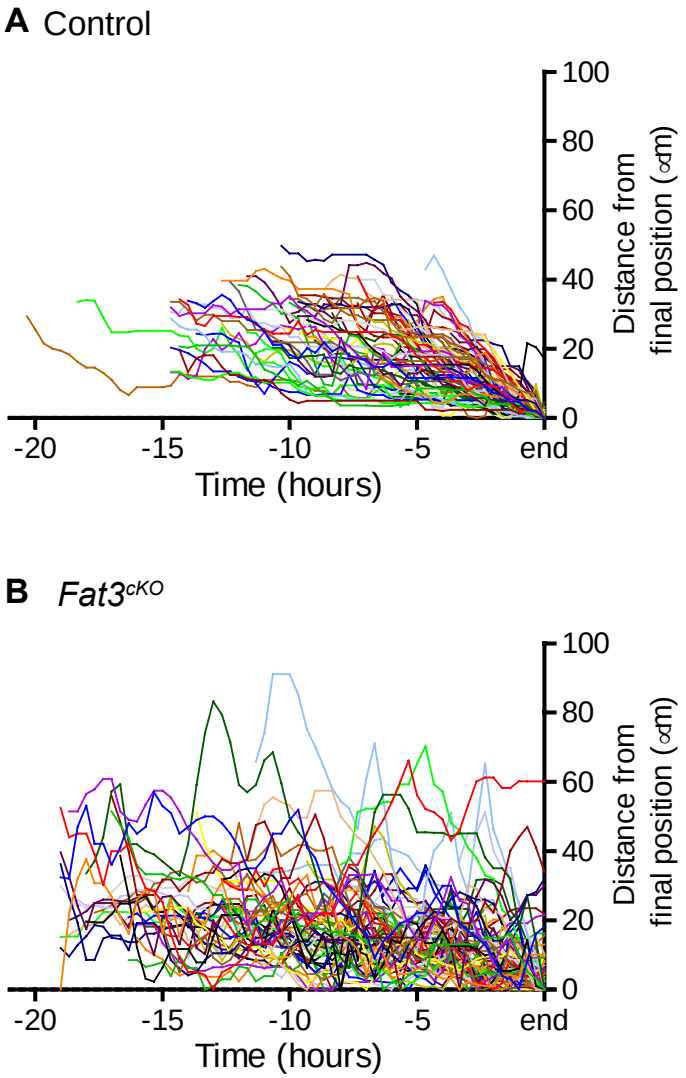


Figure S1. Migratory trajectories are disrupted in amacrine cells lacking Fat3.

For each cell body that was tracked, its position was plotted over time and normalized to the lowest position reached during the imaging period. Tracks from control (n=75) (A) and *Fat3*^{cKO} (n=75) (B) animals were aligned relative to the end time of imaging (end) since cells took variable lengths of time to traverse similar distances.

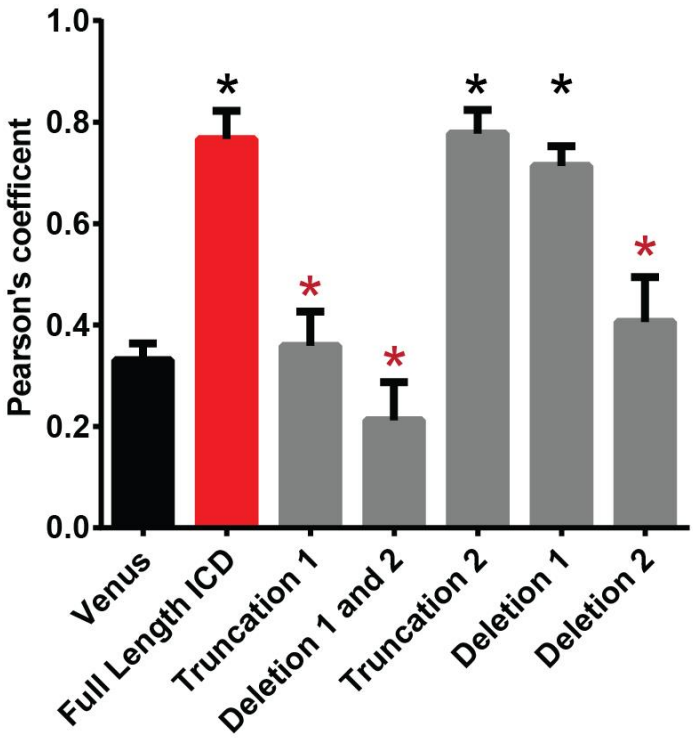


Figure S2. Quantification of colocalization.

Quantification of colocalization of mitochondrial targeted Fat3 constructs and VASP immunostaining in COS-7 cells. n=10 for each condition, from one transfection. One-way ANOVA followed by Bonferroni multiple comparisons test. Black * indicates $p<0.001$ when compared to Venus negative control. Red * indicates $p<0.01$ when compared to Fat3ICD positive control. Fat3ICD vs. Venus: $p<0.0001$; Truncation 2 vs. Venus: $p<0.0001$; Deletion 1 vs. Venus: $p=0.0001$; Truncation 1 vs. Fat3ICD: $p=0.0001$; Deletion 1 and 2 vs. Fat3ICD: $p<0.0001$; Deletion 2 vs. Fat3ICD: $p=0.001$. Bars show mean ± SEM.

Supplementary Materials and Methods

Antibody Validation

Batch numbers were not available for any of the antibodies. Rabbit anti-Mena was validated by western blot of *mena* mutant tissue (Lanier et al., 1999) and of lysates from Mena-transfected cells (Bear et al., 2000). Mouse anti-Mena was validated by western blot and by immunostaining of transfected cells (Lebrand et al., 2004). Anti-VASP was validated by western blot of VASP-transfected cells (Bear et al., 2000). Anti-EVL was validated by western blot of EVL-transfected cells and tissue lysates (Lambrechts et al., 2000) and by western blot of EVL mutant mouse brain lysates (Kwiatkowski et al., 2007). Anti-Fat3 was validated by immunostaining *Fat3* mutant retinas and by western blot of *Fat3* mutant lysate (Deans et al., 2011 and data not shown). Actin was detected using a standard commercial antibody, which revealed a band of the expected size upon western blot. tdTomato was detected using a standard commercial antibody against DsRed and revealed the same pattern evident without amplification.

Image acquisition

Electroporated retinas were imaged using an Olympus FV 1200 confocal microscope using a 60x Lens, 1.4 N.A, pinhole C.A. 90 µm, z step 0.4 µm with an x y resolution of 0.103 µm x 0.103 µm per pixel. Analysis was limited to cells with cell bodies fully present in the stack. Z stack projections are shown.

Live imaging of amacrine cells was performed using a 40X 1.1 NA objective on a Zeiss 710 NLO multiphoton microscope with an environmental chamber that regulates temperature, CO2, and humidity. The laser was tuned to 950 nm to excite both fluorophores, and fluorescence emission was captured by non-descanned detectors. Images were collected at 2 µm intervals throughout a 130-150 µm deep region within 200 µm of the optic nerve. For visualization

purposes, a median filter was applied to all images, and maximum intensity projections of a selected range of z slice images were created in order to isolate individual neurons for analysis.

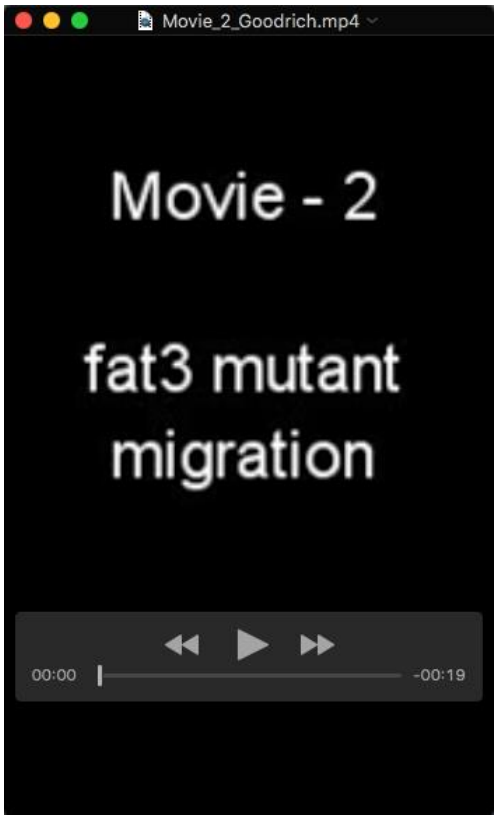
For Fat3 and Mena double immunostaining, z-projections were made of a stack of four consecutive images taken with a 60x lens, with a resolution in x.y of 0.207 µm x 0.207 µm per pixel. For visualization of actin and of Ena/VASP family members in control vs *Fat3* mutant retinas and for analysis of cell migration, single optical slices were taken with a 40x lens, with a resolution in x,y of 0.397 µm x 0.397 µm per pixel. To quantify cell distribution, images were collected within 500 µm of the optic nerve.

Pulldown Assays

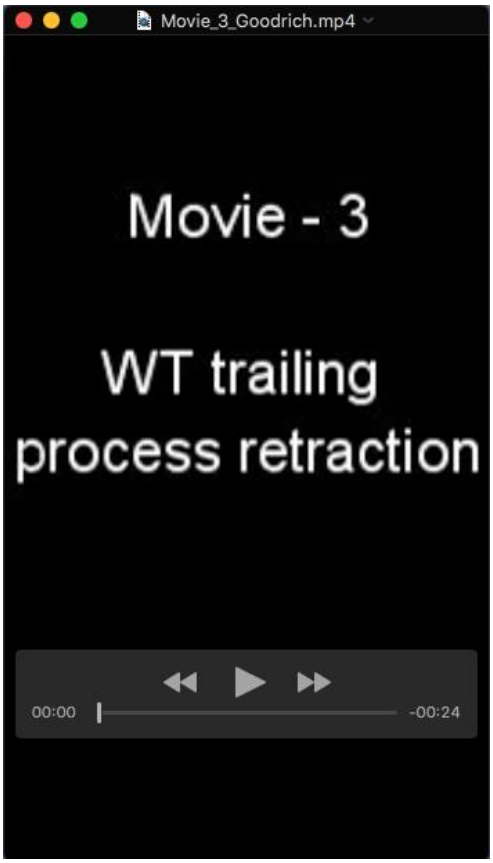
GST-Fat3ICD fusion proteins were grown in Rosetta *E. coli* (Millipore) at room temperature, induced with IPTG at OD600 = 0.5-1 and harvested 4 hours after induction. Bacterial pellets were lysed in phosphate buffered saline (PBS) containing 1% Trion X-100, 1mM EDTA, 0.1mg/ml lysozyme supplemented with 1 mM Pefabloc SC PLUS protease inhibitor (Roche, Rochester, NY), 1mM DTT. Fusion proteins in bacterial lysate were bound to glutathione Sepharose beads (GE Life Sciences)

P1 retinas were dissected and homogenized on ice in 750 µl lysis buffer (50 mM Tris ph7.5, 200 mM NaCl, 10% glycerol, 1% NP40, 1mM EDTA, 1 mM EGTA, supplemented with 1 mM Pefabloc SC PLUS protease inhibitor (Roche, Rochester, NY), 1mM Na3OV4, and 1mM DTT) per 100 mg tissue. 900 µl of lysate was added to 50 µl of beads bound to fusion protein and rotated at 4 °C overnight. Supernatant was recovered and combined with 45 µl lysis buffer and 25 µl 4x NuPage sample buffer (Invitrogen). Proteins present in the brain lysate input, supernatant, and beads were analyzed by standard western blot analysis. GST-Fat3ICD fusion proteins were expressed from the *pGEX6P-1* vector (GE Lifesciences).

Movie 1. Migration of wild type amacrine cells. Time-lapse video of a migrating wild-type amacrine cell in a retinal slice culture preparation. Cells were labeled using *ex vivo* electroporation to introduce a Cre-reporter construct expressing tdTomato into P0 retinas harvested from *Ptf1a-cre* pups. The video starts after 24 hours in culture. Yellow arrow indicates cell of interest. Time: hr:min.

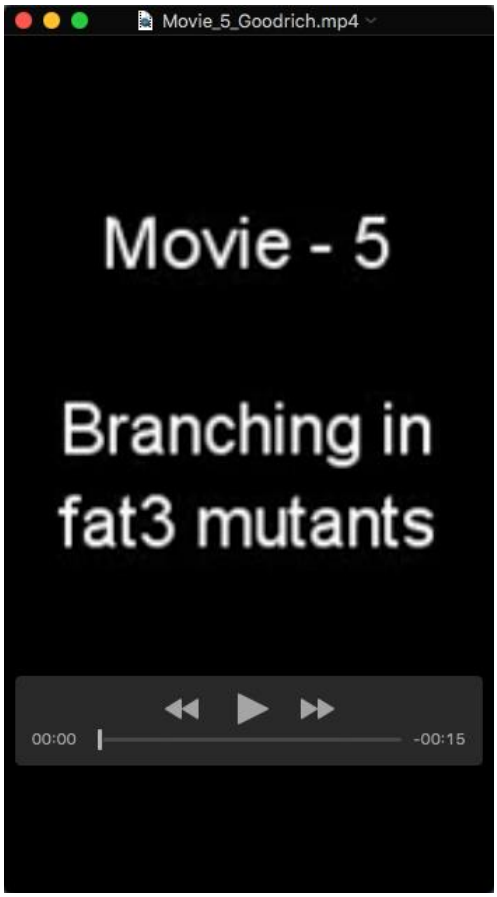


Movie 2. Migration of *fat3* mutant amacrine cells. Time-lapse of a migrating *fat3* mutant amacrine cell in a retinal slice culture preparation. Cells were labeled using *ex vivo* electroporation to introduce a Cre-reporter construct expressing tdTomato into P0 retinas harvested from *Ptf1a-cre*; *fat3*$^{fl/-}$ pups. The video starts after 24 hours in culture. Yellow arrow indicates cell of interest. Time: hr:min.



Movie 3. Trailing process retraction in wild type amacrine cells. A time-lapse video illustrating the retraction of the trailing process in a wild-type amacrine cell in a retinal slice culture preparation. Cells were labeled using *ex vivo* electroporation to introduce a Cre-reporter construct expressing tdTomato into P0 retinas harvested from *Ptf1a-cre* pups. The video starts after 48 hours in culture. Yellow arrow indicates cell of interest. Time: hr:min.

Movie 4. Trailing process retraction in *fat3* mutant amacrine cells. A time-lapse video illustrating the retraction of the trailing process in a *fat3* mutant amacrine cell in a retinal slice culture preparation. Cells were labeled using *ex vivo* electroporation to introduce a Cre-reporter construct expressing tdTomato into P0 retinas harvested from *Ptf1a-cre*; *fat3*$^{fl/-}$ pups. The video starts after 48 hours in culture. Yellow arrow indicates cell of interest. Time: hr:min.



Movie 5. Ectopic trailing process branching in *fat3* mutant amacrine cells. A time-lapse video capturing the ectopic branching that occurs in the trailing process of *fat3* mutant amacrine cells. Cells were labeled using *ex vivo* electroporation to introduce a Cre-reporter construct expressing tdTomato into P0 retinas harvested from *Ptf1a-cre*; *fat3*$^{fl/-}$ pups. The video starts after 48 hours in culture. Yellow arrow indicates cell of interest. Time: hr:min.