1   ***Drosophila* TNF modulates tissue tension in the embryo to facilitate macrophage**

2   **invasive migration**

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26 **Summary:**

27 Migrating cells penetrate tissue barriers during development, inflammatory responses

28 and tumor metastasis. We study if migration *in vivo* in such three-dimensionally

29 confined environments requires changes in the mechanical properties of the

30 surrounding cells using embryonic *Drosophila melanogaster* hemocytes, also called

31 macrophages, as a model. We find that macrophage invasion into the germband

32 through transient separation of the apposing ectoderm and mesoderm requires cell

33 deformations and reductions in apical tension in the ectoderm. Interestingly, the

34 genetic pathway governing these mechanical shifts acts downstream of the only

35 known TNF superfamily member in *Drosophila*, Eiger, and its receptor, Grindelwald.

36 Eiger-Grindelwald signaling reduces levels of active Myosin in the germband

37 ectodermal cortex through the localization of a Crumbs complex component, Patj

38 (Pals-1-associated tight junction protein). We therefore elucidate a distinct molecular

39 pathway that controls tissue tension and demonstrate the importance of such

40 regulation for invasive migration *in vivo*.

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Introduction

A full understanding of migration in the complex three-dimensional environment that exists *in vivo* requires investigating how cells move through the mechanical constraints posed by their surroundings. A number of studies have recently shown that increased stiffness of the matrix promotes invasion by cancer and immune cells (Friedl et al., 2012; Laklai et al., 2016; Levental et al., 2009; Miroshnikova et al., 2016) but little research has been conducted *in vivo* on the influence of the mechanical properties of neighboring tissues. We address such questions using the migration of *Drosophila* plasmatocytes in the embryo. Plasmatocytes, also called hemocytes, are the primary phagocytic cells in the *Drosophila* embryo and share striking similarities with vertebrate macrophages in ontogeny, functionality and migratory behavior (Evans and Wood, 2011; Gold and Brückner, 2014; Lemaitre and Hoffmann, 2007; Nourshargh et al., 2010; Ratheesh et al., 2015; Reymond et al., 2013; Vestweber, 2015; Weavers et al., 2016). They are specified in the head mesoderm at embryonic Stages 4-6 and at Stage 9 they start migrating along pre-determined paths following cues from the PDGF- and VEGF-related factors (Pvf) 2 and 3 (Brückner et al., 2004; Cho et al., 2002; Evans and Wood, 2011; Gold and Brückner, 2014; Siekhaus et al., 2010; Weavers et al., 2016) to populate the entire embryo (Figure 1A). One of these early migratory routes stretches from the head mesoderm across the yolk sac and into the extended germband (blue arrow, Figure 1A) (Ratheesh et al., 2015; Siekhaus et al., 2010) that undergoes retraction to the posterior of the embryo by late Stage 12. Our previous work demonstrated that this germband route requires macrophage movement through a tissue barrier and displays molecular parallels to vertebrate immune cell transmigration in its requirement for modulation of Integrin affinity through small GTPases in macrophages (Siekhaus et

76   al., 2010). In this study we unravel the molecular and mechanical changes that occur

77   in the germband to promote this macrophage tissue invasion.

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79   **Results**

80   ***Macrophages invade the germband between the closely apposed epithelial ectoderm***

81   ***and mesoderm***

82   We first examined in detail the environment of macrophage migration into the

83   germband as well as the nature of the constraints that they might encounter in the

84   process. We performed two-photon imaging in Stage 11 embryos, labeling both the

85   macrophages as well as the surrounding tissues and focusing on the region of the

86   germband (Figure 1B). At this stage they migrated on top of the yolk sac. While a few

87   macrophages migrated into the folds of the amnioserosa (yellow arrow, Figure 1C and

88   S1), the majority moved further along the yolk sac and entered the germband (Figure

89   S1A,B and Movie S1). Migration into the germband at late Stage 11 appeared to be a

90   multi-step, time intensive process during which the initial macrophage first inserted a

91   protrusion into and between the cells of the germband (Figure 1C, white arrow). This

92   protrusion enlarged as the initial macrophage fully penetrated the germband (Figure

93   1C arrowhead and Movie S1) followed closely by the remaining macrophages.

94   Macrophages invariably entered the germband at specific locations (Figure 1C and

95   Movie S1) which confocal imaging of fixed Stage 12 embryos revealed to be where

96   ectoderm expressing DE-Cadherin abuts visceral mesoderm expressing DN-Cadherin

97   (Figure 1D). Macrophages continue to migrate along this ectoderm-mesoderm

98   interface during Stage 12 (Figure 1D). Prior to macrophage entry the ectodermal

99   epithelium and the mesoderm lie closely apposed to one another (Figure 1D merge,

100   Figure 1E arrows). During macrophage entry into the germband, the two tissues

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101 separate concomitantly with macrophage entry and the macrophages migrate onwards

102 while confined between them (Figure 1D,E, Figure S1A,B and Movie S1).

103 To examine this tissue interface in more detail we used transmission electron

104 microscopy (TEM), identifying the ectoderm and the mesoderm using their

105 morphology, with the outer ectoderm cells having a typical columnar appearance and

106 the inner mesoderm cells appearing more rounded. TEM images confirmed that the

107 two tissues lie in close proximity prior to macrophage entry (Figure S1C,D, Figure

108 1F-H) matching our observation that macrophages are not utilizing pre-existing gaps

109 in the germband to enter. Close examination of the germband ectoderm prior to entry

110 revealed that ectodermal cells are organized in a polarized epithelium, with Adherens

111 junctions (AJ) facing the outside of the germband (Figure S1D, E), and the basal side

112 of the ectoderm forming an interface with the adjacent mesoderm. Previous studies

113 have shown that Collagen is deposited at significant levels during later embryonic

114 stages (Matsubayashi et al., 2017) however we found that the mesoderm starts

115 secreting Laminin A (LanA) in Stage 11, resulting in visible deposition at the

116 interface of the ectoderm and mesoderm (Figure 1I,J) by the time macrophages

117 prepare to enter the germband. Previous studies have shown that Matrix

118 Metalloproteases are not required for macrophage migration into the germband

119 (Siekhaus et al., 2010), suggesting that while macrophages might utilize Laminin as a

120 substrate during migration, ECM cleavage is not needed for macrophage entry. Our

121 data show that embryonic macrophages intercalate between previously closely

122 apposed tissues as they enter and continue moving within the germband (Figure 1K,

123 Figure S1A,B and Movie S1) indicating that this system is ideally suited to

124 understand the three-dimensional mechanical constraints extant during tissue invasion

125 *in vivo*.

Eiger in the amnioserosa is required for macrophage invasion into the extended germband

To identify the molecular pathways that could modulate mechanical parameters during germband invasion we searched for genes expressed in and around the tissues which macrophages enter. Given that vertebrate TNFα facilitates leukocyte ingression between vascular endothelial cells, we focused on Eiger (EDA-like cell death trigger), the sole *Drosophila* member of the Tumor Necrosis Factor (TNF) superfamily (Igaki, 2002; Kanda et al., 2002) which is expressed in the amnioserosal tissue that lies adjacent to the germband (Figure 2A, arrow) as well as along the ventral nerve cord. We tested if Eiger was important for macrophage migration into and within the germband by assessing the numbers of macrophages inside the germband in embryos from the *eiger* excision mutants, *egr¹* and *egr³,* which remove the promoter as well as the translational start site (Igaki, 2002). To be sure that any differences in macrophage numbers between genotypes were not due to differences in staging, we examined embryos with 35-40% retraction of the germband from the anterior, a discrete time period during Stage 12 (Figure S2A). Fixed embryos from *egr¹* and *egr³* mutant flies displayed a 50% decrease in the number of macrophages in the germband compared to the control, visualized using a macrophage specific driver (Figure 2B-D and Figure S2A). The *egr¹* phenotype was not enhanced over the deficiency Df(2R)BSC303 that completely removes the gene (Figure 2D) and *egr¹* embryos displayed no expression of the gene (Figure 2A). This evidence suggests that *egr¹* is a null allele and we therefore used this mutant in all of our further experiments. Changes in the timing of the initiation of the developmental movements of the germband or the speed with which these steps occur could have affected our assessment of macrophage numbers in the germband. Hence we imaged the germband live in control and *egr¹* embryos

151 and found no significant difference either in when germband extension was initiated

152 (192$\pm$2 minutes after fertilization (AF) for control and 194$\pm$2 minutes AF for *egr^1*) or

153 the time taken for its completion (34$\pm$1 minutes for control and 34$\pm$2 minutes for *egr^1*

154 embryos, n=17 embryos for both genotypes). We also found no significant difference

155 either in when germband retraction began (Figure S2B), the time taken for the initial

156 phase of retraction to 35-41% of egg length (Figure S2C) or for its full completion

157 (122$\pm$4 minutes for control and 121$\pm$3 minutes for *egr^1*, n=16 embryos for control and

158 17 embryos for *egr^1*). We therefore concluded that Eiger directly regulates the number

159 of macrophages that migrate into the germband. The amnioserosa (AS) appeared to be

160 the predominant source of the Eiger that affects macrophage tissue invasion;

161 expressing *eiger* in the AS significantly rescued the *egr^1* phenotype and driving an

162 RNAi construct against *eiger* in the AS substantially recapitulated the *egr^1* phenotype

163 (Figure 2E,F and Figure S2D,E). *egr^1* embryos displayed no changes in the number of

164 macrophages migrating along the ventral nerve cord (vnc) (Figure S2F,G,H) or in the

165 number of macrophages that end up in the anterior tip of the head (Figure S2I,J).

166 There was also no change in the total number of macrophages (Figure S2K), arguing

167 that Eiger acts specifically to affect macrophage migration into or within the

168 germband but not their movement along other routes, survival or division.

169 To confirm this conclusion we directly assessed the migratory properties of

170 macrophages in the *egr^1* null mutant. We performed two-photon imaging in live

171 *Drosophila* embryos with sufficient spatial and temporal resolution to allow us to

172 segment and track the macrophage nuclei in 4D (Movie S2 & 3). We observed no

173 significant change in speed during macrophage migration from the head up to the

174 germband when compared to the control and a small (7%) decrease in the persistence

175 (Figure 2G-I and Figure S2L,M). The conclusion that Eiger does not impede

176 migratory steps prior to germband entry was further supported by the observation in

177 both fixed analysis and live imaging of *egr¹* mutant embryos that macrophages

178 accumulated at the edge of the germband (Figure 2B,J (arrows), and Movie S2). We

179 therefore imaged macrophage entry into the germband in embryos in which

180 macrophages were visualized with cytoplasmic mCherry and the ectoderm was

181 labeled with *knock-in DE-Cadherin::GFP* (Huang et al., 2009). Macrophages paused

182 at the germband edge with the first macrophage taking ~30 min to move in after

183 initial contact (Figure 2K and Movie S4), confirming our initial assessment that

184 macrophage entry into the germband is a time-intensive process. In *egr¹* mutants,

185 germband entry took even longer, requiring 60% more time (~50 min, Figure 2K).

186 We went on to assess whether Eiger also affects macrophage migration within the

187 germband. Interestingly we found that the speed of migration of the first macrophage

188 in the area of germband which lies in contact or very close to the AS (Figure 2L), is

189 significantly slower in *egr¹* mutant embryos compared to the control, yet the speed

190 thereafter did not show a significant difference (Figure 2M,N Figure S2N,O). This

191 suggests that Eiger expression in the AS controls macrophage migration within the

192 region of the germband that lies adjacent to the AS. Thus we show that, like

193 vertebrate TNF, the *Drosophila* TNF, Eiger can regulate immune cell tissue invasion,

194 facilitating invasive entry and initial invasive migration.

195 ***The TNF receptor Grindelwald in the ectoderm regulates macrophage tissue***

196 ***invasion but not general migration***

197 We asked how Eiger, which is expressed in the AS, could regulate macrophage

198 migration into and within the neighboring germband. Confocal imaging of antibody

199 staining against Eiger in Stage 10 embryos, in which macrophages do not contact the

200 amnioserosa or germband, revealed Eiger localized in puncta at the membrane of the

201   AS cells (Figure 3A, arrowhead in Figure 3A' and Figure S3A, arrowhead in Figure

202   S3A'). However, at Stage 11, in which embryos displayed germband retraction of 29-

203   31% and macrophages had reached the AS and the germband, Eiger staining was

204   much lower in the AS (Figure 3B,C, Figure S3B, arrowhead in Figure S3B'), and

205   became evident on the germband ectoderm (arrow in Figure 3B'), displaying a three

206   fold increase when quantitated (Figure 3D), as well as becoming detectable on other

207   surrounding tissues. This suggested that Eiger is released from the AS cells in a

208   developmentally timed process that correlates with macrophage germband invasion.

209   Our data is consistent with previous studies which have shown that Eiger, like other

210   TNFs, can undergo proteolytic cleavage to permit release and diffusion of the

211   extracellular domain from the cell surface (Agrawal et al., 2016; Jo et al., 2017;

212   Kauppila et al., 2003). We next looked for potential Eiger receptors expressed in the

213   germband that could mediate its role in macrophage tissue invasion. Interestingly, the

214   transcript encoding Grindelwald, a transmembrane protein with homology to

215   members of the TNFR superfamily, is expressed at high levels in the germband

216   ectoderm in Stage 11 embryos with weaker expression throughout the caudal

217   ectoderm (Figure 3E). Grindelwald has recently been shown to act as an Eiger

218   receptor, binding Eiger through its TNF homology domain and mediating its pro-

219   apoptotic and signaling functions (Andersen et al., 2015). Ectodermal knockdown of

220   *grindelwald* using tissue specific RNAi resulted in a decrease in the number of

221   macrophages in the germband (Figure 3F), suggesting that Grindelwald functions in

222   the ectoderm to support macrophage tissue invasion. We then tested the effect of a

223   *grindelwald* null mutant (Andersen et al., 2015), $grnd^{Minos}$, on macrophage migration.

224   Similarly to egr^1, $grnd^{Minos}$ embryos showed a significant decrease in the number of

225   macrophages in the germband compared to the control (Figure 3G). This change was

226   not due to a decrease in the total number of macrophages, since that was unaffected in

227   *grnd*^{*Minos*} (Figure S3C). Live imaging and tracking of macrophage nuclei

228   demonstrated that the speed and persistence of macrophage migration up to the

229   germband was unaffected in the *grnd*^{*Minos*} mutant compared to the control (Figure 3H,

230   Figure S3D-G and Movie S3). There was also no significant change in macrophage

231   numbers along the vnc suggesting that, like Eiger, Grindelwald does not affect the

232   general migratory properties of macrophages (Figure S3H,I). Taken together this data

233   suggests that Eiger is released from AS cells and acts on the neighboring germband

234   ectodermal epithelium through its interaction with Grindelwald to facilitate

235   macrophage tissue invasion.

236   ***Eiger does not regulate E or N-Cadherin or cell-ECM signaling at the ectoderm-***

237   ***mesoderm interface***

238   We analyzed whether Eiger signaling might facilitate macrophage tissue invasion into

239   and within the first part of the germband by lowering the levels of adhesion molecules

240   present at the ectoderm-mesoderm interface in this region. To examine this, we

241   assessed wild type and *egr*^{*1*} mutant embryos both at Stage 10, before the release of

242   Eiger from the amnioserosa, and at Stage 11, when Eiger appears on the ectoderm and

243   macrophages contact the germband. We found no significant difference between wild

244   type and *egr*^{*1*} mutant embryos in DE-Cadherin or DN-Cadherin levels at either stage,

245   suggesting that increased coupling between the two adhesion molecules and thus the

246   two tissues could not account for the *egr*^{*1*} phenotype (Figure S4A-C and Figure 4B-

247   D). We then looked for potential changes in the interactions of ectodermal cells with

248   the ECM that lies on its basal side, at the interface with the mesoderm, in *egr*^{*1*}

249   mutants compared to wild type. We observed no change at either stage between

250   control and *egr*^{*1*} mutants in the levels of accumulation at the ectoderm-mesoderm

251 interface of Laminin A (Lan A) or its potential receptors Dystroglycan (Deng et al.,

252 2003) and Myospheroid which is the common Integrin β subunit present in flies, or

253 Talin which is essential for linking ligand-bound Integrins to the cytoskeleton and

254 focal adhesion assembly (Brown et al., 2002; Ellis et al., 2014; Klapholz et al., 2015)

255 (Figure 4E-K, Figure S4D-K). Our data shows that Eiger does not regulate levels of

256 Lan A or its receptors at the ectoderm-mesoderm interface, but do not preclude

257 changes in signaling that we did not assess. We also did not see any significant

258 change between Stages 10 and 11 in wild type embryos at the ectoderm-mesoderm

259 interface in the levels of E- or N-Cadherin, Dystroglycan, Myospheroid or Talin

260 (Figure S4L). The one protein for which we did see a difference was LanA, which

261 increased in Stage 11 compared to Stage 10 both at the ectoderm-mesoderm interface

262 (Supplementary Figure 4L) as well as within the mesoderm (Figure S4M). This LanA

263 increase was not Eiger dependent since we saw a similar increase in the *egr^1* mutant

264 embryos (Figure S4L,M). These findings argue that Eiger does not regulate Cadherin

265 or Integrin mediated adhesion at the ectoderm-mesoderm interface to facilitate

266 macrophage tissue invasion.

267 ***Eiger maintains apical Pals1-associated tight junction (PATJ) to regulate myosin***

268 ***activity in the ectoderm without affecting polarity during macrophage tissue***

269 ***invasion***

270 The known ability of Eiger, when overexpressed in the imaginal discs, to activate

271 apoptosis through JNK (Jun amino terminal kinase) signaling (Andersen et al., 2015;

272 Igaki, 2002; Moreno et al., 2002) led us to examine these potential responses to Eiger

273 signaling in the germband. However the level of Caspase mediated apoptosis in the

274 germband was unaffected in both *egr^1* and *grnd^{Minos}* embryos compared to the control

275 (Figure 5A, Figure S5A). We then assessed JNK signaling using the *Puc^{E69}-GAL4*

276   crossed to *UAS-GFP* as a reporter line (Adachi-Yamada, 2002). In control embryos, a

277   few cells in the AS showed expression of the JNK reporter, yet none did so in the

278   germband ectodermal epithelium (Figure S5B), and the expression level did not

279   change significantly in *egr*[1] embryos compared to controls (Figure S5C). We therefore

280   concluded that Eiger does not regulate macrophage tissue invasion through canonical

281   JNK signaling and activation of apoptosis.

282   We then turned our attention to the apical side of the germband ectoderm which is in

283   close contact with the amnioserosa where Eiger is expressed. Grindelwald has been

284   shown to interact with the Crumbs apical polarity complex (Andersen et al., 2015).

285   The complex consists of the transmembrane protein Crumbs, its adaptor protein

286   Stardust, and the Stardust associated proteins Patj (Pals1-associated tight junction)

287   (Bachmann et al., 2008; Bulgakova et al., 2008; Sen et al., 2015) and Veli (Lin-7)

288   which Grindelwald can directly bind (Fig 6I, left panel) (Andersen et al., 2015). To

289   test the importance of Veli in our system, we knocked it down in the ectoderm with

290   RNAi and observed a defect in the number of macrophages within the germband

291   (Figure 5B); this result supports the hypothesis that the association of Grindelwald

292   with the Crumbs complex through Veli is important for the regulation of macrophage

293   tissue invasion. Based on this, we tested if the Eiger-Grindelwald complex might

294   regulate the action of members of the Crumbs complex in the germband ectoderm.

295   Crumbs was apically localized in a pattern similar to the control in *egr*[1] and *grnd*[Minos]

296   mutant Stage 12 embryos and quantification of apical Crumbs levels showed no

297   significant difference between the control embryos and the *egr*[1] or *grnd*[Minos] mutant

298   embryos suggesting that ectodermal apical polarity was unaffected (Figure 5D,E and

299   Figure S5D). However, we detected a strong reduction in the apical localization of

300   Patj in both *egr*[1] and *grnd*[Minos] embryos (Figure 5F,G and Figure S5D). Importantly,

301   overexpressing *patj* using an ectodermal driver rescued the macrophage germband

302   migration defect in *egr^1* embryos (Figure 5J,K), supporting the idea that the defect in

303   macrophage invasion in *egr^1* embryos is significantly caused by the loss of Patj.

304   Next we examined how the loss of apical Patj could affect the germband ectodermal

305   epithelium, and ultimately macrophage migration. Patj has been shown to bind the

306   Myosin binding subunit (MBS) of Myosin phosphatase (Sen et al., 2012) which

307   downregulates Myosin activity by decreasing its phosphorylation (Kimura et al.,

308   1996; Lee and Treisman, 2004). Although no antibody for Myosin phosphatase exists,

309   we were able to analyze the localization and levels of active Myosin in the ectoderm

310   using an antibody that detects the mono-phosphorylated form of Myosin regulatory

311   light chain (MRLC, *sqh* in *Drosophila*) (Zhang and Ward, 2011). We found a strong

312   increase in the level of apical junctional phospho-MRLC in both *egr^1* and *grnd^{Minos}*

313   mutant embryos compared to controls in the region of the germband ectoderm along

314   which the first macrophage to enter migrates more slowly in the *egr^1* mutant (Figure

315   5H,I and Figure S5D). There was also a significant decrease in the levels of apical

316   phospho-Myosin seen in wild type embryos from Stage 10 to early Stage 11 within

317   this region suggesting that the shift in Eiger localization from the amnioserosa to the

318   germband observed between these two stages (Figure S5E,F) reduces apical phospho-

319   Myosin levels. Finally, to test if the increased active Myosin seen in the *egr^1* mutants

320   contributes to the defect in macrophage migration, we lowered Myosin levels in the

321   ectoderm in *egr^1* mutant embryos using RNAi against *MRLC (sqh)* and were able to

322   significantly rescue the *egr^1* phenotype (Figure 5J,K). This suggests that Eiger and

323   Grindelwald facilitate macrophage invasion into and within the germband by

324   maintaining apical Patj in the ectodermal epithelium, thus promoting a reduction in

325   the levels of apical activated Myosin without affecting polarity.

Eiger regulates ectodermal apical tension and tissue deformation to facilitate macrophage invasion

As Myosin activity is known to affect cellular tension (Fernandez-Gonzalez et al., 2009) we tested if increased apical phospho-MRLC correlated with increased cortical tension. We conducted apical laser ablation in the germband ectoderm (Figure S6A) in control, egr^1, and $grnd^{Minos}$ mutant embryos carrying *knock-in DE-Cadherin::GFP* to visualize the apical cortex of ectodermal cells. The laser intensity was standardized to ensure ablation of the apical Cadherin without the induction of significant damage to the embryos, which remained viable after the experiment. We measured the initial recoil velocity after the cut, which is indicative of the inherent apical tension at that location. Embryos from both mutants displayed higher recoil velocity indicating higher apical tension in the germband ectoderm compared to control embryos (Figure 6A-E, and Movie S5). This increased tension was Myosin dependent, as it was eliminated in the presence of the Rho kinase inhibitor Y27632 (Figure 6F).

We then went on to examine the net interfacial tension along the apical edge of the ectoderm cells using a recently described force inference method, named CellFIT-3D (Brodland et al., 2014; Krens et al., 2017), which assesses interfacial tension distributions based on the angles present between contacting cell surfaces at triple cell junctions (Figure S6B). We performed CellFIT-3D analysis on high-resolution 3D confocal images of DE-Cadherin labeled fixed control and egr^1 mutant embryo stacks that were oversampled in the Z-axis, analyzing the apical, basal and lateral domains of the germband ectoderm. This force inference analysis indicated that while in control embryos both apical and basal tensions were approximately 1.8 times the lateral tension, in egr^1 mutant embryos the apical tension was higher than both the lateral and the basal tensions (Figure S6D and Movie S6). This comparison of the distribution of

351 relative interfacial tensions is consistent with our laser ablation analysis, which

352 indicates that Eiger decreases apical tension in the germband ectoderm.

353 To directly assess the role of tension in regulating macrophage migration into the

354 germband, we overexpressed in the ectoderm constitutively active Rho1 (Rho1.V14),

355 a positive regulator of Rok, the kinase that phosphorylates Myosin (Kaibuchi et al.,

356 1999; Niederman and Pollard, 1975). The number of macrophages present in the

357 germband was significantly decreased in these embryos (Figure 6G and Figure S6E),

358 supporting the conclusion that high levels of ectodermal tension can impede

359 macrophage invasion into and within the germband. We therefore conclude that Egr

360 and Grnd-mediated regulation of activated Myosin levels and thus apical tension of

361 the ectodermal epithelium is critical for normal macrophage tissue invasion.

362 Finally we asked how changes in ectodermal apical tension could mechanistically

363 facilitate the entry and initial migration of macrophages, which move between the

364 basal side of the ectodermal epithelium and the adjacent mesoderm. As increased

365 tensions have been predicted theoretically and shown experimentally to increase

366 effective tissue stiffness (Koenderink et al., 2009; Kollmannsberger et al., 2011;

367 Lange and Fabry, 2013; Wang et al., 2002), we reasoned that macrophage tissue

368 invasion might require tension-dependent deformation of the columnar ectodermal

369 cells. To test this hypothesis, we analyzed whether deformation of the ectodermal

370 cells accompany macrophage entry and initial migration within the germband. We

371 quantified in live embryos the shape changes as a ratio of length/width (LWR) of all

372 the ectodermal cells in a given plane within 10μm from the macrophage edge. In

373 control embryos, ectodermal cells displayed a significant decrease in LWR during this

374 time, consistent with the hypothesis that ectodermal cells undergo compression as the

375 macrophage fully insinuates itself into the germband (Figure 6H, Figure S6F and

376   Movie S7). Crucially, this deformation was significantly less pronounced in the *egr¹*

377   mutant embryos (Figure 6H, Figure S6F and Movie S7) suggesting that the observed

378   increase in apical tension of these ectodermal cells causes them to resist the

379   deformation accompanying macrophage entry into and initial migration within the

380   germband to a higher degree than in the control, thereby delaying the macrophages.

381   Based on our results, we propose the following model for Eiger-Grindelwald

382   signaling during macrophage germband entry and migration within the germband.

383   Amnioserosal Eiger binding to ectodermal Grindelwald leads to an enrichment of the

384   Myosin-Phosphatase-MBS-Patj complex at the apical cortex where it remains bound

385   to the Crumbs polarity complex through its interaction with Veli and Stardust (Figure

386   6I). This results in a reduction in apical phosphorylated Myosin and apical tension

387   that allows ectodermal deformation and thus macrophage migration into and within

388   the region of the germband that abuts the amnioserosa.

389   **Discussion**

390   Our studies indicate an ancient conserved role for the TNF family in aiding immune

391   cells to cross a tissue barrier and suggest that this functionality and the mechanisms

392   underlying it evolved even before the vasculature did. The *Drosophila melanogaster*

393   TNF, Eiger, has been previously linked mostly to stress responses, aiding in adaptive

394   reactions to infection (Schneider et al., 2007), tumors (Ohsawa et al., 2011; Parisi et

395   al., 2014), starvation (Agrawal et al., 2016), and UV irradiation (Babcock et al.,

396   2009). Eiger can also be coopted to promote tumor invasiveness in some

397   circumstances (Cordero et al., 2010) while the Eiger receptor, Grindelwald, has been

398   shown to cause neoplastic growth as a result of polarity defects (Andersen et al.,

399   2015). These tumors are the result of the Crumbs complex signaling to Grindelwald to

400   induce JNK activation independently of Eiger (Andersen et al., 2015). Our work

401   provides evidence that during normal development the communication flows in the

402   reverse direction, with Eiger and Grindelwald required for alterations in the apical

403   localization of components normally associated with the Crumbs complex (Sen et al.,

404   2015). We also show that this relocalization of Patj correlates with reduced apical

405   Myosin activity, which underlie the macrophage migration defects in the *eiger* mutant

406   since raising Patj or lowering Myosin levels rescues the phenotype. We thus identify a

407   previously unappreciated molecular pathway by which a TNF can act, and

408   demonstrate its importance during *Drosophila* macrophage tissue invasion. Vertebrate

409   TNF α is known to facilitate leukocyte transmigration through the vasculature during

410   inflammation (Sata, 1998). Previous studies treating human endothelial cells *in vitro*

411   with 100 fold higher levels of TNF than normally found in the blood stream have

412   detected increases in Myosin phosphorylation (Damas et al., 1989; McKenzie, 2007).

413   In contrast we observe *in vivo* that endogenous amounts of Eiger decrease cortical

414   phosphorylated Myosin. Investigating if the mechanisms we describe here for a TNF

415   family member also function during vascular extravasation *in vivo* is an important

416   area of future research.

417   Little is known about the modulation of mechanical constraints that occurs when cells

418   penetrate between other cells during development (Montell, 2003; Seifert and

419   Lehmann, 2012), metastasis (Friedl et al., 2012; Reymond et al., 2013) and

420   inflammation (Nourshargh et al., 2010; Vestweber, 2015). Previous studies examining

421   the effects of stiffness of a three-dimensional environment *in vivo* on cell migration

422   have shown that increased ECM stiffness promotes invasion through stimulation of

423   mechanotransduction in the invading cells (Calvo et al., 2013; Egeblad et al., 2010;

424   Laklai et al., 2016; Levental et al., 2009). In these systems increased stiffness has

425   been shown to affect MMP secretion and/or realignment of the matrix fibers by the

426   invading cells thus aiding invasive migration (Gaggioli et al., 2007). Our data stands

427   in apparent contrast to these findings, as we discover that increased apical ectodermal

428   tension in the egr^1 mutant leads to decreased macrophage ingression and migration

429   within the germband. We have not measured ectodermal stiffness directly in the

430   embryo, but have seen that in the absence of Eiger, ectodermal cells undergo less

431   deformation as the macrophages enter, suggesting that the higher apical tension leads

432   to an overall increase in the effective stiffness of the ectoderm potentially due to

433   volume conservation. Tension has been shown to lead to strain stiffening (Koenderink

434   et al., 2009; Kollmannsberger et al., 2011; Lange and Fabry, 2013; Wang et al., 2002)

435   and we hypothesize that the increased tension on the apical side results in a stiffer

436   cortex which acts as a "cap" resisting curving out of the ectoderm both during initial

437   macrophage penetration and subsequent migration. This hypothesis is further

438   supported by the observation that a decrease in macrophage migration speed is seen

439   only as they migrate into and along the region of the germband where we detected an

440   increase in the apical levels of phosphorylated Myosin in the *eiger* mutants. Our work

441   suggests that previously identified mechanisms which enable invading cells to

442   overcome the hindrance of a stiff ECM, such as matrix metalloprotease secretion or

443   realignment of the matrix fibers (Gaggioli et al., 2007) are not sufficient when cells

444   invade between other closely apposed cells.

445   We identify here a controlled developmental switch, which modulates these

446   mechanical properties during *Drosophila* embryogenesis to facilitate macrophage

447   tissue invasion of a complex tissue structure. While the Pvf guidance factors (Cho et

448   al., 2002) and the border between the ectoderm and mesoderm appear to determine

449   the location of the route taken by macrophages into and within the germband, our

450   work suggests that Eiger helps set the timing and speed of this movement by releasing

451 the brake on macrophage entry and initial migration produced by higher cortical

452 tension. Previous *in vitro* work has indicated that substrates that are too soft impede

453 migration, by decreasing the ability of cells to exert the traction forces needed to

454 move (Pelham and Wang, 1997; Saez et al., 2005). In our system it is possible that the

455 macrophages migrate not on the ectoderm, but rather use the underlying ECM and

456 mesoderm as a substrate to move forward. Hence the final migratory properties of the

457 macrophages within the germband would depend not only on ectodermal stiffness, but

458 on the mechanical properties of the ECM and mesoderm as well. Immune cell

459 infiltration of solid tumors involves immune cell invasion into the tumor mass that

460 consists of a multitude of components of varying mechanical properties including the

461 ECM, stromal cells, angiogenic vessels and the tumor cells themselves. Our work

462 demonstrates the importance of understanding the distinctions in the mechanical

463 constraints exerted by each of these components in the *in vivo* environment cells

464 traverse and the molecular mechanisms needed to alter each of them. Such a complete

465 understanding of invasive migration has the potential to identify new strategies for

466 treatment of autoimmunity and cancer in humans.

467

468 **Author contribution**

469 AR and DS conceived of the project and AR, AMC and DS designed the experiments.

470 AR, JB, JV and AMC performed experiments with support from AG who generated

471 reagents and provided technical support. MS aided the laser ablation and migration

472 analysis. EP wrote the Matlab script to permit segmentation and deformation analysis

473 and aided with optimizing imaging protocols. GK performed CellFIT-3D analysis.

474 WK optimized EM protocols. AR, MS, EP, AMC, and DS analyzed the data. AR and

475 DS wrote the manuscript.

476

Declaration of Interests

The authors declare no competing interests.

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704

705 **Figure Legends**

706 **Figure 1. Macrophages invade the germband at the interface between the**

707 **ectoderm and mesoderm**

708 (**A**) Cartoon showing the 3 pre-determined routes of macrophage migration in the

709 embryo at Stage 12. Blue arrow marks the migration towards the amnioserosa (AS,

710 blue) and the germband. Pink arrow indicates migration along the dorsal vessel and

711 the brown arrow delineates the migration along the ventral nerve cord (vnc).

712 (**B**) Schematic of the region of the germband from a Stage 11 embryo imaged in **C**.

713 (**C**) Stills from two-photon time-lapse imaging of a Stage 11 *e22c-GAL4 srpHemo-*

714 *3xmCherry; 10xUAS-CD8::GFP* embryo in which *CD8::GFP* labels germband

715 membranes (green) and *srpHemo-3XmCherry* labels macrophages (red). The white

716 arrow pinpoints the initial protrusion of the macrophage between the ectoderm and

717 the mesoderm and the yellow arrow specifies the macrophages that migrate into the

718 amnioserosa at 10 min. The white arrowhead indicates a gap appearing between the

719 ectoderm and mesoderm once the macrophage penetrates the germband at 15 min. See

720 also Movie S1 and Figure S1.

721 (**D**) Confocal images of fixed lateral Stage 10 and 12 wild type embryos with

722 macrophages visualized by *srpHemo-3XmCherry* expression (red), ectoderm by

723 antibody staining against DE-Cadherin (green) and mesoderm by antibody staining

724 against DN-Cadherin (magenta), along with a merge of all channels. The dotted white

725 line in the green channel indicates the apical side of the ectoderm cells.

726 (**E**) A magnification of the area outlined by the dotted box in **D**. Arrows indicate the

727 interface of the ectoderm and mesoderm in Stage 11 embryos prior to macrophage

728 migration when the tissues are closely apposed. Arrowheads in Stage 12 identify

729 macrophages sitting between the two tissues that have separated.

730 (**F**) Schematic denoting with a blue box the region of the germband shown in (**G, H**)

731 in an early Stage 10 embryo. The black dotted line within the germband in the

732 schematic indicates the ectoderm-mesoderm interface.

733 (**G**) Transmission electron microscopy (TEM) image of the ectoderm-mesoderm

734 tissue interface from an early embryo in which the macrophages have not yet reached

735 the germband (overview in Figure **S1D**).

736 (**H**) A magnification of the area within the dotted box in **G,** showing that the cells at

737 the interface are in close contact before macrophage entry into the germband (yellow

738 arrow) with some extracellular spaces (blue arrowheads). Mesoderm cells are pseudo-

739 colored in magenta. See also Figure S1.

740 (**I, J**) Confocal microscopy images of a fixed lateral Stage 11 embryo stained with an

741 antibody against Laminin A (LanA), with macrophages visualized by *srpHemo-*

742 *H2A::3XmCherry* and a magnification of the area indicated in dotted box in **I** shown

743 in **J**. LanA is expressed by the mesoderm in the germband before macrophage entry.

744 Arrowhead indicates the LanA found where the macrophages traverse into the

745 germband, at the interface between the ectoderm and the mesoderm.

746 (**K**) Schematics of Stage 10 and Stage 12 embryos (grey) with box indicating the

747 region magnified below to illustrate the morphology of the germband before (Stage

748 10) and after (Stage 12) macrophage invasion. Macrophages (red) enter between the

749 caudal ectoderm (green), and the visceral mesoderm (magenta) along a track of

750 Laminin A (orange). The amnioserosa adjacent to the ectoderm is in blue and the yolk

751 in grey. See also Figure S1.

752 Embryo pictures throughout are shown with anterior to the left and dorsal up.

753 Embryos which displayed stomodeum invagination and a germband retraction away

754 from the anterior of less than 29%, were classified as Stage 10 and embryos with

755 germband retractions between 29-31% for Stage 11 and 35-40% as Stage 12.

756 Scale bar represents 40μm in **C**, 20μm in **D, I**, 10μm in **E, J**, 5μm in **G** and 2μm in

757 **H.**

758 **Figure 2. Amnioserosal Eiger (Dm-TNF) regulates macrophage invasion of the**

759 **embryonic germband**

760 (**A**) *In situ* hybridizations of lateral Stage 11 embryos reveal *eiger* expression in the

761 amnioserosa (arrow) and neural ectoderm in wild type embryos and its absence in

762 *egr^1* mutant embryos.

763 (**B**) Confocal microscopy images of z-projections of fixed lateral Stage 12 embryos,

764 stained with DE-Cadherin antibody (green). Control (con) and *egr^1* mutant embryos

765 are shown, with macrophages labeled in red by the expression of

766 *srpHemo>H2A::RFP*. The dotted line demarcates the edge of the germband. Arrows

767 in *egr^1* indicate accumulation of macrophages outside the germband.

768 (**C**) Schematic drawing of a lateral Stage 12 embryo depicting the paths taken by

769 macrophages (red dots) migrating into the germband. The blue circle indicates the

770 area analyzed to count the number of macrophages in the germband throughout the

771 manuscript.

772 (**D**) Quantification reveals that the number of macrophages that have moved into the

773 germband in Stage 12 is decreased in embryos from *egr^1, egr^3, and*

774 *egr¹/Df(2R)BSC303* which removes *egr* completely. Macrophages were labeled by the

775 expression of *srpHemo>H2A::RFP*. n=20 embryos for each genotype.

776 (**E**) Quantification of macrophages in the germband in embryos from the control

777 (con), the *egr¹* mutant, and the *egr¹* mutant expressing *UAS-eiger* under the control of

778 the LP1 amnioserosal GAL4 driver which was able to partially rescue the mutant

779 phenotype. n=25 embryos for all genotypes. See also Figure S2.

780 (**F**) Quantification of the number of macrophages that have penetrated the germband

781 in Stage 12 embryos in control (con) embryos and those in which an amnioserosal

782 (*c381-GAL4*) driver directs the expression of an RNAi against *eiger*. n=20 embryos

783 for all genotypes. See also Figure S2.

784 (**G**) Schematic drawing of the anterior half of a lateral Stage 11 embryo indicating the

785 region analyzed (area within blue box) to quantify the (**H**) speed and (**I**) persistence of

786 macrophage migration up to the germband in two-photon movies from wild type and

787 *egr¹* mutant embryos with macrophage nuclei labeled with *srpHemo-*

788 *H2A::3xmCherry*. Speed was unaffected and persistence was mildly reduced. n=3

789 embryos for each genotype. See also Figure S2 and Movies S2 and S3.

790 (**J**) Stills from two-photon movies of control (con) *srpHemo-H2A::3xmCherry* and

791 *egr¹; srpHemo-H2A::3xmCherry* embryos showing macrophage nuclei migrating

792 from the head into the germband at the indicated time points. The white dotted line

793 indicates the edge of the germband that was detected using autofluorescence from the

794 yolk. Movie areas (Movie S2) correspond to the dashed box in the schematic embryos

795 above. See also Figure S2 and Movies S2 and S3.

796 (**K**) Left: Schematic showing the entry of the first macrophage into the germband

797 (green arrow). Amnioserosa (AS) indicated in blue. The time required for macrophage

798 entry after first contact with the germband is quantified and is significantly increased

799 in *egr¹* mutants compared to the control (con). Macrophages were labeled with

800 *srpHemo::3xmCherry* and the germband with *knock-in DE-Cad::GFP*. n=5 embryos

801 for both genotypes.

802 (**L**) Schematic indicating the regions of the germband in which migration of the first

803 macrophage was quantified in **M** and **N**. (**M**) Migration within the region of the

804 germband adjacent to the AS (light blue), shown with a dark blue arrow, is

805 significantly lower whereas (**N**) further migration along the germband (brown arrow)

806 is not significantly altered. n=3 embryos for both genotypes. See also Figure S2.

807 Histograms show mean ± s.e.m. ***P<0.001, **P<0.01, ns not significant. One-way

808 ANOVA with Dunnett post test used for **D,F,H,I** and One-way ANOVA with Tukey

809 for **E**, unpaired t-test for **K,M,N**.

810 All embryos are oriented with anterior to the left and dorsal up, unless otherwise

811 noted. The black dotted line within the germband in the schematics shown in **C,J,K,L**

812 indicate the ectoderm-mesoderm interface. Embryos were staged for imaging and

813 quantification based on having germband retraction away from the anterior of 29-31%

814 for Stage 11 and 35-40% for Stage 12. Scale bar represents 50µm in **A,B** and 40µm in

815 **J.**

816

817 **Figure 3. Grindelwald (Dm-TNFR) expressed in the ectoderm is essential for**

818 **macrophage germband invasion**

819 Confocal images of a single sagittal section of (**A**) a lateral wild type Stage 10 and (**B**)

820 a Stage 11 embryo with macrophage nuclei labeled by the expression of *srpHemo-*

821 *H2A::3XmCherry* (red) and Eiger recognized with an antibody (green). The regions

822 imaged in **A** and **B** correspond to the magnified areas of the grey embryo depicted in

823 the adjoining schematics on the left, in which macrophages are shown in red and the

824   amnioserosa (AS) in blue. The black dotted line within the germband in the

825   schematics indicates the ectoderm-mesoderm interface. (**A', B'**) Right panels show a

826   magnification of the area indicated by the white dotted box in the adjoining panels on

827   the left. (**A'**) We observe punctate membrane expression of Eiger in AS cells in Stage

828   10 (arrowhead) and almost no localization in the germband ectoderm (arrow). (**B'**) In

829   Stage 11 before the macrophages enter the germband we observe some Eiger

830   remaining on the AS (also see Figure S3B,B') but also additional localization on the

831   germband ectoderm (arrow in **B'**).

832   (**C, D**) Quantification in Stage 10 and 11 embryos of the intensity of Eiger antibody

833   staining at the membrane normalized to the cytoplasm in the (**C**) AS and (**D**)

834   ectoderm. n=30 cell boundaries, 5 embryos for each genotype. See also Figure S3.

835   (**E**) *In situ* hybridizations of Stage 11 embryos reveal that in wild type *grindelwald* is

836   expressed in the ectoderm, particularly highly in the germband (arrowhead), and that

837   *grnd*^*Minos* embryos show no expression.

838   (**F**) Confocal microscopy images of z-projections of fixed lateral Stage 12 embryos,

839   from the control (con) and a line in which RNAi against *grindelwald* is driven by the

840   *e22c-GAL4* ectodermal driver. Macrophages are labeled in red by the expression of

841   *srpHemo:3xmCherry*. The white dotted line indicates the edge of the germband.

842   Quantification on the right shows that RNAi knockdown of *grindelwald* in the

843   ectoderm results in reduced macrophage entry into the germband. n=22 embryos for

844   both genotypes.

845   (**G**) Confocal microscopy images of z-projections of fixed lateral Stage 12 embryos.

846   Control (con) and *grnd*^*Minos* mutant embryos are shown, with macrophages labeled in

847   red by the expression of *srpHemo-H2A::3xmCherry*. The dotted line demarcates the

848 edge of the germband. Quantification shows that the *grind^Minos* mutant displays

849 reduced macrophage entry into the germband. n=17 embryos for both genotypes.

850 (**H**) Stills from two-photon movie of *grnd^Minos; srpHemo-H2A::3xmCherry* embryo

851 showing macrophage nuclei migrating from head into the germband at the indicated

852 time points. The white dotted line indicates the edge of the germband, which was

853 detected using autofluorescence from the yolk. The region imaged in the movie

854 (Movie S2) corresponds to the dashed boxed area in the schematic embryos above.

855 See also Figure S3 and Movies S2 and S3.

856

857 Histograms show mean ± s.e.m. Unpaired t-test in **C,D,F,G.** ****P<0.0001.

858 The black dotted line within the germband in the schematics indicates the ectoderm-

859 mesoderm interface. Embryos shown with anterior to left and dorsal up in all panels.

860 Embryos were staged for imaging and quantification based on germband retraction

861 away from the anterior of less than 29% for Stage 10, 29-31% for Stage 11 and 35-

862 40% for Stage 12. Scale bars represent 10μm in **A-B',** 40μm in **H** and 50μm in

863 **E**,F,**G**.

864

865 **Figure 4: Eiger (Dm-TNF) does not regulate adhesion at the ectoderm-mesoderm**

866 **interface.**

867 (**A**) Schematic drawings of a lateral Stage 11 embryo (grey, on top) and a

868 magnification of the region near the germband (in dotted box, middle) depicting

869 macrophages in red at the edge of the germband prior to entry. The black dotted line

870 within the germband section highlighted in the small dotted box indicates the

871 ectoderm-mesoderm interface. Below is a schematic indicating the arrangement of

872   the ectoderm (green) and mesoderm (magenta) cells in the germband. Blue box in the

873   lowest schematic indicates the region shown in **B**, **E-G**.

874   (**B,E-G**) Confocal microscopy images of a single sagittal plane at the ectoderm-

875   mesoderm interface (blue box in **A**) from control (con) and *egr^1* mutant embryos with

876   *srpHemo-H2A::3xmCherry* in the background to label macrophage nuclei. Stage 11

877   embryos were selected for imaging and quantification based on having germband

878   retraction away from the anterior of 29-31% and macrophages at or near the edge of

879   the germband. Embryos were imaged with antibodies against (**B**) DE-Cadherin

880   (green) and N-Cadherin (magenta), (**E**) Dystroglycan (green), (**F**) DE-Cadherin

881   (green) and β-PS integrin or (**G**) Talin shown in magenta. The white dotted line in the

882   left panel indicates the ectoderm-mesoderm interface. See also Figure S4.

883   (**C,D,H-K**) Quantification of the interface intensity, normalized to cytoplasmic

884   background, of (**C**) DE-Cadherin, (**D**) N-Cadherin, (**H**) Laminin A, (**I**) Dystroglycan,

885   (**J**) β-PS integrin, and (**K**) Talin in control (con) and *egr^1* mutant embryos. See also

886   Figure S4.

887   Anterior to left and dorsal up in all panels. Scale bars represent 10μm. Histograms

888   show mean ± s.e.m. ns = not significant, unpaired t-test.

889

890   **Figure 5: Eiger (Dm-TNF) supports apical Patj localization and regulates myosin**

891   **activity in the germband ectoderm.**

892   (**A**) Quantification of apoptotic corpses, labeled with an antibody against activated

893   Caspase 3, in the germband of Stage 12 shows no significant difference between

894   control (con)*, egr^1* and *grnd^{Minos}* embryos expressing *srpHemo-H2A::3xmCherry* to

895   label macrophages. n=15 embryos for all genotypes. See also Figure S5.

896 **(B)** Confocal microscopy images of z-projections of fixed lateral Stage 12 embryos

897 from control (con) embryos and those in which RNAi against *veli* is driven by the

898 *e22c-GAL4* ectodermal driver. Macrophages are labeled in red by the expression of

899 *srpHemo:3xmCherry*. The white dotted line indicates the edge of the germband.

900 Quantification reveals that RNAi knockdown of *veli* in the ectoderm leads to a strong

901 reduction in macrophage invasion into the germband. n=25 embryos for both

902 genotypes.

903 **(C)** Schematics showing a Stage 12 embryo with the region boxed represented in the

904 lower schematic, in which the black dotted line within the germband delineates the

905 ectoderm-mesoderm interface. The blue box indicates the region of the germband

906 imaged and analyzed in **D-I.**

907 **(D,F,H)** Confocal images of the germband ectoderm (blue boxed area in lower

908 schematic in **C**) from fixed lateral Stage 12 embryos in which macrophages were at or

909 near the edge of the germband. Control (con), *egr^1* and *grnd^{Minos}* embryos were

910 immunolabeled for **(D)** Crumbs, **(F)** Patj, or **(H)** the phosphorylated form of Myosin

911 Regulatory Light Chain, p-MRLC, also called Sqh-1P. See also Figure S5.

912 **(E,G,I)** Quantification of line scan analysis of apical Crumbs, Patj and Sqh-1P levels

913 normalized to their respective cytoplasmic level. **(E)** Crumbs was not significantly

914 altered, but **(G)** apical Patj levels were significantly lower, and **(I)** Sqh-1P levels were

915 significantly higher in the *egr^1* and *grnd^{Minos}* embryos compared to the control (con).

916 n=6 embryos and 30 contacts of each genotype for Crumbs analysis, 7 and 37 for Patj

917 and 7 and 40 for Sqh-1P.

918 **(J)** Confocal microscopy images of z-projections of fixed lateral Stage 12 embryos

919 from the control (con), the *egr^1* mutant, and the *egr^1* mutant expressing *UAS-Patj* or

920 an RNAi against MRLC *(sqh)* under the control of the *e22c-GAL4* ectodermal driver.

921   Macrophages were labeled in red by the expression of *srpHemo-H2A::3xmCherry*.

922   The white dotted line indicates the edge of the germband.

923   (**K**) Quantification indicates that expression of *patj* or removal of MRLC in the

924   ectoderm as described in **J** partially rescues the *egr^1* mutant phenotype. n=20 embryos

925   for control, *egr^1* and *patj* rescue. n=18 embryos for *MRLC* knockdown.

926   Embryos were staged for quantification and imaging based on having germband

927   retraction away from the anterior of 29-31% for Stage 11 and 35-40% for Stage 12.

928   Anterior to left and dorsal up in all panels. Scale bar represents 50μm in **B**,**J** and

929   10μm in **D,F,H.** ****$P<0.0001$, ***$P<0.001$, ns=not significant. One-way ANOVA

930   with Dunnett post test for **A,E,G,I**. One-way ANOVA with Tukey for **K** and unpaired

931   t-test for **B**.

932

933   **Figure 6: Eiger regulates apical tension in the germband ectoderm**

934   **(A)** Schematic drawing (left panel) of a dorsal Stage 11 embryo indicating the

935   ectodermal region where laser ablation was conducted (green box). Schematic (right

936   panel) depicting single cell ablation showing a line (red) cut perpendicular to the

937   apical DE-Cadherin (light blue) and centered between neighboring cell vertexes.

938   Displacement of apical DE-Cadherin after severing is indicated (black arrows).

939   (**B**) Stills from confocal spinning disc movies (left panel) showing ectodermal apical

940   DE-Cadherin::GFP in control, *egr^1 and grnd^{Minos}* embryos before (precut) and 1 sec

941   (s) after laser ablation (postcut). Embryos where macrophages were near or at the

942   edge of but not within the germband were chosen for ablations. Area of ablated DE-

943   Cadherin::GFP along the apical cell cortex is indicated (red arrow) and outer edges

944   for kymograph analysis in **C** are marked (light blue arrowheads). See also Figure S6

945   and Movie S5.

946   (**C**) Kymograph analysis showing apical DE-Cadherin::GFP before (pre), during (red

947   arrow) and after laser ablation (postcut) in control, *egr¹* and *grnd^{Minos}* embryos. Black

948   arrows indicate the time (s) after ablation. The open-ends of the gap left after severing

949   DE-Cadherin::GFP are highlighted with green lines to illustrate retraction behavior.

950   (**D**) Displacement of the apical cell cortex labeled with DE-Cadherin::GFP is shown

951   for each 800ms time frame post-cut and the values were curve fitted using

952   nonparametric fitting with smoothing splines. Time resolved displacement is higher

953   after the cut in *egr¹* and *grnd^{Minos}* embryos as compared to control, indicating greater

954   tension.

955   (**E**) *egr¹* and *grnd^{Minos}* embryos when compared to control show an increased initial

956   recoil velocity after the cut of the apical cell cortex labeled with DE-Cadherin::GFP.

957   n=20 embryos for control, 15 for *egr¹*, and 8 for *grnd^{Minos}*.

958   (**F**) Recoil velocity is lower in control (con) and *egr¹* embryos when injected with the

959   Rho Kinase inhibitor drug Y27632 (+Y) compared to uninjected control embryos.

960   n=20 embryos for control, 12 for control + Y27632, 15 for *egr¹*, and 7 for *egr¹* +

961   Y27632 .

962   (**G**) Quantification reveals a decrease in the number of macrophages that have

963   migrated into the germband in Stage 12 embryos expressing a dominant active form

964   of Rho1 (*UAS-Rho1.V14*) in the ectoderm under the control of the *e22c-GAL4*

965   ectodermal driver compared to the control (con). Embryos were staged for

966   quantification and imaging based on having germband retraction away from the

967   anterior of 35-40% for Stage 12. n=20 embryos for both genotypes. See also Figure

968   S6.

969   (**H**) Left: Schematic showing a Stage 12 embryo viewed dorsally. The ectodermal

970   cells in the green boxed area are shown magnified below at time point 0 when the

971 macrophage protrusion touches the basal side of the ectoderm cells and time point 3

972 when the macrophage has fully insinuated itself under the ectoderm. Right:

973 Quantification of two-photon movies of the ectodermal cell deformations that occur

974 during macrophage migration into the area of the germband shown in the green box in

975 the schematic. Ectoderm visualized with *knock-in DE-CAD::GFP* and macrophages

976 visualized with *srpHemo-3xmCherry* in wild type and *egr^1* mutant embryos.

977 Deformations are measured only for ectodermal cells within a 10μm radius of the

978 macrophage cell edge. The length/ width ratio (LWR) of the ectodermal cells, shown

979 on the y-axis is plotted over time, indicated on the x-axis. Time interval 40 seconds,

980 n=3 embryos for each genotype, 10-40 cells for each time point. See also Figure S6

981 and Movie S7.

982 **(I)** Model: Eiger binding to the Grindelwald receptor results in greater localization of

983 Patj and its binding partner, the Myosin phosphatase, MBS. This leads to a decrease

984 in the level of active phosphorylated Myosin, and lower ectodermal apical tension,

985 which facilitates macrophage invasion into the germband.

986 Anterior to left and dorsal up in all panels. Scale bar in **B** represents 10μm. Anterior

987 to left in all panels. ***P<0.001, **P<0.01, * P<0.05. One-way ANOVA with

988 Dunnett post test for **G**. Unpaired t-test for **E,F**,**H.**

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STAR Methods

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and

will be fulfilled by the Lead Contact, Dr. Daria Siekhaus (daria.siekhaus@ist.ac.at).

1000 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

1001 <u>**Fly strains and preparation**</u>

1002 Flies were raised on standard food bought from IMBA (Vienna, Austria) which

1003 contained agar, cornmeal, and molasses with the addition of 1.5% Nipagin. Adults

1004 were placed in cages in a Percival DR36VL incubator maintained at 25ºC and 65%

1005 humidity; embryos were collected on standard plates prepared in house from apple

1006 juice, sugar, agar and Nipagin supplemented with yeast from Lesaffre (Marcq,

1007 France) on the plate surface. Fly crosses and embryo collections for RNA interference

1008 experiments (7 hour collection) as well as live imaging (6 hour collection) were

1009 conducted at 29ºC. All fly lines utilized are listed below: *srpHemo-GAL4* was

1010 provided by K. Brückner (UCSF, USA) (Brückner et al., 2004) and *egr¹, egr³, UAS-*

1011 *egr* (weak) and *UAS-egr IR* lines were provided by M. Galko (MD Anderson Cancer

1012 Centre, USA) and have been previously described (Igaki, 2002). The *knock-in DE-*

1013 *Cad::GFP* line which contains GFP fused to the C terminus of Cadherin and knocked

1014 into the endogenous locus was provided by Y. Hong (Huang et al., 2009). *grnd^{Minos}*

1015 and *UAS-grnd IR* lines were provided by P. Leopold (iBV, France) and have been

1016 previously described (Andersen et al., 2015). *Puc^{E69}-GAL4* was provided by A.

1017 Classen (University of Munich, Germany). *srpHemo-H2A::3xmCherry* and *srpHemo-*

1018 *3xmCherry* lines have been previously described (Gyoergy et al., 2018). The

1019 following lines were obtained from the Bloomington Stock Centre: *UAS-Patj*

1020 (BL39735), *UAS-sqh* RNAi line (TRiP HMS00437), *Df(2R)BSC303*, *10xUAS-*

1021   *CD8::GFP, c381-GAL4, sg3-GAL4, LP1-GAL4, e22c-GAL4* and *UAS-Rho.V14*. The

1022   *UAS-grnd* RNAi line KK104538 was obtained from the Vienna Drosophila Resource

1023   Center (VDRC), Vienna, Austria.

1024   **Embryo staging**

1025   Fixed embryos which had completed germband extension were staged for imaging

1026   based on the invagination of the stomodeum as well as germband retraction away

1027   from the anterior. Embryos which showed stomodeum invagination and a germband

1028   retraction of less than 29% was classified as Stage 10 and embryos with germband

1029   retractions between 29-31% for Stage 11 and 35-40% for Stage 12.

1030   **The lines used in each Figure are listed below**

1031   **Figure 1C:** *e22c-GAL4 srp-3xmCherry*; *10xUAS-CD8::GFP.* **Figure 1D,E,G,H** and

1032   **Figure S1D,E:** +; *srpHemo-3xmCherry.* **Figure 1I,J:** +; *srpHemo-H2A::3xmCherry.*

1033   **Figure 2A** and **Figure S2B,C,** *Oregon R, egr[1].* **Figure 2B** and **Figure S2A, F-K:** +;

1034   *srpHemo-GAL4 UAS-GFP UAS-H2A::RFP, egr[1]; srpHemo-GAL4 UAS-GFP UAS-*

1035   *H2A::RFP.* **Figure 2D:** +; *srpHemo-GAL4 UAS-GFP UAS-H2A::RFP, egr[1];*

1036   *srpHemo-GAL4 UAS-GFP UAS-H2A::RFP, egr[3]; srpHemo-GAL4 UAS-GFP UAS-*

1037   *H2A::RFP, Df(2R)BSC303/ egr[1]; srpHemo-GAL4 UAS-GFP UAS-H2A::RFP.* **Figure**

1038   **2E and Figure S2D:** +; *srpHemo-3xmCherry, egr[1]; srpHemo-3xmCherry, egr[1] UAS-*

1039   *egr; LP1-GAL4 srpHemo-3xmCherry.* **Figure 2F** and **Figure S2E:** +; *srpHemo-*

1040   *3xmCherry/+, UAS-egr* IR/+; *srpHemo-3xmCherry/+; c381-GAL4/+.* **Figure**

1041   **2H,LM,N, Figure S2M,O:** +; *srpHemo-H2A::3xmCherry, egr[1]; srpHemo-*

1042   *H2A::3xmCherry.* **Figure 2K:** *knock-in DE-Cad::GFP srpHemo-3xmCherry, egr[1]*

1043   *knock-in DE-Cad::GFP srpHemo-3xmCherry.*

Figure 3A,A',B,B',C,D and Figure S3A,A',B,B': +; *srpHemo-H2A::3xmCherry.*

Figure 3E: *Oregon R*, *grnd^{Minos}*. **Figure 3F:** *e22c-GAL4, srpHemo-3xmCherry; UAS-GFP, e22c-GAL4, srpHemo-3xmCherry; UAS-grnd RNAi KK.* **Figure 3G,H** and

Figure S3C,E-I: +; *srpHemo-H2A::3xmCherry, grnd^{Minos}; srpHemo-H2A::3xmCherry.*

Figure 4B,C,D,H and **Figure S4A-E,M:** *srpHemo-H2A::3xmCherry, egr^1; srpHemo-H2A-3xmCherry.* **Figure 4 E,F,G,I,J,K** and **Figure S4 F-K:** *srpHemo-H2A::3xmCherry, egr^1; srpHemo-H2A::3xmCherry.*

Figure 5A,D,E Figure S5A,D (Crumbs immunolabeling): +; *srpHemo-H2A::3xmCherry, egr^1; srpHemo--H2A::3xmCherry, grnd^{Minos}; srpHemo-H2A::3xmCherry.* **Figure 5B:** *e22c-GAL4 srpHemo-3xmCherry; UAS-GFP, e22c-GAL4 srpHemo-3xmCherry; UAS-veli IR.* **Figure 5F-I and Figure S5D (Patj and Sqh-1P immunolabeling):** +; *srpHemo-H2A::3xmCherry, egr^1; srpHemo-3xmCherry, grnd^{Minos}; srpHemo-3xmCherry.* **5J,K:** *e22c-GAL4 srpHemo-3xmCherry; UAS-GFP, e22c-GAL4 srpHemo-3xmCherry egr^1; UAS-GFP, e22c-GAL4 srpHemo-3xmCherry egr^1; UAS-Patj, e22c-GAL4 srpHemo-3xmCherry egr^1; UAS-sqh IR.* **Figure S5B,C:** *UAS-GFP; srpHemo-3xmCherry, puc^{E69}-GAL4, egr^1 UAS-GFP; srpHemo-3xmCherry puc^{E69}-GAL4.* **Figure S5E,F:** +; *srpHemo-H2A::3xmCherry.*

Figure 6B,C,D,E and **Figure S6A:** *knock-in DE-Cad::GFP srpHemo-3xmCherry, egr^1 DE-Cad::GFP srpHemo-3xmCherry, grnd^{Minos}; knock-in DE-Cad::GFP srpHemo-3xmCherry.* **Figure 6F,H** and **Figure S6F:** *knock-in DE-Cad::GFP srpHemo-3xmCherry, egr^1 knock-in DE-Cad::GFP srpHemo-3xmCherry.* **Figure 6G and Figure S6E:** *e22c-GAL4 srpHemo-3xmCherry/+; UAS-GFP/+, e22c-GAL4 srpHemo-3xmCherry/+; UAS-Rho.V14/+.* **Figure S6C,D:** +; *srpHemo-H2A::3xmCherry, egr^1; srpHemo-3xmCherry.*

METHODS DETAILS

***In situ* hybridization and immunofluorescence**

1071 Embryos were fixed with 3.7% formaldehyde/heptane for 20 min followed by

1072 methanol devitellinization for *in situ* hybridization. The *eiger* cDNA clone, RH51659

1073 and the *grindelwald* cDNA clone RE28509 were obtained from the *Drosophila*

1074 Genomics Resource Centre (DGRC). T7 or T3 polymerase-synthesized digoxigenin-

1075 labelled anti-sense probe preparation and *in situ* hybridization was performed using

1076 standard methods (Lehmann and Tautz, 1994). Images were taken with a Nikon-

1077 Eclipse Wide field microscope with a 20X 0.5 NA DIC water Immersion Objective.

1078 For most antibody stainings, embryos were fixed with freshly prepared 4.0%

1079 paraformaldehyde and heptane for 20 min followed by methanol devitellinization as

1080 described previously (Zhang and Ward, 2011). Phalloidin, DE-Cadherin and N-

1081 Cadherin staining utilized hand-devitellinized embryos. Eiger staining was conducted

1082 on embryos devitellinized with ethanol. The following primary antibodies were used.

1083 Chicken Anti-GFP (Aves Labs GFP-1020, 1:500), Rabbit anti-E-Cadherin (Santa

1084 Cruz sc-33743, 1:25), Rat anti-DE-Cadherin (Oda et al., 1994) (DSHB DCAD2,

1085 1:25), Rat anti-DN-Cadherin (Iwai et al., 1997) (DSHB DN-Ex #8, 1:25), Mouse anti-

1086 Talin (Brown et al., 2002) (DSHB Talin A22A, Talin E16B, 1:10), Mouse anti-

1087 Integrin betaPS (Brower et al., 1984) (DSHB CF.6G11, 1:25), Mouse anti-Crumbs

1088 (Tepass and Knust, 1993) (DSHB CQ4, 1:50), Rabbit anti-Dystroglycan (Deng et al.,

1089 2003)1:50), Rabbit anti-LanA (Schneider et al., 2006)1:50), Rabbit Eiger R1 (Igaki,

1090 2002), guinea pig anti-Patj (Sen et al., 2012), guinea pig anti-Sqh1P ((Zhang and

1091 Ward, 2011). Alexa fluor 488 or 633 labelled secondary antibodies and Phalloidin

1092 (Thermo Fisher Scientific) were used at a dilution of 1:500. Embryos were mounted

1093 after immunolabeling in Vectashield Mounting Medium (Vector Labs, Burlingame,

1094   USA) and imaged with a Zeiss Inverted LSM700 Confocal Microscope using a Plain-

1095   Apochromat 20X/0.8 Air Objective or a Plain-Apochromat 63X/1.4 Oil Objective as

1096   required.

1097   **Electron Microscopy**

1098   Early embryos in which the macrophages had not yet reached the germband were

1099   collected and dechorionated before mounting on cup-shaped aluminum planchettes

1100   (cavity dimensions Ø 2mm, depth 200μm; Wohlwend, Sennwald, CH) using 2% BSA

1101   in phosphate buffer (0.1M, pH 7.4) as filler and a flat planchette as a lid. Such

1102   sandwiched samples were rapidly frozen using a HPF machine (HPM010; BalTec,

1103   Balzers, LIE) and stored in liquid nitrogen. Embryos were then freeze-substituted in

1104   an AFS1 device (Leica Microsystems) using the following FS cocktail: 1% osmium

1105   (w/v; EMS) in non-hydrous acetone plus 0.2% uranyl acetate (v/v of 20% stock in

1106   methanol; Agar Scientific). The sequence for step-wise warming was: -80°C for 48 h,

1107   temperature rise 3°C/h, -20°C for 12 h, temperature rise 3°C/h, 4°C for 1 h. Embryos

1108   were then washed in non-hydrous acetone, embedded in epoxy resin (Durcupan

1109   ACM, Fluca) and cured for 48 h at 60°C. Serial ultrathin sections (70-80 nm) were

1110   cut using an ultramicrotome (Leica Microsystems UC7), collected onto formvar-

1111   coated copper slot grids and contrast enhanced by means of 1% uranyl acetate in

1112   water (w/v) and 0.3% lead citrate.   Sections were examined in a TECNAI 10

1113   transmission electron microscope operated at 80 kV, equipped with a Morada CCD

1114   camera (Soft Imaging Systems). Alternatively, sections were cut at 250 nm, collected

1115   on formvar-coated 100-line bar grids, carbon coated (thickness 6 nm) and observed

1116   under a Jeol JEM 2800 operated at 200 kV in STEM bright-field mode.

1117

1118   **Time-Lapse Imaging**

1119   Embryos were dechorionated in 50% bleach for 4 min, washed with water, and

1120   mounted in halocarbon oil 27 (Sigma) between a coverslip and an oxygen permeable

1121   membrane (YSI). The anterior dorsolateral region of the embryo was imaged on an

1122   inverted multiphoton microscope (TrimScope II, LaVision) equipped with a W Plan-

1123   Apochromat 40X/1.4 oil immersion objective (Olympus). GFP and mCherry were

1124   imaged at 860 nm and 1100 nm excitation wavelengths, respectively, using a Ti-

1125   Sapphire femtosecond laser system (Coherent Chameleon Ultra) combined with

1126   optical parametric oscillator technology (Coherent Chameleon Compact OPO).

1127   Excitation intensity profiles were adjusted to tissue penetration depth and Z-

1128   sectioning for imaging was set at 1 or 1.5 µm for tracking and segmentation

1129   respectively. For long-term imaging, movies were acquired for 180-200 minutes with

1130   a frame rate of 40 seconds. All embryos were imaged with a temperature control unit

1131   set to 28.5°C. To assess potential changes in germband extension and retraction in the

1132 egr^1 embryos, wild type and egr^1 embryos were collected for 30 minutes and then

1133   imaged for a further 10 hours using a Nikon-Eclipse Wide field microscope with a

1134   20X 0.5 NA DIC water Immersion Objective. Bright field images were taken every 5

1135   minutes.

1136   **Analysis of macrophage cell counts:** Transmitted light images of the embryos were

1137   used to measure the position of the germband to determine the stages for analysis.

1138   Germband retraction away from the anterior was used to classify embryos into Stage

1139   11 or Stage 12. Embryos with germband retraction of between 29-31% were assigned

1140   to Stage 11. Those with 35-40% retraction (Stage 12) were analyzed for the number

1141   of macrophages that had entered the germband and those with above 50% retraction

1142   for the number along the ventral nerve cord (vnc), anterior tip of the head and in the

1143   whole embryo. Macrophages were visualized using confocal microscopy with a Z-

1144 resolution of 3 μm and the number of macrophages within the germband or the

1145 segments of vnc was calculated in individual slices (and then aggregated) using the

1146 Cell Counter plugin in FIJI. Total macrophage numbers were obtained using Imaris

1147 (Bitplane) by detecting all the macrophage nuclei as spots.

1148 **Image Processing and Analysis of macrophage migration**

1149 Embryos in which the macrophage nuclei were labeled with *srpHemo-*

1150 *H2A::3XmCherry* were imaged and 250x130x36μm^3 3D-stacks were typically

1151 acquired with a constant 0.5x0.5x1μm^3 voxel size at every 40-41 seconds for

1152 approximately 3 hours. Images acquired from multiphoton microscopy were initially

1153 processed with InSpector software (LaVision Bio Tec) to compile channels from the

1154 imaging data, and the exported files were further processed using Imaris software

1155 (Bitplane) to visualize the recorded channels in 3D. Briefly,

1156 i. The movie from each imaged embryo was rotated and aligned along the AP axis for

1157 tracking analysis.

1158 ii. To calculate migration parameters while macrophages migrate from the head

1159 mesoderm to the edge of the germband, movies were cropped in time to that period

1160 (typically 60 minutes from the original movie was used for analysis).

1161 iii. Macrophage nuclei were extracted using the spot detection function and tracks

1162 generated in 3D over time. We could not detect all the macrophages in the head

1163 mesoderm as spots because of limitations in imaging parameters. Tracks of

1164 macrophages which migrate towards the dorsal vessel, ventral nerve cord and to the

1165 anterior of the head were omitted. The edge of the germband was detected using

1166 autofluorescence from the yolk and the mean position of the tracks in X- and Y-axis

1167 was used to restrict analysis to before macrophages reach the edge of the germband.

1168   iv. Nuclei positions in XYZ-dimensions were determined for each time point and used

1169   for further quantitative analysis.

1170   v. To calculate the speed of migration of the first macrophage in the germband the

1171   track generated for the first macrophage alone was utilized to obtain the nuclei

1172   position in XYZ-dimensions. Speed calculated within the first 35-45 μm of the

1173   germband is shown in Figure 2M and within the next 35-45 μm is shown in Figure

1174   2N.

1175   Cell speeds and persistence was calculated in Matlab (The MathWorks Inc.) from

1176   single cell positions in 3D for each time frame measured in Imaris (Bitplane), as

1177   described elsewhere (Smutny et al., 2017). Briefly, instantaneous velocities from

1178   single cell trajectories were averaged to obtain a mean instantaneous velocity value

1179   over the course of measurement. To calculate persistence values, single cell

1180   trajectories were split into segments of equal length (l; $l = 10$ frames) and calculated

1181   via a sliding window as the ratio of the distance between the macrophage start-to-end

1182   distance (D) over the entire summed distance covered by the macrophage between

1183   each successive frame (d_i) in a segment. Calculated persistence values were averaged

1184   over all segments in a single trajectory and all trajectories were averaged to obtain a

1185   persistence index (I) for the duration of measurement (with 0 being the lowest and 1

1186   the maximum directionality) as follows:

$$I\,(l) = \sum_{k=1}^{n-l} \frac{\left(D_k \Big/ \sum_{i=k}^{k+l} d_i\right)}{n-l}$$

1187   where n represents the total number of frames, i the sum of frame-to-frame distances

1188   over one segment and k the sum over all segments of a trajectory.

1189   Embryos expressing *srpHemo-3XmCherry* and *knock-in DE-Cadherin::GFP* were

1190   used for calculating time for macrophage entry. Briefly, 100x130x34μm^3 3D-stacks

1191 were typically acquired with a constant $0.28\text{x}0.28\text{x}2\mu m^3$ voxel size at every 40-41

1192 seconds for approximately 3 hours. The time point when the macrophage protrusion

1193 touched the edge of the germband was defined as T0 and the time point when the

1194 entirety of the macrophage was within the germband was taken as T1 and T1-T0 was

1195 defined as time for macrophage entry. T0 and T1 were determined by precisely

1196 examining macrophage position in xy and z dimensions (examination of individual 2

1197 micron slices) over time.

1198 **Measurement of junctional fluorescence intensities**

1199 The apical junction intensity of Patj and Phospho-Myosin Regulatory Light Chain

1200 were calculated using linescan analysis as previously described (Smutny et al., 2010)

1201 with the following changes. The line length was approximately the cell length and the

1202 line was always drawn from the outside of the cell to the inside. For every line, a

1203 Gaussian fit was applied and intensities across the cell junction were then normalized

1204 against the cytosolic signal using the PeakfitProfiles plugin in Fiji. Calculation of

1205 mean intensity within the ectoderm-mesoderm interface for DE-Cadherin, N-

1206 Cadherin, Laminin A, Dystroglycan, β PS integrin and Talin, and calculation of mean

1207 intensity at the cell boundaries for Eiger was conducted as follows. Mean intensity

1208 within a line (typically of line width 10 pixels) drawn at the interface or at the cell

1209 boundary (for Eiger) was obtained in FIJI and then normalized to the cytosolic signal.

1210 The levels of Eiger in the AS was quantified at all sagittal planes including those in

1211 which the amnioserosa extends more broadly across the embryo in the anterior

1212 posterior direction, thus more extensively covering the germband ectoderm. For both

1213 junctional and interface analysis typically 30-40 interfaces / boundaries from 3-7

1214 embryos were used for the analysis.

1215

Tension measurements of cell junctions by UV laser ablation

1216

1217 The apical tension of cell-cell contacts was assessed by conducting laser ablation on a

1218 previously described Axio Observer Z1 (Zeiss) inverted microscope with a confocal

1219 spinning disc unit for high speed imaging (Smutny et al., 2015) and a Plan Apo 63x

1220 1.2 NA water-immersion lens (Zeiss). A pulsed 355 nm laser was used to ablate

1221 apical cell junctions labeled with *knock-in DE-cadherin::GF*P in control, *egr*[1] and

1222 *grnd*[Minos] mutant embryos when the macrophages were near the edge of the germband

1223 but not within. UV ablations were operated by a custom-designed LabView software

1224 to allow simultaneous control of image acquisition and laser ablation. A typical

1225 ablation experiment was performed point-wise along a 6 μm length line perpendicular

1226 to and centered on an apical junction, using a laser pulse rate of 1kHz, an area density

1227 along the line of 1 shot/μm^2, 25 pulses per ablation point with an average power of

1228 15μwatt and a total ablation duration between ~150-350 ms. Fluorescent images were

1229 acquired for one channel (488 nm wavelength) with an iXon DU-897-BV camera

1230 (Andor Technology) using exposure times and frame rates of 200ms. Laser ablation

1231 itself lasted for ~250 ms and resulted in a local depletion of DE-Cadherin at the apical

1232 cell cortex. Care was taken to ablate contacts without causing any damage to the

1233 embryo, which was confirmed by monitoring embryos over an extended time period

1234 without obvious detection of leakage of fluorescent cytoplasmic material, or cell

1235 rupture and apoptosis. To inhibit Myosin activity, 100mM Rho-kinase inhibitor (Y-

1236 27632 dihydrochloride, Tocris Bioscience) resuspended in water was injected

1237 laterally into the perivitelline space of Stage 11 embryos after germband extension

1238 was complete. To assess the recoil of apical DE-Cadherin in response to laser

1239 ablation, we first post-processed all raw images from laser ablation with Fiji software

1240 by subtracting measured mean background values and applied a Gaussian filter before

1241 using a kymograph analysis of a segmented line defining DE-Cadherin along the

1242 ablated apical cell cortex. The minimum intensity of apical DE-Cadherin was

1243 measured pre-cut to threshold the kymograph for post-cut analysis. The two open end

1244 tips of apical cortical DE-Cadherin after ablation were tracked for the first frame after

1245 opening (t_0) and every consecutive 0.8 seconds (t_n) to calculate the total distance d

1246 (μm) of the opening for every time point. The recoil distance (R) of each end tip at a

1247 given time point (t_n) was calculated as follows:

$$\frac{d(t_n) - d(t_0)}{2} = R(t_n)$$

1248 The initial recoil velocity RV (μm/s) for each cut was derived by calculating the

1249 recoil distance for the primary opening frame (t_1) post-cut as follows:

$$\frac{d(t_1) - d(t_0)}{2\,t_1} = RV$$

1250 Measurements were processed in Prism (Graphpad) and Matlab (R2013a; Mathworks)

1251 for calculations and plotting of graphs. For curve fitting of recoil distances, we used

1252 nonparametric fitting with a smoothing spline model through the calculated data.

1253 **CellFIT-3D interfacial tension analysis**

1254 CellFIT provides a general-purpose mathematical formalism for calculating the forces

1255 that produce specific observed cell and tissue motions and geometries, which was

1256 recently adapted to a 3D system (Brodland et al., 2014; Krens et al., 2017).For the

1257 CellFIT-3D analysis, we acquired confocal image stacks of fixed Stage 11 embryos

1258 that were oversampled in the z-direction (0.5 μm steps), to increase the number of

1259 triple cell junctions that could be annotated per cell. In short, to obtain estimates of

1260 the relative edge tensions, the angles at triple junctions, such as those between apical,

1261 basal and the lateral interfaces of the germ band ectoderm cells, were manually

1262 digitized using custom software. The angles along particular cells edges were

1263   digitized in multiple images within the stack in order to obtain the true angles of the

1264   cell membranes with the edge (see Movie S6). We analysed 70 control (n=5 embryos)

1265   and 79 egr^1 (n=4 embryos) triple cell junctions, an that, in average, consists of ~5

1266   annotated triplets (Z-planes) per analysed triple cell junction annotation, resulting in a

1267   total of approximately 750 cell manually annotated triplets. Force-balance equations

1268   were written for each digitized triple junction as described previously (Krens et al.,

1269   2017), and least-squares solutions were found for all such equations. The solutions to

1270   these equations provided the relative strengths of the tensions along each edge type

1271   (Krens et al., 2017). The averaged values provided were used as the relative strengths

1272   of the tensions along each edge type.

1273   **Cell segmentation and deformation analysis**

1274   Embryos expressing *srpHemo-3XmCherry* and *knock-in DE-Cadherin::GFP* were

1275   used for cell segmentation. Briefly, $100x130x34\mu m^3$ 3D-stacks were typically

1276   acquired with a constant $0.28x0.28x1.5\mu m^3$ voxel size at every 40-41 seconds for

1277   approximately 3 hours. Fragments of multiphoton movies showing the initial stages of

1278   macrophage penetration through the ectoderm were selected. To facilitate 2D

1279   segmentation of ectoderm cell bodies, we used the Ilastik 1.1.8 program. Pixel

1280   classification project was trained to distinguish between cell bodies and cell

1281   membrane, using full range of neighborhood analysis parameters presented in Ilastik

1282   1.1.8, with a deviation σ=3. After training on 15 reproducible images of the ectoderm,

1283   the remaining ectoderm images were batch-processed and images containing the

1284   probabilities of pixels belonging to the 'membrane' or the 'cytosol' area were

1285   exported and used for intensity-based segmentation. In some areas, the segmentation

1286   had to be corrected manually in the final binary images. Ectoderm membranes that

1287   appeared inside of the macrophages were manually removed from the segmented

1288   image to avoid including apoptotic cells in the final analysis.

1289   The resulting binary images were analyzed using a custom Matlab R2015b script;

1290   they were preprocessed using a set of bwmorph functions to remove spurious pixels

1291   and single-pixel bridges between the separate cells. Then the length-to width ratio of

1292   an ellipse encircling each ectodermal cell was calculated using ectodermal cells that

1293   were not adjacent to the image border. The length-to-width ratio was defined as a

1294   ratio of regionprops.MajorAxisLength to regionprops.MinorAxisLength for each cell

1295   (Figure S6F). To understand how deformation in the neighborhood of a macrophage

1296   occurs, we analyzed the length-to-width ratio of ectoderm cells with centroids located

1297   not further than 10μm from the nearest edge of a macrophage (Figure 6H). The

1298   significance of the length to width ratio distribution differences between cells in the

1299   control and the *egr^1* mutant (Figure 6H) was analyzed with an unpaired t-test

1300   assuming normal distribution.

1301   **QUANTIFICATION AND STATISTICAL ANALYSIS**

1302   **<u>Statistical Analysis</u>**

1303   Statistical tests as well as the number of embryos/ cells assessed are listed in the

1304   Figure legends. All statistical analyses were performed using GraphPad Prism and

1305   significance was determined using a 99% confidence interval. No statistical method

1306   was used to predetermine sample size and the experiments were not randomized.

1307   Data points from individual experiments / embryos were pooled to estimate mean and

1308   s.e.m. Error bars in all graphs represent the standard error of the mean. An unpaired t-

1309   test was used to calculate the significance in differences between two groups and

1310   One-Way ANOVA followed by Dunnett or Tukey post tests were used for multiple

1311   comparisons.

Repeatability

All measurements were performed in 3-40 embryos. Representative images shown in

Figure 1D,E,I,J Figure 2B, Figure 3A,A'B,B',F,G, Figure 4B,E,F,G, Figure 5B,D,J,

Figure S2A,D,E,F, Figure S3A,A'B,B',H, Figure S4C,E,I,J,K, Figure S5A,B,D,E and

Figure S6E were from experiments that were repeated at least 3 and up to 7 times.

Representative *in situ* images shown in Figure 2A were from an experiment repeated

3 times and those in Figure 3E were from an experiment repeated 2 times.

Representative TEM images shown in Figure 1E,H and Figure S1D,E were from an

experiment repeated 3 times. Representative images showing macrophage migration

tracks color coded for persistence in Figure S2M,O and 3E and stills shown in Figure

1C, Figure 2J, Figure 3H, and Figure S6F are from two-photon movies, which were

repeated at least 3 times. Representative Images shown in Figure 6B,C and Figure

S6A are stills from confocal spinning disc movies which were repeated at least 8

times.

Movies

Movie S1: Macrophages invade into the germband by transiently separating

apposing tissues, related to Figure 1C.

Two-photon time-lapse imaging *e22c-GAL4 srpHemo-3xmCherry; 10xUAS-*

CD8::GFP embryos where CD8-GFP labels membranes (green) and 3XmCherry

labels macrophages (red). The embryo is oriented with anterior to the left and dorsal

up and the area imaged in the embryo is indicated in the grey box in the schematic

Figure 2D. The time interval between each acquisition is 40 seconds and the display

rate is 10 frames per second. Scale bar represents 25µm.

1337

1338 **Movie S2: Macrophage migration into the germband in control (con), *egr¹* and**

1339 *grnd^Minos* embryos, related to Figure 2J and 3H**.**

1340 Representative two-photon movies of control (con) embryos (*srpHemo-*

1341 *H2A::3xmCherry*), *egr¹* (*egr¹; srpHemo-H2A::3xmCherry*) and *grnd^Minos* (*grnd^Minos;*

1342 *srpHemo-H2A::3xmCherry*) embryos showing macrophage nuclei (red) migrating

1343 from the head into the germband. The migration was imaged for 2 hours and the

1344 embryo is oriented with anterior to the left and dorsal up. The area imaged in the

1345 embryo is indicated in the schematic in Figure 1J. The line indicates the position of

1346 the germband at selected time points. The time interval between each acquisition is 40

1347 seconds and the display rate is 20 frames per second. Scale bar represents 25μm.

1348

1349 **Movie S3: Tracking macrophage migration from head to the germband in**

1350 **control (con), *egr¹* and** *grnd^Minos* embryos, related to Figure 2G-I, Figure S2 L,M and

1351 Figure S3D-G**.**

1352 Representative movies of control (con) embryos (*srpHemo-H2A::3xmCherry*), *egr¹*

1353 (*egr¹; srpHemo-H2A::3xmCherry*) and *grnd^Minos* (*grnd^Minos; srpHemo-*

1354 *H2A::3xmCherry*) embryos showing macrophage nuclei (red) migrating from the

1355 head up to the edge of the germband. Superimposed are the macrophage nuclei

1356 detected as spots (grey) and dragon tail tracks showing the migration of the

1357 macrophages over time. Each dragon tail shows macrophage migration behavior for 5

1358 time points. The time interval between each acquisition is 40 seconds and the display

1359 rate is 10 frames per second. Scale bar represents 10μm.

1360

1361 **Movie S4: Macrophage invasion into the germband is a time intensive process,**

1362   **related to Figure 2K.**

1363   Representative two-photon movie showing macrophage invasion into the germband in

1364   control embryos (*DE-Cad::GFP; srpHemo-3xmCherry*). A z-projection of 7 slices

1365   (2μm each) is shown. The time point when the macrophage protrusion touched the

1366   edge of the germband was defined as T0 (time point 50, arrow) and the time point

1367   when the entirety of the macrophage was within the germband (determined by the

1368   examination of the macrophage in individual slice and time) was taken as T1 (time

1369   point 90, arrowhead). T1-T0 was defined as the time for macrophage entry. T0 and T1

1370   were determined by precisely examining macrophage position in xyz dimensions over

1371   time. The time interval between each acquisition is 41 seconds and the display rate is

1372   5 frames per second. Scale bar represents 25μm.

1373

1374   **Movie S5: Laser ablation of the germband ectoderm cells, related to Figure 6A-C**

1375   **and Figure S6A.**

1376   Representative confocal spinning disc movies showing laser ablation of the

1377   ectodermal apical DE-Cadherin::GFP in control, *egr^1 and grnd^{Minos}* embryos, each

1378   carrying *srpHemo-3xmCherry* to label macrophages and *knock-in DE-*

1379   *Cadherin::GFP.* The time interval between each acquisition is 200 milliseconds and

1380   the display rate is 5 frames per second. Scale bar represents 10μm.

1381

1382   **Movie S6: CellFIT-3D analysis, related to Figure 6.**

1383   Movie showing consecutive slices from confocal microscopy image of a fixed control

1384   Stage 11 embryo with macrophage nuclei labeled by the expression of *srpHemo-*

1385   *H2A::3XmCherry* and ectoderm cells visualized by immunolabeling for DE-Cadherin.

1386   Representative cell triple interfacial junctions at apical and basal interfaces of a few
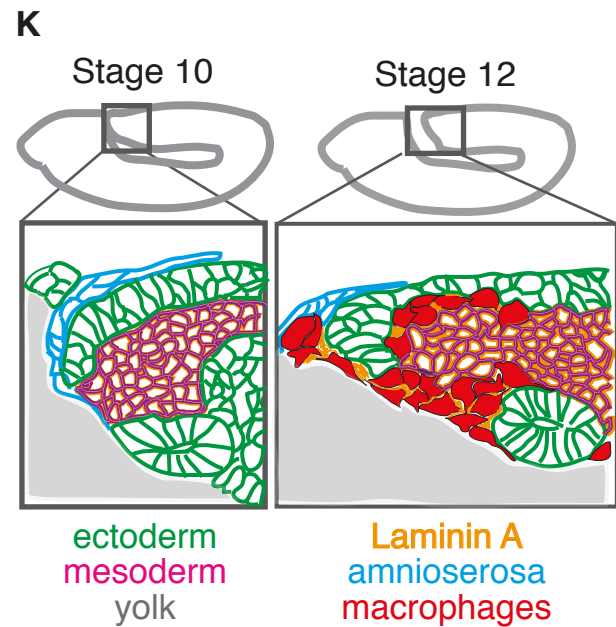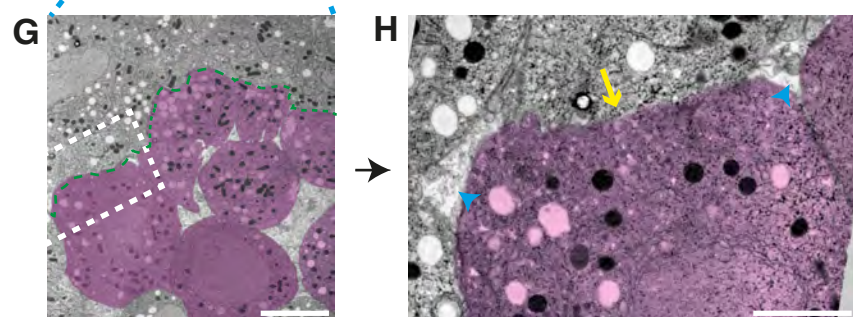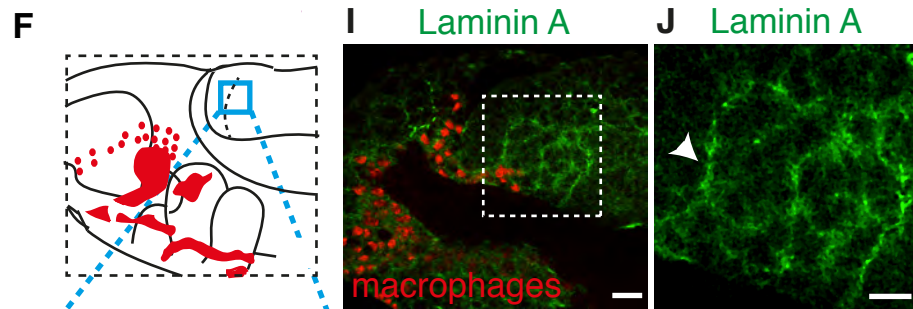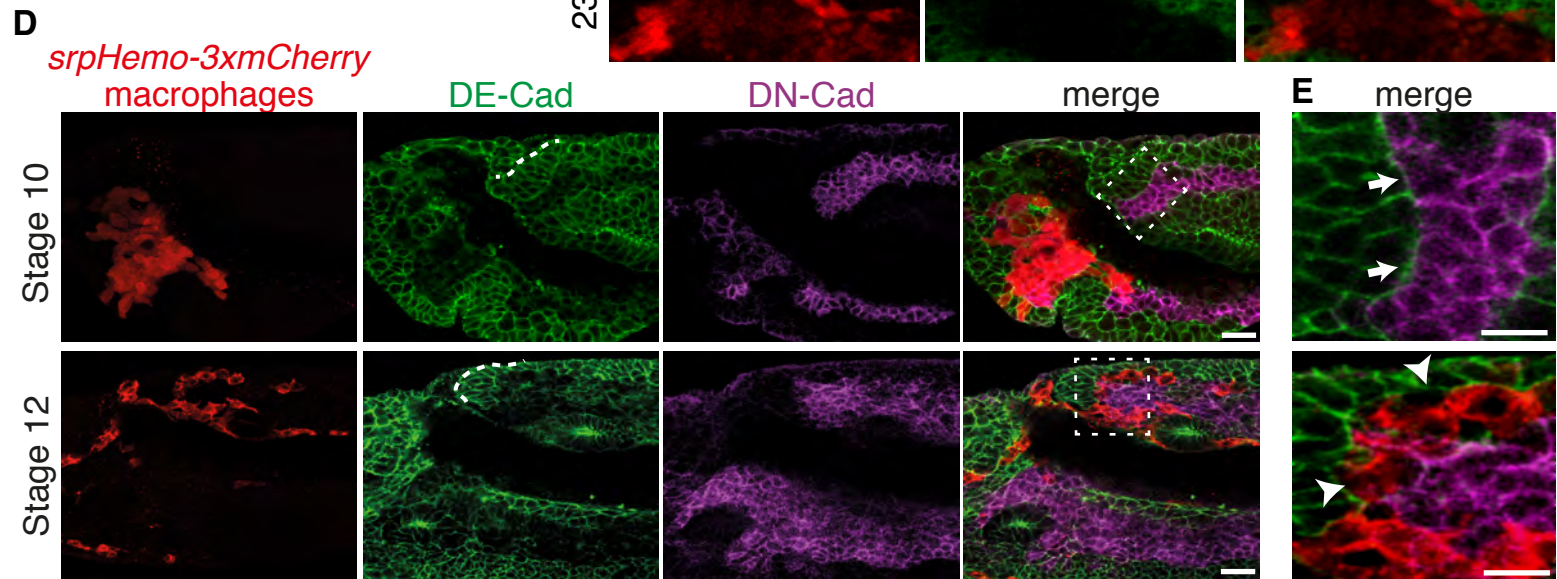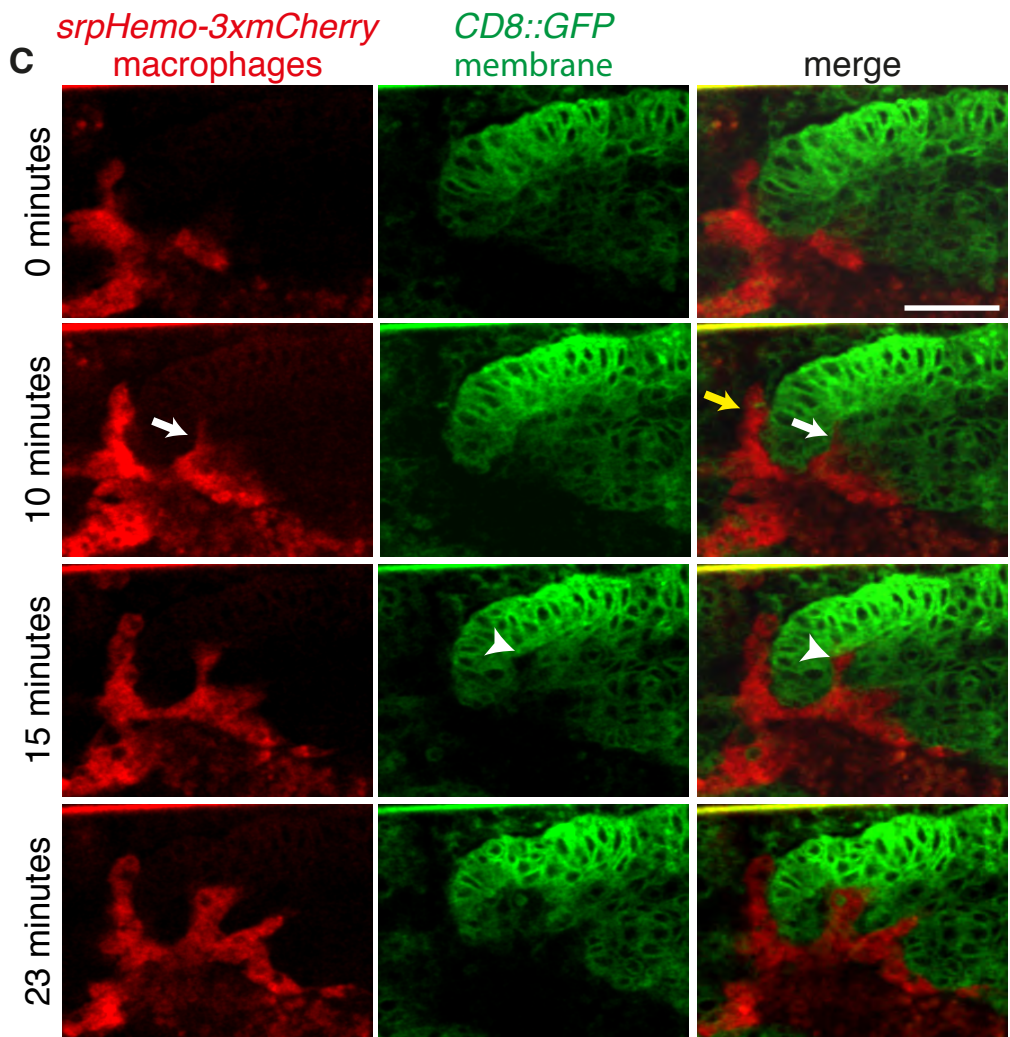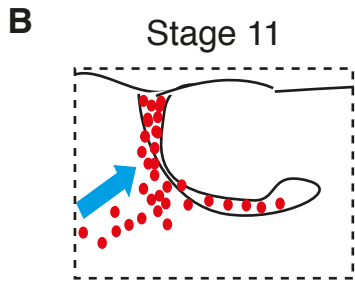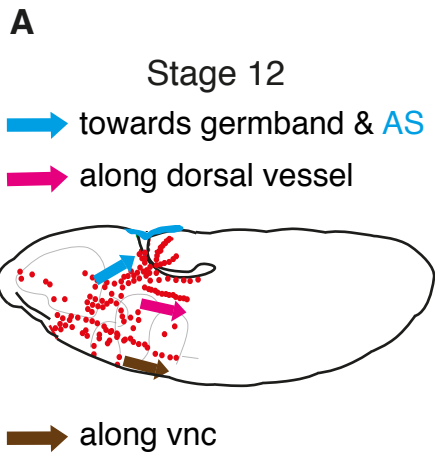
cells in the germband ectoderm used for CellFIT-3D based tension analysis are displayed. The display rate is 4 frames per second and the scale bar represents 10μm.

Movie S7: Macrophages in *egr¹* embryos are unable to effectively deform the ectodermal cells during invasion into the germband, related to Figure 6H and Figure S6F.
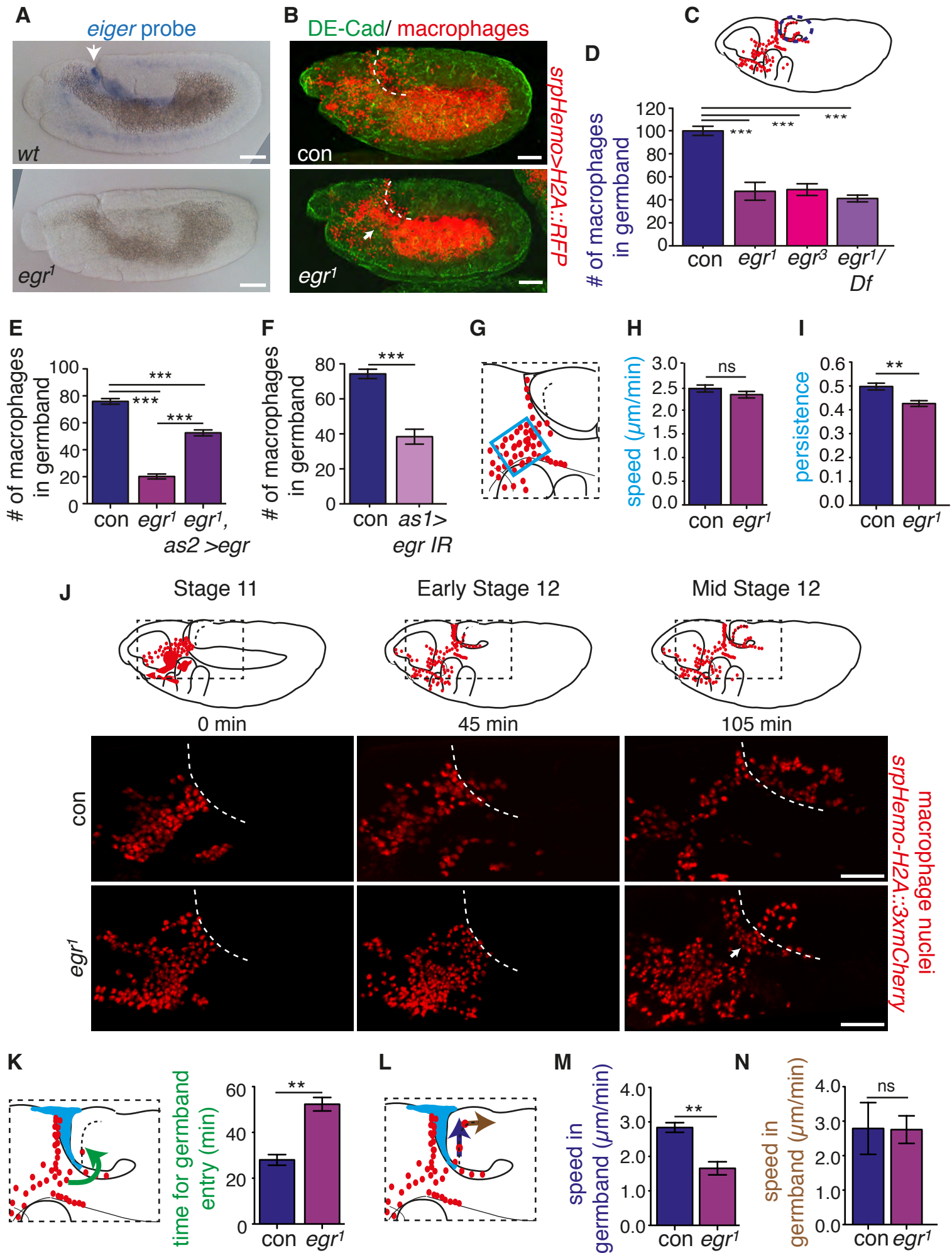
Movie showing changes in the length/ width ratio (LWR) from before to after macrophage invasion in control (con) and *egr¹* embryos carrying *srpHemo-3xmCherry* to label macrophages and the *knock-in DE-Cadherin::GFP* to visualize cell edges. The macrophage (indicated by the white ROI) appears at time point 3. The length/ width ratio (LWR) of the ectodermal cells is shown color-coded on a scale from 1 to 4, with 1 representing a circular shape. The area imaged in the embryo is indicated in the schematic in Figure 5G. The time interval between each acquisition is 40 seconds and the display rate is 5 frames per second. Scale bar represents 10μm.
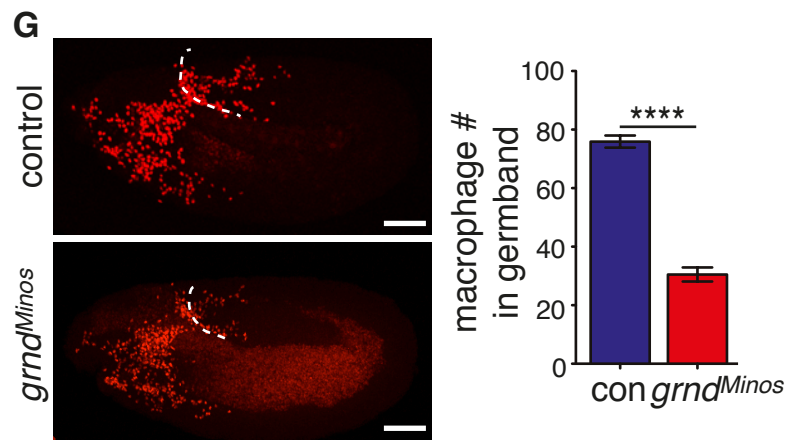
Siekhaus Figure 1

A Stage 12
- → towards germband & AS
- → along dorsal vessel
- → along vnc

B Stage 11

C *srpHemo-3xmCherry* macrophages | *CD8::GFP* membrane | merge
- 0 minutes
- 10 minutes
- 15 minutes
- 23 minutes

D *srpHemo-3xmCherry* macrophages | DE-Cad | DN-Cad | merge
- Stage 10
- Stage 12

E merge

F

I Laminin A — macrophages

J Laminin A

K Stage 10 | Stage 12
- ectoderm
- mesoderm
- yolk
- Laminin A
- amnioserosa
- macrophages

G

H

Siekhaus Figure 2

A *eiger* probe — *wt* / *egr¹*

B DE-Cad/ macrophages — con / *egr¹* — *srpHemo>H2A::RFP*

C — **D** # of macrophages in germband — con, *egr¹*, *egr³*, *egr¹/Df* — ***

E # of macrophages in germband — con, *egr¹*, *egr¹, as2 >egr* — ***

F # of macrophages in germband — con, *as1> egr IR* — ***

G

H speed (*µm/min*) — con, *egr¹* — ns

I persistence — con, *egr¹* — **

J Stage 11 — 0 min / Early Stage 12 — 45 min / Mid Stage 12 — 105 min — con / *egr¹* — macrophage nuclei *srpHemo-H2A::3xmCherry*

K time for germband entry (min) — con, *egr¹* — **

L

M speed in germband (*µm/min*) — con, *egr¹* — **

N speed in germband (*µm/min*) — con, *egr¹* — ns

Siekhaus Figure 3

Siekhaus Figure 4
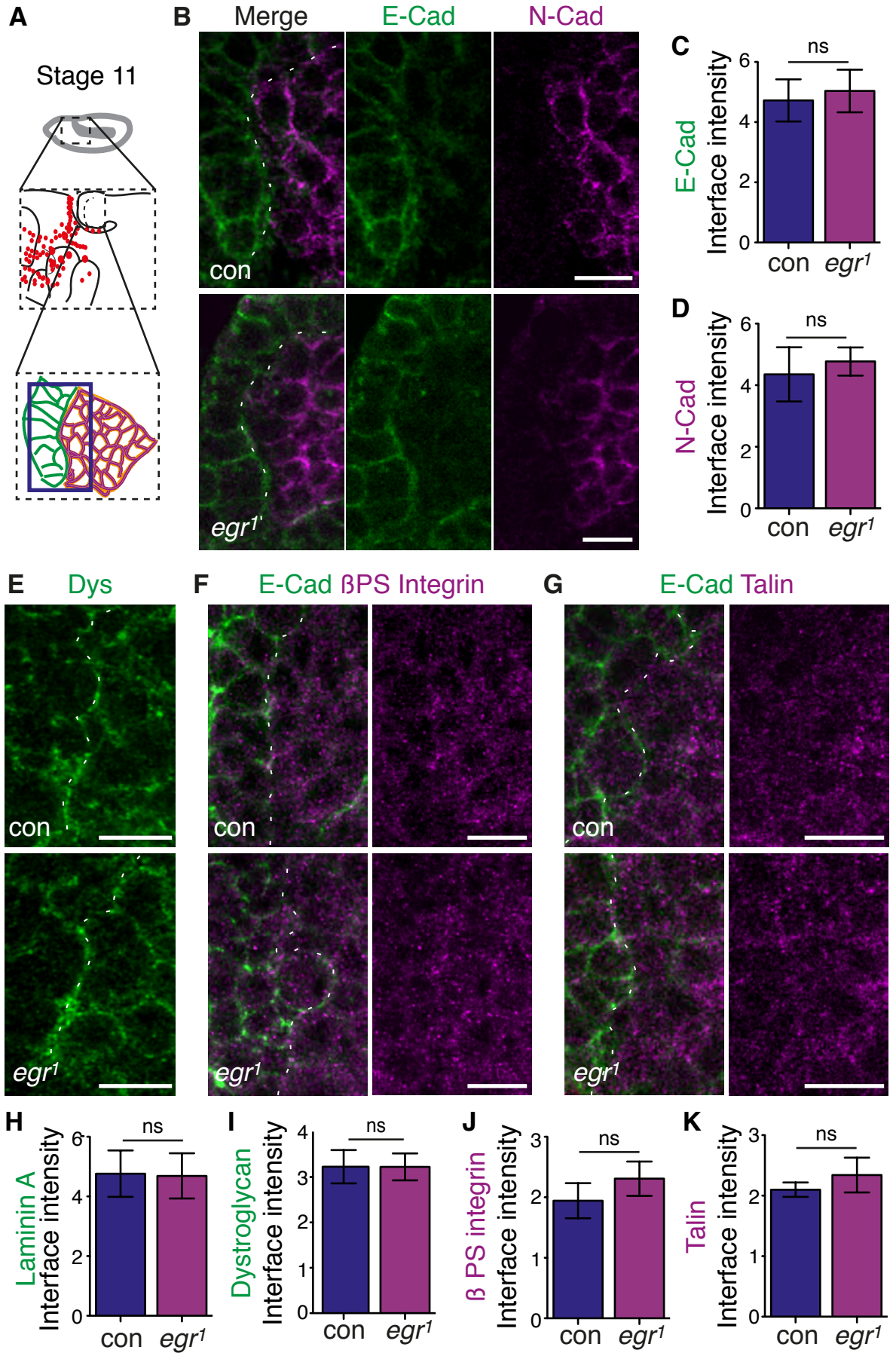
Siekhaus Figure 5

Siekhaus Figure 6

Figure S1

Stage 11

Stage 12

D
A ←→ P
V
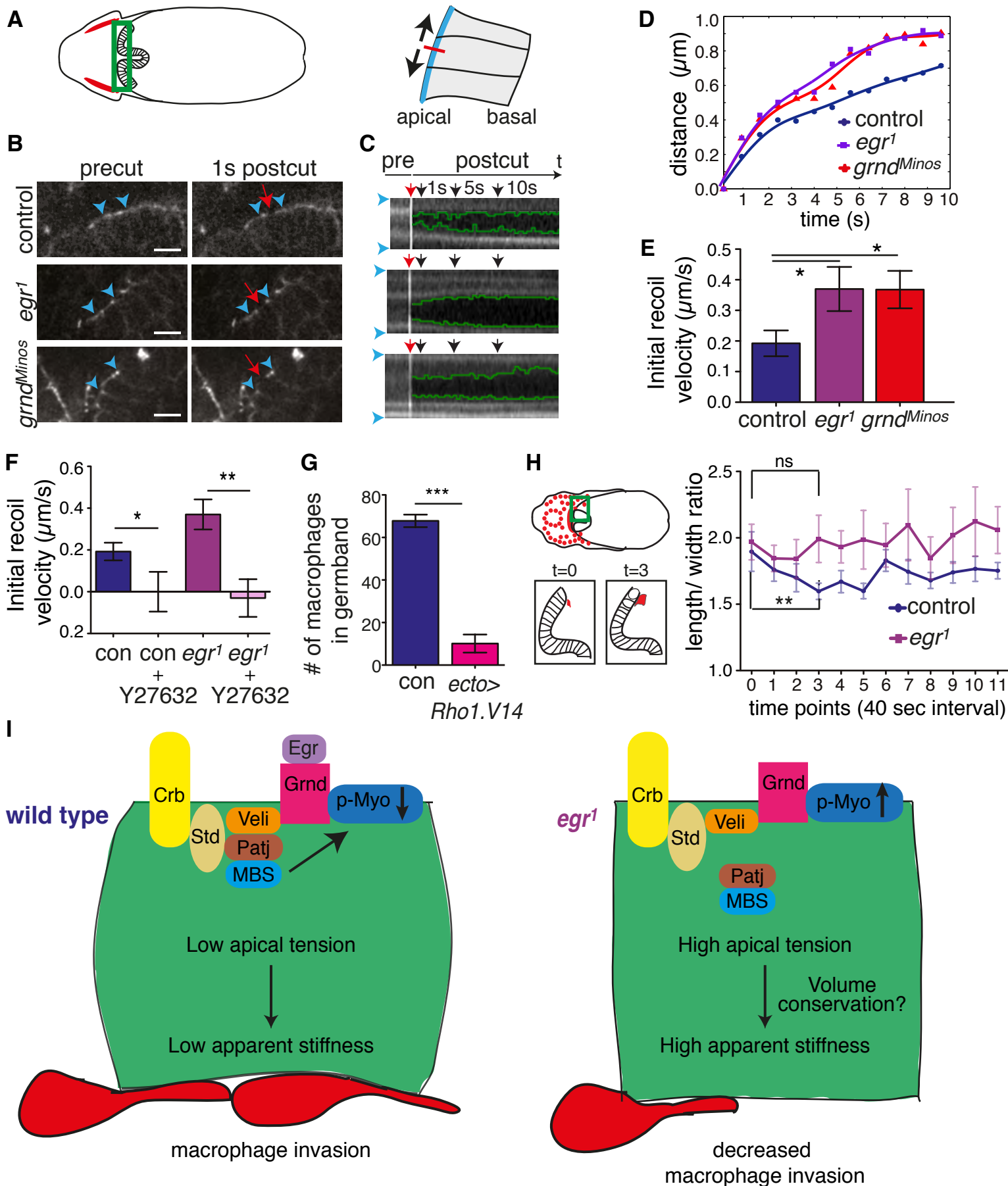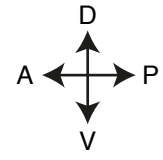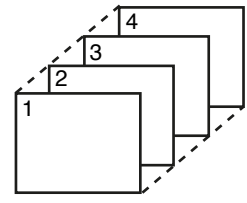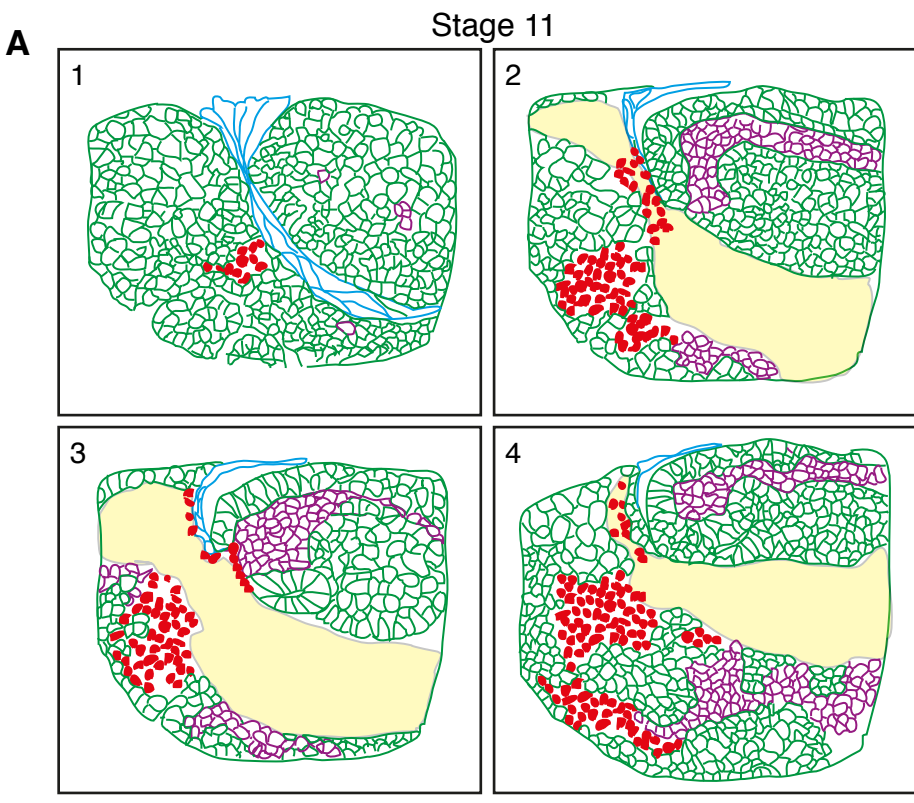
ectoderm
mesoderm
amnioserosa
yolk
macrophages

overview
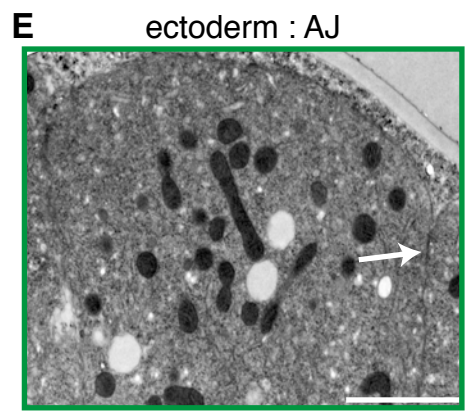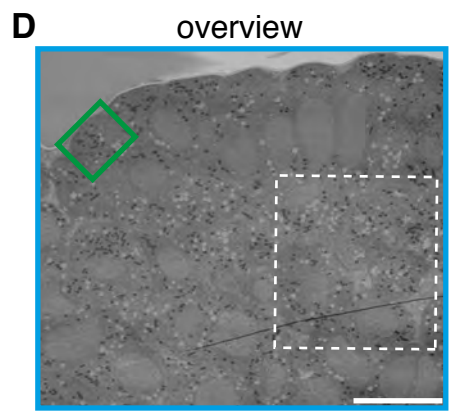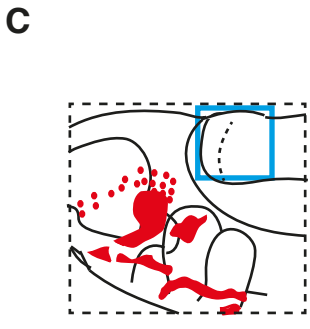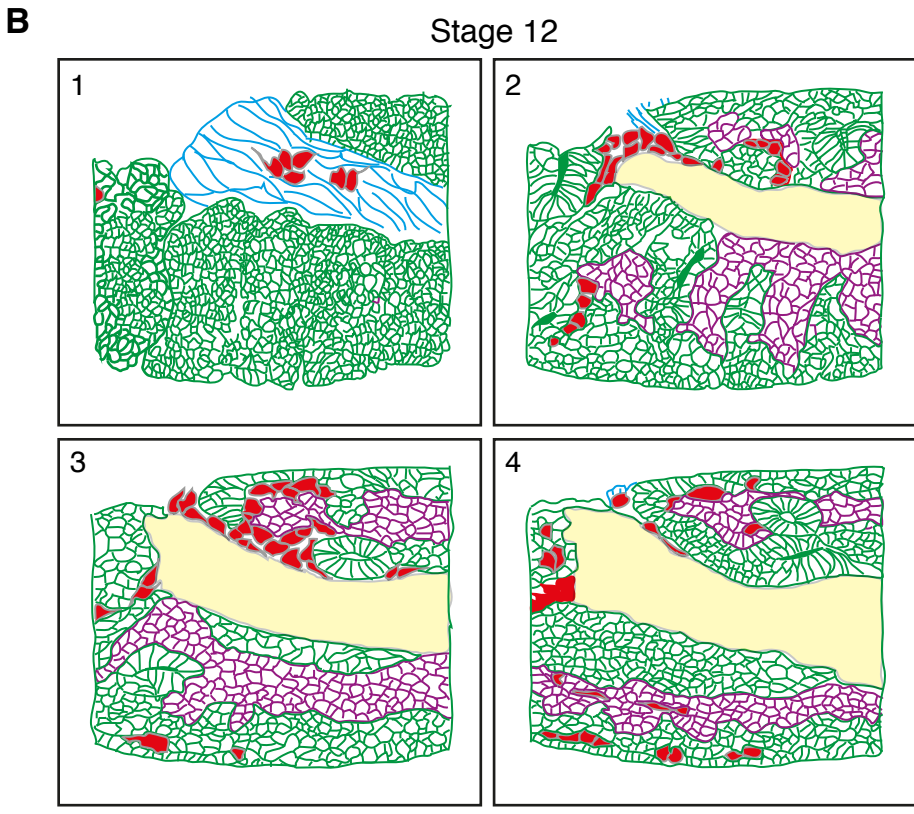
ectoderm : AJ

Figure S1: Macrophages invade at the ectoderm-mesoderm interface in the germband. Related to Figure 1.

(A,B) Cartoons showing consecutive sagittal sections (1-4) of a **(A)** Stage 11 or **(B)** Stage 12 *Drosophila* embryo. Embryo is shown with anterior to left and dorsal up. A: ante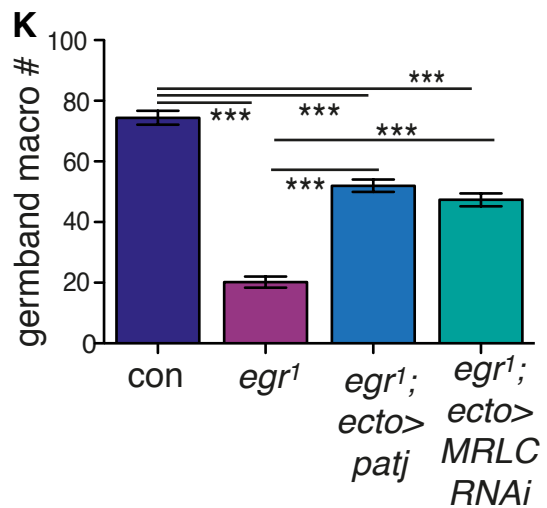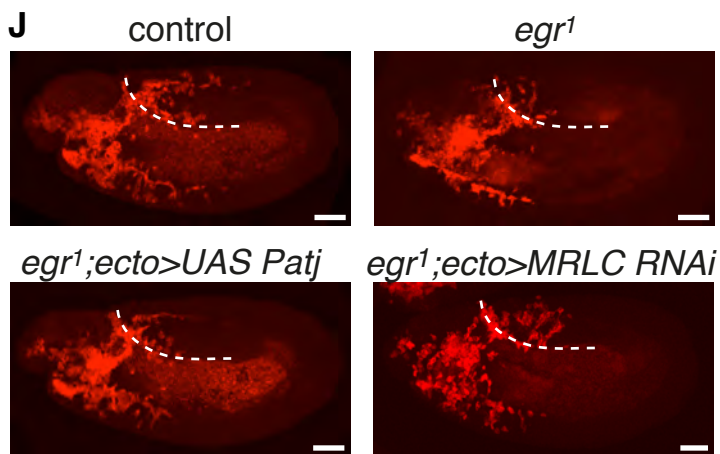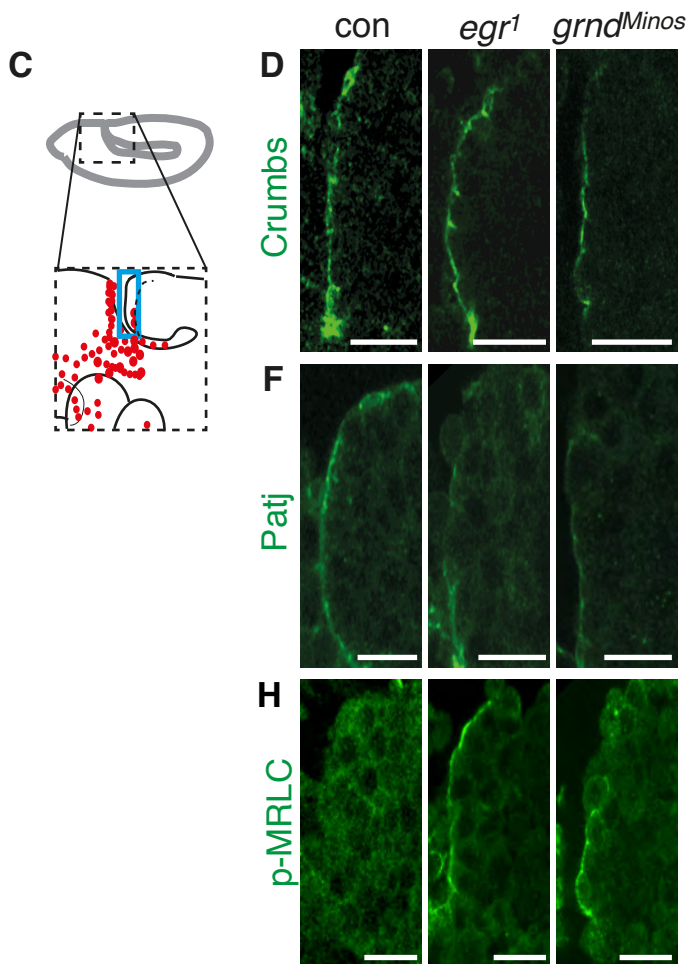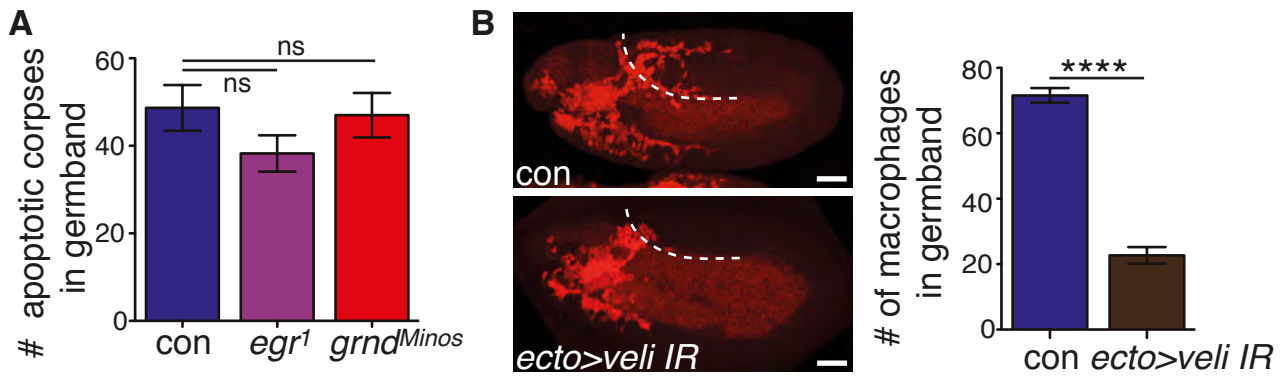rior, P: posterior, D: dorsal, V: ventral. Ectoderm (green), mesoderm (purple), yolk (yellow), amnioserosa (blue) and macrophages (red) are depicted.

(C) Schematic drawing of a lateral Stage 10 embryo with macrophages depicted as red dots. The black dotted line within the germband in the schematic indicates the ectoderm-mesoderm interface. Blue box indicates the area visualized in **(D)**, a Transmission electron microscopy (TEM) image of the germband of an early embryo in which the macrophages have not yet reached the germband**.** A magnification of the region indicated within the white dotted box is shown in Figure 1G. **(E)** TEM image showing a magnification of the area shown within the green box in **D**. White arrow indicates the presence of Adherens Junctions between ectodermal cells.

Embryo is shown with anterior to left and dorsal up. Embryos were selected for Stage 11 if they displayed germband retraction away from the anterior of 29-31% and for Stage 12 retraction of 35-40%. Scale bar represents 10μm in **D** and 2μm in **E**.

Figure S2

A

con

egr^1

B

start of germband retraction (minutes)

con   egr^1

C

time for 35-41% germband retraction (minutes)

con   egr^1

D

con   egr^1 egr^{1}AS2>egr

E

con

AS1>UAS-egr IR

F

con

egr^1

H

# of macrophages (vnc)

T1   T2   T3

ns   ns   ns

G

control
egr^1

I

J

# of macrophages in anterior tip of head

ns

control   egr^1

K

total # of macrophages

ns

control   egr^1

L

M

control   egr^1

<0.3
0.3-0.5
>0.5

<0.3
0.3-0.5
>0.5

Persistence

low
medium
high

N

O

control   egr^1

0.5   3.5   0.5   3.5
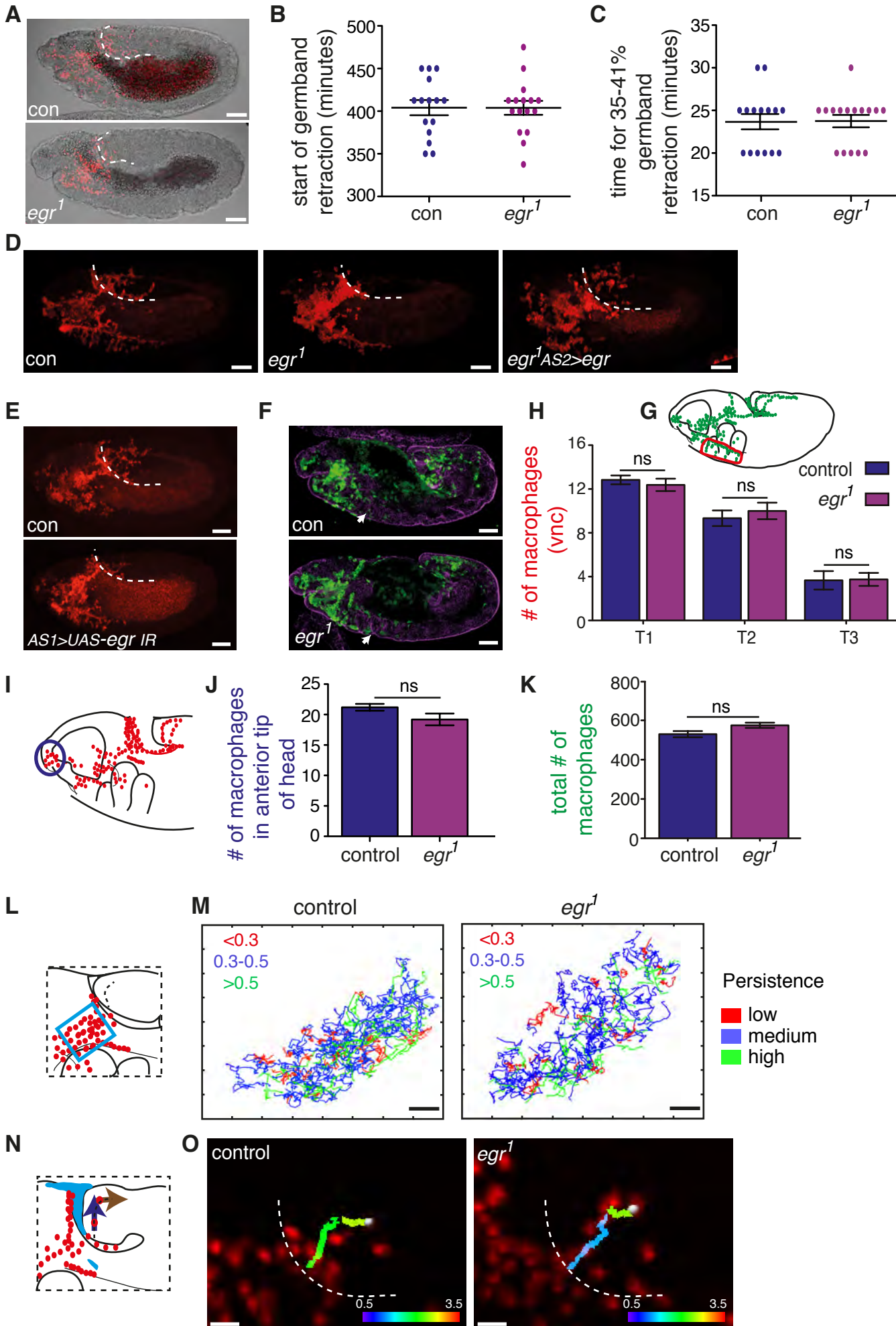
Figure S2: Amnioserosal Eiger regulates macrophage invasion of the embryonic germband but not migration along the vnc. Related to Figure 2.

(**A**) Confocal microscopy images of Z-projections of fixed lateral Stage 12 embryos. Control and *egr¹* mutant embryos are shown, with macrophages labeled in red by the expression of *srpHemo>H2A::RFP* and the embryo visualized with transmitted light. The dotted line demarcates the edge of the germband.

(**B,C**) Quantifications showed no significant change in either the start of the initiation of germband retraction (**B**, minutes after egg laying) or the time taken to complete 35-41% of germband retraction (**C**, minutes). n=15 for control and 16 for *egr¹* embryos.

(**D,E**) Confocal microscopy images of Z-projections of fixed lateral Stage 12 embryos. Macrophages are labeled in red by the expression of *srpHemo-H2A::3xmCherry*. The dotted line indicates the edge of the germband. AS stands for amnioserosa. (D) Control, *egr¹* mutant, and *egr¹* mutant embryos expressing *UAS-eiger* under the control of the LP1 amnioserosal driver, which was able to partially rescue the mutant phenotype. (**E**) Control embryos and those in which the amnioserosal *c381-Gal4* driver directs the expression of an RNAi against *eiger*.

(**F**) Confocal microscopy images of control and *egr¹* fixed lateral late Stage 12 embryos with macrophages labeled in green by the expression of *srpHemo>GFP*. Arrows point to the macrophages migrating along the vnc. (**G**) Schematic drawing of a lateral late Stage 12 embryo depicting macrophages with green dots. The red dotted area indicates the area analyzed to count the number of macrophages in the ventral nerve cord (vnc). (**H**) Quantification reveals that the number of macrophages migrating along the vnc is not significantly affected by the *egr¹* mutation. n=15 embryos for both genotypes.

(**J**) The number of macrophages in the anterior tip of the head (area within the blue circle in schematic **I**) and (**K**) the total number of macrophages within the entire embryo are not significantly affected by the *egr¹* mutation. n=20 for all genotypes in J,K.

(**L**) Schematic of the anterior half of a lateral Stage 11 embryo indicating the region analyzed (area within blue box) to assess the persistence of macrophage migration up to the germband in the two-photon movie tracks shown in the right two panels. (**M**) Representative tracks of macrophages in control and *egr¹* embryos from cell tracking in the region shown in blue box in **L**, color-coded according to low (<0.3; red), medium (0.3-0.5; blue) and high (>0.5; green) persistence of movement. n=3 embryos for both genotypes.

(**N**) Schematic indicating the regions of the germband in which the migration of the first macrophage was quantified. The amnioserosa (AS) is shown in light blue. The migration along the region adjacent to the amnioserosa is shown with a dark blue arrow and further migration along the germband with a brown arrow. (**O**) Tracks color-coded for mean instantaneous speed (µm/min) during migration within the region of the germband adjacent to the AS and further migration along the germband in control and *egr¹* embryos. n=3 embryos for both genotypes.

Anterior to left and dorsal up in all panels. The black dotted line within the germband in the schematics shown in **G,I,L,N** indicates the ectoderm-mesoderm interface. Embryos were selected for imaging and quantification as being Stage 11 if they displayed germband retraction away from the anterior of 29-31% and Stage 12 with 35-40%. Scale bar represents 50µm in **A,D,E,F** and 10µm in **M,O**. Histograms show mean ± s.e.m. ns=not significant. Unpaired t-test for **B,C,H,J,K.**

Figure S3

macrophages Eiger   Eiger

srpHemo-H2A::3xmCherry

A   **A'**

B   **B'**

C

total # of macrophages

con   *grnd^{Minos}*

ns

D   **E**   *grnd^{Minos}*

<0.3
0.3-0.5
>0.5

Persistence

low
medium
high

F

speed (*μm*/min)

con   *grnd^{Minos}*

ns

G

persistence

con   *grnd^{Minos}*

ns

H   control   *grnd^{Minos}*

I

macrophage # (vnc)

control
grnd^{Minos}

T1   T2   T3   A1   A2

ns   ns   ns   ns   ns
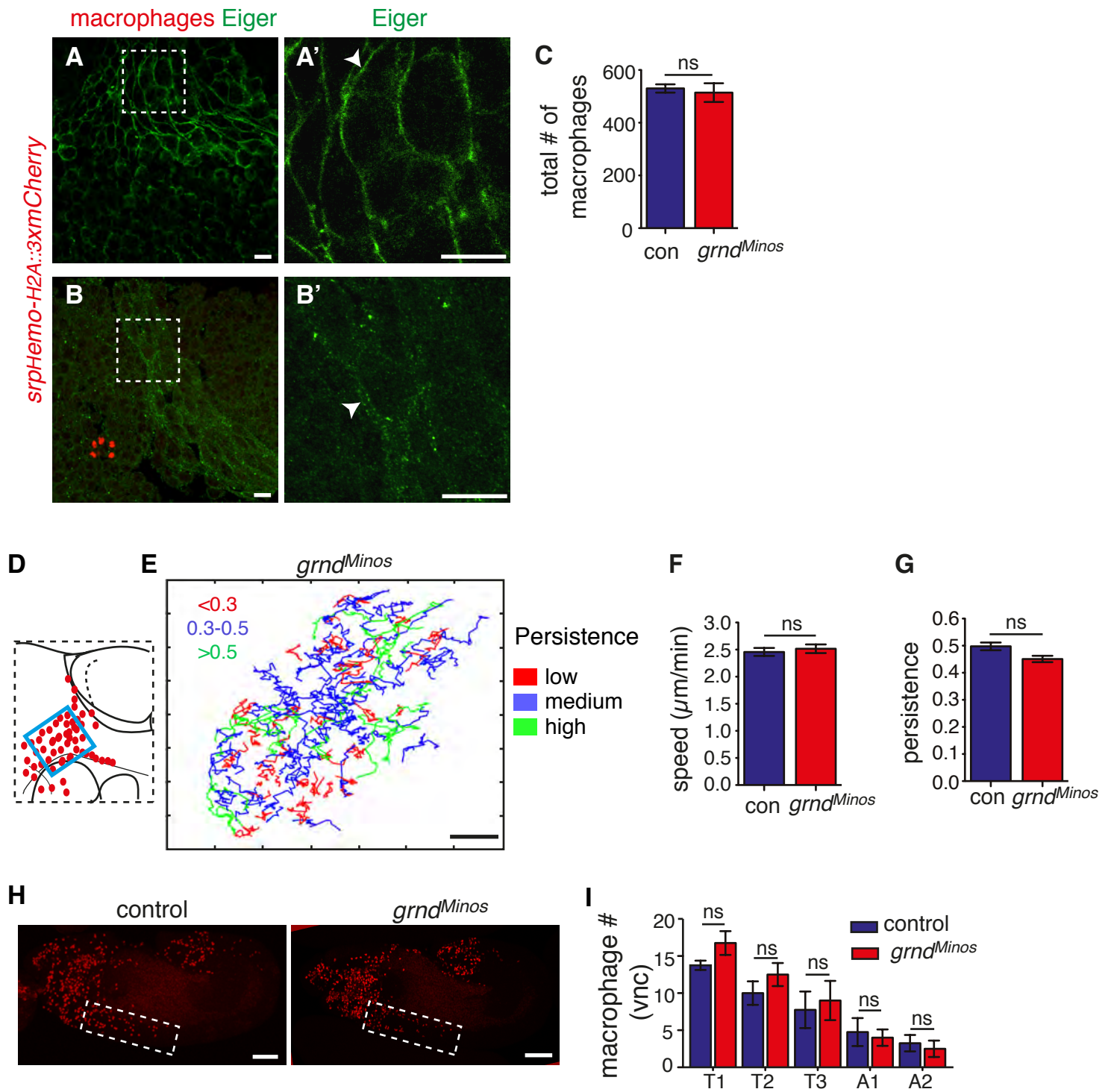
Figure S3: Grindelwald does not affect general macrophage migration. Related to Figure 3.

(**A,B**) Confocal images of a single sagittal section, from the same location as Figure 3A,B but at a position along the Z-axis that is closer to the viewer, in which the amnioserosa extends more broadly across the embryo in the anterior posterior direction, thus more extensively covering the germband ectoderm. Images are of (**A,A'**) a lateral control Stage 10 and (**B,B'**) a Stage 11 embryo with macrophage nuclei labeled by the expression of *srpHemo-H2A::3XmCherry* (red) and Eiger recognized with an antibody (green). Right panels show a magnification of the area indicated by the white dotted box in the adjoining panels on the left. (**A'**) We observe punctate membrane expression of Eiger in AS cells in Stage 10 (arrowhead) and (**B**) much less in Stage 11.

(**C**) The total number of macrophages in the embryo in late Stage 12 is not significantly (ns) affected by the *grnd*^{Minos} mutation. n=15 embryos for both genotypes.

(**D**) Schematic drawing of the anterior half of a lateral Stage 11 embryo indicating the region analyzed (area within blue box) to assess the persistence of macrophage migration up to the germband to produce the two-photon movie tracks shown on the right. The black dotted line within the germband in the schematic indicates the ectoderm-mesoderm interface. (**E**) Representative tracks of macrophages in *grnd*^{Minos} embryos from cell tracking in the region shown in the blue box in **D**, color-coded according to low (<0.3; red), medium (0.3-0.5; blue) and high (>0.5; green) persistence of movement. (**F,G**) No significant change was observed in the *grnd*^{Minos} mutant in speed or persistence. n=3 embryos for each genotype.

(H) Confocal microscopy images of Z-projections of fixed lateral late Stage 12 (greater than 45% retraction of the germband away from the anterior) control and *grnd^{Minos}* mutant embryos are shown with macrophages labeled in red by the expression of *srpHemo-H2A::3xmCherry*. The dotted area indicates the region quantitated to determine macrophage migration along the ventral nerve cord (vnc).

(I) Quantification of the number of macrophages in each indicated thoracic (T1-3) or abdominal (A1-2) segment along the vnc. No significant difference was seen when comparing control and *grnd^{Minos}* embryos. n=15 embryos for both genotypes.

Anterior to left and dorsal up in all panels. Embryos were selected for Stage 10 if they displayed germband retraction away from the anterior of less than 29%, Stage 11 if they displayed germband retraction away from the anterior of 29-31% and for Stage 12 retraction of 35-40%. Scale bar represents 10μm in **A-B',E** and 50μm in **H**. Histograms show mean ± s.e.m. ns=not significant. Unpaired t-test for **C** and **I** and One-Way ANOVA with Dunnett post test for **F** and **G**.

Figure S4

A E-Cadherin Interface intensity — con, *egr¹*, Stage 10, ns

B N-Cadherin Interface intensity — con, *egr¹*, Stage 10, ns

C E-Cad N-Cad macrophages — control, *egr¹*, Stage 11 overview

D Laminin A Interface intensity — con, *egr¹*, Stage 10, ns

E macrophages Lan A — *egr¹*, Lan A, Stage 11 overview

F Dystroglycan Interface intensity — con, *egr¹*, Stage 10, ns

G β PS integrin Interface intensity — con, *egr¹*, Stage 10, ns

H Talin Interface intensity — con, *egr¹*, Stage 10, ns

I Dystroglycan macrophages — control, *egr¹*, Stage 11 overview

J E-Cad β PS macrophages — control, *egr¹*, Stage 11 overview

K E-Cad Talin macrophages — control, *egr¹*, Stage 11 overview

L Stage 10 vs Stage 11

	control	*egr¹*
E-Cadherin	ns	ns
N-Cadherin	ns	ns
Dystroglycan	ns	ns
LamininA	**	**
β PS integrin	ns	ns
Talin	ns	ns

ns = p>0.05, ** = p<0.05

M Laminin A mesoderm junctional intensity — con, *egr¹* (Stage 10); con, *egr¹* (Stage 11); ns, **, **, ns

Figure S4: Eiger (Dm-TNF) does not regulate adhesion at the ectoderm-mesoderm interface. Related to Figure 4.

(A,B,D,F-H) Quantitation of the (**A**) E-Cadherin, (**B**) N-Cadherin, (D) Laminin A (LanA), (**F**) Dystroglycan, (**G**) β-PS integrin, and (**H**) Talin levels at the ectoderm-mesoderm interface in Stage 10 control (con) and egr^1 mutant embryos.

(**C,E,I-K**)) Confocal images of the germband ectoderm [area in schematic in (Figure 4A)] from a single sagittal plane of fixed lateral Stage 11 control and egr^1 mutant embryos showing staining with an antibody against (C) E-Cadherin (green) and N-Cadherin (magenta), (E) LanA (green), (I) Dystroglycan (green) or (J,K) DE-Cadherin in green and (J) β-PS integrin or (K) Talin in magenta. Macrophages labeled in red by the expression of *srpHemo-3xmCherry (egr^1 mutant* in C,E) or *srpHemo-H2A::3xmCherry*. Dotted lines indicate the ectoderm-mesoderm interface.
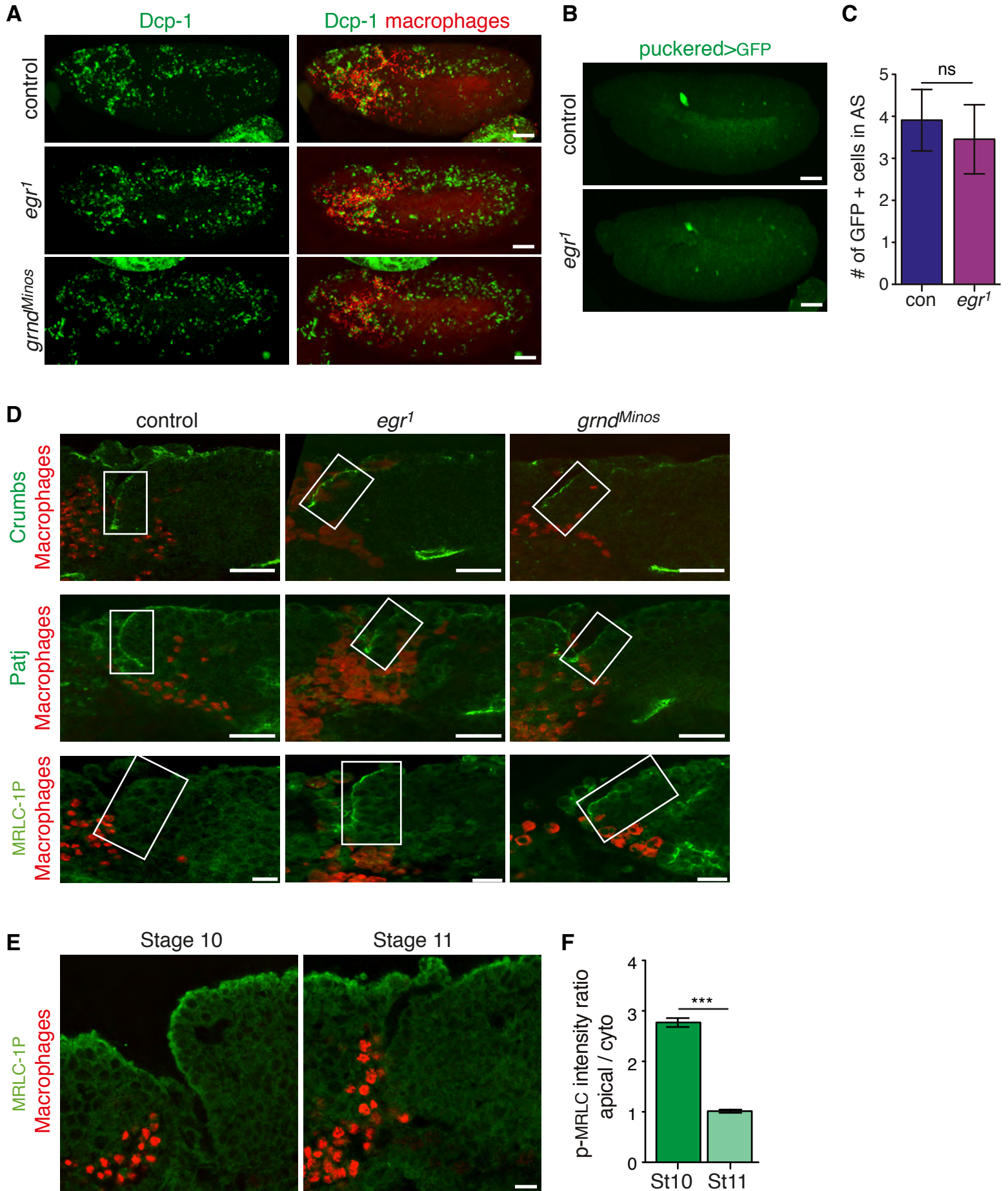
 (**L**) A table showing a comparison of the levels of adhesive proteins at the ectoderm-mesoderm interface between Stage 10 and Stage 11 for both control and egr^1 mutant embryos.

(**M**) Quantitation of the junctional LanA levels in the mesodermal junctions in Stage 10 and Stage 11 control (con) and egr^1 mutant embryos.

Anterior to left and dorsal up in all panels..Embryos were selected for Stage 10 if they displayed germband retraction away from the anterior of less than 29% and Stage 11 if they displayed 29-31% retraction with macrophages at and near the edge of the germband. Scale bar represents 20μm in left panel in **E** and 10μm in all the other images.

Histograms show mean±s.e.m. **P<0.01, ns=not significant, Unpaired t-test for **A,B,D,F-H,L** and One-Way Anova with Tukey post test in **M**.

Figure S5

A

Dcp-1 | Dcp-1 macrophages

control

egr¹

grnd^Minos

B

puckered>GFP

control

egr¹

C

# of GFP + cells in AS

ns

con   *egr¹*

D

control | *egr¹* | *grnd^Minos*

Crumbs / Macrophages

Patj / Macrophages

MRLC-1P / Macrophages

E

Stage 10 | Stage 11

MRLC-1P / Macrophages

F

p-MRLC intensity ratio apical / cyto

***

St10   St11

Figure S5: Eiger and Grindelwald do not regulate cell death and JNK activity to support macrophage germband invasion. Related to Figure 5.

(A) Confocal microscopy images of Z-projections of fixed lateral Stage 12 control, *egr¹* mutant and *grnd^Minos* mutant embryos with apoptotic nuclei visualized by antibody staining against Dcp-1 (green) and a merge showing macrophages visualized by *srpHemo-3XmCherry* expression (red) along with the apoptotic nuclei (green).

(B) Confocal microscopy images of Z-projections of fixed lateral Stage 11 control, (*UAS-GFP; srpHemo-3XmCherry pucE69-GAL4*) and *egr¹* mutant embryos (*UAS-GFP egr¹; srpHemo-H2A::3XmCherry pucE69-GAL4*) with JNK activity visualized by the *pucE69-GAL4 UAS-GFP* reporter.
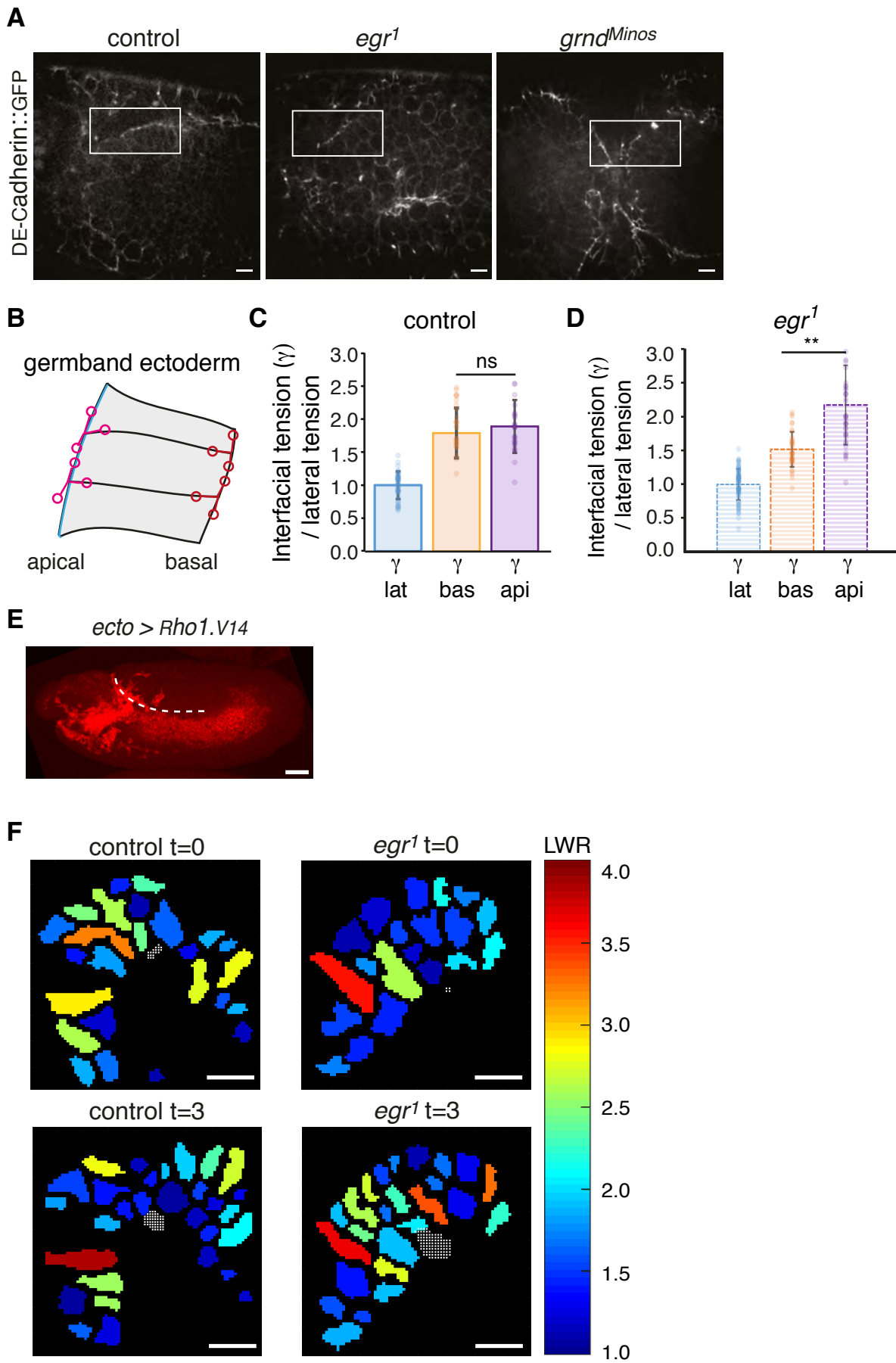
(C) Quantification of the number of GFP positive cells in the amnioserosa in the control (con) and *egr¹* mutant embryos. n=15 embryos for both genotypes.

(D) Confocal images of the germband ectoderm [area in schematic in **(Figure 5D)]** from a single sagittal plane of lateral Stage 12 fixed embryos from control, *egr¹* and *grnd^Minos* embryos immunolabeled for Crumbs, Patj or a phosphorylated form of Myosin Regulatory Light Chain (p-MRLC, also called Sqh-1P) and macrophages labeled by the expression of *srpHemo-H2A::3xmCherry* or *srpHemo-3XmCherry*. Magnifications of the boxed areas from the images are shown in Figure 5 **D,F,H.**

(E) Confocal images of the germband ectoderm [area in schematic in Figure **5C]** from a single sagittal plane of fixed lateral Stage 10 or Stage 11 embryos, showing staining with an antibody against Sqh-1P in green and macrophages labeled in red by the expression of *srpHemo-H2A::3xmCherry*. **(F)** Quantification of apical Sqh-1P levels in the germband ectoderm normalized to the cytoplasmic levels in Stage 10 and Stage 11 embryos. n=5 embryos, 42 contacts for both genotypes.

Anterior to left and dorsal up in all panels. Embryos were selected for Stage 10 if they displayed germband retraction away from the anterior of less than 29%, Stage 11 for displaying germband retraction away from the anterior of between 29-31%, with macrophages at and near the edge of the germband, and Stage 12 with 35-40% retraction. Scale bar 50μm in **A,B,** 20μm in **D,** and 10 μm in **E**. Histograms show mean ± s.e.m. ns=not significant, ***P<0.001. Unpaired t-test for **C,F**.

A

DE-Cadherin::GFP

control   *egr¹*   *grnd^{Minos}*

B

germband ectoderm

apical   basal

C control

Interfacial tension (γ) / lateral tension

ns

γ lat γ bas γ api

D *egr¹*

Interfacial tension (γ) / lateral tension

**

γ lat γ bas γ api

E *ecto > Rho1.V14*

F

control t=0   *egr¹* t=0   LWR

control t=3   *egr¹* t=3

Figure S6: Eiger regulates ectodermal cortical tension and facilitates ectoderm cell deformations. Related to Figure 6.

(A) Spinning disc images of control, *egr¹* and *grnd^Minos* embryos labeled with *knock in DE-Cad::GFP*. Boxes indicate the region where the laser cuts were conducted.

(B) Schematic of a few cells from the germband ectoderm showing representative cell triple interfacial junctions at apical (magenta) and basal (brown) interfaces, as part of the CellFIT-3D based tension analysis.

(C,D) Interfacial tensions of the germband ectoderm determined by CellFIT-3D analysis. Interfacial tensions in Stage 11 (C) control and (D) *egr¹* mutant embryos of the lateral, basal and apical regions are shown normalized to the tension of the lateral side.

(E) Confocal microscopy image of a fixed lateral Stage 12 embryo carrying *e22c-GAL4* to drive ectodermal expression of a dominant active form of Rho1 (*UAS-Rho1.V14*) in an embryo with *srpHemo-3XmCherry* labeling macrophages. n=20 embryos for both genotypes.

(F) Representative time points (t=0 and t=3, see **Figure 6H**) from time-lapse analysis of ectodermal cell deformation during macrophage entry in control and *egr¹* embryos. The length/ width ratio (LWR) of the ectodermal cells is shown color-coded according to the scale on the right; the white dotted region is the macrophage.

Embryos shown with anterior to the left and dorsal up. Embryos were selected as Stage 11 for displaying germband retraction away from the anterior of between 29-31%, with macrophages at and near the edge of the germband, and Stage 12 with 35-40% retraction. Histograms show mean ± s.e.m. **P<0.01, Unpaired t-test for **C,D**. Scale bar represents 50μm in **E** and 10μm in **A,F.**