A genetic program boosts mitochondrial function to power macrophage tissue invasion

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SUMMARY

Metabolic adaptation to changing demands underlies homeostasis. During inflammation or metastasis, cells leading migration into challenging environments require an energy boost, however what controls this capacity is unknown. We identify a previously unstudied nuclear protein, Atossa, as changing metabolism in *Drosophila melanogaster* immune cells to promote tissue invasion. Atossa's vertebrate orthologs, FAM214A-B, can fully substitute for Atossa, indicating functional conservation from flies to mammals. Atossa increases mRNA levels of Porthos, an unstudied RNA helicase and two metabolic enzymes, LKR/SDH and GR/HPR. Porthos increases translation of a gene subset, including those affecting mitochondrial functions, the electron transport chain, and metabolism. Respiration measurements and metabolomics indicate that Atossa and Porthos powers up mitochondrial oxidative phosphorylation to produce sufficient energy for leading macrophages to forge a path into tissues. As increasing oxidative phosphorylation enables many crucial physiological responses, this unique genetic program may modulate a wide range of cellular behaviors beyond migration.

Keywords: immune cell, invasion, metabolism, mitochondria, translation, oxidative phosphorylation, RNA helicase, tissue infiltration, migration

INTRODUCTION

Charged with protecting the organism against continuously changing threats, the immune system must constantly adapt, altering the location, number, and differentiation status of its different immune cell subtypes (Nicholson, 2016). Such continuous adjustment comes at a cost, as it requires high levels of energy. However, how immune cells adjust their metabolic capacities to achieve these increased metabolic requirements is just beginning to be

47 understood (Guak et al., 2020; O'Neill et al., 2016). The main energy currency in the cell is
48 ATP. The conversion of carbohydrates into ATP is mediated mostly by cytoplasmic
49 glycolysis and the mitochondrial TCA cycle that feeds electron donors into oxidative
50 phosphorylation (OxPhos) complexes I through IV. Anaerobic glycolysis is quick and does
51 not require oxygen, but respiratory OxPhos extracts considerably more ATP from a single
52 molecule of glucose, albeit more slowly (Berg et al., 2002). Amino acids and fatty acids also
53 feed into the TCA cycle and fuel OxPhos (O'Neill et al., 2016). OxPhos is most directly
54 regulated by the activity and the amount of complexes I through V that carry it out
55 (Hüttemann et al., 2007), but can also be affected by mitochondrial fusion (Rambold et al.,
56 2015) and biogenesis (Le Bleu et al., 2014). Upregulation of OxPhos is known to be required
57 for many important immune cell functions, such as B cell antibody production (Price et al.,
58 2018), pathogenic T cell differentiation during autoimmunity (Shin et al., 2020), and CD8+
59 memory T cell development and expansion (van der Windt et al., 2012; van der Windt et al.,
60 2013), T reg suppressive function (Angelin et al., 2017; Weinberg et al., 2019; Beir et al.,
61 2015) and the maturation of anti-inflammatory macrophages (Vats et al., 2006). However,
62 what genetic programs immune cells utilize to upregulate OxPhos remains unclear and how
63 such shifts in metabolism could influence immune cell migration is unexplored.
64

65 Immune cells move within the organism to enable their distribution and maturation (Kierdorf
66 et al., 2015; Masopust and Schenkel, 2013), as well as to detect and respond to homeostatic
67 challenges, injuries, tumors or infections (Woodcock et al., 2015; Luster et al., 2005;
68 Ratheesh et al, 2015). To migrate across unimpeded environments cells expend energy to
69 restructure their own actin cytoskeleton, activate myosin ATPase, and reorganize their cell
70 membrane (Bernstein and Bamburg, 2003; Cuvelier et al., 2007; Rottner and Schaks, 2019;
71 Li et. al, 2019). Even greater energy requirements exist when cells must also remodel their
72 surroundings as they move ahead against the resistance of flanking cells or extracellular
73 matrix (Zanotelli et al., 2018; Zanotelli et al., 2019, Cunniff et al., 2016; Kelley et al., 2019).
74 Most studies on the metabolism that enables immune cell migration *in vitro* or *in vivo* have
75 highlighted the importance of glycolysis, in macrophages, dendritic cells and regulatory T
76 cells (Guak et al., 2018; Kishore et al., 2018; Semba et al., 2016; Liu et al., 2019). To our
77 knowledge, only one study has demonstrated a need for a functional electron transport chain
78 (ETC) to speed neutrophil migration *in vivo* (Zhou et al., 2018) potentially through polarized
79 secretion of ATP to amplify guidance cues (Bao et al., 2015). In cancer cells an increase in
80 the transcription of mitochondrial genes, mitochondrial biogenesis and thus OxPhos by PGC-
81 1 appears to underlie enhanced invasion and metastasis (Le Bleu et al., 2014). OxPhos is
82 particularly required in the first cancer cell leading coordinated chains into challenging
83 environments *in vitro* (Khalil et al., 2010; Commander et al., 2020); these leader cells have
84 been shown to need higher ATP levels to create a path (Zhang et al., 2019). Although the
85 ability of immune cells to invade tissues or tumors also depends on movement against
86 surrounding resistance, it is not known if immune cells similarly require OxPhos for such
87 infiltration.
88

89 To identify new mechanisms governing *in vivo* migration, we study *Drosophila*
90 macrophages, also called plasmatocytes. Macrophages are the primary phagocytic and

91 innate immune cell in *Drosophila* and share remarkable similarities with vertebrate
92 macrophages in ontogeny, functional properties, and migratory behavior (Brückner et al.,
93 2004; Nourshargh and Alon, 2014; Ratheesh et al., 2015; Weavers et al., 2016; Wood and
94 Martin, 2017; Weavers et al., 2020). Phagocytic macrophages not only resolve infections,
95 but also influence development and homeostasis (Caputa et al., 2019; Riera-Domigo et al.,
96 2020; Buck et al., 2016; Bunt et al., 2010). Embryonic *Drosophila* macrophages follow
97 guidance cues to disseminate along predetermined routes (Cho et al., 2002; Brückner et al.,
98 2004; Wood et al., 2006) from their initial site of specification. During embryogenesis a
99 dynamic chain of macrophages invades into the extended germband between the closely
100 apposed ectoderm and mesodermal tissues, moving against the resistance of surrounding
101 tissues (Siekhaus et al., 2010; Ratheesh et al., 2018; Valoskova et al., 2019). Importantly,
102 the rate limiting step for this tissue invasion is the infiltration of the pioneer macrophage, a
103 process affected both by the properties of the surrounding tissues (Ratheesh et al., 2018) as
104 well as macrophages themselves (Valoskova et al., 2019; Belyaeva et al., 2021).

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106 Here we identify a program that powers the invasive capability of these pioneer
107 macrophages *in vivo*. We characterize a metabolic shift orchestrated in these immune cells
108 by a single previously unexamined nuclear factor that we name Atossa. We show that
109 Atossa induces higher mRNA levels of two metabolic enzymes and a previously unstudied
110 helicase. This helicase which we name Porthos enhances translation of a diverse set of
111 proteins, including those affecting mitochondrial and metabolic function, to increase
112 OxPhos and ATP. Our work thus reveals a detailed cellular mechanism that induces
113 concerted metabolic and mitochondrial reprogramming to support higher energy levels.
114 Given that we find that Atossa's mammalian orthologs maintain its function, our data lay
115 the foundation for mammalian studies on diverse pathological conditions, from
116 autoimmunity to cancer, as well as those independent of invasion.

117

118 **RESULTS**
119 ***CG9005* is required in macrophages for their early invasion into the extended germband**
120 To identify unknown molecular pathways mediating germband invasion, we searched for
121 previously uncharacterized genes enriched in macrophages prior to and during germband
122 tissue entry. Examining the BDGP *in situ* project (https://insitu.fruitfly.org/cgi-
123 bin/ex/report.pl?ftype=1&ftext=FBgn0033638) we identified CG9005 as a gene fitting these
124 requirements. CG9005 is enriched in macrophages from their birth through their invasion of
125 the germband. CG9005 is maternally deposited and expressed in all mesodermal cells during
126 stage 4-6 when macrophages are specified in the head mesoderm. CG9005 is further
127 upregulated in macrophages starting at Stage 7 while its expression decreases in the
128 remaining mesoderm. CG9005 continues to be expressed during Stage 9-12 in macrophages,
129 during their ingression, dissemination, and movement towards and into the germband. After
130 invasion, CG9005 is downregulated in macrophages to match the lower expression levels
131 found ubiquitously in the embryo.

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133 We examined a P element insertion allele, *CG9005^{BG02278}*, henceforth abbreviated to

134     $CG9005^{PBG}$, visualizing macrophages through expression of a nuclear fluorescent marker.

135   Quantification of the number of macrophages within the germband in fixed embryos at Stage

136   12 revealed a 36% decrease in $CG9005^{PBG}$ mutant embryos compared to the control (Figs.

137   1A-B and 1D), similar to that seen when $CG9005^{PBG}$ was placed over either $Df(2R)ED2222$

138   or $Df(2R)BSC259$ that remove the gene entirely (Fig. 1D), demonstrating that $CG9005^{PBG}$ is a

139   genetic null for macrophage germband invasion. Expressing CG9005 in macrophages in the

140

141   **Figure 1. CG9005 acts in macrophages to spur pioneer cell infiltration into the germband tissue. Fig 1A-**
142   **C.** Representative confocal images of Stage 12 embryos from the control, the $P\{GT1\}CG9005^{BG02278}$ P element
143   mutant (henceforth called $CG9005^{PBG}$), and $CG9005^{PBG}$ with $CG9005$ expression restored in macrophages.
144   Macrophages (red) and phalloidin to visualize embryo (green). "*mac*" represents the *srpHemo-Gal4* driver.
145   Germband edge indicated by dotted white line. **Fig 1D.** Quantification reveals a significant decrease in the
146   number of macrophages that have penetrated the germband in Stage 12 embryos from $CG9005^{BG}$ (n=56), and
147   from $CG9005^{BG}$ over two deficiencies (Df) that completely remove the gene ($CG9005^{PBG}/Df1(2R)$ n=25 and
148     $CG9005^{PBG}/Df2(2R)$ n=9), compared to the control (n=35). Macrophage expression of $CG9005$ rescues the
149   mutant phenotype arguing that $CG9005$ is required only in macrophages for germband penetration (n=18 for
150   rescue, p<0.0001 for control vs mutant, p=0.98 for control vs rescue, p=0.001 for mutant vs rescue).
151   *Df1(2R)=Df(2R)ED2222. Df2(2R)=Df(2R)BSC259.* **Fig 1E.** Macrophage specific knockdown of $CG9005$ by
152   *UAS* RNAi lines under the control of *srpHemo-GAL4* can recapitulate the mutant phenotype (control 1 n=22,
153   *CG9005 RNAi 1* (VDRC 106589) n=20; p<0.0001; control 2 n=21, *CG9005 RNAi 2* (VDRC 36080) n=23;
154   p<0.0001; control 3 n=35, *CG9005 RNAi 3* (BL33362) n=28, p<0.0001). **Fig 1F.** Stills from two-photon movies
155   of control and $CG9005^{PBG}$ mutant embryos showing macrophages (nuclei, red) migrating starting at Stage 10
156   from the head towards the germband and invading into the germband tissue. Elapsed time indicated in minutes.
157   The germband edge (white dotted line) was detected by yolk autofluorescence. **Fig 1G-H.** Quantification shows
158   no change in macrophage migration speed (**G**) in the head or (**H**) between the yolk sac and the germband edge
159   in the $CG9005^{PBG}$ mutant compared to the control. Head speed: control and mutant=2.2 µm/min; movie #:
160   control=8, mutant=3; track #: control=360, mutant=450, p=0.65. Between yolk sac and germband speed:
161   control=2.6 and mutant=2.4 µm/min; # movies: control=7, mutant =3; # tracks: control=46, mutant=19, p=0.62.
162   **Fig 1I.** The time required for the first macrophage nucleus to enter into the extended germband is increased by
163   65% in the $CG9005^{PBG}$ mutant compared to the control (control=22.8 min, n=7, mutant=37.4 min, n=5,
164   p<0.0001). **Fig 1J-K**. The migration speed of the first and second macrophage into the germband between the
165   mesoderm and ectoderm is significantly slower in the $CG9005^{PBG}$ mutant compared to the control. First
166   macrophage speed: control=2.5 and mutant =2.1 µm/min, movie #: control=6, mutant=5, p=0.012. Second
167   macrophage speed: control=2.9 and mutant=2.2 µm/min, movie #: control=5, mutant=5, p=0.03. **Fig 1L.** The
168   migration speed of the third to fifth macrophage nuclei along the first 25-30 um of the path between the
169   germband mesoderm and ectoderm is similar in the $CG9005^{PBG}$ mutant and the control (speed: control=2.5 and
170   mutant=2.4 µm/min, movie #: control=5, mutant=4, p=0.17). In schematics, macrophages are shown in red and
171   analyzed macrophages in light blue, the ectoderm in green, the mesoderm in purple, and the yolk in beige.
172   Macrophage nuclei visualized by *srpHemo-H2A::3xmCherry* expression. See also **Fig. S1** and **Videos 1** and **2**.
173   Throughout this work, embryos were staged based on germband retraction away from the anterior of less than
174   29% for stage 10, 29%-31% for stage 11, and 35%–40% for stage 12. In all figures and histograms show
175   mean±SEM and ns=p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. One-way ANOVA with Tukey for
176   (**D-E**), and unpaired t-test for (**G-L**). Scale bars: 50 µm in (**A-C**), 30 µm in (**F**).
177   See also **Figure S1** and **Videos 1** and **2**: representative movies of macrophage migration into the germband in
178   the control (**Video 1**) and the $CG9005^{PBG}$ (*atos*) mutant (**Video 2**). Macrophages (red) are labeled
179   with *srpHemo-H2A::3xmCherry*. Arrow indicates first macrophage moving into the germband. The time
180   interval between each acquisition is 40 s and the display rate is 15 frames/s. Scale bar: 20 µm.

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183   mutant completely restored their capacity to invade the germband (Figs. 1C-D). Depleting

184   *CG9005* by driving any one of three independent RNA interference (RNAi) lines in

185   macrophages caused a 37-40% decrease in macrophages within the germband compared to

186   controls (Fig. 1E). We also observed 24-27% more macrophages sitting on the yolk near the

187   entry site that have not yet invaded the germband in $CG9005^{PBG}$ (Fig. S1A) and in the RNAi

188   lines (Fig. S1B) compared to their controls. This finding supports the conclusion that
189   macrophages in these backgrounds migrate normally up to the germband but are less able to
190   enter. We counted macrophages migrating along the ventral nerve cord (vnc) in late Stage 12
191   embryos, a route guided by the same factors that lead into the germband (Brückner et al.,
192   2004; Cho et al., 200; Wood et al., 2006) but that does not require tissue invasion (Siekhaus
193   et al., 2010; Weavers et al., 2016). There was no significant difference in both the
194   *CG9005*^{PBG} mutant (Fig. S1C) and the *CG9005* RNAi-expressing macrophages (Figs. S1D-F)
195   compared to their controls, arguing that basic migratory processes and recognition of
196   chemotactic signals are unperturbed. Moreover, we detected no significant change in the total
197   number of macrophages for any of these genotypes (Figs. S1G-H). Taken together, these
198   results from fixed embryos clearly suggest that CG9005 is specifically required in
199   macrophages for the early steps of germband invasion.
200

201   **Atossa (CG9005) promotes efficient invasion of pioneer macrophages into the**
202   **germband tissue**
203   To directly assess CG9005's role in germband invasion, we conducted two-photon live
204   imaging. We labeled macrophages with the nuclear marker *srpHemo-H2A::3xmCherry* in
205   control and *CG9005*^{PBG} embryos (Figs. 1F and S1I, Videos 1 and 2). We observed no
206   significant change in speed or directionality during macrophage migration from their initial
207   position at Stage 9 in the head mesoderm up to the yolk neighboring the germband entry
208   point in *CG9005*^{PBG} (Fig. 1G, Figs. S1J-L) (speed in the head and yolk: 2.2 µm/min for both
209   the control and *CG9005*^{PBG}; p=0.65, p=0.78 respectively), nor in their directionality within
210   these regions (directionality: 0.39 in control and 0.37 in mutant in both regions, p=0.74 for
211   head, p=0.86 for yolk). We also observed no significant change in migration speed for
212   macrophages moving between the yolk and ectoderm (control=2.6 and *CG9005*^{PBG}=2.5
213   µm/min, p=0.62) (Fig. 1H). However, the first macrophage in *CG9005*^{PBG} required 65%
214   more time than the control to enter into the germband tissue (time to entry: control=23 min
215   and *CG9005*^{PBG}=38 min, p<0.0001) (Fig. 1I). The speed of the first two pioneering
216   macrophages is also significantly slower as they invade along the path between the mesoderm
217   and ectoderm in *CG9005*^{PBG} mutant embryos compared to the control (1st cell: control=2.5
218   and *CG9005*^{PBG}=2 µm/min, p=0.012; 2nd cell: control=2.9 and *CG9005*^{PBG}=2.1 µm/min,
219   p=0.03) (Figs. 1J-K). However, the speed of the next few cells migrating along this path was
220   not affected (3rd-5th cells: control=2.5 and *CG9005*^{PBG}=2.4 µm/min, p=0.17) (Fig.1L). We
221   conclude that CG9005 specifically regulates tissue invasion, facilitating the initial entry into
222   and subsequent movement within the germband tissue of the first two pioneer macrophages.
223   Since the macrophage stream invading the germband becomes a trickle in *CG9005*^{PBG} we
224   named the gene *atossa* (*atos*), for the powerful Persian queen whose name means trickling.
225
226

227   **Atossa (CG9005) is a nuclear protein whose conserved motifs and TADs are important**
228   **for macrophage tissue invasion, a function conserved by its vertebrate orthologs**
229   Atossa (Atos) contains a conserved domain of unknown function (DUF4210) and a
230   Chromosome segregation domain (Chr_Seg) (Fig. 2A). Atos also displays two trans-
231   activating domains (TADs) common among transcription factors as well as three nuclear

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232 localization signals (NLS) and a nuclear export signal (NES). We first tested the subcellular
233 distribution of the Atos protein, transfecting the macrophage-like S2R+ cell line with a
234 *FLAG::HA* tagged form of *atos* under the control of the macrophage promoter *srpHemo*. We
235 found Atos mainly in the nucleus colocalized with DAPI, and also partially in the cytoplasm
236 (Fig. S2A). When expressed *in vivo* in macrophages, Atos is also predominantly a nuclear
237 factor (Fig. 2B). To assess the importance of the conserved domains and TADs, we made
238 versions of Atos lacking these regions. All mutant forms were present in the nucleus similarly
239 to wild-type Atos (Fig. S2A). While expression of wild-type Atos in the macrophages of *atos*
240 embryos completely rescues germband invasion (Figs. 2C-D), Atos lacking either the
241 conserved DUF2140, the Chr_Seg domain, or either or both of the two TAD motifs failed to
242 do so (Figs. 2D, S2B-C). Consistent with a germband invasion defect, expression of these

243
244 **Figure 2. CG9005/Atossa requires conserved domains linked to transcriptional activation to enhance**
245 **tissue invasion, a function maintained by its mammalian orthologs.**
246 **Fig 2A.** Deduced protein structure of *Drosophila* CG9005/Atossa (Atos) and its murine orthologs, mFAM214A
247 and B. These proteins all contain the same conserved motifs: a domain of unknown function (DUF4210), a
248 domain associated with Chromosome segregation (ChromSeg), at least one transcriptional activation domain
249 (TAD), nuclear localization signal (NLS) and nuclear export signal (NES). FAM214A and B are 44-45%
250 identical to Atos. **Fig 2B.** Macrophages (red) near the germband in Stage 11/12 embryos display colocalization
251 of Atos tagged with HA (HA antibody, green) with the nucleus stained by DAPI (blue). *srpHemo-atos::H2A*
252 line utilized. **Fig 2C.** Representative confocal images of Stage 12 embryos from the control, *atos^{PBG}*, and
253 *atos^{PBG}* expressing Atos itself or variants lacking particular domains in macrophages. **Fig 2D.** Germband
254 macrophage quantification in control, *atos^{PBG}*, and *atos^{PBG}* expressing Atos or its altered forms in macrophages.
255 The tissue invasion defect in *atos^{PBG}* can be fully rescued by Atos expression in macrophages unless Atos lacks
256 the conserved DUF4210, ChrSeg, or TADs. Control n=32, mutant n=56, WT rescue n=18, DUF4210⁻ rescue
257 n=17, ChrSeg⁻ rescue n=21, DUF4210⁻/ChrSeg⁻ rescue n=19, TAD1⁻/TAD2⁻ rescue n=25. For control vs mutant
258 p<0.0001, for control vs rescue p=0.99, for mutant vs rescue p=0.0014. **Fig 2E.** Representative confocal images
259 of *atos^{PBG}* rescued with a murine ortholog, *mFAM214A* or *mFAM214B*, expressed in macrophages. **Fig 2F.**
260 Quantification of macrophages in the germband in Stage 12 embryos from the control, *atos^{PBG}*, and *atos^{PBG}*
261 embryos expressing *mFAM214A* or *mFAM214B* in macrophages shows that Atos's mammalian orthologs can
262 rescue *atos*'s macrophage tissue invasion defect. Control n=25, *atos^{PBG}* n=56, rescue with *atos* n=18, with
263 *mFAM214A* n=22, with *mFAM214B* n=25. For control vs *mFAM214A* and *mFAM214B* rescues p>0.05, for
264 *atos^{PBG}* vs *mFAM214A* and *mFAM214B* rescues p<0.005. *mFAM214A* or *B* are expressed under the direct
265 control of the *srpHemo* promoter. Throughout paper > indicates *GAL4 UAS* regulation. In (**C**) and (**E**)
266 macrophages (red) are visualized by *srpHemo-H2A::3xmCherry* expression and actin by Phalloidin staining
267 (green). One-way ANOVA with Tukey for (**D**) and (**F**). Scale bars are 5 µm in (**B**) and 50 µm in (**C**) and (**E**).
268 See also **Figure S2**.

269
270 Atos mutants led to a higher number of macrophages sitting on the yolk at the germband
271 entry site prior to invasion than in the rescue with wild-type Atos (Fig. S2D). These data
272 clearly show that the conserved domains and TADs are critical for the primarily nuclear
273 protein, Atos, to facilitate macrophage invasion.

274
275 Atos's uncharacterized murine orthologs, mFAM214A and mFAM214B, maintain these
276 domains, displaying 40% identity to their *Drosophila* counterpart (Fig. 2A). Expression in
277 macrophages of either mFAM214A or B in *atos^{PBG}* rescued the germband invasion defect as
278 efficiently as the *Drosophila* protein itself (Figs. 2E-F) and restored the normal number of
279 macrophages on the yolk next to the extended germband (Fig. S2E). Therefore, we conclude
280 that the molecular functions that enable Atos to promote macrophage tissue invasion are
281 maintained in vertebrates.
282

Atos raises mRNA levels of an RNA helicase and metabolic enzymes, which are each required for germband invasion

Given Atos's nuclear localization and requirement for TADs, we hypothesized that Atos might modulate transcription in macrophages to aid their initiation of germband invasion. To identify potential targets, we performed RNA-sequencing analysis on FACS isolated macrophages from wild type and *atos^{PBG}* embryos during germband invasion in early Stages 11-12 (Fig. S3A) (Supp. Data 1). Transcriptome analysis revealed 25 genes with reduced mRNA levels and 39 genes with higher ones in the absence of Atos, requiring a P value<0.05 (Fig. S3B). Gene ontology analysis (GO term) indicates that the significantly downregulated genes are involved in oxidation-reduction (redox) processes, stress responses as well as the nervous system (Fig. S3C). We therefore conclude that the presence of Atos in macrophages controls the mRNA levels of a small set of proteins.

We tested the hypothesis that the *atos^{PBG}* macrophage germband invasion defect is caused by the lower levels of the downregulated genes. We focused only on the 5 genes that had at least a >5-fold change in expression and were enriched in embryonic macrophages or had an identified molecular function (Fig. 3A). We expressed *RNAi* constructs against them in macrophages and observed a significant reduction in germband macrophage numbers for three of these 5 candidates (Figs. 3B-G, S3D-E). For all three we also observed an increase in the number of macrophages sitting on the yolk next to the germband before invasion, consistent with a specific defect in germband invasion (Figs. S3F-H). These were a predicted ATP-dependent RNA helicase (CG9253) we name Porthos (Pths) (Martin et al., 2021) (Figs. 3B, E), and two metabolic enzymes, Glyoxylate Reductase/Hydroxypyruvate Reductase (*dGR/HPR,* CG9331) (Figs. 3C and 3F) and Lysine α-Ketoglutarate Reductase/Saccharopine Dehydrogenase (*dLKR/SDH*, CG7144) (Figs. 3D, G). Downregulation of *Glycerophosphate oxidase 2* (*Gpo2*, CG2137) (Fig. S3D) and *Golgi matrix protein 130 kD* (*GM130*, CG11061) (Fig. S3E) did not produce any invasion defect. GR/HPR is highly conserved from bacteria to mammals and the *Drosophila* form shows 48% identity to its human ortholog (NCBI

Figure 3. Atos leads to higher mRNA levels of an RNA helicase and metabolic enzymes required for germband invasion.

Fig 3A. A selection of genes down-regulated in *atos^{PBG}* mutant macrophages compared to the control, chosen for having a >5 fold change in expression as well as an identified biological function. **Fig 3B-D.** Representative confocal images of early Stage 12 embryos from the control, and lines expressing an *RNAi* against (**B**) *porthos*, (**C**) *dGR/HPR* or (**D**) *dLKR/SDH* specifically in macrophages (red). *srpHemo-H2A::3XmCherry* labels macrophages. **Fig 3E.** Quantification of Stage 12 embryos reveals that expression of a *porthos RNAi* in macrophages decreases their number in the germband by 48%. Control n=36, *porthos RNAi* (BL36589) n=28, p<0.0001. **Fig 3F-G.** Quantification of Stage 12 embryos indicates that fewer macrophages have moved into the germband upon the expression in macrophages of any of (**F**) three different RNAis against *dGR/HPR* or (**G**) two different RNAis against *dLKR/SDH*, arguing that these metabolic enzymes are required in macrophages for tissue invasion. Control 1 n=18, *dGR/HPR RNAi 1* (VDRC 44653) n=18, p<0.0001, control 2 n=21, *dGR/HPR RNAi 2* (VDRC 107680) n=24, p<0.0001, control 3 n=15, *dGR/HPR RNAi 3* (VDRC 64652) n=23, p=0.08. *dLKR/SDH RNAi 1* (VDRC 51346) n=17, control 2 n=21, *dLKR/SDH RNAi 2* (VDRC 109650) n=23, p<0.0001. **Fig 3H.** Schematic illustrates how the bifunctional enzyme dGR/HPR can catalyze the reduction of glyoxylate into glycolate and convert hydroxypyruvate into D-glycerate by oxidation of the cofactor NAD(P)H. **Fig 3I.** Schematic shows the metabolic pathway in which *Drosophila* Lysine α-Ketoglutarate Reductase/Saccharopine Dehydrogenase (dLKR/SDH) catalyzes the first two steps of the lysine catabolism pathway, resulting in the production of glutamate and acetyl-CoA, a TCA substrate, through several downstream enzymatic reactions. Glu: Glutamate, α-KG: α-Ketoglutarate, AASA: α-Aminoadipate δ-semialdehyde. Unpaired t test for (**E-G**). Scale bar: 50 μm in (**B-D**). See also **Figure S3** and **Data S1. Data S1:** Annotated primary and normalized RNA sequencing data from FACS sorted control and *atos* macrophages.

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334 BLAST). GR/HPR is the linchpin of the glyoxylate cycle, catalyzing the reduction of
335 glyoxylate into glycolate and the conversion of hydroxypyruvate into D-glycerate (Fig. 3H)
336 (Booth et al., 2006). This contributes to glucose and urea synthesis. The bifunctional
337 enzyme dLKR/SDH is also highly conserved, with 71% identity to its human counterpart
338 (identified by NCBI BLAST). It catalyzes the first two steps of lysine catabolism and can
339 participate in the production of Acetyl CoA (Bhattacharjee, 1985) (Fig. 3I). We therefore
340 conclude that Atos enhances macrophage tissue invasion by increasing the levels of the
341 metabolic enzymes dLKR/SDH and dGR/HPR and the helicase ortholog Porthos.
342
343 **The nuclear RNA helicase, Porthos, functions downstream of Atos in pioneer**
344 **macrophages to allow their initiation of germband invasion**
345 Atos's target *porthos* (*CG9253*) displayed the strongest invasion defect upon RNAi
346 knockdown (KD) (Fig 3E). Porthos is a conserved DEAD-box RNA helicase (Fig. S4A)
347 sharing 71% identity and 84% similarity with its human ortholog, the helicase DDX47,
348 including the conserved DEAD motif and helicase C terminal domain, with which DDX47
349 interacts with RNA structures. *porthos* is expressed in the embryo by *in situ* analysis in a
350 pattern similar to *atos* but a few stages later, to *atos* in *Drosophila* embryos, being enriched
351 in macrophages in the head region during Stages 9-12 (https://insitu.fruitfly.org/cgi-
352 bin/ex/report.pl?ftype=1&ftext=FBgn0032919). In S2R+ cells, HA-tagged Porthos
353 colocalized with markers for the nucleus (DAPI) and the nucleolus (Fibrillarin), where
354 ribosome assembly and rRNA processing occur (Fig. 4SB). In embryonic macrophages
355 HA-tagged Porthos also localized to the nucleus, detected by DAPI (Fig. 4A).
356
357 **Figure 4. The nucleolar RNA helicase, Porthos, acts as a key downstream target of Atos to promote**
358 **pioneer macrophage germband invasion.**
359 **Fig 4A.** Macrophages (red) near the germband in Stage 11/12 embryos show partial colocalization of the HA
360 antibody labeling Porthos (green) with the nucleus stained by DAPI (blue). Embryo express *srpHemo-*
361 *porthos::HA*. **Fig 4B.** Stills starting at Stage 11 from two-photon movies of control embryos and those
362 expressing *porthos RNAi* in macrophages; stills show macrophage migration from the head mesoderm towards
363 and into the germband at the indicated time points. White dotted line indicates the germband edge. Macrophage
364 nuclei labeled by *srpHemo-H2A::3xmCherry*. *UAS-porthos RNAi* (BL36589) expressed by *srpHemo-GAL4*. **Fig**
365 **4C-H.** Quantification of macrophage migration parameters from two-photon movies. (**C-D**) Macrophages
366 expressing *porthos RNAi* migrate with a similar speed in the head and between the yolk sac and the germband
367 edge compared to the control. Speed in head: control=2.01 µm/min, *porthos RNAi*=2.09 µm/min; movie #:
368 control=4, *porthos RNAi*=6; track #: control=507, *porthos RNAi*=859, p=0.56. Speed between yolk sac and
369 germband mesoderm: control=2.17 µm/min, *porthos RNAi*=2.41 µm/min, p=0.45; movie #: control n=5, *porthos*
370 *RNAi* n=5, track #: control n=40, *porthos RNAi* n=51. **Fig 4E.** The time required for the first macrophage
371 nucleus to enter into the germband is significantly increased in embryos expressing *porthos RNAi* compared to
372 the control. Control=21.5 min, n=6, *porthos RNAi*=36.2 min, n=4, p<0.0001. Blue arrow in schematic indicates
373 route analyzed. **Fig 4F-G.** The speed of the first and second macrophage invading into the germband along the
374 path between the mesoderm and ectoderm is significantly slower in embryos expressing *porthos RNAi*
375 compared to the control. First macrophage speed: control=2.99 and *porthos RNAi*=2.0 µm/min; p=0.009; #
376 movies: control n=4, *porthos RNAi* n=4. Second macrophage speed: control=2.61 and *porthos RNAi*=1.98
377 µm/min; p=0.037; # movies: control n=6, *porthos RNAi* n=4. **Fig 4H.** The speed of the third to fifth
378 macrophages invading the germband is similar in macrophages downregulated for *porthos* and the control
379 (speed: control=2.66 and *porthos RNAi*=2.31 µm/min; p=0.21; # movies: control n=5, *porthos RNAi* n=4). **Fig**
380 **4I.** Representative confocal images of early Stage 12 embryos from control, *atos*[PBG], and *atos*[PBG] expressing
381 *atos::FLAG::HA* or *porthos::FLAG::HA* in macrophages (red) through *srpHemo-GAL4* control of *UAS*
382 constructs. Embryo detected by phalloidin staining (green). **Fig 4J.** Quantification of macrophages in the
383 germband shows that the *atos*[PBG] mutant phenotype can be substantially rescued by expressing
384 *porthos::FLAG::HA* in macrophages. Control (n=15), *atos*[PBG] (n=22), *atos*[PBG] with *srpHemo>atos::FLAG::HA*

8

385   (n=27), *srpHemo>porthos::FLAG::HA* (n=30). For control vs *atos^{PBG}* p<0.0001, for control vs *atos* rescue of
386   *atos^{PBG}* p<0.0001, for control vs *porthos* rescue of *atos^{PBG}* p=0.0007. Macrophages detected by cystoplasmic
387   *srpHemo-3xmCherry* in (**A**) and nuclear *srpHemo-H2A::3xmCherry* in movies and in (**I**). Unpaired t test for (**C**-
388   **H**), and one-way ANOVA with Tukey for (**J**). Scale bars: 50 µm in (**A**) and 30 µm in (**B**).
389   See also **Figure S4** and **Videos 3** and **4**. Representative movies of macrophage migration into the germband in
390   the control (**Video 3**) and the *porthos RNAi* embryos (**Video 4**). Macrophages (red) are labeled with *srpHemo-*
391   *H2A::3xmCherry*. Arrow indicates first macrophage moving into the germband. The time interval between each
392   acquisition is 40 s and the display rate is 15 frames/s. Scale bar: 20 µm.

393

394   To determine at which step in macrophage germband invasion Porthos is needed, we
395   examined wild type embryos and those expressing *porthos* RNAi in macrophages. In fixed
396   embryos we observed no change in migration along the non-invasive route of the vnc (Fig.
397   S4C) or in the total number of macrophages compared to the control (Fig. S4D), arguing
398   that Porthos is specifically required for migration into or within the tissues of the
399   germband. We then utilized 2-photon imaging of live embryos and tracked macrophages as
400   they moved from their initial position within the head towards the germband and then
401   during their infiltration into this tissue (Videos 3-4, Fig. 4B and 4SE). We observed no
402   significant change in speed or directionality in the head or on the yolk (Fig. 4C, Fig S4F-H)
403   (speed: in head, 2 µm/min for control and *porthos RNAi*, p=0.56, and on yolk, control=2.1
404   and *porthos RNAi*=2.2 µm/min, p=0.35; directionality: in head, control=0.35 and *porthos*
405   RNAi=0.37, p=0.27, and on yolk, control=0.42 and *porthos* RNAi=0.39, p=0.58).
406   Moreover, we detected no significant change in the speed of macrophages moving on the
407   yolk and beneath the germband beyond the entry point (control=2.2 and *porthos RNAi*=2.4
408   µm/min, p=0.45) (Fig. 4D). However, *porthos RNAi* macrophages waited 69% longer than
409   the control to enter the germband tissue, (control=21.5 and *porthos RNAi*=36.3 min,
410   p<0.0001) (Fig. 4E). Once within the germband, the first two macrophages invading
411   between the mesoderm and ectoderm progressed significantly slower than the control (Fig.
412   4F-G) (1st cell: control=3.0 and *porthos RNAi*=2.0 µm/min, p=0.009, 2nd cell: control=2.6
413   and *porthos RNAi*=2.0 µm/min, p=0.037). In contrast, the speed of the subsequent
414   macrophages was not significantly altered by *porthos RNAi* (Fig. 4H) (3rd-5th cells:
415   control=2.7 and *porthos RNAi*=2.3 µm/min p=0.21). Thus, *porthos RNAi* phenocopies
416   *atos*'s migration defect. Finally, we expressed Porthos in *atos^{PBG}* to restore its higher levels
417   in macrophages. This strongly improves the *atos* mutant phenotype (87% rescue) (Figs. 4I-
418   J). Thus, we conclude that Porthos is a key player downstream of Atos, exerting an
419   essential role in pioneer macrophages to specifically allow their initiation of germband
420   invasion.

421

422   **Porthos alters translation of a subset of mRNAs**
423   Given the helicase Porthos's nucleolar localization we hypothesized that it might modulate
424   translation. We purified ribosomes and polysomes by sucrose density gradient fractionation
425   of the control and S2R+ cells treated with *porthos* dsRNA (Fig. 5A). We observed a
426   reduction in polysomes, the 40S small subunit, and 80S ribosome fraction (Fig. 5B) along
427   with an increase in the large 60S subunit peak in the *porthos* KD. This data suggests that
428   Porthos is required for normal levels of 40S biogenesis, ribosome and polysome assembly,
429   and supports the idea that the higher levels of Porthos triggered by Atos could affect mRNA
430   translation.

431

432 **Figure 5. Porthos increases translation of an mRNA subset including many involved in mitochondrial**
433 **OxPhos and metabolic processes. Fig 5A.** Sucrose density gradient fractionation allowed purification of
434 ribosome subunits and polysomes. Polysomal or total cellular mRNA fractions were isolated following dsRNA
435 treatment and RNA sequencing libraries were prepared. **Fig 5B.** Sedimentation analysis showing the relative
436 abundance of 40S, 60S, and 80S ribosomes indicates that *porthos* depletion by dsRNA markedly reduces the
437 ratio of polysomes to monosomes. A non-targeting dsRNA was used as a control. Profiles were aligned on the
438 basis of the 40S ribosome peak's position and labeled with distinct colors, black for control and red for *porthos*
439 KD, n=3 biological replicates. **Fig 5C.** Scatter plot of Translational efficiency (TE) values from *porthos dsRNA*
440 S2R+ vs control *gfp dsRNA* cells. Red (down-regulated, DR) and green (up-regulated, UP) dots represent genes
441 with $\log_2$ TE changes that meet the 2 standard deviation cutoff. **Fig 5D.** DR mRNAs in *porthos dsRNA* treated
442 versus Control *dsRNA* treated S2R+ cells. 71% of the genes encoded proteins with predicted functions, the
443 number corresponding to a functional category is shown. Proteins involved in mitochondrial-related functions,
444 metabolic processes, and redox processes are highlighted. **Fig 5E.** Porthos modulates the translation of RNAs
445 encoding components of mitochondrial OxPhos, including subunits of mitochondrial complexes III, and the
446 ATP synthase complex V along with assembly factors for complexes I and IV. Porthos also enhances the TE of
447 mitochondrial transporting channels, structural proteins as well as those involved in mitochondrial translation.
448 **Fig 5F.** List of the proteins encoded by mRNAs that are downregulated in *porthos dsRNA* treated S2R+ cells
449 that are involved in metabolic pathways. NF: Not Found. See also **Figure S5** and **Data S2**. **Data S2**: Complete
450 set of TE values from polysome-sequencing data from control *gfp dsRNA* and *porthos dsRNA* treated S2R+cells.

451

452 To examine which mRNA transcripts depend on Porthos for their efficient translation, we
453 performed polysome-profiling, sequencing transcripts associated with highly translationally
454 active polysomes as well as all the transcripts in the S2R+ cells (Fig 5A, Supp. Data 2). We
455 calculated translational efficiency (TE) as the ratio of the normalized reads present for each
456 gene in the mRNAs from the polysome fraction to those in the total mRNA levels; this ratio
457 was determined for the data from both the control *GFP RNAi* and *porthos KD* cells. We
458 plotted the mean TE values for control (GFP KD) and *porthos* KD replicates and calculated
459 the mean change in TE (ΔTE) for each gene as the ratio of TEs between control (GFP KD)
460 and porthos KD replicates (Fig. 5C). Targets were defined as genes falling 2 standard
461 deviations from the median ΔTE as previously described (Flora et al., 2018). We identified
462 204 annotated coding genes that were less efficiently translated and 102 that were more
463 efficiently translated in *porthos KD* cells.

464   The mRNA targets whose TE Porthos enhances are involved in respiration,
465 transport and translation in mitochondria, metabolic processes, transcription, translation,
466 signal transduction, immune responses as well as redox processes (Fig. 5D-F, Fig. S5A-B).
467 The targets include several components of mitochondrial OxPhos, namely ubiquinol
468 cytochrome C reductase (complex III, UQCR-Q), ATP synthase subunit G and coupling
469 factor F(o) (complex V), predicted assembly factors for complex I and IV, and proteins
470 involved in mitochondrial translation and transport (Fig. 5E) as well as other metabolic
471 pathways (Fig. 5F).

472

473 **Porthos is required for mitochondrial oxidative respiration and energy production**
474 Mitochondria generate ATP through OxPhos mostly from the pyruvate formed by the
475 glycolytic pathway (Pavlova and Thompson, 2016; Vander Heiden et al., 2009) (Fig. S6A)
476 and thus can utilize metabolites downstream of the two enzymes we identified as Atos
477 targets, LKR/SDH and GR/HPR. To directly investigate if Porthos regulates mitochondrial
478 energy production, we generated S2R+ cells producing 56% of *porthos's* normal mRNA
479 levels with CRISPR/Cas9-mediated mutagenesis (which we call *porthos-KD* cells) (Fig.

10

480   S6B). We then utilized a Seahorse assay in which the oxygen consumption rate (OCR)
481   (Llufrio et al., 2018) is determined before and after sequential treatment with compounds
482   affecting different steps in OxPhos (Figs. 6A, S6A). By comparing the OCR observed upon
483   the different treatments we calculated OxPhos-dependent basal and maximum respiration
484   and found that both were reduced 64% in *porthos-KD* (Figs. 6A-C) (see Methods). We also
485   found significant decreases in OxPhos-dependent spare respiration capacity and as well as
486   OxPhos-independent respiration (72% and 42% reduction, respectively) (Fig. 6C). S2R+
487   cells utilize primarily mitochondrial OxPhos rather than glycolysis for ATP production
488   (Freijie et al., 2012); this remains the case even in the *porthos KD* cells (Fig. S6C) as we
489   also observed a 60% reduction in the basal extracellular acidification rate (ECAR), a
490   measure of lactate production through complete glycolysis (Fig. S6D). In totality, ATP
491   production through OxPhos was reduced by 50% upon *porthos* depletion (Fig. 6C). Given
492   that Porthos modulates the translation of subunits of mitochondrial complex III and the
493   ATP synthase complex V, our data argues that Porthos induces a shift in metabolic capacity
494   and flux that contributes to the upregulation of the OxPhos pathway and higher levels of
495   energy production.
496

497   **Mitochondrial respiration is required for metabolism and energy production in**
498   **macrophages to initiate invasion into the germband tissue**
499   We sought to directly assess the importance of the OxPhos complexes whose TE is
500   upregulated by Porthos for macrophage germband invasion in the embryo. Therefore, we
501   tested the effect of a dominant negative form of *complex V*, the ATP synthase which
502   converts the electron gradient produced during OxPhos into ATP (*CV-DN*) (Figs. 6D-F).
503   We also expressed RNAis against different subunits of *complex III* and the α subunit of
504   *complex V* in macrophages (Figs. 6G-H). Consistent with the polysome-profiling results
505   from *porthos-KD* S2R+ cells, these treatments significantly reduced macrophage numbers
506   within the germband (Figs. 6D-H) and increased them on the yolk at the germband entry
507   site (Figs. 6F, S6E), phenocopying the germband invasion defect of *atos^{PBG}* or *porthos*
508   *RNAi* in macrophages. We observed no significant difference in macrophage numbers on
509   the vnc in late Stage 12 upon expression of *CV-DN* (Fig. S6F) or of the RNAis (Fig. S6G)
510   compared to the control, indicating normal general migration. This data strongly supports
511   the conclusion that higher levels of the OxPhos complexes III and V are required
512   specifically for macrophage tissue invasion.
513
514   **Figure 6. Higher levels of mitochondrial respiration are required in macrophages to power their**
515   **germband tissue invasion. Fig 6A.** Schematic of the procedure for mitochondrial energetic profiling in wild-
516   type and *porthos KD* S2R+ cells with a Seahorse efflux assay. **Fig 6B.** The Oxygen Consumption Rate (OCR,
517   pmols O_2/min) was assessed as a representative parameter of OxPhos in control and *porthos KD* S2R+ cells by a
518   Seahorse Bioscience XF96 Extracellular Flux Analyzer. The ATP synthase inhibitor oligomycin (2 µM), the
519   uncoupler FCCP (2 µM), and the mitochondrial complex I inhibitor Rotenone (1 µM) with antimycin A (1 µM)
520   were injected sequentially (see S6A). **Fig 6C.** Calculation from relative OCR values at different stages assesses
521   basal and maximum OxPhos respiration, spare OxPhos respiration capacity, OxPhos ATP production, and non-
522   OxPhos respiration rates. At least three independent biological experiments (n>6 technical replicates in each
523   repeat). **Fig 6D-E. (D)** Representative confocal images and (**E**) quantification of Stage 12 embryos reveals that
524   the number of macrophages (red) that penetrated into the germband in Stage 12 embryos is significantly
525   decreased upon the expression of a dominant negative c-ring of the ATP synthase (CV-DN) compared to the
526   control. Control n=24, *CV-DN* n=20, p=0.003. **Fig 6F.** Quantification of macrophages on the yolk in fixed early
527   Stage 12 embryos shows a significant increase in the *CV-DN* embryos compared to the control. Control n=21,

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528 *CV-DN* n=17, p=0.003. **Fig 6G-H. (G)** Representative confocal images and (**H**) quantification of Stage 12
529 embryos indicates that fewer macrophages (red) move into the germband upon the expression in macrophages
530 of any of three different *RNAis* against mitochondrial OxPhos *Complex III* (*Ubiquinol-cytochrome c reductase,*
531 *UQCR*), or an *RNAi* against *Complex V* (*F1F0, CG3612*), arguing that these two components are required in
532 macrophages for germband tissue invasion. Control n=34; *Complex III* (*Cyt-c1, CG4769*): *RNAi 1* (VDRC
533 109809) n=20, p=0.0001; *Complex III* (*UQCR-cp1, CG3731*): *RNAi 2* (VDRC 101350) n=18, p<0.0001;
534 *Complex III* (*UQCR-cp2, CG4169*): *RNAi 3* (VDRC 100818) n=16, p=0.0027; *Complex V*: (*F1F0, CG3612*)
535 *RNAi* (VDRC 34664) n=24, p<0.0001. **Fig 6I.** A single plane confocal microscope image during germband
536 entry in early Stage 12 embryos from control (Ctrl) or *atos^{PBG}* embryos, or lines expressing *porthos* RNAi or
537 *CV-DN* in macrophages. Antibodies used against the phosphorylated at S293 and thus inactivated Pyruvate
538 Dehydrogenase (pPDH, green) or total PDH (magenta) in macrophages (red). Higher pPDH levels are usually
539 found when ATP/ADP levels are high and input into the TCA cycle is being downregulated (Patel et al., 2014).
540 **Fig 6J.** Quantification of normalized values for pPDH/PDH levels calculated from fluorescence intensities in
541 macrophages from the genotypes in (**6I**) during initial germband invasion in early Stage 12. The pPDH/PDH
542 ratio is significantly reduced in all compared to the control, arguing that decreasing the function of CV, *atos* or
543 *porthos* in macrophages results in lower cellular ATP/ADP ratios compared to the control. Control n=10, *CV-*
544 *DN* n=9, p=0.0002; *atos^{PBG}* n=13, level p=0.0002; control n=7, *macro>porthos* RNAi n=8, p=0.0002. Three
545 independent experiments. Macrophages visualized in (**C**) and (**G**) with nuclear *srpHemo-H2A::3xmCherry*
546 expression and (**I**) with cytoplasmic *srpHemo-3xmCherry*. Unpaired t test for (**B-C**), (**E-F**), and (**H-J**). Scale
547 bars: 50 μm in (**D**) and (**G**), 10 μm in (**I**). See also **Figure S6**.

Atos and its target Porthos increase macrophage bioenergetics for germband tissue invasion

550 To examine the bioenergetic state of embryonic macrophages *in vivo* in the absence of
552 Porthos or Atos, we first assessed the Pyruvate dehydrogenase complex (PDH), which allows
554 pyruvate formed by glycolysis to feed into the TCA cycle. PDH is a key point of metabolic
555 regulation (Patel et al., 2014) (see Fig. 7A). Metabolites produced by the TCA cycle increase
556 PDH's phosphorylation thereby inhibiting it and thus the running of the cycle; metabolites
557 utilized by the TCA cycle decrease PDH phosphorylation and activate it. Importantly, when
558 energy levels fall and mitochondrial ADP levels rise, PDH is unphosphorylated and active,
559 opening the gate to the TCA cycle and OxPhos (Patel et al., 2014). By antibody staining we
560 determined the levels of phosphorylated inactive PDH (pPDH) and the total amounts of PDH
561 (Lieber et al., 2019) in embryonic macrophages. We assessed the pPDH/PDH ratio; a smaller
562 number indicates less inhibition and thus more activity of PDH. As a positive control we first
563 examined macrophages expressing CV-DN, which blocks mitochondrial ATP synthase, and
564 thus increases ADP levels. Indeed we observed a lower pPDH/PDH ratio than in the control
565 (Fig. 6J). We also observed significantly lower pPDH/PDH ratios in macrophages invading
566 the germband in *atos^{PBG}* embryos as well as those expressing *porthos* RNAi in macrophages
567 compared to the control (Figs. 6I-J). Our results support the conclusion that in the absence of
568 Atos or Porthos, macrophages *in vivo* have reduced ATP/ADP ratios, leading the cells to
569 keep PDH in its active form to try to generate more ATP by converting pyruvate into acetyl
570 CoA that can feed into the TCA cycle.

Atos enhances cellular metabolism and ATP/ADP levels

573 To investigate the full complement of metabolic changes that Atos enables, we performed
574 untargeted comparative metabolite profiling by capillary liquid chromatography-tandem mass
575 spectrometry (LC-MS/MS) (Figs. S7A, 7A) characterizing extracts from control and *atos^{PBG}*
576 embryos. Most importantly, consistent with the results we had observed in the Seahorse assay

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577 and the p-PDH/PDH ratio measurement, we observed a significantly decreased ATP/ADP
578 ratio in the absence of Atos (Fig. 7B). Thus our metabolic data supports that Atos regulates a
579 set of targets that shift metabolism to enhance ATP production. Consistent with Atos's role in
580 increasing GR/HPR levels, in $atos^{PBG}$ we observed higher levels of this enzyme's substrate,
581 4-hydroxy α-ketoglutarate (4-HαKG) (Figs. S7B-C) and of hydroxy-L-proline (HLP), the
582 metabolite just upstream of 4-HαKG (Fig. 7A). We also observed significantly higher levels
583 of dipeptides containing HLP (Fig. 7D). Atos also regulates LKR/SDH; we observed a
584 reduction to 60% of control levels of its product alpha-amino adipic semialdehyde (ASAA),
585 by targeted-metabolomics profiling (Fig. 7A).
586
587 **Fig 7. Mitochondrial metabolism is enhanced by Atos and Porthos. Fig 7A.** Schematic depicting ATP-
588 generating pathways in eukaryotic cells: glycolysis, the Pentose Phosphate Pathway (PPP), fatty acid
589 oxidation (FAO), the TCA cycle, and the mitochondrial respiratory electron transport chain (ETC). Blue stars
590 mark *porthos* targets. Green indicates individual metabolites with statistically significant upregulation in
591 $atos^{PBG}$ compared to the control. **Fig 7B-F**. Cellular metabolites were measured by LC–MS-based
592 metabolomics from extracts of Stage 11 embryos (Control n=5, $atos^{PBG}$ n=7). **Fig 7B.** Normalized ATP/ADP
593 ratio values are decreased in $atos^{PBG}$ compared to control embryos. (p-value=0.028). **Fig 7C.** Quantification in
594 $atos^{PBG}$ compared to wild-type embryos shows an increase in the pyruvate/glucose ratio (p-value=0.035), but
595 none for the Lactate/Glucose ratio (p-value=0.65). **Fig 7D-F.** Heatmap of non-targeted metabolites in $atos^{PBG}$
596 compared to wild-type embryos shown with average log$_2$fold change (FC) reveals **(D)** a significant increase in
597 some dipeptides including those containing hydroxyproline, **(E)** increases in intermediates of mitochondrial
598 fatty acid β-oxidation (FAO), including different carnitine-conjugated lipids, and **(E-F)** a significant increase
599 in ketone body substituents compared to the control**. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Values
600 are obtained from untargeted metabolomic analysis in (**B**, **D-F**). **Fig 7G.** Our model: Atos raises mRNA
601 transcript levels of the helicase Porthos and the metabolic enzymes GR/HPR and LKR/SDH in macrophages.
602 Metabolic pathways downstream of GR/HPR and LKR/SDH are known to produce metabolites that feed into
603 glycolysis and the TCA cycle to produce ATP. Porthos enhances the translational efficiency of mRNAs,
604 including those encoding mitochondrial OxPhos components and a mitochondrial carnitine transporter.
605 Macrophages with elevated mitochondrial OxPhos can meet the demands for the energy needed to create a
606 path for tissue invasion. However, the absence of Atos leads to reduced levels of GR/HPR, LKR/SDH and
607 Porthos. This decreases generation of ATP through OxPhos leading to defective tissue infiltration of the
608 pioneering macrophages. Unpaired t-test for (**B-F**). See also **Figure S7** and **Data S3**. **Data S3:** Primary
609 metabolomics data from control and *atos* mutant embryos**.
610
611   As the metabolomics was conducted on embryos constitutively defective in Atos,
612 we expected to see some compensatory changes as well. Matching our previous data, no
613 indications of a metabolic shift away from mitochondrial OxPhos towards aerobic
614 glycolysis in the absence of Atos were present (Figs. 7C, S7D-F). Instead we observed
615 results consistent with a backup of some metabolites whose products would normally be
616 fed into glycolysis and the TCA cycle. We found significantly higher levels of β-
617 hydroxybutyrate, which can be broken down to acetyl-CoA (Puchalska and Crawford,
618 2017) along with increases in carnitine-conjugated fatty acids (Figs. 7E-F). There were
619 strong increases in thymidine, which can be catabolized to a product that is fed into
620 glycolysis (Tabata et al., 2017), and uridine which can be interconverted with thymidine,
621 along with other purine and pyrimidine nucleotides (Figs. S7G-H). We observed a small
622 increase in most amino acids in $atos^{PBG}$ (Fig. S7I). Additionally strong reductions occurred

13

623 in the glycine-related metabolite sarcosine (N-methylglycine) known to be a biomarker of
624 highly metastatic prostate cancer (Fig. S7J) (Sreekumar et al., 2009; Zhang et al., 2012).
625 In sum, the metabolomics profiling data in combination with our other findings strongly
626 supports the conclusion that Atos is a powerful regulatory protein, increasing the efficiency
627 and amount of OxPhos by inducing a metabolic shift that affects the ETC and complex V
628 as well as the TCA cycle (Fig. 7G).
629

630 # DISCUSSION

631 We identify a key regulator of energy levels in *Drosophila* macrophages as a highly
632 conserved and previously uncharacterized nuclear protein, that we name Atos. Atos mRNA is
633 deposited maternally and is ubiquitously expressed at low levels. However Atos mRNA is
634 also developmentally upregulated in macrophages several hours prior to tissue invasion and
635 down regulated after invasion is completed. Live imaging shows that the presence of Atos
636 speeds the tissue entry and forward movement within the germband tissue of only the first
637 two macrophages, the invasion pioneers. RNA sequencing indicates that Atos leads to the
638 upregulation in macrophages of mRNAs encoding two metabolic enzymes, dGR/HPR by 6.5-
639 fold and dLKR/SDH by 25-fold, as well as a 10-fold increase in the mRNA encoding an
640 ATP-dependent RNA helicase, named Porthos. Each of these three proteins is required for
641 normal amounts of invasion. We show in S2R+ cells that two-fold higher levels of Porthos
642 mRNA correspond to two-fold higher OxPhos activity, a process that generates ATP by
643 transferring electrons from NADH and FADH2 produced by the TCA cycle to oxygen
644 (Martínez-Reyes and Chandel, 2020). We thus favor the hypothesis that these two metabolic
645 enzymes act in pathways that ultimately feed into the TCA cycle and thus the ETC. We
646 identify an increase in the active state of the PDH enzyme in *porthos* and *atos* mutant
647 macrophages, an indirect indication that ATP could be lower without these proteins.
648 Importantly, we directly detect two-fold lower ATP/ADP levels in *atos* mutant embryos.
649 Given that Atos is much more highly expressed in macrophages at this stage than in the rest
650 of the embryo, the effects within these immune cells will be even greater. In sum our data
651 argues that the developmentally programmed upregulation of Atos triggers a metabolic shift
652 by upregulating this triad of targets, ultimately significantly increasing ATP/ADP in all
653 macrophages and thereby enabling pioneer macrophages to power the creation of a path for
654 tissue infiltration against surrounding resistance. Our findings are consistent with previous
655 work indicating that higher ATP levels are needed in the first cell to migrate through
656 extracellular matrix (Kelley et al., 2019; Zhang et al., 2019). However, to our knowledge our
657 work is the first to identify a concerted molecular pathway that can produce the higher energy
658 levels needed to speed pioneer cell invasion.
659

660 The target of Atos that we have focused on in this study is a previously
661 uncharacterized protein we call Porthos. Porthos belongs to a family of ATP-dependent
662 DEAD-box RNA helicases that have essential roles in RNA metabolism (Martin et al., 2021,
663 Bourgeois et al., 2016; Venema et al., 1997; Venema and Tollervey, 1995). We find Porthos
664 localized to the nucleolus in macrophages, suggesting a function in ribosome production or
665 assembly (Baßler and Hurt, 2019). Porthos' vertebrate ortholog, DDX47, binds rRNA
666 precursors (Sekiguchi et al., 2006); its *S. cerevisiae* ortholog, RRP3, can separate short RNA

14

667 helices and is required for the RNA processing that produces the 18S rRNA component of the
668 40S ribosomal subunit (O'Day et al., 1996; Garcia et al., 2012). Consistent with this in S2R+
669 cells treated with *porthos* dsRNA we find a lower ratio of the 40S to the 60S ribosome
670 subunits along with a strong decrease in multiple ribosomes sitting on an mRNA, called
671 polysomes. Importantly, Porthos also enhances the translational efficiency (TE) of a subset of
672 mRNAs. A significant subset of the mRNAs whose TE is enhanced by Porthos encode
673 mitochondrial proteins. These are orthologs of proteins shown to affect many aspects of the
674 organelle's biology, from its specialized translation, its import of proteins and their insertion
675 into the inner membrane where the ETC resides, to its import of fatty acids as fuel. Some of
676 these targets are also components directly involved in OxPhos. We identify two orthologs of
677 proteins that affect the assembly and function of OxPhos complexes I and IV (Formosa et al.,
678 2015; Dennerlein et al., 2015), one of which causes mitochondrial disease if mutated (Calvo
679 et al., 2010). The yeast ortholog of the complex III subunit we identify as a target, QCR9, is
680 required to strongly increase reductase activity (Brandt et al., 2017). We identify a protein
681 whose ortholog has been implicated in ATP synthase function (Belogrudov, 2002;
682 Belogrudov, 2008). Another, complex V subunit G, fosters complex dimerization, thereby
683 contributing to cristae formation (Davies et al., 2011; Hahn et al., 2016), as does another
684 target, Mics1 (Oka et al., 2008). More cristae correlate with higher levels of OxPhos (Brandt
685 et al., 2017), and have been proposed to foster ATP production (Mannella, 2020). Thus the
686 increased OxPhos we see in cells with more Porthos could result from improved efficiency
687 through multiple avenues; more translation of Porthos targets would be predicted to increase
688 the amount, localization, and assembly of OxPhos components as well as the extent of the
689 membrane folds in which they are localized. Co-regulation to increase this set of
690 mitochondrial proteins could thus allow a concerted enhancement of OxPhos and
691 mitochondrial energy production by avoiding that invidual steps become rate limiting.
692 Atos's two mammalian orthologs, FAM214A and B, can fully substitute for Atos during
693 macrophage invasion, arguing that they maintain Atos's ability to increase ATP/ADP. All of
694 Atos's targets that we show act during invasion have highly conserved human orthologs
695 whose mRNAs are broadly expressed along with FAM214A and B (Sekiguchi et al., 2006;
696 Human Protein Atlas, BioGPS). Thus Atossa's vertebrate orthologs could be utilized by
697 particular mammalian cell types in energetically demanding circumstances. In the immune
698 system FAM214A appears particularly enriched within plasmacytoid dendritic cells (pDCs)
699 and B cells (Table 1); pDCs upregulate OxPhos in response to IFN-1s during anti-viral
700 responses (Wu et al., 2016) and B cells upregulate OxPhos during differentiation for effective
701 antibody secretion (Price et al., 2018). Furthermore, FAM214A and B are well expressed in
702 the brain which utilizes large amounts of energy and produces almost all of its ATP though
703 OxPhos (Raichle and Gusnard, 2002; Hall et al., 2012). A shift from aerobic glycolysis to
704 OxPhos is required for neural stem cell differentiation and neural survival (Zheng et al.,
705 2016); many neurodegenerative diseases are associated with defects in OxPhos (Koopman et
706 al., 2013). Interestingly, four different single nucleotide polymorphisms (SNP) in FAM214A
707 introns have been linked to more severe Alzheimer's disease or neurofibrillary tangles in
708 genome wide association studies while another SNP in a transcription factor-binding region
709 was associated with increased general intelligence (p-value for all variants$\leq$5X10^{-6};
710 https://www.ebi.ac.uk/gwas/search?query=FAM214A), Sherva et al., 2020; Beecham et al.,

15

711 2014; Wang et al., 2020; Davies et al., 2018). The importance of OxPhos enhancers for brain
712 function is demonstrated by the many neurodegenerative diseases connected to defects in
713 PGC-1 (Zheng et al., 2010; Cui et al., 2006; Weydt et al., 2006). PGC-1 activates OxPhos
714 through transcription of mitochondrial genes and thus mitochondrial biogenesis (Lin et al,
715 2005). In contrast, Atossa increases translation from mitochondrial mRNAs that already exist
716 by raising levels of the helicase Porthos. The closest *Drosophila* ortholog of PGC-1 also
717 raises transcription of mitochondrial proteins and OxPhos (Tiefenbock et al., 2010) and is
718 expressed in invading macrophages at levels comparable to Atossa in our RNAseq. We
719 hypothesize that these two regulators of mitochondrial function could work in concert, with
720 Atossa allowing faster and more easily reversible control of enhanced energy production.
721 Thus investigating the mammalian version of the regulatory network that we identified in this
722 work and strategies to modulate it in the brain and immune system is of wide interest.
723   Altogether, our work uncovers a surprising molecular genetic view into the
724 physiology of the organism, revealing a heretofore unsuspected cross-regulatory mechanism
725 that spans different levels of the biological organization of cellular metabolism, cell biology
726 and the tissue invasiveness of the immune system.
727

743

Author Contributions
745 S.E., E.T.M. A.G. J.B. J-W.G. and T.K. conducted experiments, T.R.H. and A.B. provided
746 resources, S.E., T.R.H., J.B. J-W. A.G, T.K. P.R. and D.E.S. designed experiments, S.E.,
747 E.T.M., and D.E.S. wrote the paper, S.E., E.T.M. and J-W.G. conducted formal analysis, all
748 authors reviewed and edited the paper, A.B., D.E.S. and P.R. conducted Supervision and
749 Project Administration, S.E., D.E.S. and P.R carried out Conceptualization. D.E.S., P.R. A.B.
750 and T.R.H. acquired funding.
751

Declaration of Interests
753 The authors declare no competing interests.
754

755

EXPERIMENTAL PROCEDURES

757

Fly work

Flies were raised on food bought from IMBA (Vienna, Austria) which was prepared according to the standard recipe of agar, cornmeal, and molasses with the addition of 1.5% Nipagin. Adults were placed in cages in a Percival DR36VL incubator maintained at 29ºC and 65% humidity or a Sanyo MIR-153 incubator at 29ºC within the humidity controlled 25ºC fly room; embryos were collected on standard plates prepared in house from apple juice, sugar, agar and Nipagin supplemented with yeast from Lesaffre (Marcq, France) on the plate surface. Embryo collections for fixation (7-8 hour collection) as well as live imaging (4-5 hour collection) were conducted at 29ºC.

767

Fly lines obtained used in this work

srpHemo-GAL4 was provided by K. Brückner (Brückner et al., 2004). The RNA lines tested in this paper (Table S1) were obtained from the Bloomington *Drosophila* Stock Centre (Bloomington, USA) and the Vienna *Drosophila* Resource Center (VDRC, Vienna, Austria). Lines *w⁻*; *P{w[+mC] srpHemo-3xmCherry}*, *w⁻*; *P{w[+mC] srpHemo-H2A::3xmCherry}* were published previously (Gyoergy et al., 2018).

774

Embryo fixation and immunohistochemistry

Embryos were collected on apple juice plates from between 6-8.5 hours at 29°C. Embryos were incubated in 50% Chlorox (DanClorix) for 5 min and washed. Embryos were fixed with 17% formaldehyde/heptane (ThermoFisher Scientific, Waltham, MA, USA) for 20 min followed by methanol or ethanol devitellinization. PDH and p-PDH staining utilized hand-devitellinized embryos. Fixed embryos were blocked in BBT (0.1 M PBS + 0.1% TritonX-100 + 0.1% BSA) for 2 hours at RT and then incubated overnight at 4°C. Antibodies were used at the following dilutions: Mouse anti α-GFP (Aves Labs Inc., Tigard, Oregon, 1:500), Rat anti-HA (Roche, Basel, Switzerland, 1:100), Mouse anti-PDH E1α (Abcam, Cambridge, UK, ab110334, 1:200) and Rabbit antiphoshpo-PDH E1α (S293) (Abcam, Cambridge, UK, ab92696, 1:200). Afterwards, embryos were washed in BBT for 2 hours, and incubated with secondary antibodies at RT for 2 hours, and washed again for 2 hours. Secondary antibodies and Phalloidin were used at the following dilutions: anti-rat 488 1:300, anti-chicken 488 1:500, anti-mouse 488 1:500 or anti-mouse 633 1:200, anti-rabbit 488 1:300, and Phalloidin 1:300 (all from ThermoFisher Scientific, Waltham, MA, USA) (Table S2). The embryos were mounted overnight at 4°C in Vectashield mounting medium (Vector Laboratories, Burlingame, USA), which contains DAPI. Embryos were placed on a slide and imaged with a Zeiss Inverted LSM800 Confocal Microscope using a Plain-Apochromat 20X/0.8 Air Objective or a Plain-Apochromat 63X/1.4 Oil Objective.

794

S2R+ cell work and immunostaining

S2R+ cells (a gift from Frederico Mauri of the Knöblich laboratory at IMBA, Vienna) were grown in Schneider's medium (Gibco) supplemented with 10% FBS (Gibco) and transfected with the *srpHemo-HA::CG9005(atos),* or *UAS-CG9005(atos)::FLAG::HA, UAS-*

799   *CG9253*(*porthos*)*::FLAG::HA* and *srpHemo-GAL4* constructs using Effectene Tranfection
800   Reagent (Qiagen, Hilden, Germany) following the manufacturer's protocol (Table S3).
801   Transfected S2R+ cells were grown on Poly-L-Lysine coated coverslips (ThermoFisher
802   Scientific, Waltham, Massachusetts, USA) in complete Schneider's medium (Gibco)
803   supplemented with 10% FBS (Sigma-Aldrich, Saint Louis, Missouri, USA) to a confluency
804   of 60%. For antibody staining, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich,
805   St Louis, MI, USA) in PBS for 15 minutes at room temperature (RT). Cells were washed
806   three times with PBS followed by permeabilization with 0.5% Triton X-100 (Sigma-Aldrich)
807   in PBS for 15 minutes and then blocked in BBT (see above) for at least 1 hour. Antibodies
808   were diluted in blocking buffer and incubated for 2 hours at RT. Primary antibodies were
809   used at the following working dilutions: Chicken anti-GFP (clone 5G4, Ogris lab, MFPL,
810   1:100), Rat anti-HA (Roche, Basel, Switzerland, 1:50), Mouse anti-Lamin (DSHB, lamin
811   Dm0, ADL1010, 1:50), and Mouse anti-fibrillarin (gift from Rangan lab, 1:1). Cells were
812   subsequently washed three times with PBS-Triton X-100 (0.05%) for 5 minutes each,
813   followed by secondary antibody incubation in blocking/permeabilization buffer for 1 hour at
814   RT. Secondary antibodies were used at the following working dilutions: anti-rat Alexa Flour
815   488 (1:50), anti-mouse Alexa Flour 488 (1:200), and anti-mouse Alexa Flour 633 (1:100) (all
816   from ThermoFisher Scientific, Waltham, MA, USA). Cells were counterstained with DAPI
817   (ThermoFisher Scientific) for 10 minutes in PBS-Triton X-100 (0.05%). After
818   immunoblotting, cells were mounted with Vectashield (Sigma-Aldrich). Images were
819   acquired using the Zeiss inverted LSM-800 confocal microscope. Pictures were processed
820   with ImageJ.
821
DNA isolation from single flies
823   Single male flies were frozen overnight before being grounded with a pellet homogenizer
824   (VWR, Radnor, USA) and plastic pestles (VWR, Radnor, USA) in 50µl of homogenizing
825   buffer (100 mM Tris-HCl, 100 mM EDTA, 100 mM NaCL, and 0.5% SDS). Lysates were
826   incubated at 65°C for 30 minutes. Then 5 M KAc and 6 M LiCl were added at a ratio of 1:2.5
827   and lysates were incubated on ice for 10 min. Lysates were centrifuged for 15 minutes at
828   20,000xg, supernatant was isolated and mixed with Isopropanol. Lysates were centrifuged
829   again for 15 minutes at 20,000xg, the supernatant was discarded and the DNA pellet was
830   washed in 70% ethanol and subsequently dissolved in distilled water.
831
832
FACS sorting of macrophages
834   For embryo collections, adult flies of either *w+; srpHemo-3xmCherry* or *w+;*
835   *CG9005BG02278; srpHemo-3xmCherry* genotypes were placed into plastic cages topped with
836   apple juice plates with yeast for egg laying. Collections were performed at 29°C at 8h-20h
837   light-dark cycle. Macrophages were collected from Stage 11-early Stage 12, when
838   macrophages initiate invasive migration into the extended germband. Briefly, adult flies
839   laid eggs for 1 hour, then the isolated plates with embryos were kept at 29°C for an
840   additional 4 hours 45 minutes to reach the desired age. Embryos were collected for 2 days
841   with about 6-7 collections per day and stored meanwhile at +4°C to slow down
842   development. Collected embryos were dissociated and the macrophages were sorted

843 according to the procedure described in (Gyoergy et al., 2018). The cells were sorted using
844 a FACS Aria III (BD) flow cytometer. Emission filters were 600LP, 610/20 and 502 LP,
845 510/50. Data was analyzed with FloJo software (Tree Star). The cells from the negative
846 control embryos were sorted to set a baseline plotAbout. Approximately $1-1.5x10^5$
847 macrophages were sorted within 30 minutes.
848
849 **Sequencing of the macrophage transcriptome**
850 Total RNA was isolated from the FACS-sorted macrophages using the Qiagen RNeasy
851 Mini kit (Cat No. 74104). The quality and concentration of RNA was determined using the
852 Agilent 6000 Pico kit (Cat No. 5067-1513) on the Agilent 2100 Bioanalyzer: about 100 ng
853 of total RNA was extracted from 1.5×10^5 macrophages. RNA sequencing was performed
854 by the CSF facility of the Vienna Biocenter according to their standard procedures
855 (https://www.vbcf.ac.at/facilities/next-generation-sequencing/). Briefly, a cDNA library
856 was synthesized using the QuantSeq 3' mRNA-seq Library Prep kit and 4 replicates of each
857 of the genotypes (*w+; +; srpHemo::3xmCherry* or *w+; CG9005^BG02278; srpHemo-*
858 *3xmCherry)* were sequenced on the Illumina HiSeq 2500 platform.
859 The reads were mapped to the *Drosophila melanogaster* Ensembl BDGP6 reference
860 genome with STAR (version 2.5.1b). The read counts for each gene were detected using
861 HTSeq (version 0.5.4p3). The Flybase annotation (r6.19) was used in both mapping and
862 read counting. The counts were normalised using the TMM normalization from the edgeR
863 package in R (Anders and Huber, 2015; Dobin et al., 2013). (Prior to statistical testing the
864 data was transformed and then the differential expression between the sample groups was
865 calculated with the limma package in R. The functional analyses were done using the
866 topGO and gage packages in R.
867
868 **Time-lapse imaging**
869 Embryos were dechorionated in 50% bleach for 4 min, washed with water, and mounted in
870 halocarbon oil 27 (Sigma) between a coverslip and an oxygen permeable membrane (YSI).
871 The anterior dorsolateral region of the embryo was imaged on an inverted multiphoton
872 microscope (TrimScope, LaVision) equipped with a W Plan-Apochromat 40X/1.4 oil
873 immersion objective (Olympus). mCherry was imaged at an 820 nm excitation wavelength,
874 using an optical parametric oscillator technology (Coherent Chameleon Compact OPO).
875 Excitation intensity profiles were adjusted to tissue penetration depth and Z-sectioning for
876 imaging was set at 1µm for tracking. For long-term imaging, movies were acquired for
877 180-200 minutes with a frame rate of 40 seconds. Embryos were imaged with a temperature
878 control unit set to 29°C.
879
880 **Image Analysis**
881 **Macrophage cell counts**
882 Autofluorescence of the embryo was used to measure the position of the germband to
883 determine the stages for analysis of fixed samples. Embryos with germband retraction of
884 between 29-31% were assigned to Stage 11. Embryos with 35-40% retraction (Stage 12)
885 were analysed for the number of macrophages that had entered the germband. Embryos with
886 above 50-75% retraction were used for the number along the ventral nerve cord (vnc) and in

887   the whole embryo. Macrophages were visualized using confocal microscopy with a Z-
888   resolution of 2 µm and the number of macrophages within the germband or the segments of
889   the vnc was calculated in individual slices (and then aggregated) using the Cell Counter
890   plugin in FIJI. Total macrophage numbers were obtained using Imaris (Bitplane) by detecting
891   all the macrophage nuclei as spots.
892
893   **Macrophage tracking, speed, directionality and time for macrophage entry analysis**
894   Embryos in which the macrophage nuclei were labeled with *srpHemo-H2A::3XmCherry*
895   were imaged and 250X130X36 µm^3 3D-stacks were typically acquired with a constant
896   0.5X0.5X1 µm^3 voxel size at every 40-41 seconds for approximately 3 hours. Images
897   acquired from multiphoton microscopy were initially processed with InSpector software
898   (LaVision Bio Tec) to compile channels from the imaging data (Table 3). Afterwards, the
899   exported files were further processed using Imaris software (Bitplane) to visualize the
900   recorded channels in 3D and the movie from each imaged embryo was rotated and aligned
901   along the AP axis for further tracking analysis.
902   To analyze the movies by Imaris, the following analyses were applied:
903   **i.** To calculate the migration parameters while macrophages migrate from the head mesoderm
904   to the yolk zone, movies were cropped in time to that period (typically 60 minutes from the
905   original movie was used for analysis).
906   **ii.** To calculate the migration parameters of the macrophage moving on the yolk zone into the
907   edge of germband, movies were acquired from the time point of the first macrophage
908   appearing in the yolk zone and recorded until the onset of germband retraction.
909   **iii.** Macrophage nuclei were extracted using the spot detection function and tracks generated
910   in 3D over time. We could not detect all macrophages in the head mesoderm as spots because
911   of limitations in our imaging parameters. Tracks of macrophages which migrate towards the
912   dorsal vessel, ventral nerve cord (vnc) and to the anterior of the head were omitted. The edge
913   of the germband was detected using autofluorescence from the yolk and the mean position of
914   the tracks in X- and Y-axis was used to restrict analysis to before macrophages reach the
915   edge of the germband.
916   **iv.** Nuclei positions in XYZ-dimensions were determined for each time point and used for
917   further quantitative analysis.
918   **v.** The time point when the first macrophage nucleus reached the germband was defined as
919   T0 and the time point when the macrophage nucleus was within the germband and moved
920   forward along the route between the ectoderm and mesoderm was taken as T1 and T1-T0 was
921   defined as time for macrophage entry. T0 and T1 were determined by precisely examining
922   macrophage position in xy and z dimensions (examination of individual 2 micron slices) over
923   time.
924   **vi.** To measure the speed along the route between the germband edge and the yolk, tracks
925   generated from macrophages from the time when the first macrophages started to move along
926   the mentioned path until germband retraction onset were utilized.
927   **vii.** To calculate the speed of migration of the first or second macrophages in the germband
928   the track generated for the first or second macrophages alone was used to obtain the nuclei
929   position in XYZ-dimensions. Moreover, the average speed of the third through fifth
930   macrophages moving along the same route was also measured. Speed was calculated within

20

931   the first 30-35 µm of the patrh between the germband ectoderm and mesoderm. The mean
932   position of the tracks in X- and Y-axis was used to restrict analysis to either of the migratory
933   zones (head, yolk, germband entry, route along the germband ectoderm and mesoderm, route
934   along the germband mesoderm and the yolk).

935   Macrophage migratory parameters, including cell speed and directionality
936   (persistence), were calculated in Matlab (The MathWorks Inc.) from single cell positions in
937   3D for each time frame measured in Imaris (Bitplane), as described elsewhere (Smutny et al.,
938   2017). Briefly, instantaneous velocities from single cell trajectories were averaged to obtain a
939   mean instantaneous velocity value over the course of the measurement. To calculate
940   directionality values, single cell trajectories were split into segments of equal length (l; $l = 10$
941   frames) and calculated via a sliding window as the ratio of the distance between the
942   macrophage start-to-end distance (D) over the entire summed distance covered by the
943   macrophage between each successive frame (d_i) in a segment. Calculated directionality
944   values were averaged over all segments in a single trajectory and all trajectories were
945   averaged to obtain a directionality index (I) for the duration of measurement (with 0 being
946   the lowest and 1 the maximum directionality) as follows:

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

947   where n defines the total number of frames, i the sum of frame-to-frame distances over one
948   segment and k the sum over all segments of a trajectory.

949   Embryos from the control (w^+; +; srpHemo::3xmCherry) and the CG9005 mutant (w^+;
950    $CG9005^{BG02278}$; srpHemo::3xmCherry) were used for calculating the time for macrophage
951   entry. Briefly, 100X130X34µm^3 3D-stacks were typically acquired with a constant
952   0.28X0.28X2µm^3 voxel size at every 40-41 seconds for approximately 3 hours.

953

954   **Cloning of constructs**
955   Standard molecular biology methods were used and all constructs were sequenced by the
956   Mycrosynth company (Vienna, Austria) before injection into flies. The enzymes *NotI*, T4
957   Polynucleotide Kinase (*PNK*) and *DpnI* were obtained from New England Biolabs, Ipswich,
958   Massasuchetts, USA (Frankfurt, Germany). PCR amplifications were performed with GoTaq
959   G2 DNA polymerase (Promega, Madison, USA) using a peqSTAR 2X PCR machine from
960   PEQLAB, (Erlangen, Germany). All Infusion cloning was conducted using an Infusion HD
961   Cloning kit (Clontech's European distributer). The relevant oligo sequences were chosen
962   using the Infusion primer Tool at the Clontech website
963   (http://bioinfo.clontech.com/infusion/convertPcr sInit.do).

964

965   **Construction of *srpHemo*-CG9005**
966   A 3894 bp fragment containing the CG9005 ORF was amplified from the *UAS-*
967   *CG9005::FLAG::HA* construct (Table S3) (*Drosophila* Genomics Resource Centre, DGRC)
968   using relevant primers (Table S4). The fragment was cloned into the *srpHemo* plasmid (a gift

21

969 from Katja Brückner (Brückner et al., 2004) after its linearization with *NotI*, using an
970 Infusion HD cloning kit (Clontech's European distributor).

971

972 **Construction of *srpHemo-FAM214A* and *srpHemo-FAMB214B***
973 Fragments of 3225 bp and 1615 bp containing the FAM214A and FAMB214B ORFs,
974 respectively, were amplified from cDNA prepared from dendritic cells (a gift from M. Sixt's
975 lab) with FAM214A Fwd and Rev primers, and with FAM214B Fwd and FAM214B Rev
976 primers (Table S4). The fragments were cloned into the *srpHemo* plasmid using an Infusion
977 HD cloning kit after its linearization with *NotI* (NEB).

978

979 **Construction of mutant forms of *srpHemo-atossa***
980 Mutant forms of *atossa* (CG9005) were generated by removing the desired region from the
981 CG9005 cDNA sequence by using inverse PCR followed by blunt end ligation and related
982 primers (Table S4). Afterwards, *atossa* mutant constructs in the Bluescript vector were
983 amplified and cloned into the *srpHemo* plasmid after its linearization with *NotI*, using an
984 Infusion HD cloning kit.

985

986 **Transgenic fly line production**
987 The *srpHemo* and *UAS* constructs (Table S4) was injected into syncytial blastoderm stage
988 embryos of M{3xP3-RFP.attP}ZH-86Fb (BL 24749) line (obtained from Peter Duchek of
989 IMBA) to generate inserts on third chromosome by C31-mediated integration (Table S3)
990 (Bischof et al., 2007; Gyoergy et al., 2018).

991

992 **CRISPR sgRNA production and cloning**
993 sgRNA target sequences for CRISPR-Cas9 based gene knock down for CG9253 (*porthos*)
994 were designed as 20 nt sequences upstream of an NGG PAM motif in the *Drosophila* genome
995 (https://www.flyrnai.org/crispr/) (Basset and Liu, 2014). The targeting oligonucleotides
996 incorporated into *porthos* sgRNAs are given in (Table S4, The annealed oligo inserts were
997 cloned into BspQ1-digested pAC-sgRNA-Cas9 vector (Addgene, plasmid # 49330) before
998 transformation. Positive clones were confirmed by sequencing with pAC-sgRNA-Cas9-U6F
999 primer (Table S4). All CRISPR-Cas9 constructs contain three distinct cassettes for
1000 expression of Cas9, an sgRNA against *porthos*, and a puromycin resistance marker.

1001

1002 **Generation of *porthos* depleted S2R+ cells**
1003 To make the stable depleted cell lines, S2R+ Cells (2×10^5) were seeded in Schneider
1004 medium plus 10% FCS (Gibco 21720024, Sigma F9665) in a 24-well plate. Plasmid sgRNA
1005 CRISPR *porthos* was co-transfected (1 µg of total DNA per well) with Effectene Tranfection
1006 Reagent (Qiagen, Hilden, Germany) following the manufacturer's protocol. 4 hours after
1007 transfection the medium was changed and the cells were incubated for 72 hours at 25℃.
1008 Cells were then transferred to a 6-well plate before addition of 5µg/ml Puromycin. Selection
1009 with Puromycin took place for 7 days. Surviving cells were incubated without selection
1010 medium for 24 hours, after that they were added to 96-well cell culture plates in conditioned
1011 medium at a density of 1 cell/well. After 7 days we checked the wells for growing colonies to
1012 rule out that more than 1 colony was present per well. When cells were dense enough we first

1013   transferred them to a 24-, then a 12- and finally a 6-well plate. Once the cells reached
1014   confluency, we extracted the genomic DNA to perform a PCR-based prescreening of
1015   *porthos*-depleted cells to detect effective CRISPR (Table S4).

1016

1017   **Quantitative Real Time-PCR (qRT-PCR) analysis**
1018   To verify the effective knockdown of genes, we first isolated RNA from S2R+ cells ($1x10^7$
1019   for the control and KD cells) according to the manufacturer's protocol (Qiagen RNeas Mini
1020   Kit Cat No./ID: 74104). We used 500 ng of isolated RNA for cDNA synthesis, according to
1021   the manufacturer's protocol (Qiagen Omniscript RT, Cat No./ID: 205111). Afterwards we
1022   performed qPCR to assess the mRNA expression of *atossa* and *porthos,* using *RpS20* as an
1023   internal control. Primer sequences for *Drosophila atossa* (CG9005) and *porthos* (CG9253)
1024   transcripts were designed using NCBI's primer design tool
1025   (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and primer sequences for RpS20 gene, as an
1026   internal control gene, were obtained from the FlyPrimerBank
1027   (http://www.flyrnai.org/FlyPrimerBank) (Table S5). We amplified 4 µL cDNA (50 ng) using
1028   10 µL of Takyon™ No Rox SYBR MasterMix Blue dTTP (Eurogentec, Liege, Belgium), 2 µL
1029   of each reverse and forward primers (10 mM). The thermal cycling conditions were as
1030   follows: 40 cycles of amplification each consisting of 10 s at 95°C, 15 s at 60°C and 10 s at
1031   72°C, and cooling at 4°C. The experiments were carried out in technical triplicates and three
1032   biological replicates for each data point. The qPCR experiment was run on a LightCycler 480
1033   (Roche, Basel, Switzerland) and data were analyzed in the LightCycler 480 Software and Prism
1034   (GraphPad Software). To calculate the fold change in *atossa* and *porthos* mRNA levels
1035   compared to the house-keeping gene mRNA levels, we averaged the Ct values of the technical
1036   replicates of each trial. We measured Δct by subtracting the housekeeping gene Ct average
1037   from the Ct average of *atossa* or *porthos*. Afterwards, the $2^{-\Delta ct}$ was calculated for each trial.

1038

1039   **Polysome profiling in *porthos*-KD S2 cells**
1040   **RNAi treatment of S2 cells**
1041   dsRNA for *porthos* (CG9253) was prepared as described by the SnapDragon manual
1042   (https://www.flyrnai.org/snapdragon). Briefly, template was prepared from S2 cell cDNA
1043   using the following primers designed using SnapDragon 5'-
1044   TAATACGACTCACTATAGGATAAG GAAGGGGACAGCGAG-3' and the reverse
1045   primer: 5'-TAATACGACTCACTATAGGTTTGAAATGCCAGTTCCCTC-3' both of
1046   which contain a T7 polymerase promoter. As a negative control, we made non-targeting
1047   dsRNA against GFP using the following primers: 5'-
1048   TAATACGACTCACTATAGGGGAGCGCACCATCTTCTTCAA-3' and 5'-
1049   TAATACGACTCACTATAGGGCTGCTTGTCGGCCATGATATAG-3'. We performed *in*
1050   *vitro* transcription overnight at 37°C using the T7 Megascript kit (AM1334) following
1051   manufacturer's instructions (Table S4). The RNA was treated with DNAse and purified using
1052   acid-phenol chloroform extraction and ethanol precipitated. The resulting RNA was annealed
1053   by heating at 65°C for 5 minutes and slow cooling to 37°C for an hour. Knocking down in S2
1054   cells was performed using 1 µg of dsRNA as previously described
1055   (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4465107/). 0.5-1.0 X 10^6 cells were seeded
1056   30 minutes prior to transfection to adhere. Prior to transfection, the media was changed for

1057   500 µl of fresh media. The seeded cells were treated with 500 µl of transfection complexes
1058   per well of a 6-well plate. 48 hours post transfection, cells were passaged to 10 cm dishes.
1059   After 3 more days cells were harvested for further analysis.
1060
1061   **Polysome profiling and polysome sequencing**
1062   Polysome sequencing was performed as described by (Flora et al., 2018) with minor
1063   modifications. Cells were incubated with fresh medium 2-4 hours before harvesting.
1064   Cycloheximide (100 µg/ml) was first added to the medium for 3 min at RT, and the cells
1065   were subsequently centrifuged at 800 xg for 3 min. The cell pellet was afterwards washed
1066   two times with ice-cold phosphate-buffered saline (1X PBS, pH 7.4). The supernatant was
1067   discarded and the pellet was gently resuspended in 300 µl of lysis buffer A (300 mM NaCl,
1068   15 mM Tris-HCl, pH 7.5, 15 mM EDTA, 1 mg/ml heparin, 1% Triton-X100, and 100 µg/ml
1069   cycloheximide) and lysed for 15 min on ice. The lysate was clarified by centrifugation at
1070   8500 xg for 5 min at 4°C. 20% of the lysate was kept aside as an input. The clarified lysate
1071   was loaded onto a 10%-50% sucrose gradient in Buffer B (300 mM NaCl, 15 mM Tris-HCl,
1072   pH 7.5, 15 mM $MgCl_2$, supplemented with 100 µg/ml cycloheximide) and centrifuged for 3
1073   hours at 35,000 rpm in an SW41 rotor in a Beckman L7 ultracentrifuge (Beckman Coulter,
1074   Krefeld, Germany). The gradients were simultaneously fractionated on a Density Gradient
1075   Fractionation System (#621140007) at 0.75 ml/min. We added 20 µl of 20% SDS, 8 µl of 0.5
1076   M pH 8 EDTA, and 16 µl of proteinase K (#P8107S) to each polysome fraction and
1077   incubated them for 30 min at 37°C. The RNA from each fraction was extracted by standard
1078   acid phenol: chloroform purification followed by 80% ethanol precipitation. The polysome
1079   fractions were then measured for RNA content and RNAseq libraries were prepared.
1080
1081   **Polysome-seq library preparation and mRNA sequencing**
1082   The RNA was first treated with Turbo DNAse (TURBO DNA-free Kit, Life Technologies,
1083   AM1907) and then purified using DNAse Inactivation buffer. The RNA was then centrifuged
1084   for 1.5 min at 1000 xg and the supernatant was collected and centrifuged once more at the
1085   same condition. The RNA quantity was determined by measuring the absorbance at 260 nm
1086   (NanoDrop 2000 spectrophotometer; Peqlab).
1087   Poly-A selection was performed according to manufacturer's instructions (Bioo Scientific
1088   Corp., 710 NOVA-512991). Following Poly-A selection mRNA libraries were prepared
1089   according to manufacturer's instructions (Bioo Scientific Corp., NOVA-5138-08), except that
1090   the RNA was incubated at 95°C for 13 min to generate optimal fragment sizes. The
1091   sequencing library quantity was determined using Qubit (Thermo Fisher Scientific). The
1092   library integrity was assessed with a Bioanalyzer 2100 system (RNA 6000 Pico kit, Agilent
1093   Technologies). The libraries on biological duplicates from each genotype were subjected to
1094   75 base-pair single-end sequencing on Illumina NextSeq500 at the Center for Functional
1095   Genomics (CFG).
1096
1097   **Data analysis of S2 cell polysome sequencing**
1098   First the reads were assessed for their quality using FastQC. Mapping of the reads was
1099   performed against *Drosophila* Genome (dm6.01, www.fruitfly.org) using Hisat version 2.1.0.
1100   Mapped reads were then assigned to feature using featureCount version v1.6.4. To calculate

24

1101   Translation efficiency (TE), TPMs (transcripts per million) values for polysome-libraries
1102   were calculated (Flora et al., 2018). All transcripts with zero reads were discarded from
1103   libraries for further analysis. The log2 ratio of TPMs between the polysome fraction and total
1104   mRNA was measured. This ratio represents TE. The TE value of each replicate was averaged
1105   and delta TE (ΔTE) was calculated as (*porthos* dsRNA TE)/(GFP dsRNA TE). Targets were
1106   defined as transcripts falling greater or less than two standard deviations (SD) from the
1107   median of ΔTE (Table S5).
1108

1109   **Extracellular flux measurements for bioenergetic profiling**
1110   Cellular respiration was assessed using a Seahorse XF96 extracellular flux analyzer (Agilent
1111   Technologies, Santa Clara, CA USA). The oxygen consumption rate (OCR) as a measure of
1112   oxygen utilization of cells is an important indicator of mitochondrial function. The
1113   extracellular acidification rate (ECAR) is a measure of glycolytic activity measured via
1114   extracellular acidification due to lactate release, formed during the conversion of glucose to
1115   lactate during anaerobic glycolysis. Prior to measurement, wild-type and *porthos* KD cells
1116   were seeded at 10×10^5 cells per well in Seahorse XF96 polystyrene tissue culture plates
1117   (Agilent) and incubated in unbuffered Seahorse RPMI assay medium (Agilent) supplemented
1118   with glucose (25 mM; Sigma-Aldrich), sodium pyruvate (1 mM; Gibco), and glutamine (2
1119   mM; Gibco) in a non-CO2 incubator at 25°C and pH 7.4 for 1 h before the experiment.
1120   Cellular oxygen consumption was assessed in basal condition (prior to any addition) and after
1121   addition of oligomycin (2 μM; Agilent) Carbonyl cyanide-4 (trifluoromethoxy)
1122   phenylhydrazone (FCCP, 2 μM; Sigma-Aldrich), antimycin A and rotenone (both at 1 μM;
1123   Agilent). The three drugs were injected into the XF96 plate sequentially. This allowed for
1124   calculation of OCR linked to ATP production, maximal respiration capacity and spare
1125   respiratory capacity. Basal respiration was measured prior to injection of oligomycin A. Both
1126   OCR and ECAR were measured every 4 min with a mixing of 2 min in each cycle, with 4
1127   cycles in total for the first step and 3 cycles thereafter.
1128   Different parameters from the OCR graph were measured as follows. ATP turnover
1129   was calculated by subtracting the "last rate measurement before oligomycin" from the
1130   "minimum rate measurement after oligomycin injection". Maximal respiration was defined as
1131   (maximum rate measurement after FCCP) - (non-mitochondrial respiration). Spare
1132   respiratory capacity (SRC) was measured by subtracting basal respiration from maximal
1133   respiration (Mookerjee et al., 2017).
1134

1135   **Metabolomics profiling analysis**
1136   Samples for metabolomics were assessed by the VBCF metabolomics facility according to
1137   Rao et al. with slight modifications
1138   (https://www.viennabiocenter.org/facilities/metabolomics/) (Rao et al., 2019). 1 gr of wild-
1139   type or *atos* embryos were extracted using an ice-cold MeOH:ACN:H2O (2:2:1, v/v) solvent
1140   mixture. A volume of 1mL of cold solvent was added to each pellet, vortexed for 30 s, and
1141   incubated in liquid nitrogen for 1 min. The samples were thawed at room temperature and
1142   sonicated for 10 min. This cycle of cell lysis in liquid nitrogen combined with sonication was
1143   repeated three times. To precipitate proteins, the samples were incubated for 1 h at –20°C,
1144   followed by centrifugation at 13,000 rpm for 15 min at 4°C. The supernatant was removed

1145 and evaporated. The dry extracts were reconstituted in 100 μL of ACN:H2O (1:1, v/v),
1146 sonicated for 10 min, and centrifuged at 13,000 rpm for 15 min at 4°C to remove insoluble
1147 debris. The supernatants were transferred to Eppendorf tubes, shock frozen and stored at
1148 -80°C prior to LC/MS analysis. A volume of 1 μL of the metabolite extract was injected on a
1149 ZIC-pHILIC HPLC column operated at a flow rate of 100 μL/min, directly coupled to a TSQ
1150 Quantiva mass spectrometer (Thermo Fisher Scientific).

1151 We used the following transitions for quantitation in the negative ion mode: AMP 346
1152 m/z to 79 m/z, ADP 426 m/z to 134 m/z, ATP 506 m/z to 159 m/z, IMP 347 m/z to 79 m/z,
1153 GMP 362 m/z to 211 m/z, GDP 442 m/z to 344 m/z, GTP 522 m/z to 424 m/z, taurine 124
1154 m/z to 80 m/z, malate 133 m/z to 115 m/z, citrate 191 m/z to 111 m/z, pyruvate 87 m/z to 43
1155 m/z, lactate 89 m/z to 43 m/z, NADH 664 m/z to 408 m/z, NAD 662 m/z to 540 m/z, hexose
1156 phosphates 259 m/z to 97 m/z, Acetyl CoA 808 m/z to 408 m/z, CoA 766 m/z to 408 m/z,
1157 succinate 117 m/z to 73 m/z. Glutamine 147 m/z to 130 m/z, glutamate 148 m/z to 84 m/z,
1158 serine 106 m/z to 60 m/z were calculated in the positive ion mode. For all transitions, the
1159 optimal collision energy was defined by analyzing pure metabolite standards.
1160 Chromatograms were manually interpreted using trace finder (Thermo Fisher Scientific),
1161 validating experimental retention times with the respective quality controls. All
1162 measurements were within the linear range of detection.

1163 For the metabolomics analysis, the metabolite concentration was normalized using a
1164 Z-score normalization method with the formula of $y = (x-\alpha)/\lambda$, in which x refers to the real
1165 concentration, α indicates the mean value of all samples, and λ is the variance of all samples.
1166 The normalized concentrations of metabolites were applied to generate a heatmap, which
1167 showed the concentration difference of all metabolites. For KEGG (http://www.kegg.jp,
1168 Tokyo, Japan) pathway analysis, the clusterProfiler R package was employed.
1169

Statistics and repeatability

1171 Statistical tests as well as the number of embryos/ cells assessed are listed in the figure
1172 legends. All statistical analyses were performed using GraphPad Prism and significance was
1173 determined using a 95% confidence interval. Data points from individual experiments/
1174 embryos were pooled to estimate mean and SEM. No statistical method was used to
1175 predetermine sample size and the experiments were not randomized. Unpaired t-test or
1176 Mann-Whitney was used to calculate the significance in differences between two groups and
1177 One-way Anova followed by Tukey post-test followed by Conover or Dunn's post-test for
1178 multiple comparisons. All measurements were performed in 3-50 embryos. Representative
1179 images illustrated in Figures 1A-C, Figures 2B-C,E, Figures S2A-B, Figures 3B-D, Figure
1180 4A,I, Figure S4B, and Figure 6D,G,I were from separate experiments that were repeated at
1181 least 3 and up to 7 times. Stills shown in Figure 1F, Figure S1I, Figure 4B, and Figure S4E
1182 are representative images from two-photon movies, which were repeated at least 3 times.
1183 Raw data from embryo scoring and analyzed tracking output from each movie is in Data S4.
1184

Exact genotype of *Drosophila* lines used in Figures:
1186 **Figure 1 and Figure S1**

1187   **Figs. 1A-C:** Control: *w-; +; srpHemo-H2A::3xmCherry*, CG9005 mutant*: w-;*
1188   *P{EP}CG9005^{BG02278};*   *srpHemo-H2A::3xmCherry*,   CG9005   rescue*: w-;*
1189   *P{EP}CG9005^{BG02278}; srpHemo-CG9005, srpHemo-H2A::3xmCherry.* **Fig. 1D:** Control: *w-;*
1190   *+; srpHemo-H2A::3xmCherry*, CG9005 mutant: *w-; P{EP}CG9005^{BG02278}; srpHemo-*
1191   *H2A::3xmCherry*, Df1: *w-; P{EP}CG9005^{BG02278}/ Df(2R)ED2222; srpHemo-*
1192   *H2A::3xmCherry*, Df2: *w-; P{EP}CG9005^{BG02278}/Df(2R)BSC259; srpHemo-*
1193   *H2A::3xmCherry*, CG9005 rescue: *w-; P{EP}CG9005^{BG02278}; srpHemo-CG9005, srpHemo-*
1194   *H2A::3xmCherry.* **Fig. 1E:** Control 1: *w⁻ P(w+)UAS-dicer/w-; P{attP,y[+],w[3`]/+;*
1195   *srpHemo-Gal4 UAS-GFP*, CG9005 RNAi 1: *UAS-Dicer2/w-; CG9005 RNAi (v106589)/+;*
1196   *srpHemo-Gal4 UAS-GFP, UAS-H2A::RFP/+*, Control 2: *w⁻ P(w+)UAS-dicer/w-; +;*
1197   *srpHemo-Gal4 UAS-GFP*, CG9005 RNAi 2: *UAS-Dicer2/ w-; CG9005 RNAi (v36080)/+;*
1198   *srpHemo-Gal4 UAS-GFP, UAS-H2A::RFP/+*, Control 3: *w⁻ P(w+)UAS-dicer/w-;*
1199   *P{attP,y[+],w[3`]/+; srpHemo-Gal4 UAS-GFP*, CG9005 RNAi 3: *UAS-Dicer2/w-; CG9005*
1200   *RNAi (v33362)/+; srpHemo-Gal4 UAS-GFP, UAS-H2A::RFP/+.* **Figs. 1F-L:** Control: *w-; +;*
1201   *srpHemo-H2A::3xmCherry,*   CG9005   mutant: *w-;   P{EP}CG9005^{BG02278};   srpHemo-*
1202   *H2A::3xmCherry.*

1203   **Fig. S1A:** Control: *w-; +; srpHemo-H2A::3xmCherry,* mutant: *w-; P{EP}CG9005^{BG02278};*
1204   *srpHemo-H2A::3xmCherry*, Df1 cross: *w-; P{EP}CG9005^{BG02278}/Df(2R)ED2222; srpHemo-*
1205   *H2A::3xmCherry*, Df2 cross: *w-; P{EP}CG9005^{BG02278}/Df(2R)BSC259; srpHemo*
1206   *H2A::3xmCherry*, CG9005 rescue: *w-; P{EP}CG9005^{BG02278}; srpHemo-CG9005, srpHemo-*
1207   *H2A::3xmCherry.* **Figs. S1B,H:** Control 1: *w⁻ P(w+)UAS-dicer/w-; P{attP,y[+],w[3`]/+;*
1208   *srpHemo-Gal4 UAS-GFP*, CG9005 RNAi 1: *UAS-Dicer2/w-; CG9005 RNAi (v106589)/+;*
1209   *srpHemo-Gal4 UAS-GFP, UAS-H2A::RFP/+.* Control 2: *w⁻ P(w+)UAS-dicer/w-; +;*
1210   *srpHemo-Gal4 UAS-GFP*, CG9005 RNAi 2*: UAS-Dicer2/w-; CG9005 RNAi (v36080)/+;*
1211   *srpHemo-Gal4 UAS-GFP, UAS-H2A::RFP/+.* Conrol 3*: w⁻ P(w+)UAS-dicer/w-;*
1212   *P{attP,y[+],w[3`]/+; srpHemo-Gal4 UAS-GFP*, CG9005 RNAi 3: *UAS-Dicer2/ w-; CG9005*
1213   *RNAi (v33362)/+; srpHemo-Gal4 UAS-GFP, UAS-H2A::RFP/+.* **Figs. S1C,G:** Control: *w-;*
1214   *+; srpHemo- H2A::3xmCherry,* mutant: *w-; P{EP}CG9005^{BG02278}; srpHemo-*
1215   *H2A::3xmCherry.* **Fig. S1D:** Control 1: *w⁻ P(w+)UAS-dicer/w-; P{attP,y[+],w[3`]/+;*
1216   *srpHemo-Gal4 UAS-GFP*, CG9005 RNAi 1*: UAS-Dicer2/w-; CG9005 RNAi (v106589)/+;*
1217   *srpHemo-Gal4 UAS-GFP, UAS-H2A::RFP/+.* **Fig. S1E:** Control 2: *w⁻ P(w+)UAS-dicer/w-;*
1218   *+; srpHemo-Gal4 UAS-GFP*, CG9005 RNAi 2*: UAS-Dicer2/w-; CG9005 RNAi (v36080)/+;*
1219   *srpHemo-Gal4 UAS-GFP, UAS-H2A::RFP/+.* **Fig. S1F:** Conrol 3*: w⁻ P(w+)UAS-dicer/w-;*
1220   *P{attP,y[+],w[3`]/+; srpHemo-Gal4 UAS-GFP*, CG9005 RNAi 3*: UAS-Dicer2/w-; CG9005*
1221   *RNAi (v33362)/+; srpHemo-Gal4 UAS-GFP, UAS-H2A::RFP/+.* **Figs. S1I-L:** Control: *w-;*
1222   *+; srpHemo-H2A::3xmCherry*, CG9005 mutant: *w-; P{EP}CG9005^{BG02278}; srpHemo-*
1223   *H2A::3xmCherry.*

1224

1225   **Figure 2 and Figure S2**
1226   **Fig. 2B:** *w-;+; UAS-atossa::FLAG::HA, srpHemo-Gal4, srpHemo-H2A::3xmCherry.* **Figs.**
1227   **2C,D:** Control: *w-; +; srpHemo- H2A::3xmCherry, atos* mutant: *w-; atossa^{BG02278};*
1228   *srpHemo-H2A::3xmCherry,* Atossa rescue: *w-; atossa^{BG02278}; srpHemo- atossa, srpHemo-*
1229   *H2A::3xmCherry ,* rescue: *w-; atossa^{BG02278}; srpHemo- atossa^{DUF4210-}, srpHemo-*
1230   *H2A::3xmCherry ,* rescue: *w-; atossa^{BG02278}; srpHemo- atossa^{CherSeg-}, srpHemo-*

1231 *H2A::3xmCherry*, rescue: *w-; atossa ^{BG02278}; srpHemo- atossa $^{DU4210-/CherSeg-}$, srpHemo-*

1232 *H2A::3xmCherry*, rescue: *w-; atossa ^{BG02278}; srpHemo- atossa $^{TAD1-/TAD2-}$, srpHemo-*

1233 *H2A::3xmCherry*. **Figs. 2E,F:** Control: *w-; +; srpHemo- H2A::3xmCherry,* mutant: *w-;*

1234 *atossa^{BG02278}; srpHemo-H2A::3xmCherry,* rescue: *w-; atossa^{BG02278}; srpHemo-FAM214A,*

1235 *srpHemo-H2A::3xmCherry,* rescue: *w-; atossa^{BG02278}; srpHemo-FAM214B, srpHemo-*

1236 *H2A::3xmCherry.*

1237 **Figs. S2B:** Rescue: *w-; atossa^{BG02278}; srpHemo-atossa $^{TAD1-}$, srpHemo-H2A::3xmCherry*,

1238 rescue: *w-; atossa^{BG02278}; srpHemo-atossa $^{TAD2-}$, srpHemo-H2A::3xmCherry*. **Fig. S2C:**

1239 Control: *w-; +; srpHemo-H2A::3xmCherry,* atos mutant: *w-; atossa^{BG02278}; srpHemo-*

1240 *H2A::3xmCherry,* Atossa rescue: *w-; atossa^{BG02278}; srpHemo-atossa, srpHemo-*

1241 *H2A::3xmCherry,* rescue: *w-; atossa^{BG02278}; srpHemo-atossa $^{TAD1-}$, srpHemo-*

1242 *H2A::3xmCherry,* rescue: *w-; atossa^{BG02278}; srpHemo-atossa $^{TAD2-}$, srpHemo-*

1243 *H2A::3xmCherry*. **Fig. S2D:** Control: *w-; +; srpHemo-H2A::3xmCherry,* mutant: *w-;*

1244 *atossa^{BG02278}; srpHemo-H2A::3xmCherry,* rescue: *w-; atossa^{BG02278}; srpHemo-atossa,*

1245 *srpHemo-atossa $^{DUF4210-}$,* rescue: *w-; atossa^{BG02278}; srpHemo-atossa $^{DUF4210-}$, srpHemo-*

1246 *H2A::3xmCherry,* rescue: *w-; atoss ^{BG02278}; srpHemo-atossa $^{CherSeg-}$, srpHemo-*

1247 *H2A::3xmCherry,* rescue: *w-; atossa^{BG02278}; srpHemo-atossa$^{DUF4210-/CherSeg-}$, 2srpHemo-*

1248 *H2A::3xmCherry* , rescue: *w-; atossa^{BG02278}; srpHemo-atossa^{TAD1-}, srpHemo-*

1249 *H2A::3xmCherry,* rescue: *w-; atossa^{BG02278}; srpHemo-atossa^{TAD2-}, srpHemo-*

1250 *H2A::3xmCherry,* rescue: *w-; atossa^{BG02278}; srpHemo-atossa$^{TAD1-/2-}$, srpHemo-*

1251 *H2A::3xmCherry*. **Fig. S2E:** Control: *w-; +; srpHemo-H2A::3xmCherry,* atos mutant: *w-;*

1252 *atossa^{BG02278}; srpHemo-H2A::3xmCherry,* Atossa rescue: *w-; atossa^{BG02278}; srpHemo-atossa,*

1253 *srpHemo-H2A::3xmCherry,* rescue: *w-; atossa^{BG02278}; srpHemo-FAM214A, srpHemo-*

1254 *H2A::3xmCherry,* rescue: *w-; atossa^{BG02278}; srpHemo-FAM214B, srpHemo-*

1255 *H2A::3xmCherry.*

1256

1257 **Figure 3 and Figure 3S**

1258 **Figs. 3B,F:** Control (for *porthos* or *CG9253*): *w/y,w[1118]; P{attP,y[+],w[3']}; srpHemo-*

1259 *Gal4, srpHemo-H2A::3xmCherry/+,* CG9253 RNAi (*porthos*): *w-; porthos RNAi*

1260 (*v36589)/+; srpHemo-Gal4, srpHemo-H2A::3xmCherry/+.* **Fig. 3C:** Control 1 (for CG9331

1261 or *GR/HPR*): *w/y,w[1118]; P{attP,y[+],w[3']}; srpHemo-Gal4, srpHemo-*

1262 *H2A::3xmCherry/+,* CG9331 RNAi 1 (GR/HPR): *UAS-Dicer2/ w-; GR/HPR RNAi*

1263 (*v44653)/+; srpHemo-Gal4, srpHemo-H2A::3xmCherry/+.* **Fig. 3D:** Control 1 (for CG7144

1264 or *LKR/SDH*): *w/y,w[1118]; P{attP,y[+],w[3']}; srpHemo-Gal4, srpHemo-*

1265 *H2A::3xmCherry/+,* CG7144 RNAi 1 (*LKR/SDH*): *UAS-Dicer2/ w-; LKR/SDH RNAi*

1266 (*v51346)/+; srpHemo-Gal4, srpHemo-H2A::3xmCherry/+.* **Fig. 3F:** Control 1: *w/y,w[1118];*

1267 *P{attP,y[+],w[3']}; srpHemo-Gal4, srpHemo-H2A::3xmCherry/+,* CG9331 RNAi 1

1268 *(GR/HPR): UAS-Dicer2/ w-; GR/HPR RNAi (v44653)/+; srpHemo-Gal4, srpHemo-*

1269 *H2A::3xmCherry/+,* Control 2: *w/y,w[1118]; P{attP,y[+],w[3']}; srpHemo-Gal4, srpHemo-*

1270 *H2A::3xm- Cherry/+,* CG9331 RNAi 2 (*GR/HPR*): *UAS-Dicer2/ w-; GR/HPR RNAi*

1271 (*v10780)/+; srpHemo-Gal4, srpHemo-H2A::3xmCherry/+,* Control 3: *w/y,w[1118];*

1272 *P{attP,y[+],w[3']}; srpHemo-Gal4, srpHemo-H2A::3xmCherry/+,* CG9331 RNAi 3

1273 *(GR/HPR): UAS-Dicer2/ w-; GR/HPR RNAi (64652)/+; srpHemo-Gal4, srpHemo-*

1274 *H2A::3xmCherry/+*. **Fig. 3G:** Control 1: *w/y,w[1118]; P{attP,y[+],w[3'];} srpHemo-Gal4,*
1275 *srpHemo-H2A::3xmCherry/+,* CG7144 RNAi 1 (*LKR/SDH*): *UAS-Dicer2/ w-; LKR/SDH*
1276 *RNAi (v51346)/+; srpHemo-Gal4, srpHemo-H2A::3xmCherry/+,* Control 2: *w/y,w[1118];*
1277 *P{attP,y[+],w[3'];} srpHemo-Gal4, srpHemo-H2A::3xmCherry/+,* CG7144 RNAi 2
1278 *(LKR/SDH): UAS-Dicer2/ w-; LKR/SDH RNAi (v109650)/+; srpHemo-Gal4, srpHemo-*
1279 *H2A::3xmCherry/+.*
1280 **Figs. S3A-B:** Control: *w-; +; srpHemo-H2A::3xmCherry,* mutant*: w-; P{EP}CG9005^{BG02278};*
1281 *srpHemo-H2A::3xmCherry.* **Fig. S3D:** Control 1: *w/y,w[1118]; P{attP,y[+],w[3'];}*
1282 *srpHemo-Gal4, srpHemo-H2A::3xmCherry/+,* CG2137 RNAi 1 (*Gpo2*): *w-/y,w[1118];*
1283 *Gpo2 RNAi (v41234)/+; srpHemo-Gal4, srpHemo-H2A::3xmCherry/+,* Control 2:
1284 *w/y,w[1118]; P{attP,y[+],w[3'];} srpHemo-Gal4, srpHemo-H2A::3xmCherry/+,* CG2137
1285 RNAi 2 (*Gpo2*): *w-/y,w[1118]; Gpo2 RNAi (68145)/+; srpHemo-Gal4, srpHemo-*
1286 *H2A::3xmCherry/+.* **Fig. S3E:** Control 1: *w/y,w[1118]; P{attP,y[+],w[3'];} srpHemo-Gal4,*
1287 *srpHemo-H2A::3xmCherry/+,* CG11061 RNAi 1 (*GM130*): *w-/y,w[1118]; GM130 RNAi*
1288 *(v330284)/+; srpHemo-Gal4 UAS-GFP, UAS-H2A::RFP/+,* Control 2: *w/y,w[1118];*
1289 *P{attP,y[+],w[3'];} srpHemo-Gal4, srpHemo-H2A::3xmCherry/+,* CG11061 RNAi 2
1290 (*GM130*): *w-/y,w[1118]; GM130 RNAi (64920)/+; srpHemo-Gal4, srpHemo-*
1291 *H2A::3xmCherry/+.* **Fig. S3F:** Control (for CG9253 or *porthos*): *w/y,w[1118];*
1292 *P{attP,y[+],w[3'];} srpHemo-Gal4, srpHemo-H2A::3xmCherry/+,* CG9253 RNAi (*porthos*):
1293 *w-; porthos RNAi (v36589)/+; srpHemo-Gal4, srpHemo-H2A::3xmCherry/+.* **Fig. S3G:**
1294 Control 1: *w/y,w[1118]; P{attP,y[+],w[3'];} srpHemo-Gal4, srpHemo-H2A::3xmCherry/+,*
1295 *CG9331* RNAi 1 (*GR/HPR*)*: UAS-Dicer2/ w-; GR/HPR RNAi (v44653)/+; srpHemo-Gal4,*
1296 *srpHemo-H2A::3xmCherry/+, Control* 2: *w/y,w[1118]; P{attP,y[+],w[3'];} srpHemo-Gal4,*
1297 *srpHemo-H2A::3xmCherry/+,* CG9331 RNAi 2 (*GR/HPR*)*: UAS-Dicer2/ w-; GR/HPR RNAi*
1298 (*v10780)/+; srpHemo-Gal4, srpHemo-H2A::3xmCherry/+, Control 3*: *w/y,w[1118];*
1299 *P{attP,y[+],w[3'];} srpHemo-Gal4, srpHemo-H2A::3xmCherry/+,* CG9331 RNAi 3
1300 (*GR/HPR*): *UAS-Dicer2/ w-; GR/HPR RNAi (64652)/+; srpHemo-Gal4, srpHemo-*
1301 *H2A::3xmCherry/+.* **Fig. S3H:** Control 1: *w/y,w[1118]; P{attP,y[+],w[3'];} srpHemo-Gal4,*
1302 *srpHemo-H2A::3xmCherry/+,* CG7144 RNAi 1 (*LKR/SDH*): *UAS-Dicer2/ w-; LKR/SDH*
1303 *RNAi (v51346)/+; srpHemo-Gal4, srpHemo-H2A::3xmCherry/+,* Control 2: *w/y,w[1118];*
1304 *P{attP,y[+],w[3'];} srpHemo-Gal4, srpHemo-H2A::3xmCherry/+,* CG7144 RNAi 2
1305 (*LKR/SDH*): *UAS-Dicer2/w-; LKR/SDH RNAi (v109650)/+; srpHemo-Gal4, srpHemo-*
1306 *H2A::3xmCherry/+.*
1307
1308 **Figure 4 and Figure 4S**
1309 **Fig. 4A:** *w-;+; UAS-porthos::FLAG::HA, srpHemo-Gal4, srpHemo::3xmCherry.* **Figs. 4B-**
1310 **H:** Control: *w/y,w[1118]; P{attP,y[+],w[3'];} srpHemo-Gal4, srpHemo-*
1311 *H2A::3xmCherry/+,* CG9253 RNAi (*porthos*): *w-; porthos RNAi (v36589)/+; srpHemo-*
1312 *Gal4, srpHemo-H2A::3xmCherry/+.* **Figs. 4I-J:** Control: *w-; +; srpHemo-H2A::3xmCherry,*
1313 *atos* mutant: *w-; atossa^{BG02278}; srpHemo-H2A::3xmCherry,* Atos rescue: *w-; atossa^{BG02278};*
1314 *UAS-atossa::FLAG::HA, srpHemo-Gal4, srpHemo-H2A::3xmCherry,* rescue: *w-;*
1315 *atossa^{BG02278}; UAS-porthos::FLAG::HA, srpHemo-Gal4, srpHemo-H2A::3xmCherry.*

1316 **Figs. 4SC-H:** Control: *w/y,w[1118]; P{attP,y[+],w[3']}/+; srpHemo-Gal4, srpHemo-*
1317 *H2A::3xmCherry/+,* CG9253 RNAi (*porthos*): *w-; porthos RNAi (v36589)/+; srpHemo-*
1318 *Gal4, srpHemo-H2A::3xmCherry/+.*

1319

1320 **Figure 6 and Figure 6S**

1321 **Fig. 6D-F:** Control: *w-; +; srpHemo-Gal4, srpHemo-H2A::3xmCherry,* dominant negative
1322 inhibitor of Complex V (CV-DN): *w-;UAS-CVDN; srpHemo-Gal4, srpHemo-*
1323 *H2A::3xmCherry.* **Figs. 6G-H:** Control*: w-; P{attP,y[+],w[3`]}/+; srpHemo-Gal4,*
1324 *srpHemo-H2A::3xmCherry ,* Complex III (Cyt-c1, CG4769) RNAi 1: *w-; cyt-c1 RNAi*
1325 (*v109809)/+; srpHemo-Gal4, srpHemo-H2A::3xmCherry,* Complex III (UQCR-cp1,
1326 CG3731) RNAi 2: *w-; UQCR-cp1 RNAi (v101350)/+; srpHemo-Gal4, srpHemo-*
1327 *H2A::3xmCherry,* Complex III (UQCR-cp2, CG4169) RNAi 3: *w-; UQCR-cp2 RNAi*
1328 (*v100818)/+; srpHemo-Gal4, srpHemo -H2A::3xmCherry,* Complex V (ATP synthase F1F0,
1329 CG3612) RNAi: *w-; RNAi (v34664)/+; srpHemo-Gal4, srpHemo-H2A::3xmCherry.* **Fig. 6J:**
1330 Control: *w-; +; srpHemo-Gal4, srpHemo-3xmCherry, atos* mutant: *w-; atossa^{BG02278};*
1331 *srpHemo-Gal4, srpHemo-3xmCherry,* Control: *w/y,w[1118]; P{attP,y[+],w[3']};srpHemo-*
1332 *Gal4, srpHemo-3xmCherry/+,* CG9253 RNAi (*porthos*): *w-; porthos RNAi (v36589)/+;*
1333 *srpHemo-Gal4, srpHemo-3xmCherry/+,* Control: *w-; +; srpHemo-Gal4, srpHemo-*
1334 *3xmCherry, CV-DN: w-;UAS-CV DN; srpHemo-Gal4, srpHemo-3xmCherry.*

1335 **Fig. 6SF:** Control*: w-; P{attP,y[+],w[3`]/+; srpHemo-Gal4, srpHemo-H2A::3xmCherry,*
1336 Complex III (Cyt-c1, CG4769) RNAi 1: *w-; cyt-c1 RNAi (v109809)/+; srpHemo-Gal4,*
1337 *srpHemo-H2A::3xmCherry,* Complex III (UQCR-cp1, CG3731) RNAi 2: *w-; UQCR-cp1*
1338 *RNAi (v101350)/+; srpHemo-Gal4, srpHemo-H2A::3xmCherry,* Complex III (UQCR-cp2,
1339 CG4169) RNAi 3: *w-; UQCR-cp2 RNAi (v100818)/+; srpHemo-Gal4, srpHemo-*
1340 *H2A::3xmCherry,* Complex V (ATP synthase F1F0, CG3612) RNAi: *w-; CG3612 RNAi*
1341 (*v34664)/+; srpHemo-Gal4, srpHemo-H2A::3xmCherry.* **Figs. 6SG-H:** *w-; +; srpHemo-*
1342 *Gal4, srpHemo-3xmCherry, atos* mutant: *w-; atossa^{BG02278}; srpHemo-Gal4, srpHemo-*
1343 *3xmCherry,* Control: *w/y,w[1118]; P{attP,y[+],w[3']};srpHemo-Gal4, srpHemo-*
1344 *3xmCherry/+,* CG9253 RNAi (*porthos*): *w-; porthos RNAi (v36589)/+; srpHemo-Gal4,*
1345 *srpHemo-H2A::3xmCherry/+,* Control: *w-; +; srpHemo-Gal4, srpHemo-3xmCherry, CV-*
1346 *DN: w-;UAS-CV DN; srpHemo-Gal4, srpHemo-3xmCherry*

1347

1348 **Figures 7 and S7:**

1349 **Figs. 7B-H, SB-I:** Control: *w-; +; srpHemo-3xmCherry,* mutant: *w-; atossa^{BG02278};*
1350 *srpHemo-3xmCherry.*

1351 **Resource Availability:**

1352 Fly lines, plasmids and other reagents utilized are available upon request from the Lead
1353 contact: daria.siekhaus@ist.ac.at

1354 Original reads from RNA sequencing and Polysome profiling has been deposited at: (will be
1355 done once paper is in revision).

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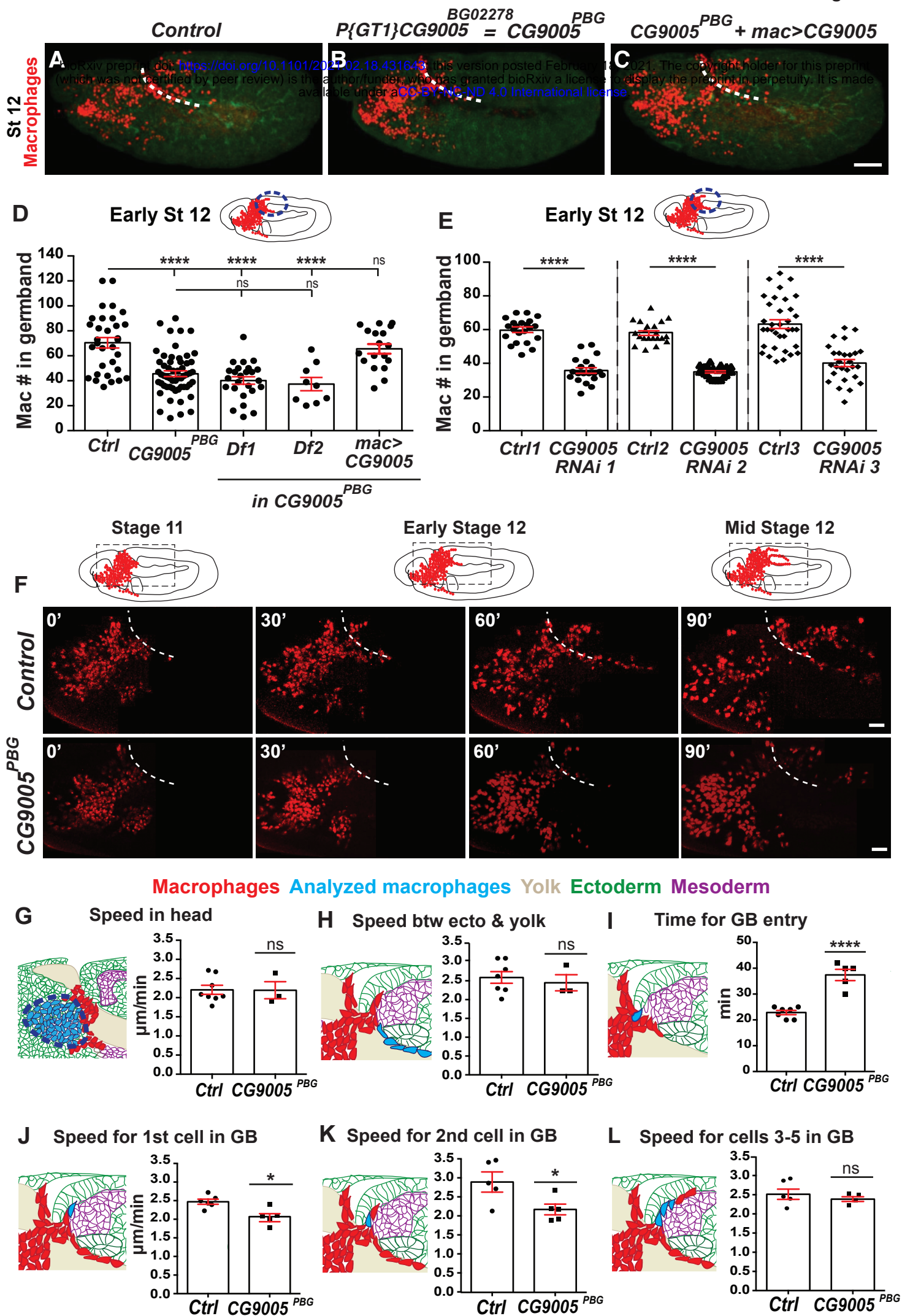1536   Cell Biol. 10, 992–1003.
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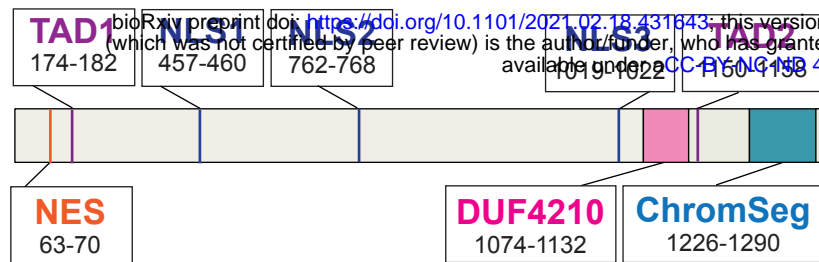1714 Wood ,W., Martin, P. (2017). Macrophage functions in tissue patterning and disease: new insights from the

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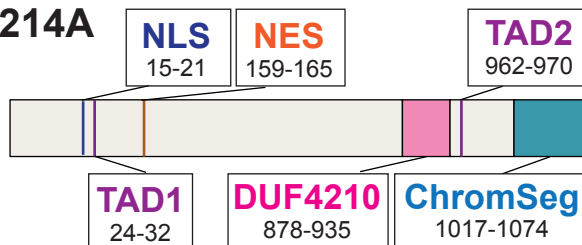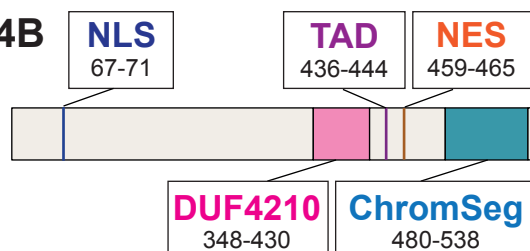
Figure 1

Control

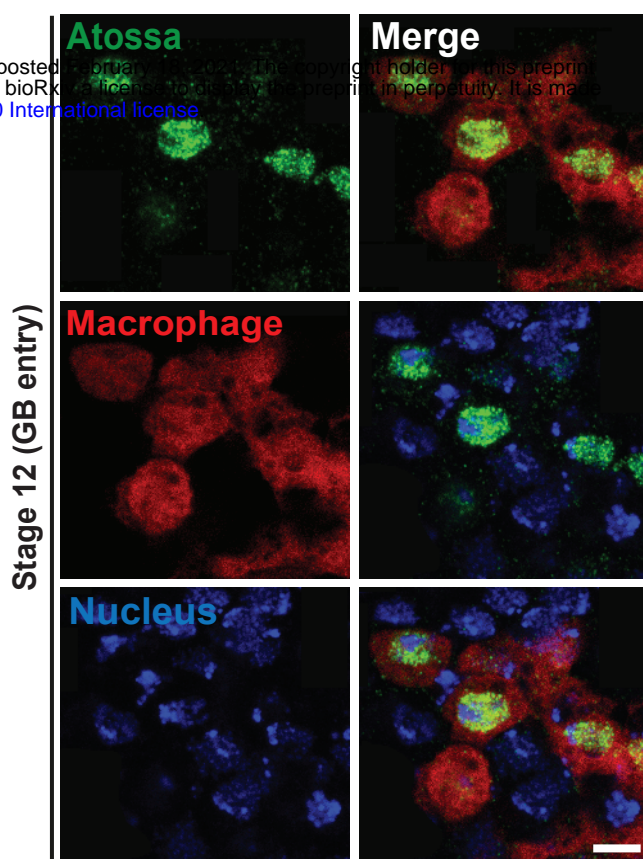*P{GT1}CG9005^{BG02278} = CG9005^{PBG}*

CG9005^{PBG} + mac>CG9005

St 12 / Macrophages

A B C

D **Early St 12**

E **Early St 12**

F

Stage 11   **Early Stage 12**   **Mid Stage 12**

Control   0'   30'   60'   90'

CG9005^{PBG}   0'   30'   60'   90'
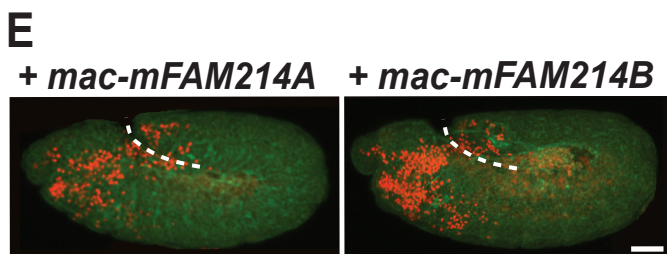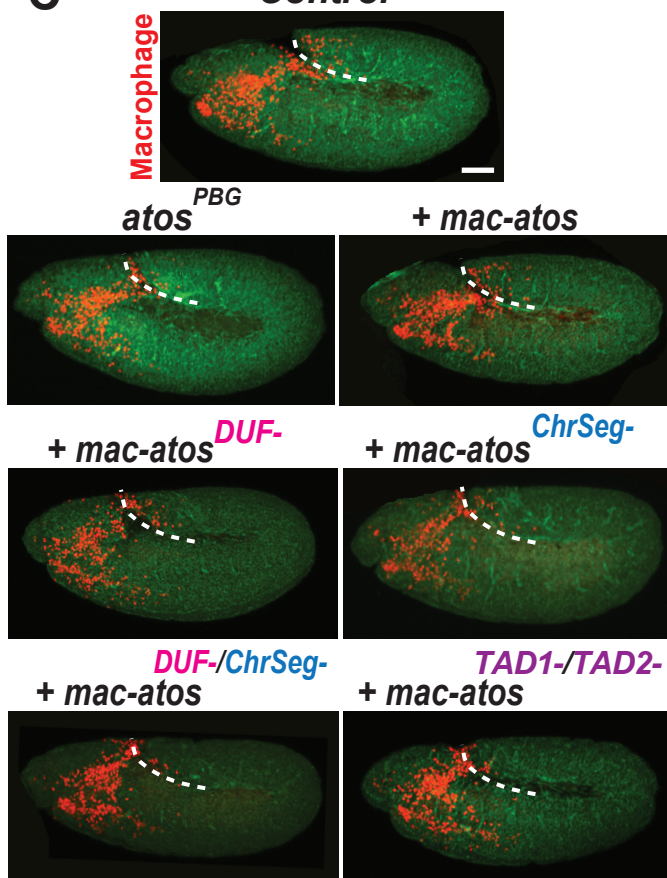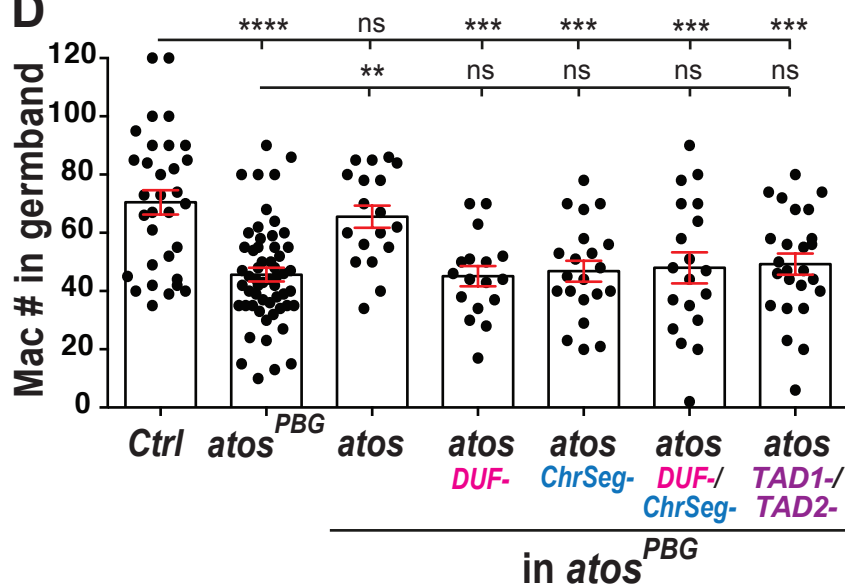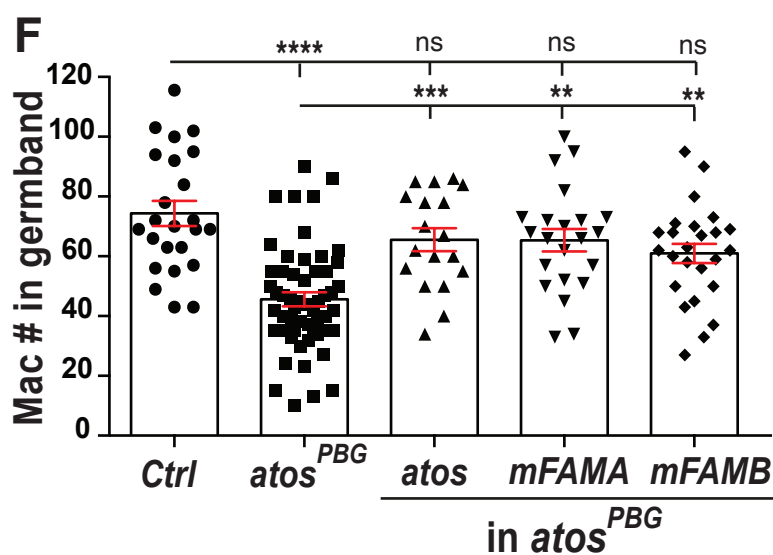
Macrophages **Analyzed macrophages** **Yolk** **Ectoderm** **Mesoderm**

G **Speed in head**

H **Speed btw ecto & yolk**

I **Time for GB entry**

J **Speed for 1st cell in GB**

K **Speed for 2nd cell in GB**

L **Speed for cells 3-5 in GB**

Figure. 2

A

CG9005 = Atossa

TAD1 174-182 | NLS1 457-460 | NLS2 762-768 | NES 1019-1022 | TAD2

NES 63-70

DUF4210 1074-1132 | ChromSeg 1226-1290

Murine FAM214A

NLS 15-21 | NES 159-165 | TAD2 962-970

TAD1 24-32 | DUF4210 878-935 | ChromSeg 1017-1074

Murine FAM214B

NLS 67-71 | TAD 436-444 | NES 459-465

DUF4210 348-430 | ChromSeg 480-538

B

Atossa | Merge

Macrophage

Nucleus

Stage 12 (GB entry)

C

Macrophage | *Control*

atos^PBG | + *mac-atos*

+ *mac-atos^DUF-* | + *mac-atos^ChrSeg-*

DUF-/ChrSeg- + *mac-atos* | *TAD1-/TAD2-* + *mac-atos*

E

+ *mac-mFAM214A* | + *mac-mFAM214B*

D

Mac # in germband

**** | ns | *** | *** | *** | ***
| ** | ns | ns | ns | ns

Ctrl | atos^PBG | atos | atos DUF- | atos ChrSeg- | atos DUF-/ChrSeg- | atos TAD1-/TAD2-

in atos^PBG

F

Mac # in germband

**** | ns | ns | ns
| *** | ** | **

Ctrl | atos^PBG | atos | mFAMA | mFAMB

in atos^PBG

A

RNA seq from control and atossa mutant macrophages

Gene	Expression			Biological functions
	WT	atos mut	FC	
CG9253 (porthos)	144	14	-10	ATP-dependent RNA heilcase
CG2137 (Gpo2)	31	0.95	-33	Glycerophosphate oxidase 2
CG11061 (GM130)	27.5	3.4	-8	Connects Golgi compartments
CG9331 (dGR/HPR)	20.7	3.2	-6.5	Glyoxylate Reductase/ Hydroxypyruvate Reductase
CG7144 (dLKR/SDH)	16.4	0.64	-25	Lysine α-Ketoglutarate Reductase/ Saccharopine Dehydrogenase

B *Ctrl*   *porthos RNAi*

C *Ctrl1*   *GRHPR RNAi 1*

D *Ctrl1*   *LKRSDH RNAi 1*

E Mac # in germband — *Ctrl*, *porthos RNAi* ****

F Mac # in germband — *Ctrl 1*, *dGRHPR RNAi 1* ****; *Ctrl 2*, *dGRHPR RNAi 2* ****; *Ctrl 3*, *dGRHPR RNAi 3* ns

G Mac # in germband — *Ctrl 1*, *dLKR/DH RNAi 1* ***; *Ctrl 2*, *dLKRSDH RNAi 2* ***

H

D-Glycerate   **dGR**   Hydroxypyruvate
NAD(P)+   NAD(P)H
Glycolate   **dHPR**   Glyoxylate
NADP+   NADPH

I

dLKR   NAD(P)+ Glutamate NAD(P)H
L-Lysine → Saccharopine → α-amino adipic semialdehyde (AASA)
α-Ketoglutarate NAD(P)H → NAD(P)+   **dSDH**
→ Acetyl-CoA

Figure. 4

Figure. 5

A

or

control dsRNA → *porthos dsRNA*

Polysome RNA   Total RNA

Sucrose gradient 10% → 50%

RNA-seq & mapping

B   Control ——   *porthos KD* ——

Abs 254 nm

0.75 / 0.50 / 0.25 / 0.00

40S   80S   Polysome

Sedimentation

C

log$_2$ (TE porthos dsRNA) vs log$_2$ (TE Control dsRNA)

● Downregulated (DR)
● Upregulated (UP)

D Porthos targets

- Mito respiration
- Mito transport/structure
- Mito translation
- Redox activity
- Sugar metabolic process
- Nucleotide metabolic process
- Amino acid/peptide metabolic process
- Lipid metabolic process
- Glycosylation
- RNA translation
- DNA/RNA regulation/processing
- Transport/channels
- Cell-cell interaction
- Signal transduction
- Activation of immune response
- Protein ubiquitination/degradation
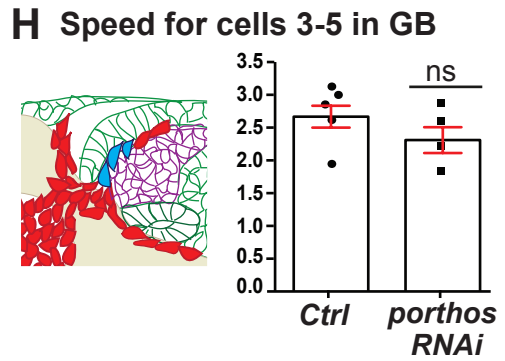- Other

Number of genes

Mito / Redox / Metabolism

E

Cellular function	Gene symbol	Description of Porthos targets	Vertebrate ortholog
Mito respiration	CG3270	Complex I, NADH:ubiquinone oxidoreductase, predicted assembly factor	Foxred1
	CG8764	Complex III, ubiquinol-cytochrome c reductase, Oxen Qcr9	Uqcr10
	CG34242	Complex IV, mitochondrial cytochrome c oxidase predicted assembly factor	Smim20
	CG6105	Complex V, ATP synthase, Fo portion, subunit G (ATPsynG)	Atp5l
	CG10731	Complex V, ATP synthase, coupling factor B	Dmac2l
Mito transport/ structure	CG1158	Tim17b1, Mito protein-transporting ATPase	Timm17a
	CG8860	Mitochondrial protein-transporting ATPase	Sec61g
	CG9090	Mpcp1,Mitochondrial phosphate carrier protein 1	Slc25a3
	CG5646	Acyl carnitine transmembrane transporter	Slc25a45
	CG1287	Mics1,Mitochondrial morphology/ cristae structure 1	Ghitm
	CG34132	Protein localization into MIM	Timm13
	CG11110	Serine peptidase, protein targeting to mitochondrion	Immp2l
	CG2854	Predicted mitochondrial T cell activation inhibitor	Tcaim
Mito translation	CG32531	mRpS14, mitochondrial ribosomal small protein S14	Mrps14
	CG13608	mRpS24, mitochondrial ribosomal small protein S24	Mrps24
	CG34147	mRpL34, mitochondrial ribosomal large protein L34	Mrpl34
	CG11679	Predicted positive regulator of mito translation	Rmnd1
Redox	CG9363	GstZ2, glutathione metabolic process	Gstz1
	CG11512	Mitochondrial GstD4, glutathione metabolic process	Gstt1
	CG6461	Ggt-1, glutathione metabolic process	Ggt1
	CG14221	Glutathione metabolic process	Nme8
	CG6762	Sulfiredoxin, response to oxidative stress	Srxn1
	CG7460	Polyamine oxidase, redox process	Paox
	CG5653	Redox process	Paox

F

Cellular function	Gene symbol	Description of Porthos targets	Vertebrate ortholog
Sugar metabolism	CG42814	UDP-sugar diphosphatase	Nudt14
	CG14212	Haloacid dehalogenase, sugar dephosphorylation	Phospho1
Nucleotide metabolism	CG30016	Hydroxyisourate hydrolase, purine metabolism	Urah
	CG3788	Uridine phosphorylase	Upp1
	CG5828	Pantothenate kinase	Pank4
	CG33514	Phosphatidylinositol bisphosphate binding	Clvs1
	CG7735	ADP ribosylation factor-like 6	Arl6
Amino acid/ peptide metabolism	CG5840	P5cr-2, Pyrroline-5-carboxylate reductase-like 2	Pycrl
	CG7768	Peptidylprolyl isomerase	Ppif
	CG11843	Acylaminoacyl-peptidase	F12
	CG34041	Methylation of glycine to generate sarcosine	P4ha2
	CG6188	Gnmt, Glycine N-methyltransferase	Gnmt
	CG14990	SPH97, serine-type endopeptidase	Tpsab1
	CG10764	Acylaminoacyl-peptidase	NF
Lipid metabolism	CG7367	Carboxylesterase	Liph
	CG17562	Fatty-acyl-CoA reductase	Far2
	CG11052	Acylphosphatase	Acyp2
	CG8303	Fatty-acyl-CoA reductase (alcohol-forming)	Far1
	CG9709	Acox57D-d, acyl-Coenzyme A oxidase	Acox1
	CG8498	Acbp1, Acyl-CoA binding protein 1	Acbd7
	CG13091	Sgroppino, fatty-acyl-CoA reductase	Far2
	CG2985	Yolk protein 1, carboxylesterase	Lipi
	CG11129	Yolk protein 3, carboxylesterase	Lipi
Glycosylation	CG31956	N-Acetylgalactosaminyltransferase 4	Galnt10
	CG33774	Protein N-linked glycosylation	Ost4
	CG3253	Glucuronosyltransferase, O-linked mannosylation	B4GAT1

Figure. 6

Figure. 7

A Altered metabolic pathways in *atos* embryos:
Significantly **upregulated** metabolites compared to the control

B ATP/ADP ratio — *Ctrl* vs *atos* (*)

C •*Control* •*atos mutant* — Normalised ratio — Pyr:Glc (*), Lac:Glc (ns)
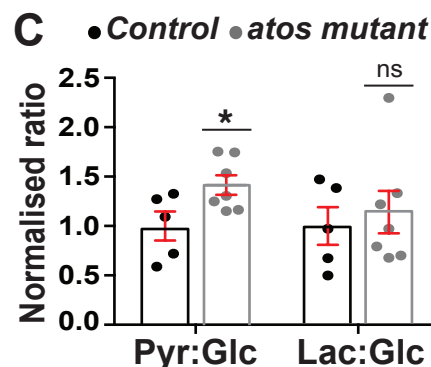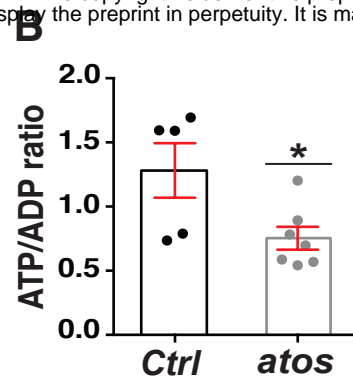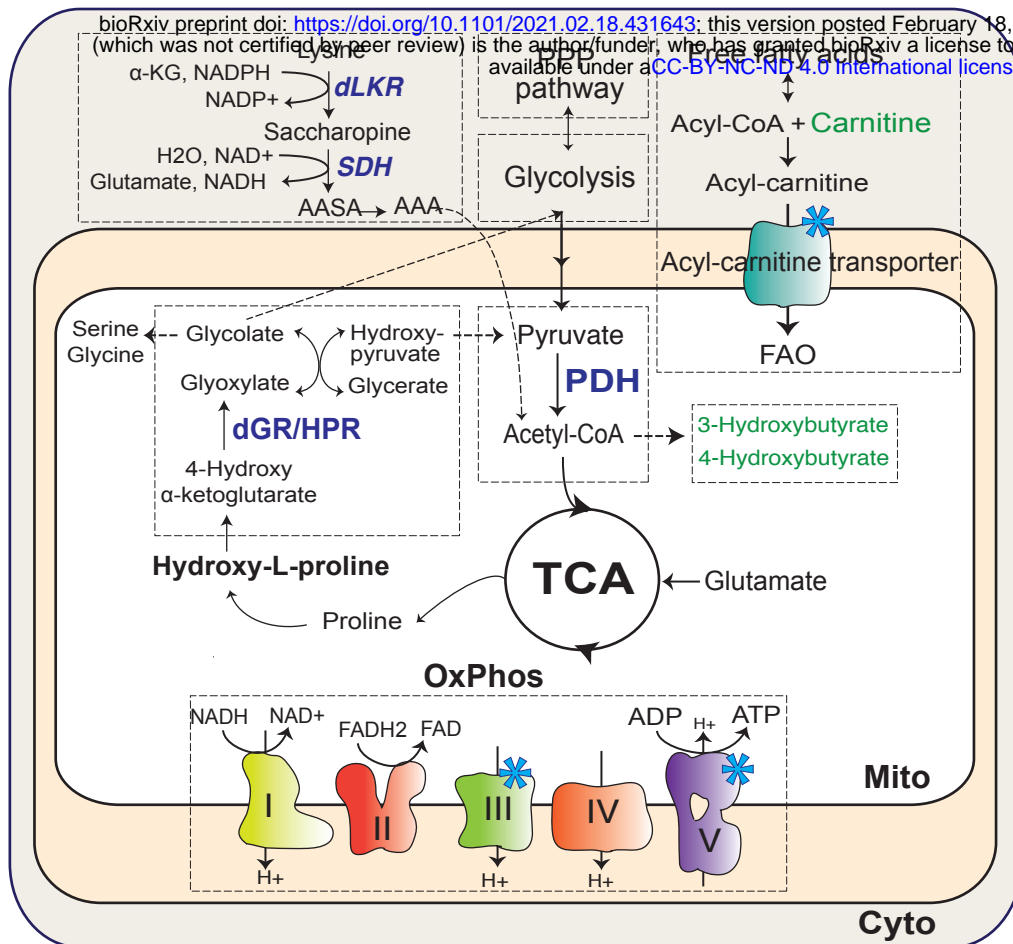
D Avg FC — Dipeptides

Avg FC	Dipeptide	
3.04	Val-HydroxyPro	****
3.02	Met-Glu	*
1.20	Ser-Tyr	*
1.17	Arg-HydroxyPro	*
0.89	Cys-Asp	
0.92	Ala-Pro	*
0.80	Arg-Glu	
0.68	Leu-Gln	
0.63	His-Pro	

E Avg FC — Carnitine metabolism

Avg FC	Metabolite	
0.50	Carnitine	*
-0.10	Acetyl-carnitine (C2)	
1.0	Propionyl-carnitine (C3)	
0.04	3-Hydroxybutyryl-carnitine (C4)	
0.52	Hexanoyl-carnitine (C6)	
1.08	Lauryl-carnitine (C12)	
1.60	Myristoyl-carnitine (C14)	
1.16	Pentadecanoyl-carnitine (C15)	
0.92	Palmitoyl-carnitine (C16)	
0.20	Stearoyl-carnitine (C18)	
0.18	Behenoyl-carnitine (C22)	
2.94	3-Hydroxybutyric acid	*

F Avg FC — Ketone bodies

Avg FC	Metabolite	
2.94	3-Hydroxybutyric acid	*
2.18	4-Hydroxybutyric acid	**

Avg fold changes (*atos* v.s. control) of analysed metabolites
-1.0 -0.5 0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0

G *atos mutant* → Slower pioneer cell invasion