Insights into *in vivo* adipocyte differentiation through cell-specific labeling in zebrafish

Running title: ***In vivo* adipocyte differentiation**

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Summary statement

Analysis of the differentiation of adipocytes *in vivo* through cell-specific labeling in zebrafish, revealed their early interaction with blood vessels as well as early lipid metabolic changes.

Abstract

White adipose tissue hyperplasia has been shown to be crucial for handling excess energy in healthy ways. Though adipogenesis mechanisms have been underscored *in vitro*, we lack information on how tissue and systemic factors influence the differentiation of new adipocytes. While this could be studied in zebrafish, adipocyte identification currently relies on neutral lipid labeling, thus precluding access to cells in early stages of differentiation. Here we report the generation and analysis of a zebrafish line with the transgene *fabp4(-2.7):EGFPcaax*. *In vivo* confocal microscopy of the pancreatic and abdominal visceral depots of transgenic larvae, revealed the presence of labeled mature adipocytes as well as immature cells in earlier stages of differentiation. Through co-labeling for blood vessels, we observed a close interaction of differentiating adipocytes with endothelial cells through cell protrusions. Finally, we implemented hyperspectral imaging and spectral phasor analysis in Nile Red labeled transgenic larvae and revealed the lipid metabolic transition towards neutral lipid accumulation of differentiating adipocytes. Altogether our work presents the characterization of a novel adipocyte-specific label in zebrafish and uncovers previously unknown aspects of *in vivo* adipogenesis.

Introduction

White adipose tissue (WAT) is present in mammals as well as in the other vertebrates, in the form of anatomically and functionally distinct depots (Zwick et al., 2018). It is formed by adipocytes, adipocyte precursors and macrophages surrounded by a collagen-rich extracellular matrix, and is highly vascularized and innervated. In humans, both visceral and subcutaneous central WAT depots primarily play energy storage and endocrine functions, which are of central importance in the regulation of energy homeostasis. Thus, WAT dysfunction, which is usually associated with obesity, contributes to the development of metabolic syndrome associated-diseases such as type II diabetes, dyslipidemia and non-alcoholic fatty liver disease (Longo et al., 2019). Meanwhile, other depots such as those in the dermis, bone marrow and mammary gland, contribute to regulation of local innate immunity and to the repair of adjacent tissues (Zwick et al., 2018).

In humans, the localization and mode of remodeling of adipose tissue have been associated with healthy or pathological phenotypes (Hepler and Gupta, 2017). Macroscopically, the expansion of subcutaneous WAT (SAT) is considered healthier than the growth of visceral WAT (VAT), as well as its localization in peripheral (extremities) vs central (abdomen in men; abdomen and hips in women) depots. Also, the accumulation of fat can occur in previously existing mature adipocytes (hypertrophy) or in newly differentiated cells (hyperplasia). Importantly, different lines of evidence support an association between adipose tissue hyperplasia with a healthier state as compared to hypertrophy (Vishvanath and Gupta, 2019). Adipogenesis is the process whereby stem cell-like precursors become committed, generating pre-adipocytes which then differentiate into mature adipocytes. While initial formation of adipogenic progenitors occur in hematopoietic tissues (Hudak et al., 2014), adult progenitors reside locally associated to blood vessels of adipose tissue (Hilgendorf et al., 2019; Tang et al., 2008). Tissue environment and cellular composition may influence the differentiation of these locally residing progenitors (for example, (Schwalie et al., 2018)). Thus, the study of adipogenesis and its relationship with other elements in the tissue *in vivo*, is critical to understand normal and pathological processes.

Work on cultured cells has provided key information about transcriptional regulation of adipogenesis (Bahmad et al., 2020). Meanwhile studies in mice have been conducted to analyze the developmental origin of adipocyte progenitors (Hepler and Gupta, 2017). More recently, the use of zebrafish to analyze adipose tissue biology has captured attention as it promises to enable the study of the tissue and its cellular biology *in vivo*.

77   Zebrafish develops only white adipose tissue, which first appears in visceral depots in

78   early larval stages (Flynn et al., 2009; Minchin and Rawls, 2017b). In contrast, mice

79   develop first SAT depots in embryonic stages while VAT appears postnatally (Hudak et

80   al., 2014). Importantly however, zebrafish adipocytes show the same subcellular

81   characteristics, gene expression patterns and final distribution (visceral and

82   subcutaneous) as in mammals (Flynn et al., 2009). Moreover, it has been reported that

83   factors affecting body fat distributions in humans have comparable effects in zebrafish

84   (Loh et al., 2020; Minchin et al., 2015). Thus, taking advantage of its fast external

85   development and optical transparency, zebrafish is an ideal system to study cellular

86   and tisular aspects of WAT development.

87   Current methods to label adipose tissue *in vivo* rely on the use of lipophilic dyes such

88   as LipidTOX or Nile Red (Minchin and Rawls, 2017a). Nile Red is particularly useful

89   because its absorption and emission spectral characteristics are modified according to

90   the polarity of the environment surrounding the probe (Greenspan and Fowler, 1985).

91   The emission of Nile Red in the context of neutral lipids is blue-shifted in comparison to

92   when it is in the presence of polar lipids. This spectroscopic characteristic has been

93   extensively used to label lipid droplets and to estimate the amount of adipose tissue in

94   live larvae as well as to classify depots (Minchin and Rawls, 2017b). However, Nile

95   Red stains all cell membranes, including the endoplasmic reticulum in which

96   biogenesis of lipid droplets takes place (Olzmann and Carvalho, 2019). Lipid stores are

97   composed of neutral lipids such as triacylglycerols and sterol esters, while polar lipids

98   are present during droplet formation as well as during lipolysis. Maulucci et al.

99   developed an approach to generate a lipid metabolic index using hyperspectral imaging

100   of Nile Red and spectral phasor analysis (Di Giacinto et al., 2018; Maulucci et al.,

101   2018). Thus, this method allowed them to differentiate among cells forced to carry out

102   lipid movement (lipid storage or lipolysis) and those in a resting state. The application

103   of this method to live larvae as well as the study of the interaction of adipocytes with

104   other cells in the tissue would require to specifically label adipocytes independently of

105   their fat load.

106   To address this we decided to generate an adipocyte specific reporter zebrafish line.

107   Up to date, no factor has been identified that is expressed in all fat depots in mice

108   (Cleal et al., 2017). However, there are several genes that are upregulated in

109   adipocytes in different stages during differentiation, like those used extensively in cell

110   culture of mammalian cells to monitor differentiation progress (Tang and Lane, 2012).

111   As expected, much less information is available from zebrafish. We therefore selected

112   early and late genes which are commonly used as adipocyte differentiation markers in

113   mammalian cell culture models and with previous evidence of being expressed in

114   zebrafish adipose tissue (Imrie and Sadler, 2010), cloned their putative promoter

115   regions and generated transgenesis constructs. We show here that a *fabp4a* -2,7kb

116   proximal genomic region effectively drives the expression of EGFP in adipocytes both

117   previous to and during the accumulation of fat. Membrane tagging of GFP allowed us

118   to observe the early interaction of adipocytes with blood vessels through adipocyte

119   membrane protrusions. Furthermore, we adapted the method of Maulucci et al. by

120   incorporating a three-component analysis in the phasor plot, which enabled us to

121   analyze the lipid metabolism of EGFP-positive cells in live larvae before the formation

122   of lipid droplets. Thus, this new zebrafish transgenic line is a valuable tool which will

123   open new possibilities to study adipocytes and adipose tissue biology *in vivo*.

Results

fabp4a(-2.7):EGFPcaax transgene is expressed in early and mature adipocytes.

Based on previously reported data on cell culture models and expression patterns in zebrafish we selected four different genes to work with: *adipoqb, cebpa, cfd, fabp4a.* All of them were previously reported to be expressed in zebrafish adipose tissue in larvae and/or adults (Imrie and Sadler, 2010). Taking into account our analysis of the promoter regions, we cloned approximately 2 kb of the proximal part of the promoter for each gene (see Materials and methods), and generated transgenesis constructs using the Tol2 system bearing the cardiac light chain myosin reporter gene, *cmlc2:GFP*, as an early selection marker (Fig. 1A). These constructs were injected together with mRNA coding for Tol2 transposase in the cytoplasm of one-cell stage embryos. Twenty-four hours post-fertilization (hpf) embryos with GFP expressing-cells in their hearts were selected for further breeding. We then analyzed larvae of 15-21 days post-fertilization (dpf) in the stereomicroscope, observing the presence of labelled cells for the constructions with *cebpa* and *fabp4a* promoters. However, only in the latter case the EGFPcaax signal coincided with lipid droplets in mature adipocytes recognizable through transmitted light. Moreover, only in the case of larvae injected with the *fabp4a(-2.7):EGFPcaax* construct we observed mature adipocytes labelled along several generations (Fig. 1B). Therefore, we decided to continue working only with the *fabp4a(-2.7):EGFPcaax* line.

First, to assess EGFP expression in live *fabp4a(-2.7):EGFPcaax* larvae we stained individuals of different stages with the lipophilic dye LipidTOX-Red and analyzed them using epifluorescence microscopy. We observed EGFPcaax signal in the surface of mature adipocytes, both in the pancreatic and abdominal depots (PVAT and AVAT respectively) (Fig. 1B, asterisks). Of note, expression levels varied among cells and this effect remained even after several outcrosses with the wild type fish line. We also analyzed other early-forming depots (renal, ocular and subcutaneous in fins) but failed to detect cells with expression of EGFP. Interestingly, both in PVAT and AVAT, besides mature adipocytes with readily visible lipid droplets, we observed EGFPcaax-positive (EGFP+) cells that had smaller lipid accumulations as well as cells that had no detectable LipidTOX-Red signal (Fig. 1B, single and double arrows respectively). Based on the fact that mammalian *fabp4* is expressed during adipocyte differentiation (Tang and Lane, 2012), these results indicated that EGFP+ cells with no visible lipid droplets (to which we refer as EGFP+/LD- from now on) were likely adipocytes at initial stages of differentiation.

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fabp4a(-2.7):EGFPcaax transgene expression pattern recapitulates the adipose tissue expression domain of endogenous fabp4a

Embryonic *fabp4a* expression has been reported to be restricted to the lens, midbrain and the blood vessels of the head and trunk in 48 hpf embryos (Liu et al., 2007). Meanwhile, 15 dpf larvae present *fabp4a* expression in trunk vessels and in early adipocytes (Flynn et al., 2009). To determine the expression pattern of the *fabp4a(-2.7):EGFPcaax* transgene, we analyzed several developmental stages and compared it with endogenous *fabp4a expression*. For this, endogenous *fabp4a* expression was assessed using fluorescent whole mount *in situ* hybridization (WMISH), and *fabp4a(-2.7):EGFPcaax* transgene expression through immunolabeling with an anti-GFP antibody.

First, we checked the specificities of WMISH probes using *fli1:EGFP* transgenic embryos, which express EGFP in blood vessels, useful as an anatomical reference. For that, 2 dpf wild type embryos were fixed and processed for WMISH as described in the "Materials and methods" section. As specificity controls, we used the *fabp4a* sense probe (negative control) and an antisense probe for *slit2* (additional specificity control). To detect EGFP, we performed a final immunolabeling step. We observed a clear signal corresponding to *fabp4a* transcripts when the WMISH was performed with the antisense probe, which co-localized with *fli1:EGFP* immunodetection almost completely (Fig. S1; note the presence of brain cells positive for *fabp4a* antisense probe without *fli1:EGFP* labeling). Neither of the other two probes generated similar patterns: no specific signal was observed with the *fabp4a* sense probe while the *slit2* antisense probe labeled the ventro-medial part of the neural tube as reported previously (Davison and Zolessi, 2020). These results corroborated the specificity of the *fabp4a* antisense probe and validated the post-WMISH immunofluorescence procedure and reagents.

To compare the distribution of transgenic *fabp4a(-2.7):EGFPcaax* expression with that of endogenous *fabp4a* in larvae, we performed WMISH in individuals of around 21 dpf, immunolabeled them with anti-GFP and analyzed them *in toto* using confocal microscopy. Endogenous expression was observed in the PVAT and AVAT areas with the *fabp4a* antisense probe, coinciding with cells expressing EGFP (Fig. 2A). The specific signal was not observed with the *fabp4a* sense probe (Fig. 2B). Also, EGFP expression was evidenced in pigment cells in live embryos (Fig. S2). This expression pattern was not described before for endogenous *fabp4a* and, in larvae processed for WMISH, expression of endogenous *fabp4a* was not observed in superficial pigment

194   cells (Fig. 2C, single arrows). Instead, we did observe staining for endogenous *fabp4a*

195   in blood vessels (Fig. 2C, double arrows). In conclusion, the expression pattern of the

196   transgene recapitulates the endogenous pattern of *fabp4a* in the adipose tissue, but

197   not within blood vessels or in the brain.

198

199   ***Development of early adipocytes in vivo and their relationship with blood***

200   ***vessels.***

201   Our results therefore supported the hypothesis that the *fabp4a(-2.7):EGFPcaax*

202   transgene marks adipocytes during differentiation. To test this we analyzed live larvae

203   and embryos of different stages using confocal microscopy. First, we analyzed

204   embryos in search of early expression of *fabp4a(-2.7):EGFPcaax*. *In vivo* confocal

205   analysis of 2 dpf and 5 dpf embryos showed expression of EGFP in cells along the

206   antero-posterior axis at dorsal, lateral and ventral positions, all reminiscent of pigment

207   cells (Fig. S2A and C, double arrows). The presence of early labeling of pigment cells

208   suggested that this expression domain corresponded to ectopic expression of *fabp4a(-*

209   *2.7):EGFPcaax* in the transgenic embryos. To discard autofluorescence or dispersion

210   of light by pigments, we immunostained fixed embryos with anti-GFP antibody. We

211   observed immunostaining co-localizing with EGFP fluorescence (Fig. S2B and D,

212   double arrows), thus confirming that in our fish line pigment cells are labeled by

213   *fabp4a(-2.7):EGFPcaax.*

214   Previously, it has been reported that lipid droplets are first evident at the right side of

215   the abdomen of early larvae, in ventral and posterior position with respect to the swim

216   bladder (Flynn et al., 2009; Minchin and Rawls, 2017b). Thus we turned our attention

217   into that region in larval stages and stained lipid droplets with LipidTOX-Red.

218   Interestingly, early larvae of standard length (SL) 4.5 mm (8 dpf) show labeling of small

219   cells within the abdomen. At higher magnification we observed the expected surface

220   localization of EGFP. However, these cells did not present lipid droplets (Fig. 3A). We

221   also observed labeling of cells within the trunk in dorsal positions which corresponded

222   to the presence of pigment cells in transmitted light images. Other signals in the

223   images corresponded to autofluorescence of gut contents in the ventral-most part of

224   the larvae and light scattering of pigments in the dorsal half of the swim bladder (Fig.

225   3A). Despite taking several actions to diminish these confounding signals (16 h food

226   restriction previous to imaging; incubation with epinephrine), they were persistent.

227   Nevertheless, based on the surface localization and intensity of the EGFP signal it was

228   possible to clearly distinguish these EGFP positive and lipid droplet-free cells

229   (EGFP+/LD-).

230   Next, we evaluated older larvae to assess whether these EGFP+ cells in the abdominal

231   region could accumulate lipids. Importantly, larvae of SL 5 and 6.3 mm (12 and 16 dpf

232   respectively) clearly showed cells with surface EGFP signal and lipid droplets of

233   various diameters (Fig. 3B and C). We also noted that some of these cells had irregular

234   forms and projections (Fig. 3C, double arrows). Larvae of these ages also had rounder

235   cells almost completely filled with one big lipid droplet (Fig. 3B and C). In mice, pre-

236   adipocytes have been found to reside near blood vessels (Tang et al., 2008). Thus, we

237   analyzed the relationship of early adipocytes with the vasculature by crossing *fabp4a(-*

238   *2.7):EGFPcaax* fish with the *kdlr:mCherry* line, which labels endothelial cells. We

239   observed EGFP+ cells both in close apposition and at some distance of vessels (Fig.

240   4A). Moreover, when cells with lipid accumulation were observed in the PVAT or AVAT

241   depots, some usually appeared in close contact with vessels, sometimes with

242   extensions surrounding them (Fig. 4B).

243   We expanded our analysis until 21 dpf larvae (larvae of SL 7-8mm) and observed

244   EGFP+ positive cells with different morphologies (Fig. 5A and B). We observed

245   rounded cells filled with a single lipid droplet which likely correspond to mature

246   adipocytes, in some cases having cell projections. Other cells, usually located at the

247   periphery of the depots, typically showed one or more smaller lipid droplets and more

248   irregular morphologies, also with membrane projections (Fig. 5A). In many occasions

249   we observed cells in close proximity to vessels or with extensions surrounding them

250   (Fig. 5B). Remarkably, in all stages analyzed in this work, EGFP+/LD- cells were

251   present. These cells could be observed not only in the abdominal region in AVAT and

252   PVAT but also surrounding the gut in different positions (Fig. 3B), including in the

253   cloaca region (Fig. S3). Interestingly, using transmitted light and high magnification we

254   observed inclusions within EGFP+/LD- cells (Fig. 5C). We performed time lapse

255   acquisitions of those cells for a short period of time. During these time lapse movies we

256   observed that inclusions moved within the cytoplasm (Movie 1). Furthermore, we

257   observed that cells could remain static or have directional movement over a cell

258   diameter distance (Movie 1). This behavior was accompanied by the formation of

259   protrusions which were evident also in our single time point observations (Fig. 3B and

260   5C).

261   Altogether, our results show that *fabp4a(-2.7):EGFPcaax* expressing cells are present

262   in different larval stages, from just before the beginning of the accumulation of fat to

263   later stages where lipid depots are readily visible. Furthermore, we found labeled cells

264   with lipid droplets of different sizes, confirming that cells expressing *fabp4a(-*

265   *2.7):EGFPcaax* in the abdominal region of larvae are adipocytes in different stages of

266   differentiation. Even though our fish line also expresses EGFP in pigment cells, *in vivo*

267   3D analysis of the abdominal region efficiently allowed us to distinguish early and

268   mature adipocytes based on localization and cell shape. Furthermore, the results

269   underscore a tight relationship between adipocytes and vessels during their

270   differentiation, and the coexistence of EGFP+ lipid-filled cells with EGFP+/LD- cells in

271   the tissue.

272

Analysis of the lipid metabolic profile of early adipocytes with Nile Red fluorescence and spectral phasor plot analysis.

275   As mentioned before, we hypothesized that EGFP+/LD- cells were in fact early

276   adipocytes. Early adipocytes initiate lipid accumulation as part of its differentiation

277   program, and thus would show a mixed lipid environment with neutral and polar

278   components. The quantification of these components has been carried out before in

279   cultured cells through Nile Red fluorescence analysis using spectral phasors (Di

280   Giacinto et al., 2018; Maulucci et al., 2018). In our transgenic larvae, the fluorescence

281   of EGFP could be used as a third component to identify the cells of interest (EGFP+

282   cells). Thus, we took advantage of the spectral phasor analysis to study the Nile Red

283   spectral shift in the presence of EGFP fluorescence. A similar approach has been used

284   to study membrane polarity using LAURDAN in the presence of mRuby fluorescence

285   (Sameni et al., 2018). For the cellular lipid metabolic profile, wild type larvae stained

286   with Nile Red or *fabp4a(-2.7):EGFPcaax* larvae with or without staining with Nile Red

287   were imaged using hyperspectral detection and the images were analyzed using the

288   advantages of the model-free spectral phasors approach (Fig. S4, the analysis

289   procedure is described in depth in the "Material and methods" section) (Malacrida et

290   al., 2017).

291   Wild type larvae stained with Nile Red or *fabp4a(-2.7):EGFPcaax* larvae without

292   staining were analyzed first to set the extremes of the distributions in the phasor plot

293   (Fig. S4B). Notice that the Nile Red fluorescence was spread in a trajectory due to the

294   heterogeneity in the polarity of Nile Red environments provided by the intracellular

295   membranes. The position along the trajectory represents pixels with different fractions

296   of membranes with more or less polarity. In the EGFP+ cells labeled with Nile Red, the

297   linear combination for the Nile Red was dragged toward the EGFP position (Fig. S4C).

298   Thus, the extremes of the Nile Red trajectory can be considered as two components

299   and the EGFP as the third component. This strategy enabled us to generate masks for

300   individual EGFP+ cells and to analyze their lipid polarity profile, avoiding the Nile Red

301   signal from other cells. An example image is shown in Fig. S4C. Two cells, one with

302   lipid droplets (cell A) and another without them (cell B), generated clusters at the

303   phasor plot with unequivocally different distribution profiles. To analyze the lipid polarity

304   profile on each of them, we obtained the polarity fractional plot (Fig. S4D). The analysis

305   of cell A yielded a multimodal distribution with higher representation of intermediate

306   zones, whereas cell B gave a single peak in the polar lipid region.

307   Using this approach, we analyzed EGFP+ cells in larvae at different stages.

308   Representative examples of the observed profiles are shown in Fig. 6A and B.

309   Seemingly mature adipocytes with a big lipid droplet showed a peak in Nile Red profile

310   in regions corresponding to the accumulation of neutral lipids as expected (Fig. 6A and

311   B, "cell D"). Interestingly, it was possible to observe a small peak towards longer

312   wavelengths, representing polar lipid components in the same cells such as the plasma

313   membrane. This was corroborated by the localization of these pixels: the former were

314   localized centrally and the latter surrounded the whole cell (Fig. 6A, see Nile Red

315   profile of "cell D"). EGFP+/LD- cells usually showed distributions enriched in polar

316   components (Fig. 6A and B, "cell A"). Nevertheless, we imaged cells with several

317   peaks or flatter distributions, probably representing transitions between polar and

318   neutral lipid environments (Fig. 6A and B, "cell B" and "cell C"). To summarize and

319   present all the profiles we observed, the center of mass (CM) and distribution range

320   (RD) of the lipid polarity profiles, were calculated and used as characteristics of each

321   distribution for comparison purposes (Materials and methods; Fig. S4D). Within the plot

322   of center of mass vs distribution range (Fig. 6C) it is possible to separate a subgroup of

323   cells with statistically distinct median (for the center of mass and distribution range) and

324   variability (only for the center of mass) compared to those cells outside this region (Fig.

325   6C and D, dashed line). The low CM and low DR means that the cells within this group

326   are constituted mostly by polar lipids. These cells represent over 50% of the cells

327   analyzed from 8 to 16 dpf (Fig. 6E). The rest of the cells analyzed lay outside the low

328   CM-low DR region due to increasing accumulation of neutral lipids, which extend the

329   DR and bias the CM towards higher values. The percentage of cells analyzed that can

330   be classified in this sub-group increased with larvae age (Fig. 6E). Of note, we

331   observed cells with distinct lipid polarity profiles among larvae of similar standard

332   length and in some cases within the same larvae (Fig. 6E). These observations imply

333   the coexistence of several stages of adipocyte differentiation within the same larvae

334   and suggest that differentiation *in vivo* is continuous and asynchronous. These results

335   indicate that our zebrafish *fabp4a(-2.7):EGFPcaax* transgenic line together with

336   hyperspectral imaging and the spectral phasor analysis shown here is a powerful tool

337   to study changes of the intracellular lipid environment in differentiating adipocytes in

338   live zebrafish larvae.

Discussion

340 A number of studies have started to address the role of compounds on lipid metabolism

341 and storage using zebrafish as a model system (Landgraf et al., 2017). Current

342 knowledge on zebrafish adipose tissue is restricted to fat accumulation capacity using

343 lipophilic dyes due to the lack of specific cell markers. Here we generated and

344 analyzed a new zebrafish line to specifically label adipocytes along their differentiation

345 *in vivo*.

Expression pattern of fabp4a(-2.7):EGFPcaax

347 We generated a new transgenic line cloning the proximal part of the promoter of the

348 lipid transporter gene *fabp4a* and used it to direct the expression of a membrane form

349 of EGFP. It was shown previously that *fabp4a* is expressed in the lens, midbrain as

350 well as in blood vessels in the head and trunk of 2 dpf embryos (Liu et al., 2007), an

351 expression pattern that we also observed with our WMISH assay in 2 dpf embryos.

352 However, the *in vivo* analysis of 2 and 5 dpf embryos showed that *fabp4a(-*

353 *2.7):EGFPcaax* transgene is not expressed in either blood vessels, midbrain or lens

354 cells. In the case of 15 dpf larvae, *fabp4a* mRNA was previously reported to be

355 expressed in abdominal cells with or without neutral lipid accumulation as well as in

356 trunk vessels (Flynn et al., 2009). Our WMISH assay in larvae showed the expression

357 of endogenous *fabp4a* in PVAT and AVAT depots, co-localizing in some cells with

358 expression of *fabp4a(-2.7):EGFPcaax* transgene. Thus, the expression pattern of

359 *fabp4a(-2.7):EGFPcaax* recapitulates primarily the adipose tissue domain of the

360 endogenous expression pattern of *fabp4a*.

361 We also found that our *fabp4a(-2.7):EGFPcaax* transgene is expressed in surface

362 pigment cells, a domain that does not coincide with the endogenous expression of

363 *fabp4a*. One possible explanation for the lack of expression in some domains as

364 mentioned earlier and the presence of an extra expression domain may be due to the

365 lack of regulatory elements in the cloned region of the promoter. In that case, it would

366 be possible to generate an improved fish line using BAC transgenesis. Alternatively,

367 these extra domains of expression may be due to the action of enhancers present in

368 the genomic region where the transgene was integrated. This is a common drawback

369 of using random insertion of transposons for transgenesis and highlights the

370 importance of using complementary strategies such as insulators or targeted transgene

371 insertion (Caldovic et al., 1999; Roberts et al., 2014). Importantly however, this extra

372 domain of expression did not hinder the utility of the transgenic line as it can be clearly

373   separated from the adipose-related signal by considering the relative position of cells in

374   3D images.

375   Another particular aspect of this new transgenic fish line is that the level of expression

376   of EGFP varies among cells. This variability remained even after three outcrosses with

377   the wild type fish line, discarding a mosaicism-based effect. Thus, variation among

378   cells may reflect different cellular states along differentiation which affect transcription

379   from the cloned region of the *fabp4a* promoter, already known to be under a complex

380   regulatory circuit. For example, binding sites for Pparγ and NF-Kβ p50 which modulate

381   transcription in reporter assays, have been reported in the promoter region used in our

382   transgenic line (Laprairie et al., 2017). Moreover, Pparγ has been shown to bind the

383   *fabp4* promoter in a brown adipocyte cell line (Tontonoz et al., 1994). Also, it has been

384   reported that *fabp4* is regulated by VEGFA-DLL4/NOTCH and insulin-FOXO1

385   pathways in endothelial HUVEC cells (Harjes et al., 2014). In humans, plasma levels of

386   Fabp4, which is mainly produced by adipocytes, have been positively correlated with

387   cardio-vascular disease, type-II diabetes and also with the progression of other

388   diseases by a still undefined mechanism (Prentice et al., 2019). Thus, it would be

389   interesting to analyze which factors contribute to the expression levels of *fabp4* as they

390   may be of clinical relevance, and our results showing significant variability among

391   adipocytes suggests this zebrafish line could be useful to this end.

392   ***Characterization of early adipocytes and their relationship to blood vessels***

393   As mentioned, our detailed microscopic analysis of *fabp4a(-2.7):EGFPcaax* larvae

394   showed labelled cells with different characteristics. In the abdominal region, where

395   WAT depots form, we observed both EGFP+ cells with lipid droplets of various sizes

396   and others without them. These observations are in agreement with previous reports

397   showing that *fabp4a* is expressed in cells with and without lipid droplets in early larvae

398   (Flynn et al., 2009). Accordingly, EGFP+/LD- were present in early larvae (8-10 dpf)

399   well before the initiation of fat accumulation. Notably, we also observed them in older

400   larvae, coexisting with mature adipocytes in fat depots. Our analysis of Nile Red

401   emission of EGFP+ cells/LD- revealed polar lipid or intermediate profiles. Thus, our

402   results indicate that the *fabp4a(-2.7):EGFPcaax* transgene labels adipocytes ranging

403   from early stages of differentiation to mature differentiated cells.

404   EGFP+/LD- cells presented inclusions when observed with transmitted light at high

405   magnification. These inclusions were LipidTOX-negative and time lapse acquisitions

406   showed that they were highly motile within the cell. Further experiments are required to

407   determine the nature of these inclusions. One interesting possibility is that they may

408   represent initial stages of lipid droplet formation in which the amount of accumulated

409   neutral lipids is not enough to be observed through LipidTOX labeling. LD are formed

410   through accumulation of neutral lipids within the lipid bilayer of the ER, initially forming

411   structures denominated lenses which grow and bud becoming lipid droplets (Olzmann

412   and Carvalho, 2019). Genetic labeling tools that have been developed to evidence

413   initial neutral lipid accumulations may be implemented to study the conservation of

414   early lipid droplet formation mechanisms in zebrafish (Kassan et al., 2013; Wang et al.,

415   2016).

416   WAT progenitors expressing PPARγ have been reported to reside in the mural

417   compartment of adipose blood vessels in mice (Hilgendorf et al., 2019; Tang et al.,

418   2008). As an analogy to mammals, some authors have hypothesized that WAT

419   progenitors in zebrafish may derive from perivascular pre-adipocytes or, alternatively,

420   from hematopoietic tissue located in the caudal region (Salmerón, 2018). In double

421   labelled larvae, we found EGFP+/LD- cells both in contact and at a distance of blood

422   vessels. In contrast, all EGFP+ cells with lipid droplets were observed in contact with

423   blood vessels. EGFP+/LD- cells were also present surrounding the gut at different

424   positions along the antero-posterior axis. Furthermore, our time lapse acquisitions

425   revealed that these cells had the capacity to migrate. Thus, our results are consistent

426   with the previous formulated hypothesis and *in vivo* time lapse microscopy of EGFP+

427   cells combined with cell tracing may provide further information. For this, new methods

428   to maintain larvae alive through extended periods of time will be needed, since in our

429   hands, larvae remained alive only for a few hours after mounting in agarose.

430   Our work also provides information about adipocytes during differentiation and in their

431   mature state. As our transgenic approach included a membrane associated form of

432   EGFP, we could clearly identify the presence of membrane protrusions in early and

433   mature adipocytes. In double-labelled larvae we could appreciate that these membrane

434   protrusions reached blood vessels, suggesting the presence of physical connections.

435   Whether this interaction is direct between cell membranes or indirect through another

436   cell remains to be determined. Extensive evidence supports that several soluble factors

437   coordinate adipogenesis and angiogenesis in obesity as well as in adipose-derived

438   stem cell therapy (Hutchings et al., 2020; Lemoine et al., 2013). Furthermore, secretion

439   of factors by peri-arterial adipocytes can mediate protection or inflammation of the

440   adventitia and atherosclerosis development (Kim et al., 2020). Much less information is

441   available on the interaction of adipocytes and vessels during formation of the adipose

442   tissue (Cao, 2007). Our results suggest an intimate relationship of early adipocytes with

443   blood vessels, probably through cell surface molecules. We hypothesize that these

15

444 interactions may be instrumental in acquisition of lipids from blood vessels as well as in

445 regulation of growth of the adipose depot.

Nile Red and phasor approach to characterize in vivo cell lipid metabolism

447 We used the new *fabp4a(-2.7):EGFPcaax* line to implement a tool for *in vivo* analysis

448 of lipid environment using Nile Red hyperspectral imaging and its analysis through

449 spectral phasor plots (Maulucci et al., 2018). Early adipocytes initiate lipid accumulation

450 as part of their differentiation program, and thus would show a mixed lipid environment

451 with neutral and polar components in their profile. Indeed, as mentioned before,

452 EGFP+/LD- cells showed different profiles, ranging from polar-lipid environment to

453 intermediate polarity-lipid environment. Several groups have studied the lipid

454 composition of *in vitro* differentiating adipocytes of different origins through disruptive

455 methods (Miehle et al., 2020). For example, human undifferentiated adipocytes were

456 enriched in membrane phospholipids such as phosphatidylethanolamines,

457 phosphatidylcholines and sphingomyelins. Meanwhile completely differentiated cells

458 were shown to present diacylglycerols, lysophosphatidylethanolamines and

459 triacylglycerols in addition to membrane phospholipids. Thus our results are consistent

460 with previous analysis, and importantly, provide a base to build on the metabolic

461 analysis of individual cells in their natural context.

462 Our data show that the technique is sensitive enough to detect lipid environment

463 changes in a non-invasive way and for a specific cell identity, opening the possibility of

464 using this tool to evaluate the progression of differentiation *in vivo* or the effect of drugs

465 on lipid metabolism or genetic interventions. Future development of other fish lines

466 using earlier molecular markers will improve the observation of cells in different stages.

467 For example, work in mice have used *pref1* and *zfp423* to mark adipose tissue

468 progenitors and pre-adipocytes (Gupta et al., 2010; Hudak et al., 2014). Both of these

469 genes are present in zebrafish and may be useful to track the origin of the adipocyte

470 lineage.

Conclusions and perspectives

472 In this work we introduced a new zebrafish line labeling adipocytes from early stages

473 up to fully differentiated cells. Furthermore, we described the interaction of early and

474 differentiated adipocytes with blood vessels and evidenced early lipid metabolic

475 changes *in vivo.* We anticipate that the new transgenic line described here will be a

476 useful tool to study the cell biology of adipocytes in the context of the tissue and the

477 whole organism, their interaction with blood vessels and their differentiation *in vivo.*

478 Adipogenesis is highly variable among depots in mammals (Hepler and Gupta, 2017),

16

479   thus it may be an advantage to use zebrafish for analysis of common conserved

480   cellular mechanisms. Recently, new fish lines labeling lipid droplets have been

481   generated (Lumaquin et al., 2020; Wilson et al., 2021) which may be combined with the

482   *fabp4a(-2.7):EGFPcaax* line presented here for screening approaches focused on

483   genetic and environmental factors affecting early adipocyte differentiation. The *fabp4a(-*

484   *2.7):EGFPcaax* fish lines and the genetic tools available in zebrafish, combined with

485   two-photon and multiplexing microscopy will surely provide a powerful platform to gain

486   in depth information on adipogenesis and its *in vivo* determinants.

Materials and methods

Zebrafish maintenance, breeding and diets.

We worked with TAB5 (wild type fish line), *kdlr:mCherry* (blood vessel labeling (Wang et al., 2010)) and *fli1:EGFP* (blood vessel labeling (Lawson and Weinstein, 2002)). *Danio rerio* adults were maintained in a stand-alone system (Tecniplast), at 28 ºC, 800 µS/cm^2, and pH 7.5, with a diet based on live 48 hour-post eclosion *Artemia salina* (artemia cyst from Artemia International) and pellet (TetraMin, tropical flakes, Tetra). Embryos were raised in petri dishes with aquarium water at 28.5 ºC (50 larvae per 10 cm petri dish) and bleached at 24 hours post-fertilization. For growth of larvae we used Larval AP100-1 (<50 µm; Zeigler) from 5 to 30 dpf and Golden Pearl Reef & Larval Diet (100-200 µm; Brine Shrimp Direct) from 15 to 30 dpf. Dry food were administered twice per day plus one extra feed of live 24 hour-post hatching *Artemia salina.*

Embryonic staging was performed according to Kimmel (Kimmel et al., 1995) up to 5 dpf and larvae staging (after 5 dpf) was done according to Parichy (Parichy et al., 2009). Standard length (SL) is the distance between the tip of the nose and the caudal peduncle, and it correlates linearly with the growth of adipose tissue as well as the development of other characteristics in larval zebrafish (Minchin and Rawls, 2017b). All protocols (n° 007-19, 009-19, 010-19, 011-19) were approved by the Institut Pasteur de Montevideo ethics committee for the use of animal models (CEUA) and performed by trained, certified staff.

Promoter cloning

For identification of the potential promoter regions we combined manual analysis and a trial version of Gene2Promoter software (Genomatix). We then designed primers using the Primer-Blast tool from NCBI (Table 1). Candidate primers were blasted against the whole zebrafish genome using the BLAT tool from UCSC Genome Browser. Restriction sites were added at their 5`end to enable directional cloning (underlined in Table 1).

We used zebrafish high-molecular weight genomic DNA, extracted as previously described (Green and Sambrook, 2014) from 48 hpf TAB embryos. For *fabp4a*, the BAC DKEY-241P5 (Source BioScience) was used as a template. Each region was first cloned into pCRII plasmid using TOPO-TA Cloning Kit (Thermo). After sequencing, they were sub-cloned into p5´Entry-MCS plasmid from Tol2 Kit (Kwan et al., 2007) through digestion and ligation with T4 ligase (Thermo). We recombined each p5´Entry vector with pMiddle Entry vector coding for EGFPcaax (caax is a prenylation signal, directing EGFP to the plasma membrane), p3´Entry Vector with poly-A signal and the

18

521   pDestTol2-CG2 backbone (with cardiac myosin light chain promoter directing the

522   expression of GFP, *cmlc2:GFP*; and tol2 sites for insertion into the genome).

523   **Table 1.** Primers used for amplification of the selected promoter regions, its position in

524   relation to the transcription start site and the size of the amplification product. The

525   underlined regions correspond to the restriction enzyme site.

gene	forward primer	reverse primer	cloned region referenced to transcription start site	size (bp)
adipoqb	ACGTCTCGAGCCAGCTGTTCTTGTGTAAATCC	ACGTGGATCCTCAAAGATTCTATATTAGCACAATCAA	-1892 to +114	2006
cfd	AGCTGGTACCTCTGAACCAGACAGGGAATAAAGTC	ACGTGGATCCGTGTGCTTTAGCCTCTTGCC	-1032 to +234	1266
cebpa	ACGTGTCGACTCCGCTCGGGTAAATAAAGA	ACGTGGATCCAGCAACCTGTCGTGACTGTG	-1903 to +107	2010
fabp4a	ACGTGTCGACGTGGTGTTTTGCAGTGGATG	ACGTGGATCCTGCACAAATTCAGTCACGAAA	-2367 to +336	2703

526

Transgenic line generation

528   TAB5 embryos at one cell stage were injected with 10-20 pg of the desired vector plus

529   10-20 pg of Tol2 Transposase mRNA . We then selected 24 hpf embryos showing GFP

530   fluorescence in the heart. We grew these embryos until 15-21 dpf when we analyzed

531   the presence of fluorescence in adipose tissue. Selected individuals were outcrossed

532   with the wild-type line until the third generation.

Fixation, permeabilization and immunolabeling

534   To decrease pigmentation, embryos were treated with PTU 0.3 % starting at 8 hpf. For

535   the same purpose larvae were anesthetized using tricaine 0.04 g/L, incubated in

536   epinephrine 10 mg/mL plus tricaine 0.04 g/L, mounted in methylcellulose and observed

537   using the stereomicroscope to select larvae expressing GFP. Fixation was carried out

538   in 4 % PFA in PBS overnight at 4 °C.

539   Fixed embryos and larvae were permeabilized and immunolabeled following the

540   protocol described by Inoue and Wittbrodt with minor modifications (Inoue and

541   Wittbrodt, 2011). Briefly, all steps were carried out at room temperature with agitation

542   unless stated otherwise. Fixed embryos and larvae were washed in PBS plus 1 %

543   Triton X100 (PBST) (3 x 10 mins), dehydrated in a methanol series (50:50 and 100:0

544   methanol:PBST) (1 x 10 min each) and incubated in 100 % methanol at -20 °C for 20

545 min. After rehydration in the same methanol series, we performed an antigen retrieval

546 step with 150 mM Tris-HCl pH 9 (5 min at RT and 15 min at 70 °C). After a wash step

547 in PBST (10 min) and two washes in distilled water (5 min each) we further

548 permeabilized samples incubating them in 100 % acetone at -20 °C for 20 min. Finally

549 we washed the samples in PBST several times (6 x 5 min each). For

550 immunofluorescence on WMISH embryos and larvae we followed the same protocol

551 without the acetone permeabilization step.

552 For immunolabeling all steps were performed with agitation. We incubated

553 permeabilized embryos and larvae in the blocking buffer (10 % FBS plus 1 % BSA in

554 PBST) for 1 h at RT. Primary and secondary antibodies were diluted in the incubation

555 buffer (1 % FBS plus 1 % BSA in PBST). Antibody incubations were performed at 4 °C

556 for 3 days, and washes at RT with PBST. The antibodies used in this work were: anti-

557 GFP (Invitrogen, 1/500), anti-rabbit-633 (Invitrogen, 1/1000).

558 **Fluorescent Whole-Mount *in situ* Hybridization (WMISH)**

559 We cloned a region of *fabp4a* previously used for probe generation (Flynn et al., 2009)

560 using the following primers: fwd: GATCAAATCTCAATTTACAGCTGTTG; rv:

561 TTCAAAGCACCATAAAGACTGATAAT and oligodT retro-transcribed cDNA as a

562 template. The amplified region was ligated into pGEM T-easy vector (Thermo).

563 Selected clones were checked through digestion and sequencing. The selected clone

564 has the region 195 to 648 from *fabp4a* mRNA sequence, spanning the 3´ half of the

565 CDS and part of the 3´UTR, flanked by T7 and SP6 promoters in 5' and 3' respectively.

566 To synthesize the probes we amplified the template using T7 and SP6 primers and

567 afterwards generated digoxigenin (DIG) labeled probes by in vitro transcription with T7

568 or SP6 polymerases, using Digoxigenin-11-UTP (Merck). As an additional specificity

569 control we used a *slit2* antisense probe which has already been tested (generously

570 provided by C. Davison (Davison and Zolessi, 2020).

571 The WMISH technique was performed as previously described (Koziol et al., 2014) with

572 modifications following Flynn et al. and Elizondo et al. (Elizondo et al., 2005; Flynn et

573 al., 2009). A detailed protocol is available upon request. Briefly, embryos and larvae

574 were fixed in 4 % PFA prepared in PBS-DEPC water overnight at 4°C. PFA was then

575 replaced twice with 100 % methanol and samples were stored at -20°C until used. After

576 rehydration in an ethanol series, larvae were permeabilized with 15 ug/mL Proteinase

577 K (Fermentas) in PBS-0.1 % Tween-20 (PBS-T) for 10 min (for embryos) or 30 min (for

578 larvae) at room temperature. After a rinse with triethanolamine buffer (0.1 M, pH 8),

579 they were treated twice with acetic anhydride (0.25% v/v for five minutes each),

580   washed with PBS-T, refixed with 4 % PFA in PBS-T for 20 min and washed extensively

581   with PBS-T at room temperature. Pre-hybridization was performed overnight at 60°C in

582   hybridization buffer (50 % formamide, 5X SSC, 1 mg/mL Torula RNA, 100 ug/mL

583   heparin, 1x Denhardt's solution, 0.1 % Tween-20, 0.1 % CHAPS, DEPC treated water).

584   DIG labelled probes were denatured at 80°C for 3 min and diluted to 0.2 ng/uL in the

585   hybridization buffer. Hybridization was performed at 58°C for 2 days with agitation.

586   Washing steps were done in hybridization buffer at 58°C with agitation, twice for 10 min

587   each, then three times with 2X SSC plus 0.1 % Tween-20 at 58°C for 20 min each,

588   three times with 0.2X SSC plus 0.1% Tween-20 at 58°C for 30 min each, and finally

589   twice with maleic acid buffer (MAB) at room temperature for 15 min. Samples were

590   then blocked overnight at 4°C in 1 % blocking reagent (Roche) plus 5 % sheep serum

591   in MAB and incubated with anti-DIG conjugated to Peroxidase (1/50; Merck) in 1 %

592   blocking reagent diluted in MAB for 3 days at 4°C. Washing steps were done in MAB

593   (three washes of five minutes, followed by three washes of one hour). Fluorophore

594   deposition was carried out with fluorescein-tyramide, prepared and developed as

595   described by Hopman et al. (Hopman et al., 1998). After washes in PBS-T, samples

596   were stored in 80% glycerol in PBS at -20°C until used.

597   ***In vivo labeling and imaging***

598   For *in vivo* lipid labeling, selected larvae were incubated in a 10 cm petri dish (when

599   labeled in group) or 12-well plate (when labeled individually) containing the lipophilic

600   dye diluted in system water. We incubated the larvae with LipidTox Red (Invitrogen,

601   1/5000) for 1 h at 28 °C or Nile Red (Sigma, 0.78 µM for adipose area quantification

602   and 0.078 µM for emission spectra analysis) for 1 h at 28°C. Labeled individuals were

603   anesthetized and incubated in epinephrine as described above and mounted in 0.8 %

604   low melting point agarose in a 3.5 mm glass bottom petri dish. After solidification, the

605   sample was covered with tricaine 0.04 g/L in system water. To ensure viability during

606   the observation period, a block of agarose covering the region of the gills and the lower

607   jaw was removed using a needle.

608   *In vivo* images were acquired using epifluorescence or confocal microscopy. For

609   epifluorescence we used an Olympus IX81 with 10x UPlan FLN 0.3 NA and 20x UPlan

610   FLN 0.5 NA Olympus objectives. Confocal microscopy images were acquired with

611   either a Zeiss LSM 800 or Zeiss LSM 880 with a 25x LD LCI Plan-Apochromat 0.8 NA

612   Imm Corr DIC M27 (glycerol, oil, water, silicone) Zeiss objective. Hyperspectral imaging

613   of Nile Red fluorescence was done using the lambda module in the Zeiss LSM 880 with

614   the 25x objective, excitation the 488 Argon laser line was used and the spectra

615   acquisition involved 22 step with 10 nm bandwidth (from 493 nm to 713 nm) using a

616   PMT-GaAsP detector.

Image analysis

618   Length and area measurements as well as brightness-contrast adjustments were done

619   using Fiji software (Schindelin et al., 2012).

620   For Nile Red hyperspectral data analysis we used the spectra phasor approach using

621   Globals for Images SimFCS 4 software (G-SOFT Inc, Champaign, IL-USA). This

622   method transforms the spectral data in each pixel to the real and imaginary component

623   of the Fourier transform, as described earlier by Malacrida et al. (Malacrida et al.,

624   2016):

625    $$G(\lambda) = \frac{\int_{\lambda min}^{\lambda max} I(\lambda) \, cos\left(\frac{2\pi n \, (\lambda - \lambda i)}{\lambda max - \lambda min}\right) d\lambda}{\int_{\lambda min}^{\lambda max} I(\lambda) d\lambda} \qquad (1)$$

626    $$S(\lambda) = \frac{\int_{\lambda min}^{\lambda max} I(\lambda) \, sin\left(\frac{2\pi n \, (\lambda - \lambda i)}{\lambda max - \lambda min}\right) d\lambda}{\int_{\lambda min}^{\lambda max} I(\lambda) d\lambda} \qquad (2)$$

627   I(λ) is the intensity at each step, n the harmonic number and λ_i is the initial wavelength.

628   Each pixel in the image will be located at a single (G, S) position at the spectral phasor

629   plot, yielding a cluster of points due to all pixels in an image. This transformation does

630   not modify the original data and does not involve any fitting or any assumption of

631   components. The position at the phasor depends on the spectrum maximum (phase

632   angle, Θ) and the full width at half maximum (Modulation, M) (Fig. S4A), as:

633    $M = \sqrt{S^2 + G^2}$ (3)

634    $\theta = arctan\,(S/G)$ (4)

635   While, red spectral shift implies increasing phase angle, the band narrowing moves the

636   position toward the spectral phasor perimeter (modulation increases).

637   The spectral phasor plot enables the use of vector properties, such as the linear

638   combination and the reciprocity principle. The linear combination allows the

639   quantification of multiple components in a mixture as a sum fractions of single emitters.

640   In our experiments, Nile Red presented complex photophysics that involved the

641   emission from polar and neutral environments (membrane and lipid droplets,

642   respectively). Furthermore, our phasor plots had an extra component from expression

643   of EGFP. Using the three-component analysis developed by Ranjit and collaborators,

644   we decomposed the fraction of Nile Red in the pixels with EGFP signal (Ranjit et al.,

645   2019). We defined two individual cursor positions (two of the vertices) from the Nile

22

646 Red trajectory extremes using images from wild-type larvae labelled with Nile Red, and

647 the third position using images of unlabeled *fabp4a(-2.7):EGFPcaax* larvae. The

648 reciprocity principle enables to trace back a region of interest from the spectral phasor

649 (imaginary space) to the original image (real space; the opposite, from a segmentation

650 in the real image to the phasor plot, is also possible). Using this property, we

651 segmented individual cells selecting the corresponding pixels in the phasor plot. Then,

652 we obtained the fractional contributions for the Nile Red trajectory as explained in detail

653 elsewhere (Ranjit et al., 2019). For comparison purposes between different treatments

654 we used the center of mass (CM) for the Nile Red fraction histogram as a central

655 tendency value and the range of the distribution as a dispersion value. The CM for the

656 distribution of each cell Nile Red fraction was calculated as

657 $\sum b.f(x)/\sum f(x)$ (5)

658 with "*b*" being the percentage of pixels at the particular fraction "*f(x)*" of the component

659 "*x*" (Malacrida and Gratton, 2018). The range of the distribution or distribution range

660 (DR) was considered as the *f(x)* interval which contains 96% of the pixels. For its

661 calculation we used the accumulated distribution for *f(x)* and determined the difference

662 between the *f(x)* values corresponding to 2% and 98% of the accumulated distribution.

663 ***Statistical analysis***

664 The statistical analysis was performed using PAST software (Hammer et al., 2001) or

665 Real Statistics Resource Pack software (Release 7.6, Copyright (2013 – 2021),

666 Charles Zaiontz, www.real-statistics.com, accessed on March 2021). For group

667 comparisons we analyzed normality using Shapiro-Wilk test and homogeneity of

668 variances using Levene test. Non-normal and homoscedastic distributions were

669 compared with non-parametric tests (Kruskal-Wallis or Mann-Whitney with Bonferroni

670 correction) as indicated in each case. Non-normal and heteroscedastic samples were

671 rank transformed (Conover and Iman, 1981) and compared using Welch test and

672 Games-Howell post-hoc test. For the comparison of coefficient of variation we used the

673 Fligner-Killeen test. The Reduced Major Axis (RMA) method was used for regression of

674 bivariate data. For comparison of slopes we used the method explained in (Warton et

675 al., 2006).

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686   **Data availability:** All the data generated in the study is presented in the manuscript.

687   **List of Symbols and Abbreviations:** hpf: hours post-fertilization; dpf: days post-

688   fertilization; WMISH: whole mount in situ hybridization; PVAT: pancreatic visceral adipose

689   tissue; AVAT: abdominal visceral adipose tissue; SL: standard length.

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865

866

867 **Figure legends**

868

869 **Figure 1. *fabp4a(-2.7):EGFPcaax* is expressed in early and mature adipocytes.**

870 **A.** The upper scheme shows the endogenous *fabp4a* gene in chromosome 19 with the

871 transcription start site (TSS), exons (boxes), introns, and the coding sequence (CDS) in

872 red. The cloned region is denoted between dashed lines. The lower scheme represents

873 the vector used for transgenesis (tol2: tol2 sites; pA: SV40_late_polyA; cmlc2: cardiac

874 myosin light chain 2 upstream region).

875 **B.** Epifluorescence microscopy images of live *fabp4a(-2.7):EGFPcaax* larvae from the

876 incross of the F3 generation labelled with LipidTOX-Red. EGFP+ cells were present in

877 the PVAT and AVAT depots. Asterisks indicate mature adipocytes stained with

878 LipidTOX-Red with low (*) or high EGFP expression (**). Single arrows denote early

879 adipocytes expressing EGFP with small lipid droplets. Double arrows indicate early

880 adipocytes with EGFP expression without LipidTOX-Red staining. sb: swim bladder; g:

881 gut. Scale bars: B: panoramic views: 200 μm; insets: 50 μm.

882

883 **Figure 2. Comparison of the expression pattern of *fabp4a(-2.7):EGFPcaxx* and**

884 **endogenous *fabp4a* mRNA in larvae.**

885 Images of *fabp4a(-2.7):EGFPcaax* larvae of 21 dpf processed for WMISH,

886 immunolabeled with anti-GFP and analyzed *in toto* through confocal microscopy.

887 **A.** Panoramic (upper row) and magnified image (lower row) of the abdominal region of

888 a larvae labeled with *fabp4a* antisense probe. Yellow arrows denote the coincidence of

889 EGFP (immunofluorescence) and WMISH signal. Arrows with asterisks show regions

890 with WMISH labeling and no EGFP signal.

891 **B.** Images of the abdominal region of a larvae labeled with *fabp4a* sense probe. Yellow

892 double arrows indicate regions with EGFP signal without WMISH labeling.

893 **C.** Images of the trunk of a larvae labeled with *fabp4a* antisense probe. Blue single

894 arrows indicate pigment cells with EGFP signal. Blue double arrows show WMISH

895 labeling in blood vessels.

896 Scale bars: A: 100 μm (upper row), 50 μm (lower row); B and C: 100 μm.

897

31

898 **Figure 3. Distribution of labeled cells in the abdominal region of live *fabp4a(-***
899 ***2.7):EGFPcaax* larvae of different stages.**

900 Larvae of the indicated stages were stained with LipidTOX-Red, mounted in agarose
901 and imaged using confocal microscopy.

902 **A.** Transmitted light and 3D projection images of a larvae of SL 4.5mm (8 dpf). Yellow
903 rectangles denote cells with transgene labeling. Insets A1 and A2 show confocal
904 sections of these regions. Note membrane localization of EGFP and lack of LipidTOX-
905 Red labeling. Cyan double arrows indicate pigment cells expressing the transgene.
906 Cyan single arrows indicate pigments that scatter light.

907 **B.** 3D projection images of larvae of SL 5 mm (12 dpf). Yellow rectangles denote
908 EGFP+ cells, magnified in B1 and B2. Cells with lipid droplets as well as without them
909 (yellow arrows) can be seen in the same larvae in different positions.

910 **C.** 3D projection images of larvae of SL 6.3 mm (16 dpf) with initial PVAT depot
911 formation. Note the presence of EGFP+ cells with unique cell-filling lipid droplets,
912 irregular cells with several lipid droplets (yellow double arrows) and small cells without
913 lipid droplets (yellow single arrows). Asterisks indicate cells without EGFP expression.

914 Scale bars: A: 100 μm (panoramic view); 10 μm (insets); B: 100 μm (panoramic view);
915 20 μm (insets); C: 100 μm (panoramic view); 20 μm (insets).

916

917 **Figure 4. Interaction of early adipocytes with blood vessels.**

918 Live larvae from the cross of *fabp4a(-2.7):EGFPcaax* and *kdlr:mCherry* fish lines were
919 imaged through confocal microscopy. Images presented here are 3D projections or
920 single sections, as indicated.

921 **A.** Larvae of SL 6 mm (13 dpf) with many EGFP+ cells in its abdominal area, a few of
922 them having lipid droplets (inset A1). Some of the cells are in contact with blood
923 vessels (double arrows) and some of them are not (single arrows).

924 **B.** Larvae of SL 7 mm (16 dpf), with PVAT and AVAT depots (only some cells of each
925 depot expresses EGFP). Insets B1, B2 and B3 show EGFP+ cells with lipid droplets in
926 close apposition to blood vessels and in some cases surrounding them (B2). 3D
927 projections and sections through the position indicated by the dashed line are shown.

928 Scale bars: A: 100 μm; B: 100 μm (panoramic view), 20 μm (insets).

929

32

930 **Figure 5. Different cell morphologies observed in *fabp4a(-2.7):EGFPcaax* larvae.**

931 **A.** *fabp4a(-2.7):EGFPcaax* larvae of 21 dpf were labeled with LipidTOX-Red and

932 imaged *in vivo* through confocal microscopy. Images are 3D projections of confocal

933 stacks, to show different cell morphologies found in these larvae*.*

934 **B.** Images of *fabp4a(-2.7):EGFPcaax*; *kdlr:mCherry* larvae of SL 8 (19 dpf). Note

935 labeled cells in the AVAT depot with cytoplasmic projections which lay in close

936 apposition to blood vessels. Images are 3D projections or sections as indicated.

937 **C.** High magnification confocal sections of EGFP+/LD- cells. Note the cytoplasmic

938 inclusions observed in transmitted light.

939 Scale bars: A: 20 µm; B: 20 µm; C: 20 µm.

940

941 **Figure 6. Larvae in different stages present cells with distinct lipid metabolic**

942 **profiles.**

943 **A.** Representative hyperspectral images of adipocytes ("cell A" to "cell D") in different

944 stages of differentiation. Raw images are presented in gray and intensity based color

945 scale. Images generated after phasor plot analysis make evident the EGFP and Nile

946 Red profiles which are represented separately by different color scales. Scale bars: A:

947 20 µm.

948 **B.** Plot with the normalized distribution of the image pixels with respect to the fraction

949 (expressed as percentage) of neutral lipids for each cell in (A).

950 **C.** Scatter plot showing the distribution range and center of mass of the cells analyzed

951 in different larval stages. Both variables are expressed as the percentage of neutral

952 lipids. The group of data coming from the same larval stage were enclosed by a

953 manually generated convex hull shape just for visualization purposes. The dashed lines

954 (DR=30; CM=60) generates the two regions considered in (D) and (E). The numbers in

955 brackets indicate the total number of larvae and the total number of cells analyzed in

956 each stage.

957 **D.** Data within (DR<30 / CM<60) and outside (DR>30 / CM>60) the region considered

958 in (C) were separated and compared. For center of mass: (*) $p=2.06 \times 10^{-13}$ for median

959 comparison (Mann-Whitney test), $p=1.62 \times 10^{-4}$ for coefficient of variation comparison

960 (Fligner-Killeen test); (**) $p=2.24 \times 10^{-16}$ for median comparison (Mann-Whitney test),

961 $p=7.1 \times 10^{-6}$ (Fligner-Killeen test).

33

962   **E.** Representation of the percentage of cells in each group (DR<30 / CM<60 and

963   DR>30 / CM>60) with respect to the larval stage presented in days post-fertilization

964   (dpf).

965   **F.** Representation of the distribution range (as the size of the dots) and center of mass

966   for the cells within some of the larva analyzed; each larva had a different standard
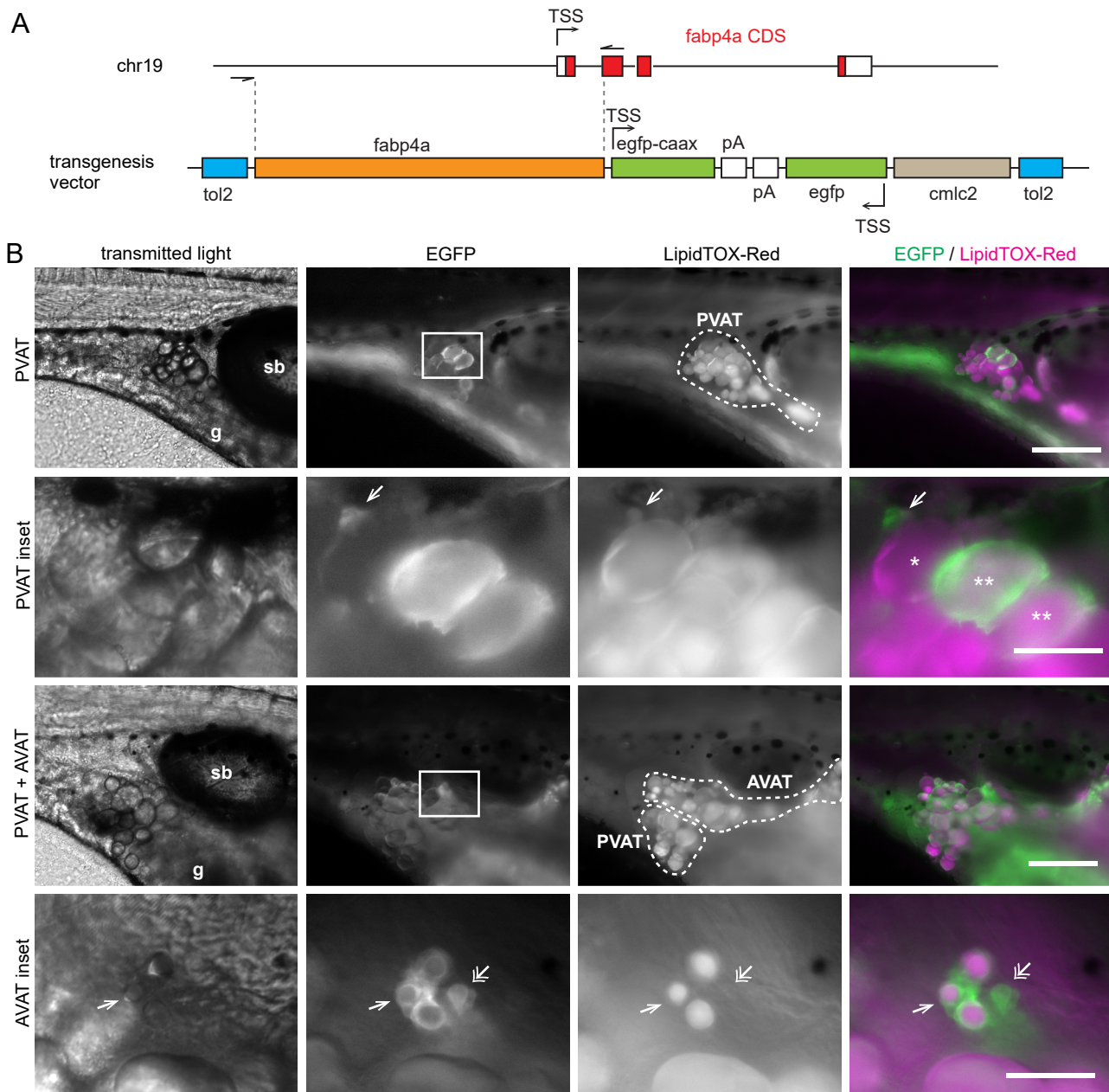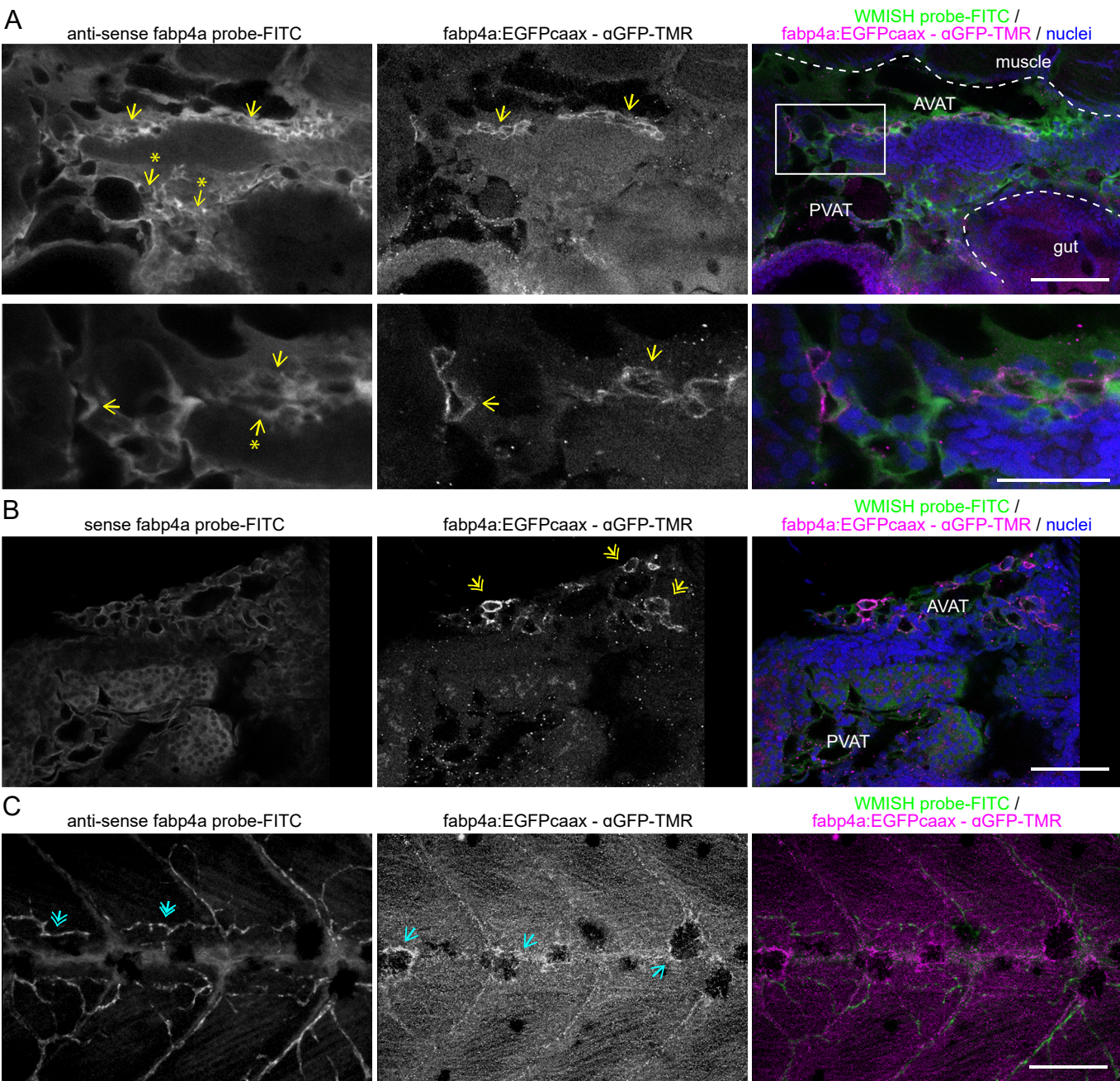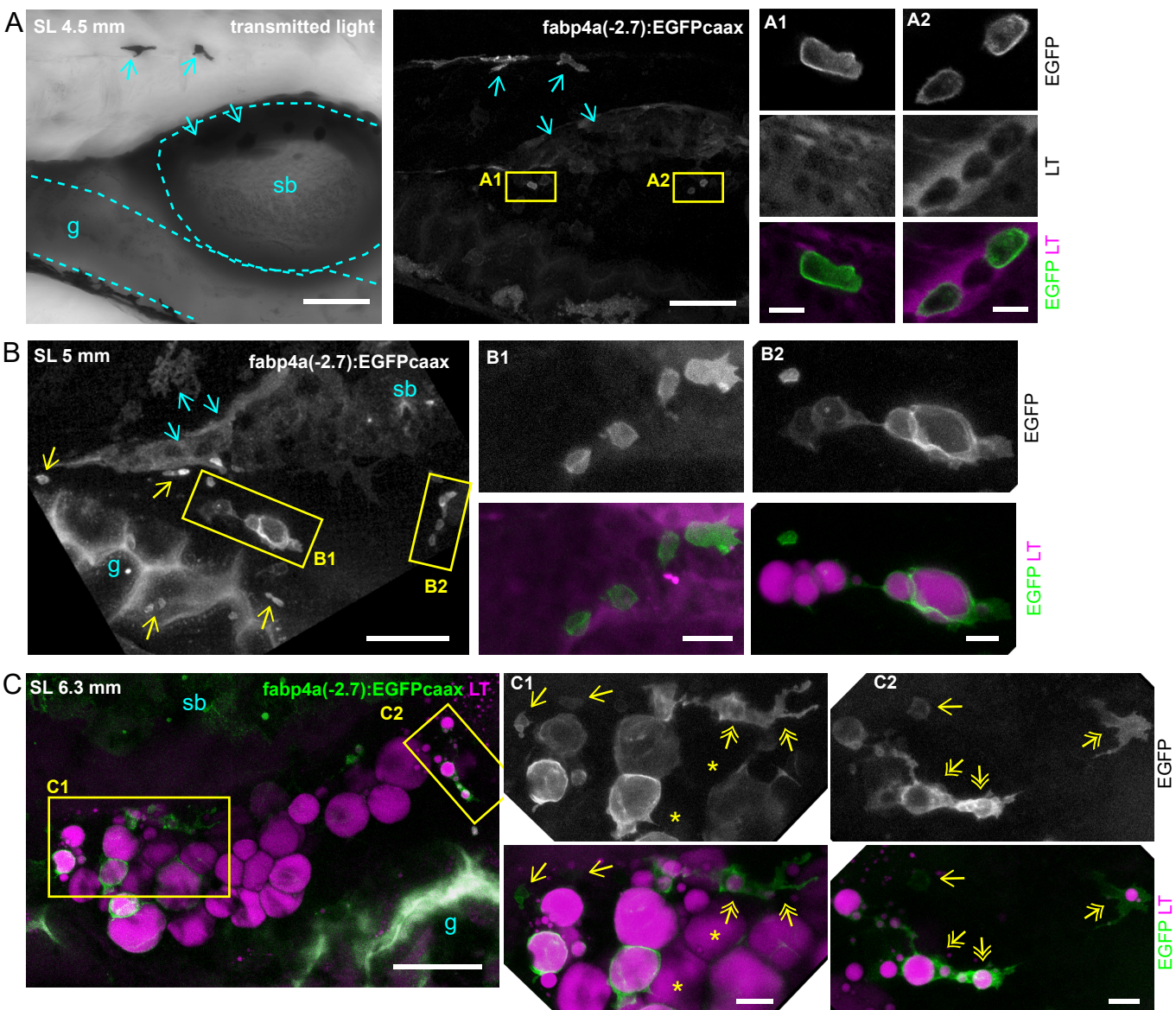
967   length.

968

Figure 1

A chr19

TSS

fabp4a CDS

transgenesis vector — tol2, fabp4a, TSS, egfp-caax, pA, pA, egfp, cmlc2, tol2, TSS

B

	transmitted light	EGFP	LipidTOX-Red	EGFP / LipidTOX-Red
PVAT	sb, g		PVAT	
PVAT inset				* ** **
PVAT + AVAT	sb, g		AVAT, PVAT	
AVAT inset				

Figure 2

Figure 3

A SL 6 mm fabp4a(-2.7):EGFPcaax kdlr:mCherry

A1 - section

A1

3dproj

kdlr fabp4a(-2.7)   transmitted light   merge

B fabp4a(-2.7):EGFPcaax kdlr:mCherry

B2   B3

B1

SL 7 mm 3dproj

fabp4a(-2.7)

B1   B2   B3

kdlr

fabp4a(-2.7) kdlr

transmitted light

Figure 4

Figure 5

A fabp4a(-2.7):EGFPcaax   fabp4a(-2.7):EGFPcaax / LT

A1

A2

A3

B

B1   B2   fabp4a(-2.7)

3dproj   3dproj

fabp4a(-2.7) kdlr   section   section

fabp4a(-2.7) kdlr transmitted light   section   section

C   transmitted light   fabp4a(-2.7)   transmitted light fabp4a(-2.7)

C1

C2

Figure 6

WMISH probe-FITC | fli1:GFP - αGFP-TMR | fli1:GFP - αGFP-TMR, probe-FITC / nuclei

fabp4a anti-sense probe — head, trunk

fabp4a sense probe — head, trunk

slit2 anti-sense probe — head, trunk

969 **Figure S1. Set up of MWISH and immunofluorescence in embryos.**

970 Images of 2 dpf *fli1:EGFP* embryos. WMISH was performed with antisense and sense

971 probes against *fabp4a*, and with previously validated antisense probes for *slit2*. After

972 immunolabeling with anti-GFP, embryos were imaged *in toto* through confocal

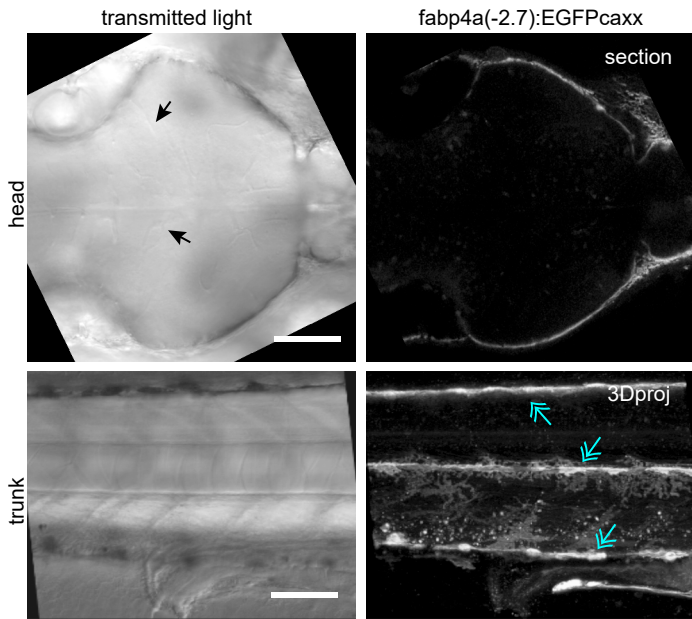973 microscopy. 3D projections generated from confocal stacks are shown. Scale bars: 100

974 μm.

975

A live 2 dpf embryos
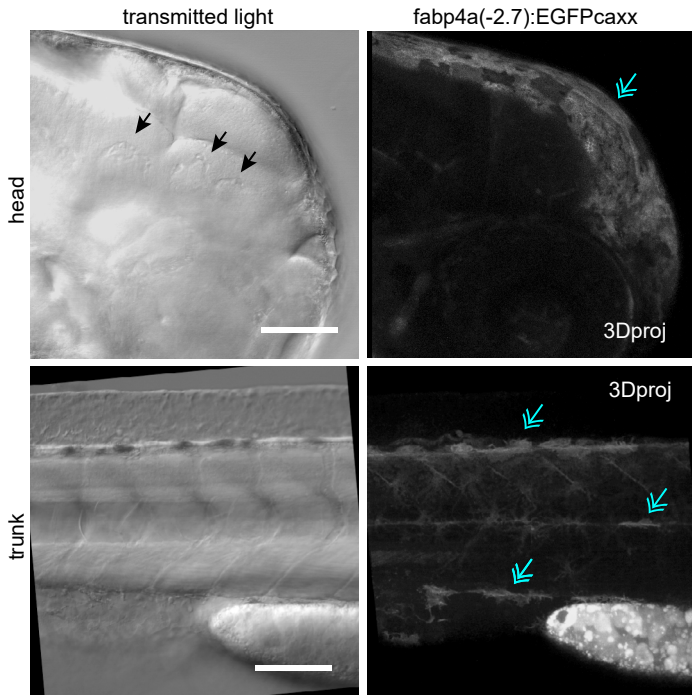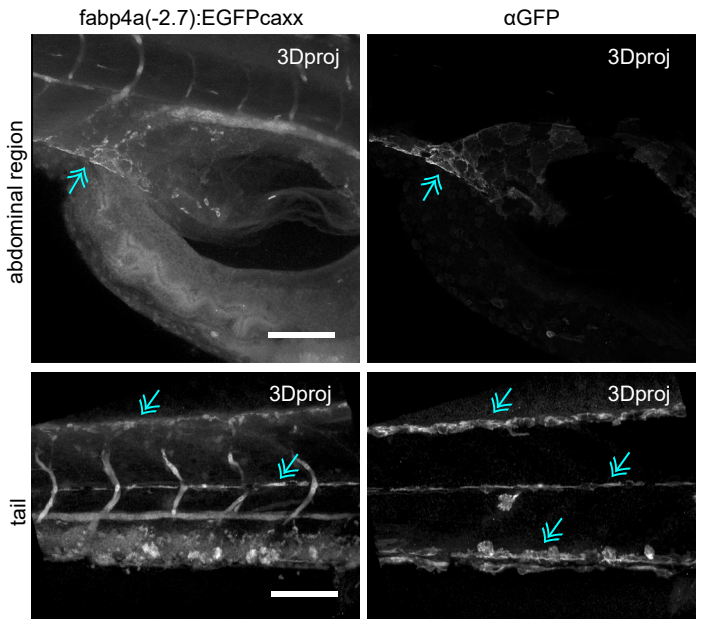
B 2 dpf - whole mount immunofluorescence
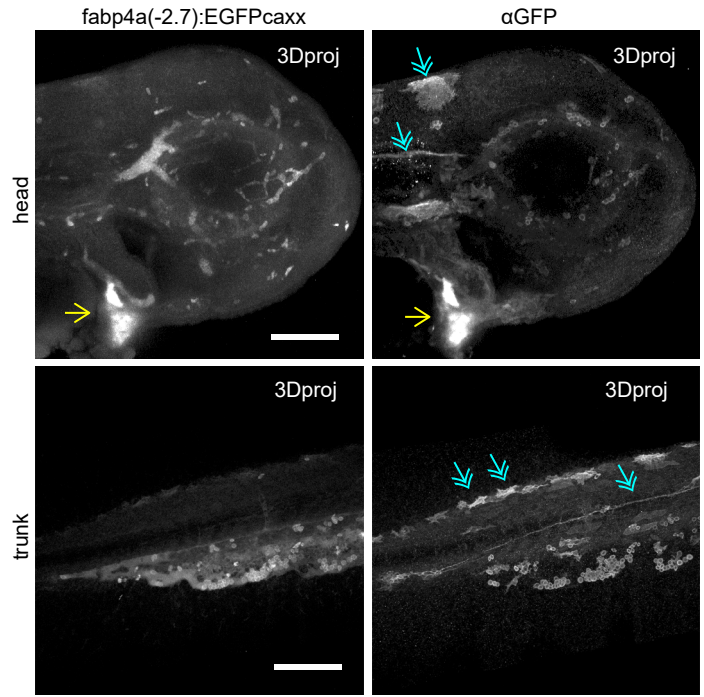
C live 5 dpf embryos

D 5 dpf - whole mount immunofluorescence

976   **Figure S2. Expression domains of *fabp4a(-2.7):EGFPcaax* in embryos.**

977   **A and C.** Images of live embryos of 2 and 5 dpf. Transmitted light or fluorescence

978   images were acquired through confocal microscopy and presented either as single

979   sections or 3D projections (3Dproj). In transmitted light it is possible to observe blood

980   vessels (black arrows) and the lack of fluorescence colocalization.

981   **B and D.** Images of fixed embryos of 2 and 5 dpf immunostained with anti-GFP

982   antibody. Both endogenous GFP and immunolabeling signals are shown. Yellow

983   arrows show the positive immunolabeling signal in heart cells. Blue-double arrows

984   indicate the presence of fluorescence in pigment cells, both in live and fixed embryos,

985   as well as through immunolabeling.

986   Scale bars: A-D: 100 µm.
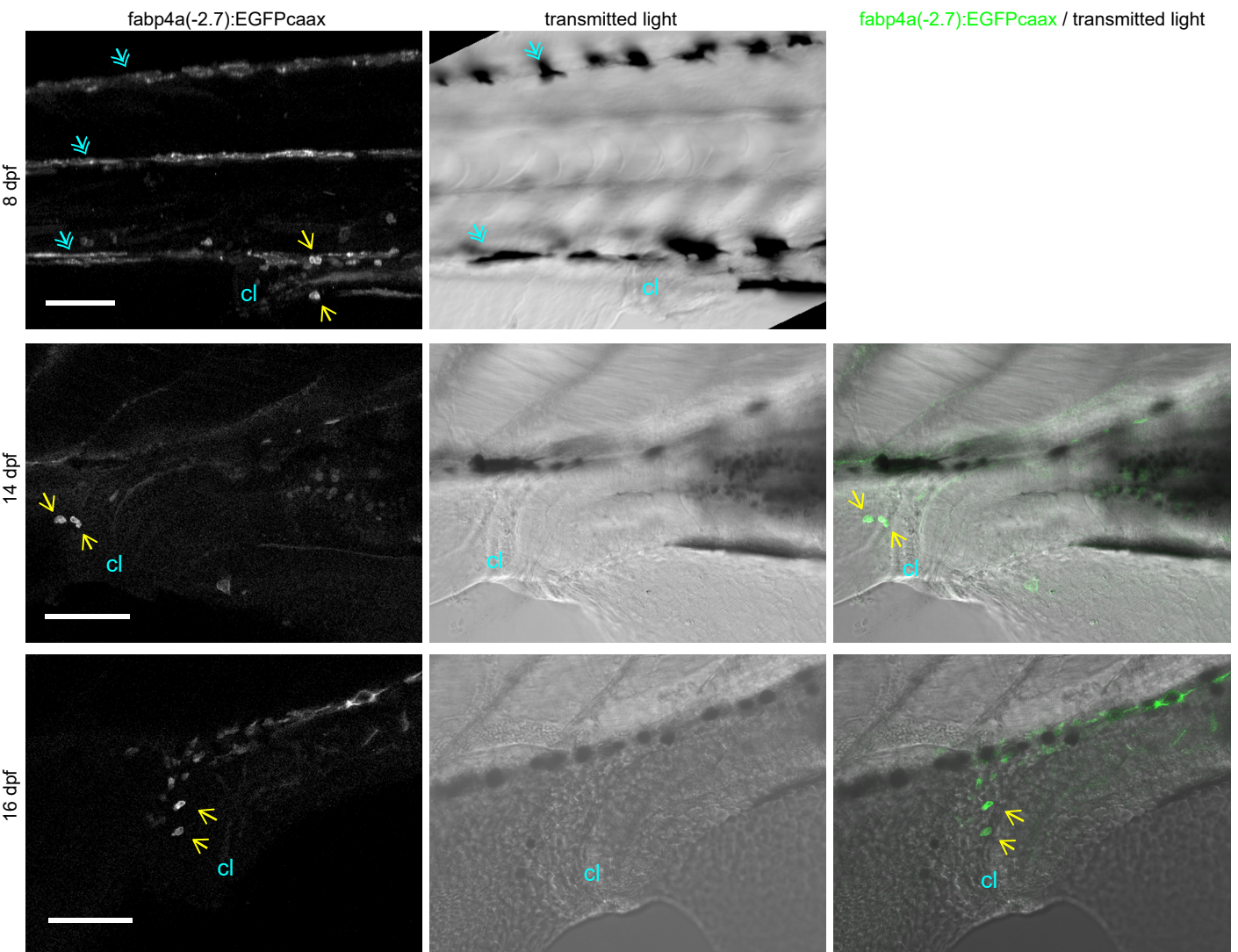
Figure S3

987 **Figure S3.** *fabp4a(-2.7):EGFPcaax expressing* **cells in the tail region of larvae at**

988 **different stages.** Images of live larvae analyzed through confocal microscopy at

989 different stages (expressed as dpf). EGFP+/LD- cells were present in all stages

990 analyzed, including in the cloaca region (yellow arrows). The fluorescence image of 8

991 dpf larvae is a 3D projection to note the presence of labeled pigmentary cells (cyan

992 double arrows). cl: cloaca. Scale bars: 100 µm.
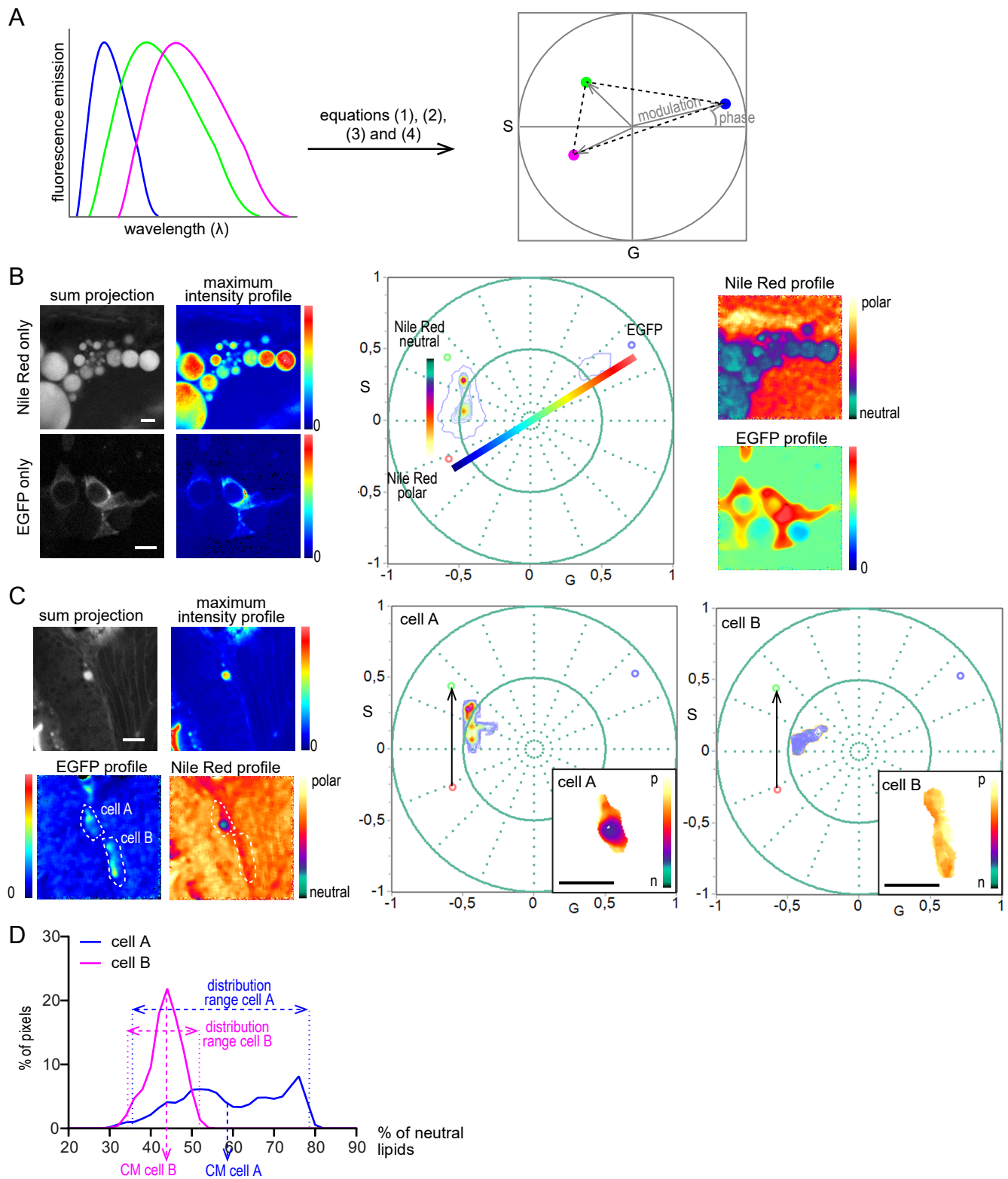
Figure S4

993 **Figure S4. Analysis of the lipid metabolic profile of EGFP+ cells.**

994 **A.** Schematic representation of the transformation of the individual spectra of three

995 different fluorophores into the same phasor plot using equations (1) to (4) indicated in

996 "Materials and Methods" section. Dashed lines indicate the area (triangle) in the phasor

997 plot in which pixels with the combination of the three different fluorophores will appear.

998 **B.** Examples of hyperspectral images of control cells and the localization of each pixel

999 in a phasor plot. Images in the left are projections of spectral images and were colored

1000 according to pixel intensity maximum. Images of wild type larvae stained with Nile Red

1001 ("Nile Red only") lay within a line-shaped trajectory, corresponding to regions of

1002 different lipid polarity within the cell, were used to define the position of two of the

1003 components (Nile Red in a polar environment: red circle; Nile Red in a neutral

1004 environment: green circle). Instead, images of cells in *fabp4a(-2.7):EGFPcaax* larvae

1005 without staining ("EGFP only") appear in a defined region with low phase angle, which

1006 was used to define the position of the third component (EGFP: blue circle). The images

1007 in the right were colored according to the position of pixels in the phasor plot (the color

1008 scales were superimposed to the Nile Red trajectory and the EGFP axis for improving

1009 clarity).

1010 **C.** Example of cells in a *fabp4a(-2.7):EGFPcaax* larvae of 8 dpf stained with Nile Red.

1011 Images were colored according to pixel intensity or to the position of pixels in the

1012 phasor. Phasor plots corresponding to the thresholded cells are presented in the right

1013 side. The direction of the Nile Red axis used for plots in (D) is denoted by a black

1014 arrow.

1015 **D.** Normalized distribution of pixels along the Nile Red axis in the phasor plot for the

1016 cells in (C). The "Nile Red axis" corresponds to the percentage of polar lipids in the

1017 region of the cell analyzed. The center of mass and range of these distributions were

1018 calculated as described in the "Material and methods" section and are schematically

1019 represented in the plot. Scale bars: B: 20 μm; C: 50 μm.

1020

1021   **Movie 1.** Time-lapse imaging of EGFP+/LD- cells. The membrane EGFP signal is in

1022   green and the transmitted light in grey. Time is showed in minutes:seconds format.

1023   Scale bar: 10 µm