1 **Identification of a specific granular marker of zebrafish eosinophils enables development of**

2 **new tools for their study**

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16 **Data Sharing Statement:** For original data or access to the novel transgenic line, please contact the

17 corresponding author.

Abstract

18

19   Eosinophils control many aspects of the vertebrate innate immune response. They contribute to

20   homeostasis, inflammatory conditions and defense against pathogens, yet, their function in disease

21   often remains enigmatic. The zebrafish has emerged as a useful model organism for human diseases

22   but tools to study eosinophils in this model are severely limited. Here, we characterize a new and highly

23   specific marker for eosinophils in zebrafish and report a new transgenic reporter line to visualize

24   eosinophils *in vivo*. In addition, we created a polyclonal antibody that allows the identification of

25   eosinophils *ex vivo*. These new tools expand the toolkit to study eosinophils in the zebrafish model.

26   Using our tools, we have been able to identify the likely ortholog of eosinophil major basic protein, a

27   critical mediator of eosinophil function, in zebrafish. These advances will allow for the investigation of

28   eosinophil biology in the zebrafish model organism, allowing researchers to identify the contribution

29   of eosinophils to the many diseases that are modeled within zebrafish and also shed light on the

30   evolution of eosinophils within vertebrates.

Introduction

Zebrafish (*Danio rerio*) are an increasingly popular model organism in biomedical research and have been used to model a variety of human diseases[1–3]. They possess an evolutionarily conserved vertebrate immune system containing all major cell types, including eosinophils[4]. The zebrafish's naturally transparent larval stage, high fecundity and genetic tractability make it an excellent model organism to study hematological and immunological questions[5,6]. While eosinophils are a recognized part of the zebrafish immune repertoire, the limited array of existing tools for the study of eosinophil function in zebrafish has restricted the use of this model for the study of eosinophil biology.

Eosinophils belong to the group of granulocytes and are characterized by large cytoplasmic granules, that store a plethora of pre-formed signaling and effector molecules, which can be stained with acidic dyes like eosin[7]. They are well known effector cells in parasitic infections as well as mediators of allergic or inflammatory conditions, e.g. asthma. Although they are only found in low numbers under homeostatic conditions, they have been associated with both beneficial as well as detrimental effects in health and disease[8–10]. Yet, their contributions often remain elusive.

Past work characterizing eosinophil responses in zebrafish have mostly relied on classical histological staining approaches and the transgenic *Tg(gata2:eGFP)* line[11]. In this transgenic line, the *gata2a* promoter drives the expression of EGFP. Since eosinophils express this marker highly, this transgenic line can be used to isolate eosinophils using fluorescence assisted cell sorting (FACS). However, *gata2a* is not expressed exclusively in eosinophils and therefore this reporter line cannot be used to identify eosinophils *in vivo*.

The broad importance of eosinophils in a range of conditions as well as their evolutionary conservation highlight the critical role played by this cell type and the need for comprehensive methods to further investigate their function in a range of settings. Here, we identify an eosinophil granule-specific marker in zebrafish that we use to create both a transgenic fluorescent reporter line as well as a polyclonal antibody to visualize zebrafish eosinophils both *in vivo* and *ex vivo*. We found that our eosinophil

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56   reporter line and antibody could be used to identify eosinophils in tissue as well as by flow cytometry,

57   facilitating the study of this immune cell population. Using electron microscopy, we found that this

58   marker localizes specifically to eosinophil granules. Based on our findings, we are able to identify our

59   marker gene as the zebrafish orthologue of eosinophil major basic protein *(embp).* To further

60   demonstrate the utility of our tools, we visualized the localization of eosinophils to mycobacterial

61   granulomas, validating the use of this marker during inflammatory conditions.

62   **Results**

63   <u>Embp is a granular marker for zebrafish eosinophils</u>

64   Previous studies into zebrafish eosinophils have often used broadly expressed markers, like the

65   *Tg(gata2:eGFP)* line, or histological properties to identify eosinophils. Single cell RNA-Seq (scRNA-Seq)

66   data allow clustering and annotation of cell types based on transcriptomic profiles. These analyses

67   facilitate an unbiased investigation into uncharacterized gene expression patterns. Using previously

68   published zebrafish scRNA-Seq data sets (GSE161712, GSE112438), we searched for differentially and

69   highly expressed genes in eosinophil populations. One gene that stood out was *dkeyp75b4.10* (also

70   known as *eslec*), a previously uncharacterized protein with a C-type lectin domain that is highly and

71   differentially expressed in eosinophils. A recent report established that the promoter of this gene is

72   eosinophil-lineage specific[23]. Based on our findings described below, we have renamed this gene *embp*

73   (for eosinophil major basic protein) which we will use throughout for clarity purposes.

74   Our aim was to create a transgenic reporter line with a precise knock-in at the endogenous gene locus

75   using CRISPR Cas. By tagging the C-terminus of Embp with a fluorescent protein, this approach will

76   result in the generation of a full-length, tagged version of the protein under the control of the

77   endogenous promoter, allowing us to visualize both eosinophils, and the localization of the Embp

78   protein itself. A donor vector was designed (Fig 1A) to generate this transgenic line. Next, a sgRNA was

79   designed to create a double-strand break with CRISPR Cas9 at the before-last intron of *embp*. The

80   donor DNA fragment reconstitutes the remaining intronic sequence as well as the two last exons

81   downstream of the cut site upon integration. Furthermore, it introduces a tdTomato sequence as a 3'

82   tag of our gene of interest, followed by a polyA sequence. The construct also includes a transgenesis

83   marker using an eye-specific *cryaa* promoter to drive EGFP enabling facile identification of embryos

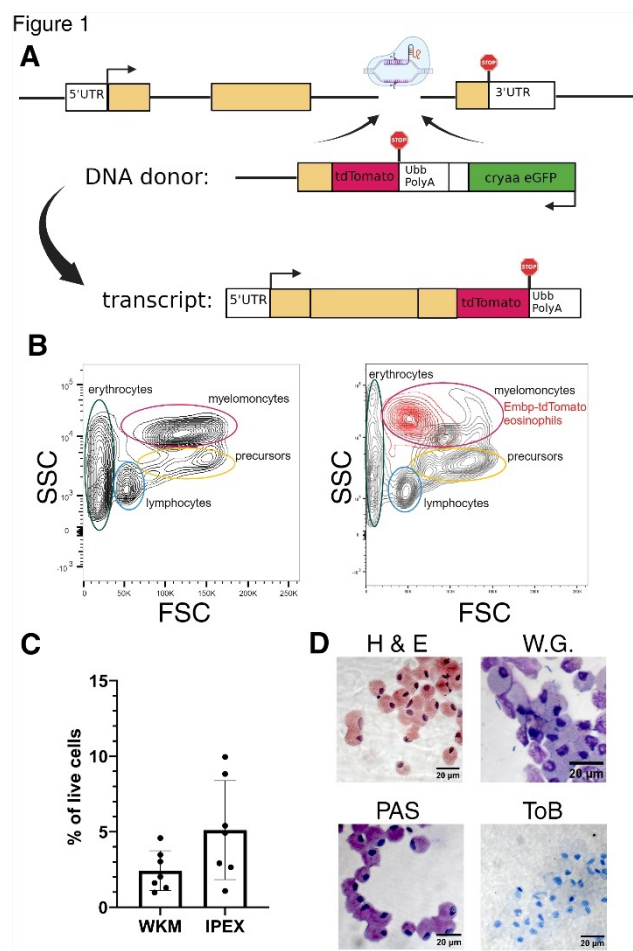84   carrying this transgene prior to the emergence of eosinophils, by bright green fluorescence in the lens.

85

86   Figure 1. Generation of *TgKI(embp-tdTomato, cryaa:EGFP)* transgenic reporter line and initial
87   validation. (A) Schematic of genetic locus and DNA donor fragment inserted in the *embp* locus.
88   Transcription leads to a fusion construct of Embp and tdTomato. (B) Traditional gating scheme by light-
89   scatter characteristics of a representative wildtype WKM as previously reported[24]. Side by side
90   visualization with the Embp-tdTomato+ cell population backgated onto the entire WKM in FSC/SSC. (C)
91   Quantification of Embp-tdTomato+ cells in WKM and IPEX samples of this transgenic line, measured by
92   flow cytometry and presentation as proportion of live cells: WKM 2.4% (± 0.38%) and IPEX 5.1% (±
93   2.7%) mean with SD. (D) FACS sorted Embp-tdTomato+ cells stained with Hematoxylin & Eosin (H&E),
94   Wright Giemsa (WG), Periodic Acid Schiff (PAS) and Toluidine Blue (ToB).

95   Wildtype zebrafish eggs were injected at one cell stage, screened for cryaa:GFP expression at 3 dpf and

96   raised to adulthood. F0 founders were spawned and screened to identify founders transmitting the

97   cryaa:GFP phenotype. cryaa:GFP-positive F1 offspring with stable integration of the transgene were

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98   analyzed using flow cytometry for tdTomato expression in the granulocyte compartment. In zebrafish,

99   eosinophils are commonly isolated from two sites: whole kidney marrow (WKM), the bone marrow

100   equivalent of zebrafish, and intraperitoneal exudate (IPEX), cells collected from the peritoneal cavity.

101   tdTomato+ cells were identified within both WKM and IPEX populations. These tdTomato-positive cells

102   were brightly fluorescent and appeared within the expected myelomonocytic gate using forward

103   scatter/side scatter gating as has previously been done in zebrafish[24] (Fig 1B). The proportions of

104   tdTomato+ cells in WKM (2.4% ± 0.38%) and IPEX (5.1% ± 2.7%) (Fig 1C) corresponded well to the

105   proportions of eosinophils observed in previous studies with Gata2a^{high} cells. Using FACS, tdTomato+

106   cells were isolated and stained with a panel of histological stains to corroborate the identity of the

107   isolated cells. Using Hematoxylin & Eosin (H&E), Wright Giemsa (WG) and Periodic Acid Schiff (PAS)

108   staining, we found that tdTomato+ cells showed the histological hallmarks of zebrafish eosinophils[11]

109   including a "round to oval shaped nucleus typically located peripherally"[25] (Fig 1D). We also observed

110   that these cell populations were negative for Toluidine Blue (ToB) staining, which identifies highly

111   granular mast cells which are also known to be present in zebrafish[26]. Confocal imaging of transgenic

112   tdTomato+ eosinophils isolated from *TgKI(embp-tdTomato, cryaa:EGFP)^{ber1}* animals revealed brightly

113   fluorescent cells with the tagged transgene having a punctate localization that is consistent with

114   targeting of this gene to the granules (Fig 2A). This transgenic reporter could be visualized not only in

115   isolated cell populations, but could also be used to observe eosinophil localization in FFPE sections in

116   conjunction with a commercial mCherry antibody (Fig 2B). Using IF and histological staining of adjacent

117   sections, we found that these tdTomato+ transgenic eosinophils localized to the same sites in the

118   zebrafish body as their wildtype counterparts under homeostasis and possessed the histological

119   characteristics of zebrafish eosinophils, suggesting that our tagging approach did not impact eosinophil

120   homeostasis.
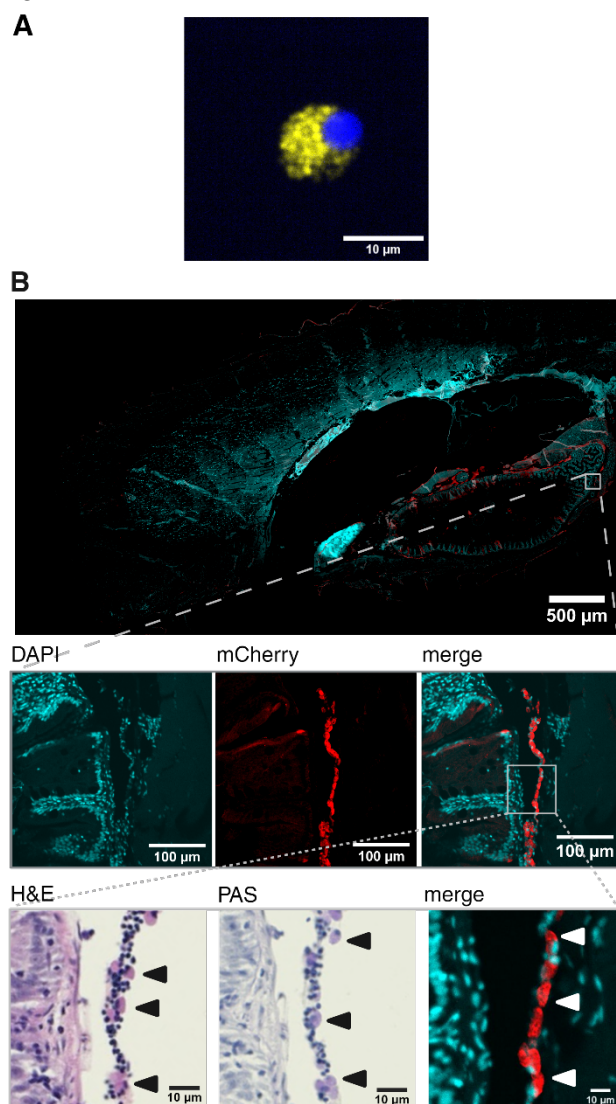
Figure 2

A

B

Figure 2. Confocal fluorescent microscopy of *TgKI(embp-tdTomato, cryaa:EGFP)* samples. (A) 40X imaging of a FACS isolated Embp-tdTomato$^+$ (yellow) eosinophil. The fluorescent transgene is localized within likely eosinophil granula. Nuclear counterstain with DAPI. (B) Detection of the Embp-tdTomato transgene in 5 μm FFPE sections using mCherry antibodies. Eosinophils are found along the lamina propria lining the intestine and in-between organs, confirmed by H&E and PAS staining of adjacent sections.

In conclusion, endogenous tagging of *embp* with tdTomato created a transgenic reporter line that enabled us to isolation and visualization of zebrafish eosinophils. The transgenically tagged cells showed eosinophil morphology and characteristics by flow cytometry, histological staining and confocal microscopy. Additionally, staining tissue sections with an mCherry antibody confirmed that tdTomato$^+$ eosinophils localize like their wildtype counterparts under homeostatic conditions, demonstrating that this targeting approach did not alter eosinophil localization in homeostasis.

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134   Further, using this reporter line, we found that this transgene appeared to localize to granules,

135   suggesting that Embp may be one of the limited subset of eosinophil granular proteins.

136   <u>Visualizing Embp-tdTomato[+] eosinophils and wildtype Embp both *in situ* and *ex vivo*</u>

137   While our transgenic reporter line was useful to isolate eosinophils and visualize eosinophils *in vivo*,

138   we also sought to establish a solution to visualize eosinophils in tissue samples that lack the transgenic

139   reporter. To this end, we generated a custom polyclonal antibody targeting Embp (Fig 3A). To confirm

140   eosinophil specificity, the antibody was tested on WKM, IPEX and isolated eosinophils of both

141   *Tg(gata2:eGFP)* and *TgKI(embp-tdTomato, cryaa:EGFP)* lines (Fig 3B+C). Positive staining was observed

142   in 92.45%/87.47% (Embp/Gata2a line) of sorted eosinophils, 4.62%/3.58% (Embp/Gata2a line) in WKM

143   and 12.63%/9.86% (Embp/Gata2a line) in IPEX samples, consistent with previous reports on the

144   proportion of eosinophils in such samples. Importantly, staining of GFP[-] and tdTomato[-] fractions from

145   *Tg(Gata2a:GFP)* or *TgKI(embp-tdTomato, cryaa:EGFP)* revealed no staining in eosinophil-depleted

146   fractions by the antibody highlighting its specificity (Fig S1B).

Figure 3

A

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MWISAAFLLFALAVNGVKSDVPNISRRCPSGWEKFGSQCFKFFSEYKTWAEAEKHCVDLGGNLASIQSDITHNFLIAYLKR
QEKGITRTWIGAHDATQADIWFWSDGSKFEYSAWHSGEPNNGGNAERCAEMGFGDEQRWNDARCETRLNFICYRMTRINM
```
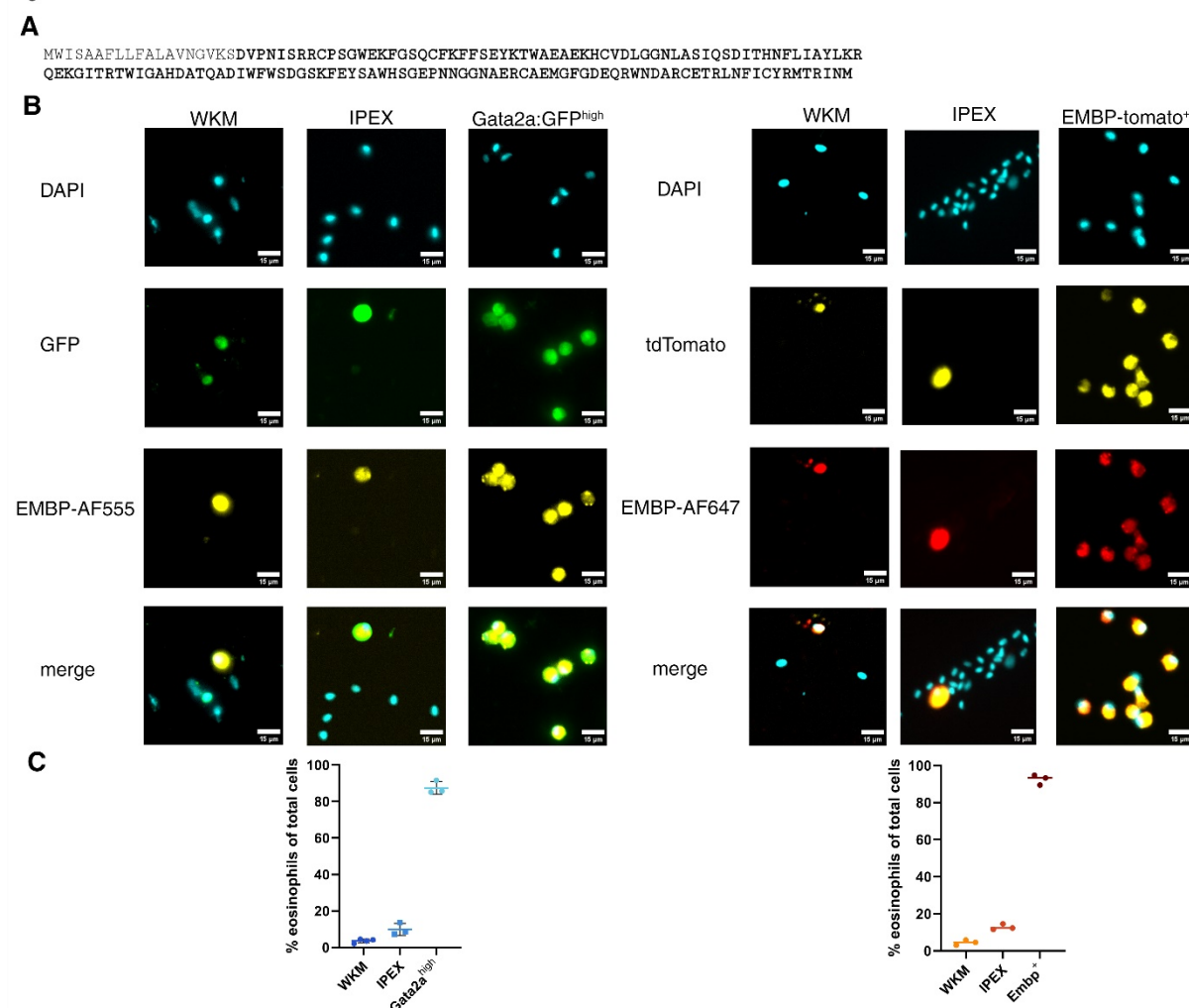
B

C

Figure 3. Novel polyclonal antibody targeting Embp using in immunofluorescence staining. (A) Amino acid sequence of Embp in single letter code. In bold: the part of the antigen used to generate the polyclonal antibody. (B) Staining of isolated WKM, IPEX and eosinophil cell populations of both the *Tg(gata2:eGFP)* and *TgKI(embp-tdTomato,cryaa:EGFP)* lines. Eosinophils were sorted by transgenic reporter expression (either Gata2a[high] or Embp[+]) (C) Quantification of cells stained positively for Embp with polyclonal antibody in the afore mentioned sorted cell populations. Positive staining was assessed in a minimum of six random fields of view across three independent experiments. 300-500 cells were evaluated per sample. Data represented as percentage of positive Embp staining in total cell population, mean with SD of each independent experiment.

We also sought to determine whether this antibody could also be used to identify eosinophils in paraffin embedded tissues. Antibody staining of formalin-fixed paraffin-embedded (FFPE) tissue sections (Fig 4) demonstrated that this antibody could detect eosinophils within the tissue, with the staining patterns corresponding with the expected distribution of eosinophils within the tissue. To further corroborate stained cells as eosinophils, we stained adjacent sections with H&E and PAS (Fig 4A) confirming that cells positive for Embp staining localized to the same locations as eosinophils

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163   detected by H&E and PAS. Eosinophils were found mainly in clusters in between organs of the

164   peritoneal cavity, along the basal lamina propria in the gut and intestine as well as in the WKM.

165   Negligible antibody staining was observed in regions of the animal where few if any eosinophils were

166   observed, confirming the specificity of the antibody binding and efficacy of this reagent for the

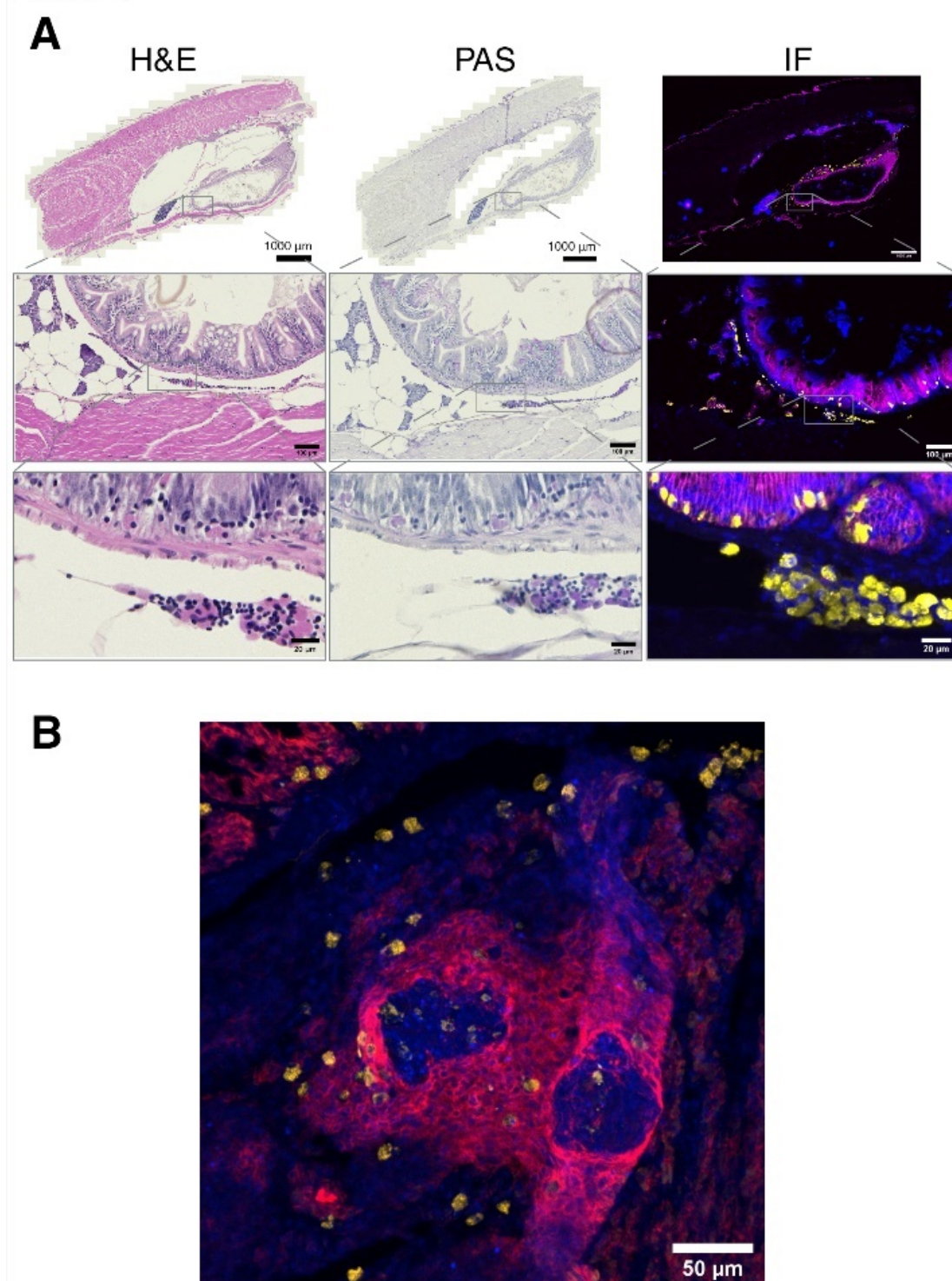167   detection of endogenous eosinophils.

Figure 4. Embp antibody staining in FFPE tissue samples. (A) H&E, PAS and IF staining with Embp antibody in healthy *TgKI(embp-tdTomato,cryaa:EGFP)* adults. Blue: DAPI Yellow: eosinophils Magenta: E-Cadherin staining epithelial cells. (B) Staining of eosinophils in *M. marinum* infected zebrafish FFPE sections. Eosinophils can be found in physiological sites in the body as well as in granuloma. Magenta: E-cadherin, marking epithelioid macrophages surrounding the necrotic core of the granuloma. Yellow: eosinophils stained by Embp antibody. Blue: DAPI.

175   During inflammation, immune populations including eosinophils undergo profound transcriptional

176   changes and eosinophils can also undergo degranulation, processes that can shift reporter gene

177   expression and localization. To confirm that our antibody and the broader reporter approach could

178   still be used to detect eosinophils within inflamed tissues, we visualized eosinophil localization in

179   *Mycobacterium marinum* infected FFPE samples of adult zebrafish (Fig 4B). *M. marinum* infection

180   causes granulomatous inflammation in zebrafish and eosinophils have been found within the zebrafish

181   granuloma[27]. We found that eosinophils could be stained within the mycobacterial granuloma as well

182   as in surrounding tissue indicating that even under inflamed conditions, this marker could be used to

183   identify eosinophils. This finding is also consistent with previous studies demonstrating recruitment of

184   eosinophils to mycobacterial granulomas, providing further evidence for eosinophils within this

185   structure and the spatial distribution of the cells throughout the granuloma.

186   Our previous experiments have indicated that not only is Embp an effective marker of eosinophils, but

187   have also supported a putative eosinophil granular localization for Embp. In mammals, eosinophil

188   granules contain a limited array of highly expressed effector genes. To definitively establish Embp as a

189   granular protein we employed cryo-immunogold electron microscopy (EM) to localize Embp within the

190   eosinophil. Embp-tdTomato[+] eosinophils were isolated from *TgKI(embp-tdTomato, cryaa:EGFP)*

191   animals by flow cytometry and stained with both our polyclonal Embp antibody to detect the native

192   antigen and an mCherry antibody, to visualize transgene localization with the same cells. We found

193   that both the endogenous Embp and the tdTomato transgene are almost entirely localized within the

194   granules of the eosinophils, with only sporadic protein detection in the cytoplasm (Fig 5). Thus our

195   findings establish Embp as a *bona fide* eosinophil granular protein in zebrafish, and a potential role for

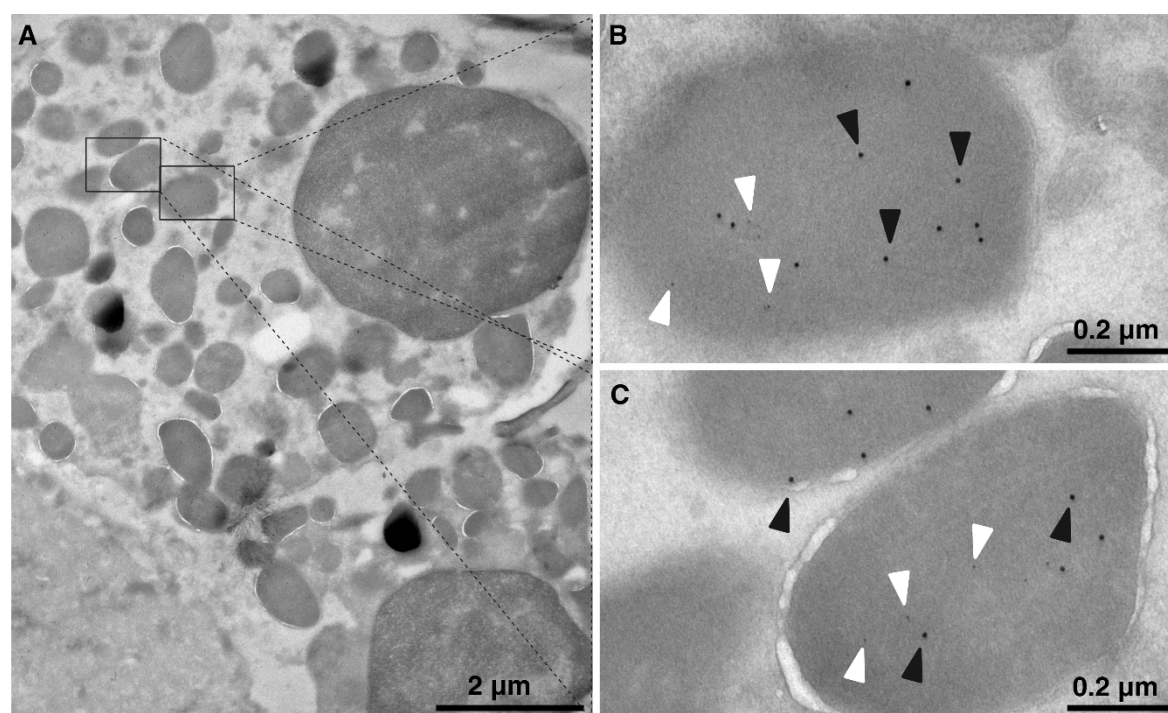196   this protein as an eosinophil effector protein.

Figure 5

Figure 5. Cryo-immunogold EM of FACS isolated Embp-tdTomato[+] eosinophils. (A) Overview of an eosinophil. (B+C) Zoom in on two different granula of this cell. Embp antibody is visualized by a 12 nm gold secondary antibody (black arrow heads) and mCherry antibody is visualized by 6 nm gold secondary antibody (white arrow heads). Both target antigens detected in the same granule.

Discussion

Eosinophils are critical effectors of the vertebrate immune response, contributing to the control of helminth pathogens and tissue remodeling but also leading to detrimental pathology in diseases such as asthma[28]. Eosinophils are broadly conserved in vertebrates, but despite their recognition in zebrafish[25], tools to study this cell population and their specialized effectors are lacking in this emerging infection model[29]. In this study, we identified a novel highly specific marker for zebrafish eosinophil granules, Embp. We initially identified Embp from analysis of previously published RNA-Seq data sets, which suggested that *embp* (also known as *dkeyp75b4.10* ENSDART00000143719, ENSDART00000110749) was both highly and differentially expressed in eosinophilic granulocytes, with minimal expression in other hematopoietic-derived lineages. This eosinophil-specific expression of Embp is consistent with recent observations of this gene and its promoter[23], though the localization and function of this gene was unclear. We generated a transgenic reporter line tagging the endogenous

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214 Embp with a fluorescent protein on its 3' end by CRISPR insertion to investigate the localization of this

215 gene resulting in the endogenous promoter of *embp* driving the expression of a full-length transgenic

216 Embp-tdTomato construct. Using this zebrafish line, we found that *embp* was specifically expressed

217 within eosinophils and not in other immune populations, demonstrating that this line could be used to

218 specifically mark eosinophils in zebrafish. This marker line could be used for flow cytometry, confocal

219 microscopy and histochemistry to identity eosinophils, expanding the toolkit available to study the

220 function of these cells in zebrafish. In addition, a newly generated polyclonal antibody targeting Embp

221 allows visualization of endogenous eosinophils *in vivo* and *ex vivo*.

222 Comparing the proportions of eosinophils in the previously characterized *Tg(gata2:eGFP)* line and our

223 new *TgKI(embp-tdTomato, cryaa:EGFP)* line, we find very similar levels of our cells of interest. While

224 numbers were close between the two lines, we did find a slight statistically significant increase in the

225 number of eosinophils only in the WKM population of *TgKI(embp-tdTomato, cryaa:EGFP)* (Fig S1) but

226 not in IPEX populations. Considering the low number of eosinophils in WKM and the high variability in

227 eosinophil number per animal in our data and the data of other groups[25], we expect that this could

228 potentially be driven by stochastic differences in this small group of animals. A second possibility is

229 that the distinct fluorophores and fluorescent intensity of each reporter could lead to subtle

230 differences in the detection frequency. Overall, however, the relative number of eosinophils in the

231 new *TgKI(embp-tdTomato, cryaa:EGFP)* line falls within the range of previously reported eosinophil

232 counts[11].

233 Structurally Embp possesses a c-type lectin domain. Although there are a number of C-type lectin

234 proteins expressed in mammalian eosinophils including major basic protein 1 (MBP-1)[30], major basic

235 protein 2 (MBP-2)[31], Dectin-1[32] and C-type lectin domain family 4, member a4 (Clec4a4)[33], MBP-1 and

236 MBP-2 are the only C-type lectins that are canonical granule proteins in mammalian eosinophils[8]. Our

237 cryo-EM data (Fig 5) together with confocal microscopy (Fig 2), demonstrate that in zebrafish Embp is

238 an eosinophil granule protein, likely acting as an eosinophil effector molecule. MBP-1 and MBP-2 are

239 amongst the most highly expressed proteins in mammalian eosinophils. Similarly, *embp* was identified

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240   as one of the most highly expressed genes in previous scRNA-Seq data and both our reporter and

241   antibody show robust positivity indicating high level expression. Based on the strong expression of

242   Embp and localization within the granules, its exclusivity to eosinophils and its C-type lectin domain,

243   we expect that Embp is a zebrafish ortholog of the MBP family. Thus, in light of our data, we believe it

244   is accurate to name this gene Embp rather than the previous gene name, *dkeyp75b4.10*, or the recently

245   proposed eslec.

246   In mammals, eosinophil major basic protein 1 is, as the name suggests, characterized by a strongly

247   basic pI. Surprisingly, the pI of Embp is relatively neutral (pI 6.8). However, while hMBP-1 is highly basic

248   (pI 11.4), hMBP-2 is known to also have a pI much closer to neutral (pI 8.7). As we saw in zebrafish for

249   Embp, hMBP-2 is exclusively expressed in eosinophils[34], while hMBP-1, although predominantly

250   expressed in eosinophils is also expressed in basophils and mast cells, suggesting that *embp* may be

251   the ortholog of hMBP-2. Interestingly, in ruminants, MBP-2 is the only MBP present[35]. Also, the

252   relatively neutral pI of Embp suggests that the characteristically strongly basic pI of hMBP-1 may be a

253   relatively recent evolutionary occurance. Indeed, the pIs of MBP orthologues vary considerably across

254   species[34,35]. Although further studies into the biochemical and functional properties of this zebrafish

255   orthologue will be needed, zebrafish *embp* will likely offer an interesting window into the evolution of

256   MBP proteins.

257   Addressing a critical gap in the study of zebrafish eosinophils, the new tools presented here will also

258   allow to study the development and maturation of eosinophils as well as function of eosinophils in

259   diverse diseases. Recent efforts using zebrafish to model allergic inflammation and food allergies have

260   identified granulocytes as significant contributors to these conditions[36], but they have not been able

261   to identify the specific cell types responsible. By facilitating studies of eosinophil function in zebrafish,

262   our tools will enable researchers to discover new roles for eosinophils that can be conserved in human

263   disease. As with the MBP protein family, the similarities and differences in eosinophil responses in

264   zebrafish will also offer a window into the evolution of these cells, as zebrafish are one of the most

265   distant human ancestors to have eosinophils. Use of zebrafish for comparative immunology

266   approaches will highlight centrally conserved immune responses and eosinophil behavior that function

267   across vertebrate species.

268

269   **Methods**

270   **Zebrafish housing and breeding**

271   Animals were housed and bred under standard conditions. All experiments were conducted in

272   accordance with institutional (Max Planck Institute for Infection Biology), state (LAGeSo Berlin), and

273   German ethical and animal welfare guidelines and regulations. Male and female animals were used in

274   approximately the same ratios for all experiments.

275   **Zebrafish lines**

276   The wild-type strain in this study was *AB and all zebrafish strains used this background. Transgenic

277   lines used in this study: *Tg(gata2:eGFP)* that has been previously been described[12] and the newly

278   created *TgKI(embp-tdTomato,cryaa:EGFP)ber1* line (see below). Fluorescent reporter lines were

279   maintained as heterozygotes.

280   **Generation of transgenic zebrafish line**

281   The new eosinophil reporter line was created using a CRISPR Cas9 based knock-in approach inserting

282   into a non-coding region as previously described[13]. The transgenic insert used to generate *TgKI(embp-*

283   *tdTomato, cryaa:EGFP)* zebrafish was assembled using the Golden Gate cloning system[14]. The insert

284   combines the last two exons of *embp* (also known as *dkeyp75b4.10* or *eslec*, ENSDARG00000079043)

285   amplified from bacterial artificial chromosome (BAC) clone CH211-226G7 (BACPAC Genomics), a

286   tdTomato sequence followed by UbB polyA and a transgenesis marker encompassing the *cryaa*

287   promoter[15] driving EGFP in the reverse orientation. The DNA donor fragment was PCR amplified and

288   cleaned up prior to injection using the Gel & PCR Clean-Up Kit (Macherey-Nagel). One cell stage

289   zebrafish embryos were injected with ~1-2 nl of 2.5 ng/µl DNA donor fragment together with 12.5

16

290   ng/μl sgRNA (GGACAATTTTCATGACAAGA, ordered from IDT) and 75 ng/μl Cas9 protein (IDT). 3 dpf

291   larva were screened for GFP expression in the retina and raised to adult hood. Mosaic F0 founders

292   were outcrossed to *AB fish and screened for germline transmission of the transgenesis marker (GFP[+]

293   eyes). GFP[+] F1 fish were raised to adulthood and screened for tdTomato[+] eosinophils using flow

294   cytometry.

295   **Flow cytometry and cell sorting**

296   Immune cells were isolated from whole kidney marrow (WKM) and intraperitoneal exudate (IPEX) in

297   L-15 (Thermo Fisher Scientific) + 5% FCS as previously described[11]. In brief, an incision was made on

298   the ventral side of the fish and the peritoneal cavity washed with 100 μl L-15 + 5% FCS to collect IPEX

299   cells. This process was repeated ~3-4 times to collect a sufficient number of cells. Next, the inner

300   organs were removed and WKM isolated as previously described[16]. Single cell suspensions were made

301   using 100 μm and 40 μm cell strainers (VWR). The single cell suspensions were centrifuged at 300xg

302   for 5 min at room temperature (RT) and resuspended in 1 ml 1X PBS + 5% FCS. Cells were stained with

303   DAPI (Thermo Fisher Scientific) for 15 min at RT before flow cytometric analysis. Flow cytometry was

304   performed on a BD FACS Canto II (Becton Dickinson) and cell sorting was performed on a Sony MA900

305   cell sorter (Sony). Data were analyzed with FlowJo version 10.6.2.

306   **Cytology**

307   Cytospin preparations were carried out using a Cytospin3 (Shandon) centrifuge. Sorted eosinophils

308   were resuspended in 150 μl of 1X PBS with 5% FCS. Cytospin chambers were pre-wetted with 100 μl

309   of 5% FCS-PBS. The cells were then centrifuged at 800 rpm for 3 min at low acceleration. Slides were

310   air-dried overnight at RT before fixation with 4% paraformaldehyde (PFA) in PBS and subsequent

311   staining.

312   **Zebrafish sectioning**

313   Zebrafish were euthanized by hypothermic shock in ice cold water. Subsequently, the fish were fixed

314   in 4% PFA solution for 2 days at RT, decalcified in 0.35 M EDTA solution for 7 days and paraffin

17

315 embedded as previously described[17]. Paraffin embedded zebrafish were sectioned into 5 µm thin

316 sections using a Microm HM355S Rotary Microtome (Thermo Fisher Scientific), floated onto polysine

317 slides (VWR) and dried at 37°C overnight.

Histological staining

319 Paraffin sections were deparaffinized in two changes of 100% xylene for 10 min each and then

320 rehydrated in two changes of 100% EtOH, two changes of 95% EtOH and one change each of 70% EtOH

321 and 50% EtOH. Sections were washed with deionized water.

322 Histological staining was carried out according to the manufacturer's protocols (Sigma-Aldrich).

323 Samples were mounted using DPX new mounting media (Sigma-Aldrich).

Immunofluorescent (IF) Staining

325 Single cell suspensions were obtained as described in the "Flow cytometry and cell sorting" section.

326 Tissue sections were obtained as described under "Zebrafish sectioning" and deparaffinized as

327 described in "Histological staining". The *M. marinum* infected, paraffin-embedded zebrafish were a gift

328 from David M. Tobin.

329 Single cell suspensions were fixed with 4% PFA in PBS in a 96 well plate for 15 min at RT. After washing

330 3x with 1X PBS, cells were permeabilized with 0.1% (v/v) Triton-X/PBS for 15 min at. Blocking was done

331 with 3% goat serum/PBS for 1 h at RT and subsequent primary antibody (Table S1) staining was done

332 in 3% goat serum/PBS overnight at 4°C. Cells were washed 3x with 1X PBS and stained with secondary

333 antibody (Table S1) solution in 3% goat serum/PBS for 1 h at RT. Cells were washed again 3x with 1X

334 PBS and counterstained with DAPI (0.2 µg/mL). After a final wash with 1X PBS, cells were transferred

335 to 50% (v/v) glycerol in 20 mM TRIS mounting media for subsequent imaging.

336 For histology slides, antigen retrieval was performed by incubating deparaffinized slide in antigen

337 retrieval solution of 10 mM Tris/1 mM EDTA, pH 9.0 followed by heat treatment in a pressure cooker

338 (Krups) for 10 min. After cooling down to RT in antigen retrieval buffer, slides were blocked with 3%

18

339   goat serum/PBS for min. 30 min and subsequently stained as described above. Selected samples were

340   additionally treated with Vector TrueVIEW Autofluorescence Quenching Kit (Vector Laboratories)

341   according to the manufacturer's protocol and mounted using the hard-curing mounting medium

342   provided with the kit.

343   **Microscopy**

344   Color images of classical histological staining were acquired using a Leica DMR microscope and ProgRes

345   SpeedX core3 camera (Jenoptik) or AxioScan Z1 (Zeiss).

346   Fluorescence images were taken on a Nikon ECLIPSE Ti2 microscope (Nikon) with a Dragonfly 200

347   confocal system (Oxford Instruments) and 10X, 20X or 40X objectives (Nikon). Image files were

348   processed with Fiji ImageJ[18] and Zeiss Zen Blue (Zeiss).

349   **Cryo-Immunogold Electron Microscopy (EM)**

350   Embp-tdTomato$^+$ eosinophils were isolated and sorted as described above. Cells were fixed with 4%

351   PFA and 0.05% GA in PBS. The cells were harvested, gelatin-embedded and infiltrated with 2.3 M

352   Sucrose according to the method described[19]. Ultrathin sections were cut at -110°C with a RMC

353   MTX/CRX cryo-ultramicrotome (Boeckeler Instruments Inc., Tucson AZ, USA) transferred to carbon-

354   and pioloform-coated EM-grids and blocked with 0.3% BSA, 6 mM Glycin, 3% CWFG in PBS. The

355   sections were incubated with appropriate dilutions in the same buffer of rabbit polyclonal antibody

356   directed against Embp (Table S1) and chicken monoclonal antibody against mCherry (Table S1).

357   Secondary antibody-incubations were carried out with goat-anti-rabbit or anti-chicken antibodies

358   coupled to 12 or 6 nm gold particles, respectively (Jackson Immuno Research, West Grove, PA, USA).

359   Specimens were then contrasted and embedded with uranyl-acetate/methyl-cellulose following the

360   method described before[20] and analyzed in a Leo 912AB transmission electron microscope (Zeiss,

361   Oberkochen, Germany) at 120 kV acceleration voltage. Micrograph-mosaics were scanned using a

362   bottom mount Cantega digital camera (SIS, Münster, Germany) with ImageSP software from TRS

363   (Tröndle, Moorenweis, Germany). Images were processed with FigureJ[21] in Fiji ImageJ.

Statistics

All experiments were repeated at least three times independently. Group sizes and statistical tests used are indicated in the figures and figure legends. Statistical tests were performed and visualized using Prism 9 (GraphPad).

Authorship Contributions

MH designed research, performed research, collected data, analyzed and interpreted data, performed statistical analysis, wrote the manuscript. CG performed research, collected data, analyzed and interpreted data. VB performed research, collected data, analyzed and interpreted data. CD performed research and collected data. MRC designed research, performed research, collected data, analyzed and interpreted data, wrote the manuscript and supervision.

Conflict of Interest Disclosures

The authors have no conflicts of interests to declare.

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