von Willebrand factor D and EGF domains is an evolutionarily conserved and required feature of blastemas capable of multitissue appendage regeneration

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20   **Abstract**
21   Regenerative ability varies tremendously across species. A common feature of regeneration of

22   appendages such as limbs, fins, antlers, and tails is the formation of a blastema--a transient

23   structure that houses a pool of progenitor cells that regenerate the missing tissue. We have

24   identified the expression of *von Willebrand Factor D and EGF Domains* (*vwde*) as a common

25   feature of blastemas capable of regenerating limbs and fins in a variety of highly regenerative

26   species. Further, *vwde* expression is tightly linked to the ability to regenerate appendages.

27   Functional experiments demonstrate a requirement for *vwde* in regeneration and indicate that

28   Vwde is a potent mitogen in the blastema. These data identify a key role for *vwde* in regenerating

29   blastemas and underscore the power of an evolutionarily-informed approach for identifying

30   conserved genetic components of regeneration.

Introduction

31

32   The underlying reasons why some animals have the ability to regenerate complex

33   structures, while others cannot, remains an important and open question. This knowledge gap has

34   led to intense study of how regeneration-competent species are able to perform complex multi-

35   tissue regeneration, with a particular focus on the ability to regenerate paired appendages, such

36   as limbs and fins. However, this has long been a pursuit without an understanding of whether this

37   ability was present when paired appendages first evolved or was acquired by certain

38   phylogenetic lineages (e.g. urodele amphibians).

39   Recent work regarding the evolutionary origins of regenerative capacity has indicated

40   that the ability to regenerate paired appendages is an inherited feature of the fin-to-limb

41   transition [1–4]. Evidence found in the fossil record [3,4], functional studies across species [2],

42   and comparisons of gene expression profiles of regenerating tissue [1,2] support the notion that

43   paired appendage regeneration is a feature lost by certain lineages and was not a newly derived

44   capacity in highly regenerative lineages. This indicates that the amniote lineage (which includes

45   humans) has lost regenerative tendencies in appendages over evolutionary time. Therefore, the

46   ability to stimulate regeneration in non-regenerative species, potentially in a therapeutic context,

47   may require the re-initiation of a core, evolutionary conserved program.

48   All species that are able to regenerate appendages share a conserved trait: the ability to

49   form a blastema. The blastema is the morphological structure that forms at the amputation plane

50   and houses the progenitor cells responsible for regeneration. Recent efforts have focused on

51   elucidating the molecular definition of the blastema, with many of these efforts aimed at the

52   axolotl limb blastema due to the ease of tissue acquisition and the ability to perform

53   experimentation in the lab [5–13]. These studies provide a wealth of information about

54   transcriptomic changes over time, cell types, and blastema-enriched genes. More recently,

55   sequencing efforts of non-model species have allowed for comparisons to the axolotl limb

56   blastema and indicate a core molecular signature that is shared between the blastemas of

57   distantly related species [1,2]. Due to these similarities and the common evolutionary origin of

58   limb regeneration capacity, we can use an evolutionarily-informed approach to understand what

59   constitutes a blastema and for identifying core features required for regeneration.

60   A recent approach to identify the unique gene expression in the axolotl limb blastema

61   compared blastema gene expression to a variety of homeostatic and embryonic tissues and

62   identified over 150 blastema-enriched genes [11]. These blastema-enriched genes may help to

63   explain the unique functions of the blastema, but the question remains as to whether these genes

64   represent a core program or are functionally required for regeneration. One of the most blastema-

65   enriched genes in this dataset was *von Willebrand Factor D and EGF Domains* (*vwde*), which to

66   date has not been functionally studied in any context.

67   We decided to apply an evolutionary framework to determine if *vwde* fit the description

68   of an evolutionary-conserved, blastema-enriched gene and if such an approach may help to

69   identify genes required for regeneration. We found that *vwde* expression is a common feature of

70   both fin and limb blastemas and was highly enriched in regenerating appendages as compared to

71   pre-amputation intact appendages. In addition, using the natural regeneration-competent and

72   regeneration-refractory periods during *Xenopus laevis* development, we observed that *vwde*

73   expression was tightly linked to the regeneration-component environment. This suggests that

74   *vwde* may be a critical factor in the regenerative niche. Finally, we found that *vwde* is

75   functionally required for axolotl limb regeneration, with transient knockdown of protein levels

76   resulting in aberrant regeneration. These data suggest that an evolutionarily-informed approach

77   can help to prioritize target genes and that genes that are blastema-enriched across different

78   species may prove to be critical factors in the ability to regenerate appendages.

79

80

81   **Results**

82   With the goal of identifying genes enriched to the regenerating blastema, a tissue-mapped

83   axolotl transcriptome was recently published [11]. Of particular interest are blastema-enriched

84   genes with high expression in the blastema and relative low expression in all other tissues

85   sampled. We found that *vwde* was highly enriched to the axolotl limb blastema (Figure 1A). This

86   analysis, however, was limited to one time point, the medium-bud blastema, and it did not

87   provide spatial information about the expression of *vwde* across the regenerating limb. To

88   understand the spatial and temporal regulation of *vwde*, we performed RNA *in situ*

89   hybridizations over a time course of axolotl limb regeneration. We found *vwde* expression to be

90   tightly tied to the presence of a blastema and expressed exclusively in the blastema and not the

91   overlying wound epidermis   (Figure 1B-E). Thus, *vwde* fits the description of an axolotl limb

92   blastema-enriched gene and we were interested in pursuing whether *vwde* may be a core

93   component of blastemas able to regenerate appendages.

94   We next sought to determine if *vwde* was present in a selection of deuterostomes,

95   including species with various regenerative abilities. Using a comparative genomics approach

96   [14], we found *vwde* to have orthologs across deuterostomes (though no ortholog was detected in

97   *Ciona intestinalis*), as well as a non-blastema-enriched paralogous gene in axolotl and other

98   species (Figure 1F, Supplementary Figure 1). We compared axolotl VWDE to proteins from

99   other species, and we found putative orthologs harboring predicted von Willebrand Factor D

100   domains and EGF-like domains. The number of EGF domains may be more variable across

101   species. However, since this gene has not yet been studied in-depth in any species, additional

102   experimental work may be required to fully characterize the expressed transcripts and proteins

103   for individual species. Using these identified orthologs, we moved forward to ask whether *vwde*

104   was a blastema-enriched gene during paired fin regeneration.

105   We explored the possibility that *vwde* could be a common feature of blastemas

106   responsible for regenerating paired fins, which share a deep homology with limbs [15], and

107   likely share an inherited gene regulatory program for regeneration [1,2]. We chose two highly

108   regenerative, but distantly related, fish species to determine if *vwde* expression was a conserved

109   feature of blastemas capable of regenerating paired appendages. These include a species in the

110   sister group to tetrapods, the Lungfish (*Lepidosiren paradoxa*), which is a lobe-finned fish, and

111   *Polypterus senegalus*, a ray-finned fish that is capable of regenerating after amputation through

112   skeletal elements that develop by endochondral ossification. We first inspected publicly available

113   transcriptome datasets of lungfish and *Polypterus* regenerating fins for the *vwde* orthologs we

114   previously identified (Figure 1F). The lungfish LG29893_g1_i1 contig was upregulated in

115   blastemas 21 days post-amputation (dpa) relative to uninjured fins [1], and the *Polypterus*

116   PS64836c0_g1_i1 contig was upregulated in 9 dpa blastemas relative to uninjured fins [2].

117   Assessment of expression levels via qPCR at various regeneration stages showed an upregulation

118   of *vwde* coinciding with blastema formation during lungfish fin regeneration (Supplemental

119   Figure 2A).

120   A similar pattern was seen for *Polypterus* fin regeneration, with expression reaching

121   highest levels at 5 dpa (Supplemental Figure 2B). Next, we assessed the spatial pattern of *vwde*

122   in histological sections of regenerating fins. Lungfish 21 dpa blastemas show distal

123   mesenchymal expression of *vwde* (Figure 2A). In 5 dpa *Polypterus* blastemas, expression is

124   observed distal to the amputation plane in mesenchymal cells but also in the epithelium,

125   suggesting that *Polpyterus* may use *vwde* in both compartments (Figure 2C). *In situ* hybridization

126   with control sense probes did not yield specific signal (Supplemental Figure 2C-D).

127   Histologically, these samples are similar to the medium-bud blastema time point in which we

128   identified *vwde* in the axolotl limb (Figure 2B, 2D). Together, these data indicate that *vwde* is

129   expressed in regenerating fins and limbs and that *vwde* expression is a conserved feature of

130   blastemas.

131   To further investigate *vwde* during regeneration, we took advantage of the regeneration-

132   competent and regeneration-refractory periods during *Xenopus laevis* tail development [16]. A

133   blastema forms in response to amputation during both distinct developmental stages, but only in

134   the regeneration-competent setting is full regeneration accomplished. This developmental feature

135   provides an ideal situation to compare regeneration-component versus regeneration-refractory

136   environments. We reasoned that finding factors that differentiate these two contexts may provide

137   clues for identifying the core requirements for successful regeneration. We probed for the

138   expression of *vwde* during the regeneration-competent and regeneration-refractory periods of

139   *Xenopus laevis* tail regeneration. Interestingly, we found that *vwde* expression was present in

140   tails prior to amputation in both the regeneration-competent and regeneration-refractory setting

141   (Figure 3A-B, 3G-H). We found robust *vwde* expression along the peripheral edge of the

142   amputation plane and near the blastema in regeneration-competent tails (Figure 3C-F). In

143   contrast, in the regeneration-refractory setting, *vwde* expression was restricted to the peripheral

144   edges of the amputation plane and was not detected near the blastema (Figure 3I-L). This

145   indicated a striking correlation between *vwde* expression and regeneration, providing evidence

146   that *vwde* may be an important factor in forming a pro-regenerative niche. These expression data

147   across a range of species indicate that *vwde* fits the profile of an evolutionarily-conserved,

148   regeneration-enriched gene and that *vwde* may play an important role in the blastema niche.

149   To investigate if *vwde* is required for regeneration, we performed morpholino-mediated

150   knockdown at its peak expression in the medium-bud limb blastema. We found a substantial

151   reduction in the length of the blastemas when Vwde was knocked down with two separate

152   translation-blocking morpholinos (Figure 4A-B). Fluorescent reporter constructs with *vwde*-

153   morpholino binding sites confirmed that both unique *vwde*-targeting morpholinos were capable

154   of blocking translation (Supplemental Figure 3). Due to the dramatic reduction in blastema

155   length, we investigated if *vwde* was important for blastema proliferation and/or cell survival. We

156   found that knockdown of Vwde substantially reduced blastema cell cycle entry (Figure 4C-D)

157   and did not alter cell survival compared to control limbs (Supplemental Figure 4). Due to the

158   observed delay in blastema growth, we questioned whether blastemas treated with translation-

159   blocking morpholinos were capable of recovering from the transient knockdown of Vwde and

160   produce fully regenerated limbs. We therefore performed the same Vwde morpholino-mediated

161   knockdown on a separate group of axolotls, and then allowed for the full course of regeneration

162   to complete, harvesting limbs more than eight weeks post-amputation. We observed that one-

163   time injection of Vwde-targeting morpholino caused substantial abnormalities in regenerated

164   limbs, suggesting an essential role for *vwde* during limb regeneration (Figure 4E-G). We found

165   defects in 4.2% (1/24) control limbs compared to 46% (13/28) of limbs treated with *vwde* MO1

166   and 25% (5/20) of limbs treated with *vwde* MO2 (Fisher's exact test $P < 0.05$) (Figure 4F, Table

167   1, Supplemental Figure 5). A second experiment yielded similar results, with defects at endpoint

168   in 27.6% (8/29) of control (*vwde* MO1 inverted) treated limbs compared to 47% (18/38) of limbs

169   treated with *vwde* MO1 (Fisher's exact test P < 0.05) (Figure 4G, Table 2, Supplemental Figure

170   6). We found a variety of defects, some of which are reminiscent of limb development

171   phenotypes where limited distal elements are present such as has been observed in *fgf4,8*-double-

172   knockout mice [17] and in the absence of *sonic hedgehog* (*ssh*) [18]. In addition, these

173   phenotypes also resemble the defective regenerative spike characteristic of *Xenopus* limb

174   regeneration [19]. Altogether, these data highlight the functional requirement for *vwde* during

175   limb regeneration.

176
177   **Discussion**

178   Recent work, most notably next generation sequencing, has led to a plethora of

179   information about the genes and cells that define the blastema [1,2,5–13]. However, it is difficult

180   to determine which genes may have functional relevance based purely on their expression. We

181   decided to investigate a single blastema-enriched gene, *vwde*, using an evolutionarily-informed

182   approach, assuming that a gene whose expression is enriched in blastemas of multiple, distantly-

183   related, species is likely a key factor during regeneration.

184   The *in vivo* assays used here place Vwde as a critical regulator of cell cycle entry during

185   axolotl limb regeneration. Proliferation is a complex, but fundamental, aspect of regeneration, as

186   there are many different cell types and potential origins of proliferative signals. Previous work

187   indicates that mitogenic signals are produced directly following amputation independent of the

188   nerve or wound epidermis [20,21], but are also provided by the nerve [22,23] or wound

189   epidermis [24]. There are thus multiple sources of mitogenic signals in the regenerating limb, but

190   it is unclear if mitogenic signals from multiple tissues are required simultaneously or perhaps in

191   a more stepwise fashion to maintain blastema proliferation. Our data indicate that Vwde may be

192   a blastema progenitor cell-derived mitogen, which adds to the potential sources of proliferative

193   signals in the regenerating limb. It has been previously postulated that nerve-derived signals are

194   required early on during blastema formation and growth, but a fibroblast-derived factor is

195   required for complete regeneration [25]. We speculate that *vwde*, which appears to be expressed

196   across the majority of cells in the blastema including likely fibroblasts, may provide one of the

197   essential fibroblast-derived factors required after the nerve has provided sufficient input. While

198   there is limited knowledge of fibroblast- or blastema cell-derived mitogens, *in vitro* cultures have

199   shown that blastema protein extracts are able to drive blastema cell proliferation [26]. More

200   generally, a global and temporally-based view of the cellular origins of mitogens and the cell

201   types that require these mitogens will provide a better understanding of what is driving

202   proliferation during different stages of regeneration.

203   In addition to the dramatic reduction in proliferation, we observed striking end point

204   phenotypes after transient knockdown of Vwde. The loss of distal elements and spike-like

205   phenotypes observed after Vwde knockdown suggest that Vwde plays a role in proximal-distal

206   determination in the regenerating limb. These phenotypes showing similarities to *ssh* and *fgf4,8*-

207   double-knockout mice, suggest that Vwde may be working similarly to—or in concert with—

208   FGFs during regeneration. Though many FGFs are epidermal factors during limb development in

209   mice and chick, FGFs are expressed in the mesenchyme during axolotl limb development [27]

210   and regeneration [28]. Thus, it may be that during axolotl limb regeneration, blastema-derived

211   factors are primarily responsible for proximal-distal patterning and that Vwde is working to

212   promote the formation of distal elements. Intriguingly, *vwde* has remained unexplored in highly

213   studied, but less regenerative species such as mouse and human, so whether *vwde* plays a role in

214   limb development in these species is unknown.

215   It is interesting to speculate on what has been lost in amniotes that prevents appendage

216   regeneration. One possibility is genes that are lost in amniotes and present in anamniotes can

217   explain differences in regenerative capacity [29]. However, the absence of a gene in amniotes is

218   not necessarily a prerequisite when considering which candidate genes might be responsible for

219   high regenerative capacity. Alternative scenarios include, but are not limited to, genes that have

220   lost ancestral pro-regenerative function or have altered expression domains/kinetics. *Vwde* may

221   fit the paradigm of a gene that is present in both regeneration-competent and regeneration-

222   incompetent species, but may exclusively be used in the blastema, a structure that cannot be

223   produced by most regeneration-incompetent species.

224   While the blastema is required for regeneration, wound healing and activation of

225   progenitor cells required for formation of the blastema must precede blastema formation. Based

226   on the expression profile, we do not expect *vwde* to be a driver of blastema formation, but more

227   likely a downstream effector once a blastema has been established. In most cases of amputation

228   in less regenerative species, the blastema is not able to form, and thus we suspect that a more

229   upstream or systemic factor may prevent blastema formation. While there may have initially

230   been one primary cause of the loss of regenerative ability, such as the rise of adaptive immunity

231   [30] or trade-offs associated with endothermy [31], it is likely that other aspects of the

232   regenerative response have now been lost due to their lack of utility. If *vwde* played a relatively

233   specialized function in the blastema and blastemas generally do not exist in less regenerative

234   species then the use for *vwde* decreases. This could explain why 42.7% of human genomes have

235   a predicted loss-of-function copy of *VWDE*, leading to speculation that *VWDE* is potentially

236   drifting towards inactivation in the human population [32]. While the blastema remains the

237   elusive feature required for appendage regeneration, this work illustrates that taking an

238   evolutionarily-informed approach can lead to identification of functionally important genes. This

239   also suggests that further work to understand the similarities between different species blastemas

240   may help to elucidate the core molecular program of the blastema.

241   **Methods**
242
243   **Animal Experimentation**
244   All axolotl experiments were performed in accordance with Brigham and Women's Hospital

245   Institutional Animal Care and Use Committee in line with Animal Experimentation Protocol

246   #04160. All animals were bred in house, but the colony was originally derived from animals

247   obtained from Ambystoma Genetic Stock Center (Lexington, KY, NIH grant P40-OD019794).

248   For amputations, animals were narcotized in 0.1% MS-222, confirmed to be fully narcotized by

249   pinch test, amputated mid-zeugopod and the bone was trimmed. Animals were allowed to

250   recover overnight in 0.5% sulfamerazine. For all functional experiments, all four limbs were

251   amputated and injected individually. Functional experiments were performed on animals ranging

252   from 3.8-8cm.

253   *Polypterus senegalus* and *Lepidosiren paradoxa* were maintained in individual tanks in a

254   recirculating freshwater system. Animals were anesthetized before amputations: *P. senegalus* in

255   0.1% MS-222 (Sigma) and *L. paradoxa* in 0.1% clove oil diluted in the system water. Experiments

256   and animal care were performed following animal care guidelines approved by the Animal Care

257   Committee at the Universidade Federal do Para (protocol no. 037-2015). Pectoral fins in both

258   species were bilaterally amputated. For *L. paradoxa* fins were amputated at approximately 1 cm

259   distance from the body, and for *P. senegalus*, fins were amputated across the fin endoskeleton.

260   Amputated fins (regenerating and uninjured) were used for histology, *in situ* hybridization and

261   qRT-PCR analysis.

262   **Electroporation**
263   Electroporation was performed while axolotls were narcotized in 0.1% tricaine and subsequently

264   immersed in ice cold 1x PBS using a NepaGene Super Electroporator NEPA21 Type II

265   electroporator. Settings for electroporation included: 3 poring pulses at 150 Volts with a pulse

266   length of 5 milliseconds, a pulse interval of 10 milliseconds, a decay rate of 0 %, and a positive

267   (+) polarity. Transfer pulse consisted of 5 pulses at 50 Volts with a pulse length of 50 milliseconds,

268   a pulse interval of 950 milliseconds, a decay rate of 0 %, and a positive (+) polarity.

269   **qRT-PCR**.

270   Total RNA from regenerating or uninjured pectoral fins was extracted using TRIzol reagent

271   (Thermo Fisher Scientific). Residual DNA removal and RNA cleanup were performed following

272 the RNeasy Mini Kit (Qiagen) protocol. cDNA was synthesized from 0.5 µg RNA using the

273 Superscript III First-Strand Synthesis Supermix (Thermo Fisher Scientific) with oligo-dT. For

274 qPCR, amplification reactions (10 µl) prepared with the GoTaq Probe qPCR Master Mix

275 (Promega) were run in a StepOnePlus Real-Time PCR System (Applied Biosystems). Gene-

276 specific oligos (Table 3) for qRT-PCR assays were designed using Primer 3.0

277 (http://bioinfo.ut.ee/primer3/) and used in a final concentration of 200 nM to each primer. Each

278 qPCR determination was performed with three biological and three technical replicates. Relative

279 mRNA expressions were calculated with the $2^{-\Delta\Delta CT}$ method [33], using *sdha* (*P. senegalus*) or

280 *polrc1* (*L. paradoxa*) genes as endogenous control and the uninjured fin (mean ΔCT value of the

281 three biological replicates) as reference sample.

In Situ Hybridization

283 For *in situ* hybridization using axolotl samples a gene fragment from the 3' UTR was amplified

284 from blastema cDNA and cloned into the pGEM-T Easy vector and sequenced. Depending upon

285 orientation, T7 or Sp6 polymerase was used to transcribe the probe. Primers for *in situ* probes

286 against axolotl *vwde* (contig c1084387_g3_i1 from [11]) can be found in Table 3. Colorimetric

287 in situ hybridization in axolotl tissue harvested from animals with snout to tail lengths of 9.5-

288 11.5cm and was performed as previously described at protocols.io

289 (https://www.protocols.io/view/rna-in-situ-hybridization-p33dqqn).

290 For *in situ* hybridizations with fish samples, fins of *P. senegalus* (5 dpa and uninjured) and *L.*

291 *paradoxa* (21 dpa and uninjured) were amputated, embedded in TissueTek O.C.T compound

292 (Fisher Scientific), and maintained at −80°C until use. Frozen sections of 20 µm were obtained on

293 a Leica CM1850 UV cryostat, positioned on slides (Color Frost Plus/Thermo Fisher Scientific)

294 and fixed as previously described [1]. Riboprobe templates containing a gene-specific segment

295 (400-500 bp) and a T7 promoter sequence were produced by a 2-round PCR strategy (primers are

296 listed in Table 3). Riboprobes were synthesized with T7 RNA polymerase (Roche) and DIG-

297 labeling mix (Roche). Controls probes (sense riboprobes) were synthesized from a template

298 containing the T7 promoter in a reverse orientation. A total of 300 ng of DIG-labeled riboprobe

299 was used per slide during *in situ hybridization* performed as previously described [1]. Images were

300 obtained on a Nikon Eclipse 80i microscope and processed using the NIS-Element D4.10.1

301 program.

302   **Whole mount RNA-FISH**

303   *Xenopus laevis* eggs were obtained, fertilized, and cultured as embryos at 18 °C using standard

304   methods as in [34]. All experimental procedures using *Xenopus laevis* were approved by the

305   Institutional Animal Care and Use Committee (IACUC) and Tufts University Department of

306   Laboratory Animal Medicine (DLAM) under protocol number M2017-53. Once embryos reached

307   regeneration-competent (Stage 40) or regeneration-incompetent (Stage 46) stages, animals were

308   anesthetized using 0.005% MS222 in 0.1X MMR and tails were amputated at the posterior third

309   of the tail and allowed to regenerate for 24 hours. Embryos at both stages, which had not been

310   amputated, were also collected as intact controls. Regenerating and intact control embryos were

311   anesthetized in 0.005% MS222 and then fixed at 4℃, rocking overnight, in either 4%

312   paraformaldehyde in 1X DEPC PBS or MEMPA buffer (0.1 M MOPS (pH 7.4), 2 mM EGTA, 1

313   mM MgSO4, 3.7% paraformaldehyde). We used a slightly modified whole-mount mouse protocol

314   [35] using hybridization chain reaction v3.0 [36] with slight modifications. After overnight

315   incubation, embryos were washed 3 times for 5 min in PBST and then taken through a methanol

316   series on ice. This series consisted of 10 min washes on ice in ice cold 25%MeOH/75% PBST,

317   50%MeOH/50% PBST, 75%MeOH/25%PBST, 100%MeOH, and then finally stored in a fresh

318   100%MeOH solution. Dehydrated embryos were then stored at -20℃ until use. For *in situ*,

319   embryos were subsequently rehydrated via a reverse methanols series, on ice, with 10 min washes

320   of 75% MeOH/25% PBST, 50% MeOH/50% PBST, 25% MeOH/75% PBST, 100% PBST, and

321   another final wash in 100% PBST. Embryos were then digested with proteinase K (10μg/mL) in

322   DEPC PBS for 5 minutes at room temperature. Post-fixation was then performed in 4% PFA in

323   1X DEPC PBS for 20 minutes at room temperature. Next, three five minutes washes with PBST

324   at room temperature was followed by 5 minutes at 37℃ in hybridization solution (50% formamide,

325   5x sodium chloride sodium citrate (SSC), 9 mM citric acid (pH 6.0), 0.1% Tween-20, 50μg/mL

326   heparin, 1x Denhardt's solution, and 20% dextran sulfate). Samples were pre-hybridized by full

327   immersion in hybridization solution without probes for 30 min at 37℃. Hybridization was

328   performed overnight at 37℃ with samples immersed in hybridization solution containing twenty

329   probe pairs against *vwde.L* (XM_018267342.1) diluted at 1:200 of 1 μM (hybridization chain

330   reaction v3.0 RNA fluorescent in situ probes were ordered from Molecular Instruments

331   (https://www.molecularinstruments.com/). The following day, samples were washed four times at

332   37℃ in probe wash buffer (50% formamide, 5X SSC, 9 mM citric acid (pH 6.0), 0.1% Tween-20,

333 and 50μg/mL heparin). Samples were then washed two times in 5X SSC at room temperature. Pre-

334 amplification was then performed at room temperature for 30 minutes in amplification buffer (5X

335 SSC, 0.1% Tween-20, 10% dextran sulfate). During pre-amplification, hairpin probes (ordered

336 from https://www.molecularinstruments.com/) compatible with *vwde.L* probe pairs were heated

337 individually at 95℃ for 30 seconds and then snap cooled for 30 min at room temperature in the

338 dark. After 30 minutes, probe pairs were added to amplification buffer at 1:50 (3 µM stock) and

339 this probe containing buffer was subsequently added to samples, ensuring that samples were fully

340 immersed. Incubation was performed overnight at room temperature. The next day, samples were

341 washed for 5 min in 5X SSCT, twice for 30 min in 5X SSCT, and a 5 min wash in 5X SSCT.

342 Samples were then stained with DAPI for 5 min in 1X PBS, washed for 5 min in 1X PBS, and then

343 stored in 1X PBS. Samples were then mounted in low melt agarose and imaged on a Zeiss LSM

344 880 Upright. A median 3x3 filter followed by maximum projection was applied to all images.

345 **Morpholino design and administration**

346 Morpholinos were designed and synthesized by GeneTools. Morpholino sequences can be found

347 in Table 3. About 1.25 µl of morpholino was injected in the blastema and electroporation was

348 performed as described in Electroporation. All morpholinos were 3' fluorescein conjugated to

349 allow for visualization. Morpholinos were reconstituted to 1 mM in 2X PBS and diluted to a

350 working concentration of 500 μM in 1X PBS prior to injection.

351
352
353 **EdU staining**

354 Stock solutions of 5-ethynyl-2'-deoxyuridine (EdU) dissolved in dimethyl sulfoxide were

355 prepared per manufacturer's instructions (Thermo Fisher). Axolotls (3-6cm tail to snout) were

356 narcotized in 0.1% tricaine at 7 days post amputation and control or Vwde-targeting morpholino

357 was injected and subsequently electroporated as described in Electroporation section of methods

358 into the blastema. At 9 dpa, intraperitoneal injections with 400 µM EdU in 0.7X PBS at a

359 volume of 20µL/g were performed. 18 hours later blastemas were harvested, fixed for 1-2h in

360 4% PFA and then taken through a sucrose gradient to 30% sucrose in 1x PBS. Tissue was then

361 embedded in OCT and frozen in a dry ice/ethanol bath. Sections were cut at 16 µm with a

362 cryostat, collected on Superfrost Plus slides (Fisher), and stored at -80℃. EdU staining was

363   performed with the Click-iT EdU Alexa Fluor 594 Imaging Kit per manufacturers instructions

364   (Thermo Fisher).

365

366   **TUNEL assay**

367   TUNEL assays were performed as previously described [11]

368

369   **Skeletal preparations and scoring**

370   Limbs were stained with Alcian blue/Alizarin red according to [37]. In brief, limbs were

371   incubated with rocking overnight in 95% ethanol and then rocking overnight an acetone. Limbs

372   were then incubated for at least 7 days in alcian blue/alizarin red at 37 °C. Limbs were then

373   cleared by incubation in 1% (wt/vol) KOH, followed by 1% (vol/vol) KOH/25% glycerol, 1%

374   KOH/50% glycerol, and 1% KOH/75% glycerol. Limbs were imaged in 1% KOH/75% glycerol.

375   Alcian blue stock was 0.3% alcian blue in 70% ethanol; alizarin red stock was 0.1% alizarin red

376   95% ethanol; the working solution was 5% alcian blue stock/5% alizarin red stock/5% glacial

377   acetic acid/volume in 70% ethanol.

378   Definitions for limbs after regeneration. Normal: All digits and carpals present, zeugopod and

379   stylopod intact. Spike: Single outgrowth from amputation plane without obvious turn at joint. Loss

380   of distal elements: Distal elements without obvious autopod. Oligodactyly: Loss or reduction in

381   size at least one digit. Syndactyly: Fusion of digits. Additional elements: Extra bones in stylopod

382   or zeugopod. For statistical analysis normal was compared to all of the above listed abnormalities.

383   **Ortholog analysis**

384   The following proteomes were downloaded from uniprot.org, human (*Homo sapiens,*

385   UP000005640, accessed 5/18/2019), zebrafish (*Danio rerio*, UP000000437, accessed

386   5/18/2019), mouse (*Mus musculus*, accessed 5/18/2019), amphioxus (*Branchiostoma floridae*,

387   accessed 8/27/2019), chick (*Gallus gallus*, accessed 8/27/2019), sea squirt (*Ciona intestinalis*,

388   accessed 8/27/2019), lamprey (*Petromyzon marinus*, accessed 8/27/2019), green anole (*Anolis*

389   *carolinensis*, 8/27/2019), frog (*Xenopus laevis*, UP000186698, accessed 7/11/2019), frog

390   (*Xenopus tropicalis*, UP000008143, accessed 7/11/2019). The South American lungfish

391   transcriptome was downloaded from https://www.ncbi.nlm.nih.gov/Traces/wgs/?val=GEHZ01

392   and converted to a putative reference protein using TransDecoder (version 5.3.0) like so:

393   `TransDecoder.LongOrfs -t`. The *Polpyterus* transcriptome can be found here:

394   https://www.ncbi.nlm.nih.gov/bioproject/480698 and converted with TransDecoder as referenced

395   above. The axolotl (*Ambystoma mexicanum*) predicted proteome was obtained from

396   https://data.broadinstitute.org/Trinity/SalamanderWeb/Axolotl.Trinity.CellReports2017.transdec

397   oder.pep.gz [11]. Cloning of axolotl *vwde* revealed a sequencing error in the axolotl

398   transcriptome which eliminated the first ~500bp of the sequence. We manually changed the

399   axolotl proteome to include this corrected version of *vwde* (Supplementary File 1).

400   To predict orthologs, we used OrthoFinder2.0 (version 2.3.3) [14]. Orthofinder was implemented

401   as follows:

402   `orthofinder -f /path/to/proteomes -M msa -A mafft  -T fasttree -t 20 -o /path/to/output/directory`

403   **Protein domain diagrams**

404   The R package, drawProteins [38] was used to draw protein domains for different species Vwde.

405   For all genes contained within Uniprot, these were downloaded directly with drawProteins. For

406   genes not available via Uniprot (https://www.uniprot.org/)[39] (e.g. *Polpyterus*, axolotl, and

407   Lungfish), the amino acid sequence of the protein was queried via Interpro with default settings

408   (https://www.ebi.ac.uk/interpro/)[40] and positions and domain annotations were extracted and

409   made into a matrix that matched the required structure for drawProteins. The Uniprot version of

410   mouse *vwde* (Uniprot ID: Q6DFV8) in the proteome used did not co EGF-like domains, so we

411   manually searched UCSC genome broswer to confirm this lack of EGF-like domains. This

412   revealed a full length Vwde (ENSMUST00000203074.2), which was then fed into Interpro and

413   domains were manually input into drawProteins.

414

415   **Vwde knockdown confirmation**

416   Two separate constructs to test the target specificity of each MO used. GFP was removed and

417   from pCAG-GFP (pCAG-GFP was a gift from Connie Cepko (Addgene plasmid # 11150 ;

418   http://n2t.net/addgene:11150 ; RRID:Addgene_11150)[41] and replaced with vectors containing

419   td-Tomato sequence and the morpholino binding site (Supplementary File 2). To confirm

420   knockdown we co-injected and electroporated into medium-bud blastemas the generated

421   constructs and the appropriate fluorescein-conjugated morpholino. Fluorescein fluorescence was

422   used to confirm injection efficiency and td-tomato expression was used to measure ability to

423   block translation.

424

425 **Statistics**

426 Nested one way ANOVA was used to determine significance between blastema lengths. Each

427 limb was considered a technical replicate within one biological (i.e. animal) replicate. Nested t

428 tests were used to determine significance in EdU and TUNEL experiments, again treating each

429 limb as a technical replicate and placing limbs from the same animal within one biological

430 replicate. Fisher's exact tests (control vs. treated) were used to determine significance of

431 outgrowth phenotypes. Significant results were considered as $P < 0.05$.

432 **Contributions**

	NDL	SS	ACD	DPD	JFS	ANA	KJ	GSD	BJH	ML	IS	JLW
Wrote the paper	■											■
Initiation/conception	■								■			■
Corresponding author												■
Animal experiments/in situ	■	■	■	■	■	■	■	■		■		■
Orthology analysis	■											
Experimental design	■	■	■	■	■			■			■	■
Manuscript editing	■	■	■	■	■	■	■	■	■	■		
Contributed resources											■	■

433

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567   **Figure Legends**

568

569

570   **Figure 1: *von Willebrand Factor D and EGF-like Domains* (*vwde*) is a blastema-enriched**
571   **gene that is found across deuterostomes.** (A) *vwde* (contig c1084387_g3_i1) expression in
572   FPKM across tissues sampled from Bryant et al. Cell Reports 2017. Proximal and distal blastema
573   samples are combined. (B-E) RNA *in situ* hybridization for *vwde* at (B) wound healing, (C)
574   early-bud blastema, (D) medium-bud blastema, and (E) palette stage regenerating limbs. Black
575   arrows indicate *vwde* expression, scale bar is 100 μm. (F) OrthoFinder 2.0 phylogeny with
576   corresponding protein domain structure for putative Vwde orthologs. Protein domain pictures
577   were generated with drawProteins [38]. Species and Uniprot ID, transcriptome contig number, or
578   Ensembl ID are included. *Polypterus vwde* contained multiple splice isoforms and the closest
579   match to axolotl Vwde is shown here. Axolotl Vwde is denoted with (*) and other species Vwde
580   that are described in this manuscript are marked with (#). Orthologs to the Vwde studied in this
581   work are indicated with brackets, paralog is also denoted with brackets.

582

583   **Figure 2: *vwde* is enriched in the regenerating fin of Lungfish (*L. paradoxa*) and *Polypterus***
584   **(*P. senegalus*).** Expression pattern of *vwde* in the fin blastema tissues *of L. paradoxa* and *P.*
585   *senegalus*. Longitudinal histological sections of fins from *L. paradoxa* at 21 dpa (A-B), and from
586   *P. senegalus* at 5 dpa (C-D). (A and C) *In situ* hybridization using an anti-sense riboprobe to
587   *vwde*. (B and D) H&E staining on sequential sections. All panels show posterior view, dorsal to
588   the top. Dotted lines indicate amputation site (Scale bars, 1 mm in all panels).

589

590   **Figure 3: *vwde* expression is tightly linked with the regeneration-component environment.**
591   *In situ* hybridization chain reaction probing for *vwde* in (A-F) regeneration-competent *Xenopus*
592   *laevis* tails (A-B) prior to amputation, (C-D) blastema 24 hours post-amputation, and (E-F) the
593   peripheral edge of the amputation plane 24 hours post-amputation. (G-L) Regeneration-
594   refractory tails (G-H) prior to amputation, (I-J) blastema 24 hours post-amputation, and (K-L)
595   the peripheral edge of the amputation plane 24 hours post-amputation. White arrows indicate the
596   location of *vwde* expression. Scale bars are 100 μm.

597

598 **Figure 4: Vwde is essential for limb regeneration.** (A) Representative images of blastemas 16

599 days post-amputation (9 days post-morpholino administration) from control morpholino

600 (Standard control MO), *vwde*-targeting morpholino 1 (*vwde* MO1), and *vwde*-targeting

601 morpholino 2 (*vwde* MO2). Dotted line indicates amputation plane, blastemas are all tissue distal

602 to amputation plane. Scale bars at 1mm. (B) Quantification of blastema length 16 days-post

603 amputation. Median and quartiles noted with dotted lines, ** indicates P< 0.01, * is P< 0.05 by

604 nested T test. (C) Representative EdU stained sections of blastemas 10 dpa (3 days post-

605 electroporation) of control morpholino (*vwde* morpholino 1 inverted, *vwde* MO1 INV) and *vwde*-

606 targeting morpholino 1. Scale bars are 100μm (D) Quantification of percent of blastema cells

607 positive for EdU in control (*vwde* MO1 INV) and knockdown (*vwde* MO1). Each dot represents

608 a limb, 4-5 animals per group. Median and quartiles noted with dotted lines,** indicates P< 0.01

609 by nested T test. (E) Representative skeletal preparations of limbs after full regeneration after

610 knockdown of Vwde at 7 dpa. From left to right, normal forelimb, normal hindlimb, spike, and

611 loss of distal elements. Scale bars are 5 mm. (F) Donut plots of regenerative outcomes, pooled as

612 abnormal versus normal from experiment with standard control morpholino, *vwde* MO1 and

613 *vwde* MO2. Asterisk (*) indicates P< 0.05 by Fisher's exact test comparing control versus *vwde*

614 MO1 and control versus *vwde* MO2. (G) Donut plots of regenerative outcomes, pooled as

615 abnormal versus normal from experiment with *vwde* MO1 INV and *vwde* MO1. Asterisk (*)

616 indicates P< 0.05 by Fisher's exact test comparing outcomes from *vwde* MO1 INV compared to

617 *vwde* MO1.

618   Table 1. Phenotypic outcomes of morpholino-mediated knockdown in axolotl (Standard control
619   vs. *vwde* MO1, *vwde* MO2)

	Normal	Spike	Loss of distal elements	Oligodactyly	Polydactyly	Syndactyly	Additional elements
Control	23	0	0	0	0	1	0
vwde MO1	15	4	5	4	0	0	0
vwde MO2	11	0	0	3	1	1	0

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622   Table 2. Phenotypic outcomes of morpholino-mediated knockdown in axolotl (Inverted control vs.
623   *vwde* MO1)

	Normal	Spike	Loss of distal elements	Oligodactyly	Polydactyly	Syndactyly	Additional elements
Vwde MO1 INV	30	0	4	4	0	0	0
Vwde MO1	20	6	9	1	0	1	1

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634   Table 3. Oligos used for qPCR, morpholinos, and ISH probe templates.

| Primer	Application	Sequence (5'- 3')
Ax_vwde_ISH_F	Riboprobe template	TGTGGAAAGAAACTTGTGCATCA
Ax_vwde_ISH_R	Riboprobe template	TTTAATCTGAAAATGGACCAGTAGATT
vwde MO1	Anti-sense morpholino	ATATCCCATACATCCTTGCGTTGGC
vwde MO2	Anti-sense morpholino	AGAAACCATCACAGTTCCTCACAGT
vwde MO1 INV	Sense (control) morpholino	CGGTTGCGTTCCTACATACCCTATA
Standard control MO	Control morpholino	CCTCTTACCTCAGTTACAATTTATA
Ps_Vwde-qPCR_F	qPCR	AGAATTCCTGTGACTGTGCGA
Ps_Vwde-qPCR_R	qPCR	TTCTGGTGTTGTTGGTGAGGG
Ps_Vwde-ISH_F	Riboprobe template	GGCCGCGGGCATGCGGAATAATGTGTGCT
Ps_Vwde-ISH_R	Riboprobe template	CCCGGGGCAGTCCAGTCTTCAGCAGTGTG
Lp_Vwde-qPCR_F	qPCR	TTCTTCTTGGAGACCCCTGAT
Lp_Vwde-qPCR_R	qPCR	GGTCTTGCTGGCTAGTGTCAG
Lp_Vwde-ISH_F	Riboprobe template	GGCCGCGGAGCTAACAGCCTGTGCAACAT
Lp_Vwde-ISH_R	Riboprobe template	CCCGGGGCATCAGGGGTCTCCAAGAAGAA
3'_T7 universal	Riboprobe template, 2nd-round PCR	AGGGATCCTAATACGACTCACTATAGGGCC CGGGGC
5'_T7 universal	Riboprobe template, 2nd-round PCR	GAGAATTCTAATACGACTCACTATAGGGCC GCGG

635
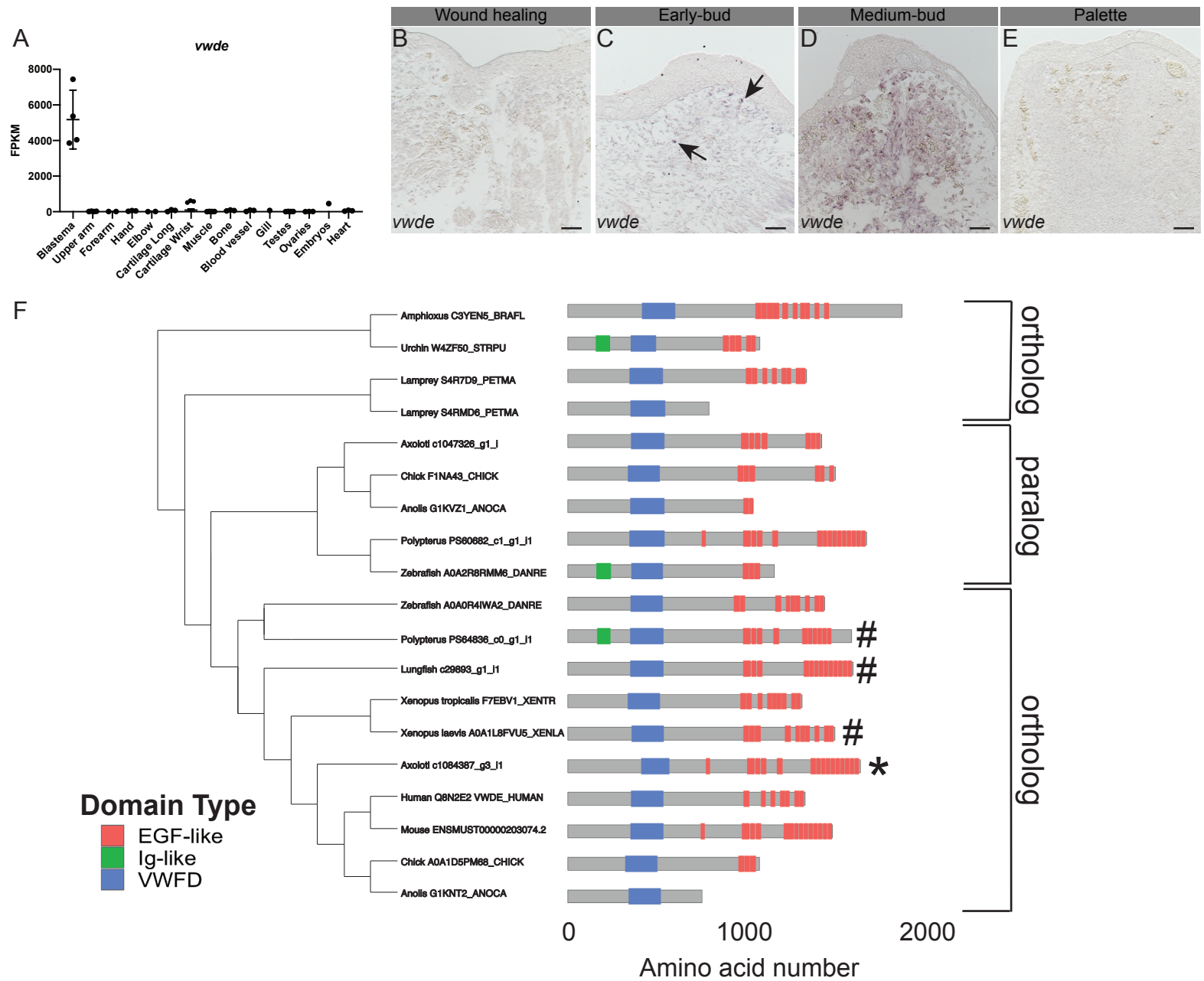
Figure 2

L. paradoxa
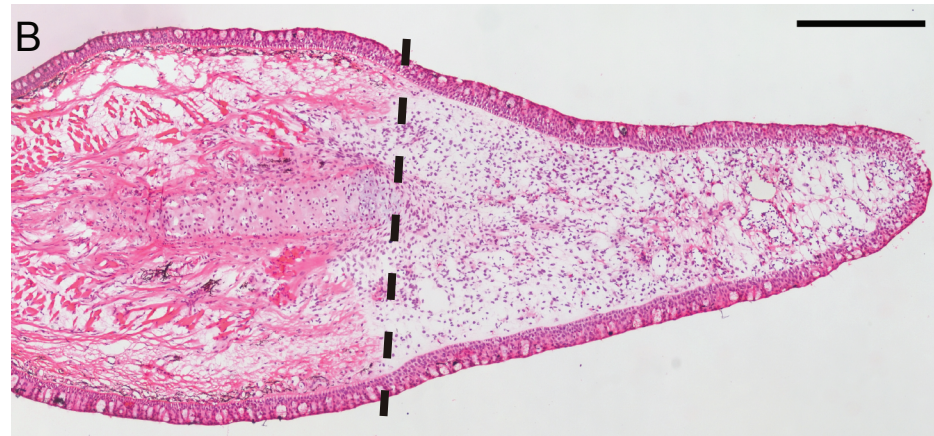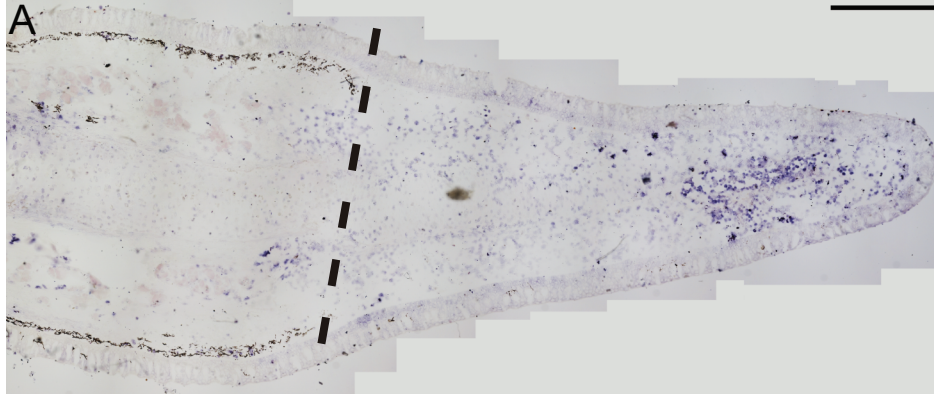
P. senegalus

Figure 3

Figure 4