Characterization of *Drosophila Nidogen/entactin* reveals roles in basement membrane stability, barrier function and nervous system plasticity

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Summary Statement

In this study we characterize *Drosophila Nidogen/Entactin* (*Ndg*) mutants revealing that loss of Ndg impairs basement membrane (BM) stability and permeability as well as proper function of the nervous system.

Abstract

Basement membranes (BMs) are specialized layers of extracellular matrix (ECM) mainly composed of Laminin, type IV Collagen, Perlecan and Nidogen/entactin (NDG). While the essential and evolutionary conserved functions of Laminin, Collagen and Perlecan are well documented in *Drosophila* and other species, the proposed role of NDG as the major ECM linker molecule has been challenged by several *in vivo* studies revealing that NDG is dispensable for viability and BM formation. Here, we report the characterization of the single *Ndg* gene in *Drosophila.* Embryonic *Ndg* expression differed from that of other BM components and was primarily observed in mesodermal tissues and the chordotonal organs, whereas NDG protein localized to all BMs. While loss of Laminin strongly affected BM-localization of NDG, *Ndg* null mutants exhibited no overt changes in the distribution of BM core components. However, loss of NDG led to ultrastructural BM defects compromising barrier function and stability *in vivo*. Although *Ndg* mutants were viable, loss of NDG led to decreased fecundity in flies as well as impaired crawling behavior and reduced response to vibrational stimuli in larvae. Further morphological analysis revealed accompanying defects in the larval peripheral nervous system especially in the chordotonal organs and the neuromuscular junction (NMJ), where *Ndg* genetically interacted with the *Leukocyte-antigen-related-like* (*Lar*) *receptor* gene to regulate NMJ extension and synaptic differentiation. Taken together, our analysis suggests that NDG is not essential for BM assembly but mediates BM stability and ECM-dependent neural plasticity during *Drosophila* development.

Introduction

Proteome analysis of isolated BMs has identified over 100 associated proteins highlighting the complex nature of these specialized ECM sheets (Uechi et al., 2014). However, BMs mainly assemble from a small subset of ECM proteins referred to as the "BM toolkit" which is a highly conserved feature of most metazoan species (Hynes, 2012). BMs consist of two different mesh-like networks formed by self-

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66   assembly of either heterotrimeric Laminin molecules or type IV Collagens which are

67   then linked by their binding partners Nidogen (NDG), the heparan sulfate

68   proteoglycan (HSPG) Perlecan and additional Collagen XV/XVIII homologs (Jayadev

69   and Sherwood, 2017; Yurchenco, 2011). The *Drosophila* genome harbors a minimal

70   set of nine genes encoding components of the "BM toolkit" (Hynes, 2012). Four

71   genes encode for two Laminin α-, one β- and one γ-subunit which form the only two

72   Laminin heterotrimers in the fly, two adjacent loci encode for type IV Collagens

73   (*Col4a1* and *viking*), one for a ColXV/XVIII homolog named *Multiplexin*, a single

74   *Nidogen/entactin* gene, and the *terribly reduced optic lobes* (*trol*) locus that encodes

75   for the Perlecan protein core. With the exception of *Ndg,* all mayor BM components

76   of *Drosophila* have been functionally characterized (Borchiellini et al., 1996; Datta

77   and Kankel, 1992; Friedrich et al., 2000; Henchcliffe et al., 1993; Lindsley and Zimm,

78   1992; Martin et al., 1999; Meyer and Moussian, 2009; Urbano et al., 2009; Voigt et

79   al., 2002; Wolfstetter and Holz, 2012; Yarnitzky and Volk, 1995; Yasothornsrikul et

80   al., 1997).

81   In 1983, Timpl et al (Timpl et al., 1983) reported the identification of an 80 kDA

82   protein from mouse Engelbreth-Holm-Swarm (EHS) sarcoma cell lines which was

83   named Nidogen due to its ability to self-aggregate into "nest-like structures". Although

84   this protein was later identified as a proteolytic fragment of Entactin, a 150 kDa

85   sulfated glycoprotein described by Carlin et al. (Carlin et al., 1981), the terms

86   Nidogen or Nidogen/Entactin are now commonly used to refer to the non-cleaved

87   protein (Martin and Timpl, 1987). The Nidogen protein consists of three globular

88   domains (G1 to G3), a rod-like segment between G1 and G2 as well as an EGF-like-

89   domain-containing segment that connects G2 and G3 (Durkin et al., 1988; Fox et al.,

90   1991). Nidogen forms stable complexes with Laminin through binding of its globular

91   G3 domain to the Laminin γ-subunit, whereas the G2 domain mediates binding to

92   Collagen IV and Perlecan (Fox et al., 1991; Hopf et al., 2001; Mann et al., 1989;

93   Reinhardt et al., 1993). Therefore it has been suggested that Nidogen supports the

94   formation of ternary complexes within the BM and functions as an "ECM linker

95   molecule" connecting the Laminin and Collagen IV networks (Aumailley et al., 1993;

96   Ho et al., 2008; Mayer et al., 1993; Mayer et al., 1995).

97   Functional analysis of the two Nidogen family members in mammals reveals that the

98   single knockouts of *Nidogen 1* (*NID1*) and *Nidogen 2* (*NID2*) in mice neither result in

99   lethality nor cause any gross defects in BM formation and morphology. However,

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100    $NID1^{-/-}$ animals exhibit neurological phenotypes like spontaneous seizures and hind
101   limb ataxia (Dong et al., 2002; Murshed et al., 2000; Schymeinsky et al., 2002).
102   Moreover, elevated NID2 levels in BMs of $NID1^{-/-}$ mice suggest a functional
103   redundancy between the two members of the mammalian Nidogen family (Miosge et
104   al., 2000). Analysis of double mutant $NID1^{-/-}$ $NID2^{-/-}$ mice does not reveal an
105   essential function during embryogenesis and embryonic BM formation but complete
106   absence of Nidogen causes perinatal lethality due to impaired lung and heart
107   development accompanied by defects in the organ-associated BMs (Bader et al.,
108   2005). Moreover, a varying degree of syndactyly and occasional twisting of forelimbs
109   is observed in $NID1^{-/-}$ $NID2^{-/-}$ animals (Bose et al., 2006). In accordance with these
110   results a non-essential function of Nidogen for invertebrate development is revealed
111   by the analysis of *C. elegans nidogen-1* (*nid-1,* the single *Nidogen* homolog in the
112   nematode) mutants that are viable and fertile and do not display abnormalities during
113   BM assembly. In addition to a slight reduction in fecundity, *nid-1* mutant animals
114   exhibit aberrant guidance and positioning of longitudinal nerves, movement defects in
115   a body bending assay, and altered neuromuscular junction (NMJ) organization which
116   suggests a role in nervous system patterning (Ackley et al., 2003; Hobert and Bulow,
117   2003; Kang and Kramer, 2000; Kim and Wadsworth, 2000; Kramer, 2005). A recent
118   study by Zu et al. (Zhu et al., 2017) reports body length reduction in *Danio rerio* upon
119   *nid1a* gene depletion but also suggests that *Ndg*-associated phenotypes are likely to
120   be obscured by functional redundancy and genetic compensation (Rossi et al., 2015)
121   between the four predicted *Nidogen* family members in zebrafish.

122   In this work, we report the characterization of the single *Nidogen/entactin* (*Ndg*) gene
123   in *Drosophila*. We found that *Ndg* was highly expressed in the *Drosophila* embryo
124   and NDG protein was abundant in all basement membranes (BMs) suggesting an
125   essential function during development. In contrast, analysis of *Ndg* mutants revealed
126   that loss of NDG did not affect viability and fertility in general, arguing for a negligible
127   role during development. This was further confirmed by examination of BM assembly
128   in *Ndg* mutant embryos revealing no overt changes in overall BM protein distribution.
129   Interestingly, ultrastructural SEM analysis and permeability assays revealed a porous
130   BM surface and severe defects in BM barrier function accelerating the disruption of
131   imaginal disc BMs under osmotic stress conditions. Moreover, *Ndg* mutant larvae
132   displayed a range of strong behavioral phenotypes such as reduced responses to
133   external stimuli, impaired motility and climbing performance as well as altered

134 crawling behavior and gravitaxis. Our morphological analysis of the larval nervous
135 system indicated that these phenotypes correlated with improper positioning of
136 neurons, defasciculation of nerves as well as severe defects in chordotonal organ
137 and neuromuscular junction (NMJ) organization. We also found that *Ndg* genetically
138 interacted with the *Leukocyte-antigen-related-like* (*Lar*) receptor gene to promote
139 NMJ differentiation and plasticity. Taken together, our analysis of *Drosophila Ndg* did
140 not support an essential role of NDG for the assembly of BMs but suggested that
141 NDG essentially contributes to the robustness of the BM barrier and ensures ECM
142 plasticity in response to external cues.

143

Results and Figures

Ndg transcript expression and protein localization

146 Developmental Northern blot analysis revealed high levels of *Ndg* expression in
147 *Drosophila* embryos after gastrulation (Sup. Fig. 1). We therefore applied
148 fluorescence *in situ* hybridization on *white*^{1118} (*w*^{1118}) embryos to analyze *Ndg* mRNA
149 distribution in comparison to NDG protein localization revealed by antibody staining
150 (Fig. 1). In line with our Northern blot analysis, *Ndg* expression was apparent at stage
151 11/12 and could be detected in single cells of the head, especially in the gnathal
152 segments (Fig. 1A, asterisk) as well as in segmentally located patches of cells in the
153 dorsal mesoderm (arrowhead, Fig. 1A). Moreover, the midline-associated,
154 mesodermal dorsal median cells (DMC, Fig. 1B, C) as well as four surrounding
155 somatic myoblasts (sMB, Fig. 1B) exhibited strong *Ndg* expression whereas we
156 detected only very weak signals in the amnioserosa (AS, Fig. 1C). At this time NDG
157 protein, embedding DMCs and sMBs, formed a thin sheet between ecto- and
158 mesoderm and outlined the region of the prospective anal plate. After germ band
159 retraction (Fig. 1D) mRNA expression had increased and was now visible in the
160 forming dorsal and ventral muscles, the amnioserosa (AS) and the segmentally
161 located chordotonal organs (Cho). This was in agreement with the expression profile
162 obtained from developmental Northern blot analysis which revealed a strong *Ndg*
163 mRNA increase in older embryos (Sup. Fig. 1). Internal views indicated a decrease of
164 expression in the DMCs (arrow) whereas *Ndg* was now expressed in the esophageal
165 visceral muscle primordium (eVM, arrowhead) and the joint region between hind- and
166 midgut (Fig. 1E). At this time, accumulation of NDG protein in the forming BMs
167 around the developing brain and ventral nerve cord (VNC), the differentiating tracheal

168 system, the future digestive tract as well as the forming somatic muscles was

169 observed (Fig. 1D, E). Embryos at stage 16 displayed strong mRNA expression in

170 somatic and visceral muscles and in the cap cells (CC in Fig. 1H) of the chordotonal

171 organs, whereas the protein localized to all embryonic BMs (Fig. 1F-I). Interestingly,

172 whereas *Ndg* mRNA expression was absent from neuronal tissues, the BMs

173 surrounding the chordotonal neurons (CN), the VNC and the brain were highly NDG-

174 positive (Fig. 1H, I). In summary, *Ndg* mRNA expression during embryogenesis

175 followed a dynamic and distinct pattern involving mostly mesodermal cells and

176 tissues but also chordotonal cap cells and the amnioserosa whereas the protein was

177 highly abundant in all embryonic BMs. Interestingly, no transcript expression was

178 observed in hemocytes or fat body cells although these tissues strongly express

179 other BM components like Laminin or type IV Collagen (Le Parco et al., 1986; Pastor-

180 Pareja and Xu, 2011; Rodriguez et al., 1996; Wolfstetter and Holz, 2012).

181

182 **Localization of NDG to BMs depends on Laminin**

183 We next asked whether the absence of other major BM components affects the

184 incorporation and localization of NDG within the BM. Therefore we performed NDG

185 antibody staining on late stage 16 embryos lacking either Laminin, type IV Collagen

186 or Perlecan (Fig. 2). Control siblings exhibited strong NDG staining of all embryonic

187 BMs and around the chordotonal organs (Fig. 2A). To differentiate between the NDG

188 localization properties of both Laminin trimers we employed either *LanA* or *wing*

189 *blister* (*wb*) loss of function mutations that impair formation and secretion of only one

190 Laminin trimer (Urbano et al., 2009). Interestingly, we found a strongly disrupted and

191 punctuated NDG pattern in transheterozygous *LanA$^{9-32}$*/Df *LanA* embryos lacking the

192 Laminin A-yielding trimer (Fig. 2B), whereas we did not detect changes in *wb^{HG10}*/Df

193 *wb* embryos in which the Wb-containing trimer is absent (Fig. 2C). The loss of all

194 secreted Laminin trimers in embryos lacking the only Laminin γ-subunit (*LanB2^{knod}*/Df

195 *LanB2*) resulted in a dramatic loss of NDG localization (Fig. 2D) indicating a

196 redundant function of both Laminin trimers for the localization of NDG to BMs. On the

197 other hand, embryos deficient for *trol* (the Perlecan encoding locus) as well as

198 embryos carrying a deficiency for the two *Drosophila* type VI Collagens (*Col4a1* and

199 *viking*) displayed no changes in NDG localization and distribution (Fig. 2E, F)

200 suggesting that Laminin is the primary crucial component for NDG distribution and

201 localization to BMs.

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Generation of *Ndg* deletion mutants

204 In order to investigate the function of *Ndg* in *Drosophila*, we employed imprecise
205 excision of the Minos transposon insertion *Mi{ET1}MB04184* to generate deletions in
206 the *Ndg* locus (Sup. Fig. 2A). Two excision lines were obtained in which 0.4 kb
207 respectively 1.4 kb of the *Ndg* locus including parts of the 5' UTR were deleted
208 (henceforth referred to as $Ndg^{\Delta 0.4}$ and $Ndg^{\Delta 1.4}$, see also Material and Methods).
209 Further analysis employing NDG-specific antibodies revealed that the strong NDG
210 staining observed in control siblings (Sup. Fig. 2B) was completely absent in
211 homozygous $Ndg^{\Delta 1.4}$ embryos (Sup. Fig. 2C), whereas faint NDG expression could
212 be detected in the $Ndg^{\Delta 0.4}$ mutant (Sup. Fig. 2D). Therefore, we concluded that the
213 deletion in $Ndg^{\Delta 1.4}$ resulted in a protein null allele while the smaller molecular lesion
214 in $Ndg^{\Delta 0.4}$ caused a hypomorphic *Ndg* allele characterized by strongly reduced NDG
215 expression.

216

Phenotypic analysis of *Ndg* mutants

218 *Nidogen* mutants generated in *C. elegans* and mouse are viable and display only
219 mild phenotypes (Bader et al., 2005; Bose et al., 2006; Dong et al., 2002; Kang and
220 Kramer, 2000; Murshed et al., 2000; Schymeinsky et al., 2002), a surprising finding
221 which is in strong contrast to the essential developmental roles demonstrated for the
222 other BM molecules (Arikawa-Hirasawa et al., 1999; Clay and Sherwood, 2015;
223 Poschl et al., 2004; Yao, 2017). In agreement with these former observations,
224 *Drosophila* Ndg^{Δ} mutants were viable and fertile and could be maintained as
225 homozygous stocks. Despite the lack of any obvious developmental function we
226 noticed several peculiarities in Ndg^{Δ} homozygous stocks that were more apparent in
227 the $Ndg^{\Delta 1.4}$ line when compared to $Ndg^{\Delta 0.4}$. In non-crowded, standard culture
228 conditions, Ndg^{Δ} pupal cases were preferentially formed in the lower half of the vial
229 or even directly on the food, indicating potentially impaired larval climbing abilities
230 (Sup. Fig. 3A, B). We also noticed a difference in the orientation of the pupal cases
231 which was shifted towards the horizontal axis in Ndg^{Δ} vials (Sup. Fig. 3B). Moreover,
232 we observed a decrease in fecundity in homozygous Ndg^{Δ} animals compared to the
233 w^{1118} control (Sup. Fig. 3C) and $Ndg^{\Delta 1.4}$ flies additionally exhibited incompletely
234 inflated wing blades at low (~5 %) penetrance rates (Sup. Fig. 3D, E).

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Distribution of major BM components is not altered in *Ndg* mutant embryos

To analyze the proposed function of NDG as universal ECM cross linker that connects Laminin and Collagen layers of BMs (Timpl and Brown, 1996), we studied the distribution of known BM components in $Ndg^{\Delta 1.4}$/Df Ndg embryos. Therefore, we applied antibodies raised against Laminin subunits and trimers, type IV Collagens and Perlecan as well as the Collagen-associated molecule SPARC (Fig. 3 and Sup. Fig. 4). In addition, we employed GFP-trap insertions in the loci of *viking* (*vkg::GFP*), one of the two *Drosophila* type IV *Collagen* genes, and *terribly reduced optic lobes* (*trol::GFP*), encoding for *Drosophila* Perlecan (Sup. Fig. 4).

In case of Laminin, all antibodies employed stained embryonic BMs indicating reactivity with secreted Laminin (Fig. 3A, B). Laminin A (LanA) outlined the BMs of all internal organs in control and *Ndg* mutant embryos (Fig. 3A, E), whereas antibody staining against the Laminin α1, 2 subunit Wing blister (LanWb) was found mainly in the BMs around the digestive tract and the apodemes of control and *Ndg* mutant embryos (Fig. 3B, E). This was in agreement with antibody staining against secreted K-cell Laminin (LanKc) which appeared similar in *Ndg* mutants compared to control embryos (Sup. Fig. 4A, D). Additionally employed antibodies against the Laminin β- and γ-subunits (referred to as LanB1 and LanB2, Sup. Fig. 4B, C, E, F) demonstrate BM staining of control and *Ndg* mutant embryos and due to the strong reactivity with non-secreted Laminin intermediates also labeling of hemocytes and fat body cells.

Further analyses employing antibodies against Collagen IV (COLL IV, Fig. 3C, G), the *vkg::GFP* protein trap (Sup. Fig. 4G, J), and the collagen-binding protein SPARC (Sup. Fig. 4H, K) revealed that these proteins were present in embryonic BMs of *Ndg* mutants. Finally, we did not observe obvious differences in Perlecan staining of control and *Ndg* mutant embryos detected by either PCAN-specific antibodies (Fig. 3D, H) or *trol::GFP* (Sup. Fig. 4I, L). Taken together, the complete loss of NDG seems not influence the formation or overall assembly of embryonic BMs.

Loss of NDG affects BM ultrastructure and function

The viability of Ndg^{Δ} mutant flies and the presence of all BM proteins analyzed so far (Fig. 3 and Sup. Fig. 4) suggested that BM assembly was not severely affected by the absence of NDG, therefore questioning its proposed role as universal ECM linker molecule. However, the loss of NDG might impair BM stability and function. To address this in more detail we dissected w^{1118} and $Ndg^{\Delta 1.4}$ 3rd instar larvae and

270 employed a Dextran-based permeability assay in addition to SEM ultrastructural

271 analysis (Fig. 4). Spot-like Texas Red (TexRed)-coupled Dextran plaques were

272 observed across the surface of the somatic muscles of w^{1118} control larval filets (Fig.

273 4A). In comparison, $Ndg^{\Delta 1.4}$ larval filets appeared much brighter after dextran staining

274 and we observed a strong leakage of TexRed-Dextran into the muscle proper (Fig.

275 4B) indicating enhanced diffusion through the BM tissue barrier in the absence of

276 NDG.

277 Further ultrastructural analysis of w^{1118} control 3rd instar larvae revealed that the

278 visceral BM which covers the surface of the larval midgut appeared as smooth sheet

279 spread across the underlying longitudinal and circular visceral muscles (Fig. 4C). In

280 comparison, the surface of the visceral BM in homozygous Ndg^{Δ} mutant larvae

281 exhibited holes (Fig. 4D-F), which were frequently found in close vicinity to the

282 longitudinal visceral muscles (LVM) but also appeared in other areas. We also

283 observed a variation in phenotypic strength between individual larvae. While most

284 null mutant $Ndg^{\Delta 1.4}$ animals displayed strong BM phenotypes, a minor fraction also

285 exhibited weaker defects resembling $Ndg^{\Delta 0.4}$ larvae. On the other hand, BM defects

286 observed in some of the analyzed $Ndg^{\Delta 0.4}$ larvae appeared similar to the null allele.

287 Interestingly, examination of heterozygous Ndg^{Δ}/CyO larvae (Sup. Fig. 5B-D) already

288 revealed a weak BM phenotype characterized by few and small surface holes which

289 was never observed in w^{1118} (Fig. 4C) or in additional control larvae from the w^*; Kr^{If-}

290 ^{1}/CyO balancer stock (Sup. Fig. 5A), indicating a dose-dependent effect of NDG on

291 BM morphology.

292

Loss of *Ndg* leads to BM disruption under osmotic stress

294 To further investigate whether the absence of NDG comprises the mechanical

295 stability of the BM, we exposed wing imaginal discs to an osmotic shock by

296 incubating them in deionized water. When water diffused into the hypertonic interior

297 of the imaginal discs it created balloon-like swelling of the disc before eventual

298 bursting. Imaginal discs of larvae resembling the genetic background of balanced

299 and homozygous Ndg^{Δ} animals (w^{1118} and w^*; Kr^{If-1}/CyO) withstand the resulting

300 osmotic pressure for ~430 s respectively ~388 s before bursting. In case of wing

301 discs derived from balanced, heterozygous Ndg^{Δ} animals we observed a slight but

302 not significant increase ($Ndg^{\Delta 0.4}$/CyO = 472 s, $Ndg^{\Delta 1.4}$/CyO = 520 s; and *Df Ndg*/CyO

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303   = 407 s). In contrast to this $Ndg^{\Delta 0.4}$ wing discs burst after ~213 s and $Ndg^{\Delta 1.4}$ or

304    $Ndg^{\Delta 1.4}$/$Df\ Ndg$ discs tore after ~240 s indicating decreased BM stability (Fig. 5).

305

306   **Altered crawling behavior and NMJ morphology in *Ndg* mutant larvae**

307   When placed on agar plates supplemented with yeast paste as food source $Ndg^{\Delta 1.4}$

308   1st instar larvae were often observed outside the food and exhibited overall lethargic

309   and reduced crawling movements (Fig. 6A, B). To monitor and compare their

310   behavioral repertoire we recorded age-matched w^{1118} controls and $Ndg^{\Delta 1.4}$ larvae

311   that were placed on Agar plates in the absence of food (Fig. 6C, D). Crawling of

312   control w^{1118} larvae was dominated by smooth forward movement with occasional

313   turns and pauses that allowed the larvae to explore the whole experimental arena

314   (Fig. 6C, E and Sup. Movie). In contrast, *Ndg* mutants generally moved within a much

315   smaller area and displayed a variety of crawling defects including excessive head

316   turning as well as exaggerated rolling and bending motions (Fig. 6D, F and Sup.

317   Movie). Interestingly, the phenotypic strength exhibited some variation since 70 % of

318   2nd instar larvae (n > 40) and half of the analyzed 3rd instar larvae (n > 40) displayed

319   rather strong behavioral abnormalities whereas the remaining animals exhibited

320   weaker crawling defects. We further analyzed crawling of Ndg^{Δ} 3rd instar larvae in

321   more detail, investigating the velocity and the stride frequency of the undisturbed

322   crawling pattern (Fig. 6G, H). Comparison of the mean crawling velocity and the

323   stride frequency showed comparable differences between the analyzed genotypes.

324   The mean crawling velocity of $Ndg^{\Delta 0.4}$ mutant larvae was slightly but not significantly

325   reduced whereas $Ndg^{\Delta 1.4}$ larvae showed a significantly reduced mean velocity

326   compared to w^{1118} larvae (Fig. 6G). Also the stride frequency in both Ndg^{Δ} strains

327   was significantly lower when compared to w^{1118} larvae (Fig. 6H). However, $Ndg^{\Delta 1.4}$

328   mutants displayed the more severe phenotype in terms of reduced crawling speed

329   and stride frequency (Fig. 6G, H).

330   These crawling phenotypes prompted us to examine neuromuscular junction (NMJ)

331   morphology in wandering 3rd instar larvae. We therefore dissected larval filets and

332   stained them with anti-Bruchpilot (BRP) antibodies to label T-zones in the synaptic

333   boutons and employed anti-HRP staining to reveal the overall NMJ morphology (Fig.

334   6I, J). We focused on the NMJ at muscle 6/7 in the 2nd abdominal segment because

335   it covers a considerably large area, innervates two muscles, and contains many

336   synaptic boutons. While these NMJs in w^{1118} control larvae appeared to be equally

9

337 sized, we observed shape and size differences of the corresponding NMJs in Ndg^{Δ}

338 mutants, even within the same animal (Fig. 6J). We also noticed a higher degree of

339 synaptic branching and increased bouton density (Fig. 6J and 8B, C) which appeared

340 to be independent of the NMJ size. Taken together, these results suggest that loss of

341 NDG leads to defects in NMJ organization presumably resulting in the climbing and

342 crawling defects observed in Ndg^{Δ} mutant larvae.

343

344 **Loss of NDG leads to reduced reaction to vibrational stimuli and aberrant**

345 **chordotonal organ morphology**

346 Our previous analyses revealed that developing chordotonal organs highly expressed

347 Ndg in their cap cells and eventually became embedded in a NDG-positive BM.

348 Notably, some of the larval crawling defects and the altered orientation of pupal

349 cases observed in the absence of NDG could be interpreted as a result of impaired

350 proprioception. Therefore, we wanted to characterize chordotonal function in Ndg^{Δ}

351 mutants on a behavioral level and additionally analyze larval PNS morphology with

352 respect to the chordotonal organs (Fig. 7). Stimulus-induced, relative body length

353 reduction upon applied vibrational stimuli was compared between 3rd instar w^{1118}

354 control and Ndg^{Δ} larvae (Fig. 7A, B). The body length reduction of control larvae in

355 response to vibrational stimuli was on average ~14 % whereas homozygous $Ndg^{\Delta 0.4}$

356 and $Ndg^{\Delta 1.4}$ larvae showed significantly reduced retraction values (Fig. 7B). $Ndg^{\Delta 1.4}$

357 mutants again displayed the most severe phenotype with a nearly abolished reaction

358 (~4 % body length reduction) to vibrational stimuli (Fig. 7B).

359 To reveal potential morphological changes underlying the observed behavioral

360 phenotypes, we examined the chordotonal organs of 3rd instar larvae. Therefore, we

361 utilized the $Jupiter::GFP$ fusion protein which localizes to the microtubule network

362 (Karpova et al., 2006). In addition, we employed anti-NDG and horseradish

363 peroxidase (HRP) staining (Jan and Jan, 1982) to reveal the chordotonal BM and

364 associated neurons respectively (Fig. 7C, D). A NDG-positive BM surrounded the

365 whole mechanosensory organ of $Jupiter::GFP$ control larvae including the bulging cell

366 bodies of the chordotonal neurons (Fig. 7C, arrowhead) which closely aligned to the

367 scolopale cell. A NDG-positive sheet also surrounded the row of aligned sensory cilia

368 (Fig. 7C, arrow). In contrast to this regular alignment, $Ndg^{\Delta 1.4}$ mutant larvae exhibited

369 detachment of the chordotonal neurons and dislocation of their cell bodies (Fig. 7D,

370 arrowhead) from the scolopale cell as well as disrupted alignment of the sensory cilia

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371 (Fig. 7D arrow). We noticed that the penetrance of these phenotypes varied even
372 between the chordotonal organs of corresponding hemisegments in a single larva
373 (Sup. Fig. 6B, C). However, we did not observe chordotonal organs in *Ndg* mutant
374 larvae that resembled wild-type morphology (Sup. Fig. 6A).

375 In addition to the chordotonal organ defects we noticed aberrations in other parts of
376 the larval peripheral nervous system (Fig. 7F). The cell bodies of the dorsal dendritic
377 arborization sensory neurons (ddA neurons) failed to cluster in *Ndg*$^{Δ1.4}$ mutant larvae
378 (Fig. 7F circles). Moreover, transmitting nerves frequently exhibited defasciculation
379 (Fig. 7E and F arrow) and positioning of neuronal cell bodies (Fig. 7F asterisks) was
380 often altered when compared to the controls (Fig. 7E asterisks).

381

382 **Ndg interacts genetically with the Lar receptor at the larval NMJ**

383 The differences in shape and size between corresponding NMJs 6/7 in the 2nd
384 abdominal segment and the higher degree of synaptic branching in *Ndg*$^{Δ1.4}$ mutants
385 (Fig. 7E, F) prompted us to analyze this phenotype in more detail with regard to
386 potential genetic interaction partners. Upon closer examination of the NMJ geometry
387 in *Ndg*^{Δ} mutant larvae we observed that in contrast to wild-type NMJs (Fig. 8A) the
388 overall branching was increased and major branching points were shifted towards the
389 center of the junction (Fig. 8B). We also observed bouton clustering and a
390 significantly enhanced number of boutons per square at *Ndg*^{Δ} junctions suggesting
391 that NDG actually suppresses NMJ maturation (Fig. 8C). Notably, these defects were
392 found to be independent from the variable NMJ size observed in *Ndg*^{Δ} animals.
393 Moreover, heterozygous *Ndg*$^{Δ1.4}$/*CyO* NMJs exhibited increased bouton clustering
394 and a tendency to branch prematurely (Fig. 8D) suggesting that these phenotypic
395 characteristics could be modified upon genetic interaction.

396 Homozygous as well as heterozygous null mutants of the *receptor protein tyrosine*
397 *phosphatase* (*RPTP*) *Leukocyte-antigen-related-like* (*Lar*) have been described to
398 exhibit a strongly reduced bouton number at the larval NMJ (Kaufmann et al., 2002).
399 In agreement to this we found drastically reduced NMJs in the rarely occurring
400 homozygous *Lar*$^{13.2}$ 3rd instar larvae (data not shown), whereas heterozygous
401 *Lar*$^{13.2}$/CyO animals (Fig. 8E) exhibited reduced branching and bouton numbers. In
402 double-heterozygous *Lar*$^{13.2}$, *Ndg*$^{Δ1.4}$/*CyO* larvae, we observed mutual repression of
403 the phenotypes caused by one copy of either *Lar*$^{13.2}$ or *Ndg*$^{Δ1.4}$ leading to NMJs that

404 resembled a more "wild-type" morphology (Fig. 8F). These results indicate that *Ndg*

405 and *Lar* function together to promote differentiation of the larval NMJ in *Drosophila*.

406

Discussion

Analysis of *Ndg* expression and NDG protein distribution

409 In this work we have characterized the single *Nidogen/entactin* gene in *Drosophila*

410 *melanogaster*. In the developing embryo, *Ndg* expression followed a highly dynamic

411 pattern involving diverse mesodermal cell types like somatic muscle founder cells,

412 mature visceral and somatic muscles as well as dorsal median cells. *Ndg* expression

413 in a subset of somatic muscle founder cells had been previously reported by (Artero

414 et al., 2003) and detailed analysis of an intronic "*Ndg* muscle enhancer" revealed a

415 complex interplay of Forkhead and Homeodomain transcription factors and their

416 binding sites that are required to drive *Ndg* expression in the different mesodermal

417 cell types (Busser et al., 2013; Busser et al., 2012; Philippakis et al., 2006; Zhu et al.,

418 2012). Our analysis further revealed *Ndg* expression in the extraembryonic

419 amnioserosa and in chordotonal cap cells indicating the presence of additional

420 regulatory elements. We did not detect *Ndg* expression in the embryonic CNS but in

421 the closely associated dorsal median cells (DMCs), which express other BM

422 components and ECM receptors such as Perlecan, Dystroglycan, Glutactin as well as

423 Laminin and type IV Collagen (Friedrich et al., 2000; Mirre et al., 1992; Montell and

424 Goodman, 1989; Olson et al., 1990). DMCs provide cues for proper axonal

425 pathfinding during transverse nerve outgrowth and regulate bifurcation of the median

426 nerve in *Drosophila* (Chiang et al., 1994). Therefore, studying a potential role of the

427 secreted ECM in this context would be an interesting topic for future investigations.

428 Interestingly, *Ndg* expression was absent from mesoderm-derived hemocytes and fat

429 body cells although these cells strongly secrete Laminin, type IV Collagens and other

430 ECM components (Le Parco et al., 1986; Pastor-Pareja and Xu, 2011; Rodriguez et

431 al., 1996; Urbano et al., 2009; Wolfstetter and Holz, 2012) indicating that *Ndg*

432 expression is regulated independently from other BM genes.

433 CEL-Seq transcriptome analysis in *C. elegans* reveals high *nid-1* mRNA expression

434 in mesodermal tissues upon gastrulation (Hashimshony et al., 2015) and NDG

435 proteins strongly associate with body wall muscles in *Drosophila* (this study), *C.*

436 *elegans*, Ascidians and mice (Ackley et al., 2003; Fox et al., 2008; Kang and Kramer,

437 2000; Nakae et al., 1993) suggesting an important, evolutionary conserved function

12

438   in this particular tissue. Indeed, *C. elegans* Nid-1 and NID2 in mice are enriched at

439   neuromuscular junctions and, in agreement with our analyses in *Drosophila*, required

440   for proper NMJ structure and function (Ackley et al., 2003; Fox et al., 2008).

441

NDG is not required for BM assembly

443   Our analysis of NDG distribution in the absence of major BM components revealed a

444   dependency of NDG localization from Laminin but not from type IV Collagen or

445   Perlecan. This is in agreement with studies on γ1III4-mice in which the NDG-binding

446   site in the Laminin γ1 chain had been deleted. This analysis revealed a severe

447   reduction of NDG from BMs as well as perinatal lethality and organ defects similar to

448   those observed in *NID1*^{-/-} *NID2*^{-/-} double mutant mice which further strengthens the

449   assumption that Laminin plays an essential role in localizing NDG to forming BMs

450   (Bader et al., 2005; Halfter et al., 2002; Willem et al., 2002). The two Laminin

451   heterotrimers in the fly function in a redundant manner to localize NDG to embryonic

452   BMs. In a single loss of function background, however, only absence of the LanA

453   trimer (containing the *Drosophila* Laminin α3/5 homolog) affected NDG localization,

454   suggesting different binding and polymerization activities of the *Drosophila* Laminins.

455   In line with these findings, combined reduction of LanA and NDG around the

456   developing gonad was also observed in βPS-integrin mutant embryos (Tanentzapf et

457   al., 2007). No obvious changes in the distribution of BM core components (Laminins,

458   Collagens and Perlecan) were found in our immunohistochemical analysis after

459   complete loss of NDG, reflecting its non-essential role during development. This

460   finding, further supported by similar analyses in other species (Ackley et al., 2003;

461   Bader et al., 2005; Kang and Kramer, 2000; Kim and Wadsworth, 2000; Rossi et al.,

462   2015) contradicts the proposed role for NDG as linker between inner and outer ECM

463   networks during BM formation, suggesting a negligible role for NDG in overall BM

464   assembly (Ho et al., 2008; Hohenester and Yurchenco, 2013; Jayadev and

465   Sherwood, 2017; Kramer, 2005; Timpl and Brown, 1996; Yurchenco, 2011).

466

Loss of NDG affects BM stability and function

468   Although BM changes are barely detectable in *Ndg*[Δ] mutants by conventional

469   immunohistochemistry, ultrastructural analysis revealed that BM continuity is

470   compromised if NDG is absent or reduced. Our findings that BM barrier function and

471   stability were decreased in *Ndg*[Δ] larvae suggests that NDG, or NDG-mediated

13

472   crosslinking of other ECM molecules, plays an important role in sealing the

473   assembled BM. Intriguingly, Matsubayashi et al. (Matsubayashi et al., 2017) observe

474   holes in the forming BM around the embryonic *Drosophila* CNS which are

475   progressively closed during hemocyte migration and concurrent Col IV deposition. In

476   addition, studies on mice demonstrate that although Laminin is sufficient for the

477   assembly of early BM scaffolds, incorporation of type IV Collagen is essentially

478   needed for BM integrity and stability (Poschl et al., 2004). Therefore, it is tempting to

479   speculate that crosslinking of hemocyte-deposited Col IV might be impaired in *Ndg*

480   mutants leading to decreased BM stability and barrier function.

481   The increased number of BM holes in close proximity to the visceral longitudinal

482   musculature in *Ndg* mutants and the reduced tolerance to osmotic shock further

483   implies that NDG protects BM integrity from mechanical stress therefore separating

484   and maintaining different compartments in the larval body. While our experiments

485   demonstrated reduced BM stability in Ndg^{Δ} larvae, the BM defects observed in the

486   background of different *Ndg* alleles exhibited some phenotypic variation. An

487   explanation for this could be that, although the genetic background of the larvae

488   determines overall BM stability, individual behavior, physical activity, and motility

489   levels could modulate *Ndg* loss of function phenotypes. A comparable finding was

490   made by Tsai and colleagues (Tsai et al., 2012) who reported a process in which

491   larval NMJ size changes in response to LanA levels at the NMJ which are influenced

492   by crawling activity as well as nervous responses to environmental cues. Given the

493   NDG-localizing properties of LanA as well as the variations in NMJ size observed in

494   Ndg^{Δ} animals, it is intriguing to think of a feedback mechanism that employs

495   spatiotemporal reorganization of the larval BM by NDG to adapt NMJ morphology to

496   altered environmental conditions.

497

498   Loss of NDG affects behavioral responses and PNS morphology

499   With the exception of $NID1^{-/-}$ $NID2^{-/-}$ double mutant mice that die at birth, loss of NDG

500   is generally associated with a range of rather subtle morphological and behavioral

501   phenotypes. Notably, abnormalities described in the absence of NDG are not always

502   fully penetrant or exhibit phenotypic variation (Bader et al., 2005; Bose et al., 2006;

503   Dong et al., 2002; Hobert and Bulow, 2003; Kang and Kramer, 2000; Kim and

504   Wadsworth, 2000; Murshed et al., 2000; Schymeinsky et al., 2002; Zhu et al., 2017).

505   Moreover, subtle behavioral phenotypes can be enhanced by adjusting the

14

506   experimental conditions (Ackley et al., 2003). In agreement with these analyses,

507   characterization of *Drosophila Ndg$^\Delta$* mutants did not reveal an essential

508   developmental function but a range of less overt, often variable phenotypes. After

509   vibrational disturbance of a normal crawling phase, wild-type *Drosophila* larvae show

510   a complex sequence of behavioral pattern. The larvae discontinue their forward

511   movement and show a head retraction ("hunch") followed by a head turning phase

512   ("kink") (Bharadwaj et al., 2013; Ohyama et al., 2013; Wu et al., 2011). This

513   sequence of behavior is initiated upon mechanical stimuli delivered to the substrate

514   which are detected by segmental chordotonal organs, mediated by stimulus

515   processing in the central nervous system, and executed under neuromuscular

516   control. Our analysis of the vibrational response behavior in *Ndg$^\Delta$* larvae shows a

517   statistically significant reduction in body contraction. This finding points to a crucial

518   role of NDG in vibrational response behavior. However, the precise level of NDG

519   action cannot be inferred from these behavioral data. NDG expression in chordotonal

520   cap cells, the morphological defects observed in larval chordotonal organs as well as

521   the altered geotaxis of *Ndg$^\Delta$* larvae suggest a contribution to mechanosensation.

522   Defective wing inflation and the aberrant NMJ morphology observed in *Ndg$^\Delta$* animals

523   additionally indicate that loss of NDG affects the neuromuscular system. Indeed,

524   previous research on *C. elegans* and mouse has uncovered a role of NDG for the

525   structural development of neuromuscular junctions as well as altered locomotor

526   behavior upon loss of NDG (Ackley et al., 2003; Bader et al., 2005; Dong et al., 2002;

527   Fox et al., 2008). Given the high conservation of NDG throughout the animal kingdom

528   (reviewed in (Ho et al., 2008)), this finding could hint at a possible comparable

529   function for NDG in insects.

530   The receptor protein tyrosine phosphatase (RPTP) Leukocyte-antigen-related-like

531   (Lar) and its cytoplasmic binding partner Liprin-α are required for proper synaptic

532   morphology at the larval NMJ in *Drosophila* (Kaufmann et al., 2002). Interestingly, a

533   specific splice form of LAR has been shown to bind a Laminin/Nidogen-containing

534   protein complex in Hela cells and LAR-like RPTPs genetically interact with *nid-1* to

535   promote presynaptic differentiation in *C. elegans* (Ackley et al., 2005). Our analysis

536   also reveals that a genetic interaction between *Lar* and *Ndg* is conserved at the larval

537   NMJ in *Drosophila*. However, in contrast to the observations in *C. elegans* both

538   factors affected overall NMJ morphology and acted antagonistically in this process.

539 In conclusion, our initial characterization of *Ndg* mutants in *Drosophila* neither
540 revealed an essential developmental function nor did it support the proposed role for
541 NDG as universal ECM-linker molecule. However, NDG appears to be important for
542 BM sealing and stability, proper mechanosensation and neuromuscular function.
543 Notably, our analyses suggest that NDG could be an important factor to ensure ECM
544 plasticity in response to environmental cues.

545

546 ## MATERIALS AND METHODS

547 **Fluorescence antibody staining**

548 Antibody staining of *Drosophila* embryos and larvae was essentially performed as
549 described by Mueller in (Dahmann, 2008). For antibody staining of wandering 3rd
550 instar larvae we adapted the protocol from Klein in (Dahmann, 2008) with the
551 following modifications: animals were relaxed before dissection by briefly dipping
552 them into 60 °C water and a permeabilization step (10 min wash in PBS supplied with
553 1 % Triton-X100) was added before blocking and primary antibody incubation. The
554 following primary antibodies were used in their specified dilutions: mouse anti-
555 Bruchpilot (Brp nc82, 1:100, (Wagh et al., 2006), DSHB), guinea pig anti-Collagen IV
556 (COLLIV, 1:500, (Lunstrum et al., 1988)), sheep anti-Digoxygenin alkaline
557 phosphatase conjugated Fab fragments (DIG-AP, 1:4.000, Roche Applied Science),
558 mouse anti-Green fluorescent protein (GFP, 1:500, Roche Diagnostics), rabbit anti-
559 Green fluorescent protein (GFP, 1:500, abcam), chicken anti-Green fluorescent
560 protein (GFP, 1:500, abcam), goat anti-Horseradish peroxidase, Cy3-conjugated
561 (HRP, 1:200, Jackson ImmunoResearch), guinea pig anti-Laminin A (LanA, 1:500,
562 (Harpaz and Volk, 2012)), mouse anti-Laminin A (LanA, 1:500, (Takagi et al., 1996)),
563 rabbit anti-Laminin B1 (LanB1, 1:400, (Kumagai et al., 1997), abcam), rabbit anti-
564 Laminin B2 (LanB2, 1:400, (Kumagai et al., 1997), abcam), rabbit anti-K-cell Laminin
565 (LanKc, 1:500, (Gutzeit et al., 1991)), rabbit anti-Wing blister (LanWb, 1:100, (Martin
566 et al., 1999)), rabbit anti-Nidogen (NDG, 1:1.000, (Wolfstetter et al., 2009)), rabbit
567 anti-Perlecan (PCAN, 1:1.000, (Friedrich et al., 2000)) and rabbit anti-SPARC (1:500,
568 (Martinek et al., 2008)). Alexa Fluor-, Cy-, Biotin-SP-, and HRP-coupled secondary
569 antibodies were purchased from Dianova and Jackson ImmunoResearch, DAPI from
570 Sigma Aldrich. Embryos and larval tissues were embedded in Fluoromount-G
571 (Southern Biotech) before visualization under Leica TCS SP2 or Zeiss LSM 800
572 confocal microscopes.

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573

Developmental Northern blot

574

575 Northern blot analyses were performed by standard procedures (Maniatis, 1982).

576 RNA was extracted by the guanidine thiocyanate/phenol/chloroform extraction

577 method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Poly(A)-tailed

578 RNA was isolated using a Pharmacia Kit (Pharmacia Biotech). A 2.5 kb fragment of

579 the *Ndg* cDNA (*cNdg5*, Stefan Baumgartner unpublished) was radioactively labelled

580 and hybridized to the Northern filter. Exposure time for Northern blots was 1.5 d. The

581 blot was re-probed with a *Drosophila 19S* probe to evaluate equimolar loading. The

582 *Ndg* blot shown in this work was identically conducted as those for *wing blister*

583 (*LanWb*) and *LanA* (Martin et al., 1999) allowing scarce comparison of the expression

584 levels of these different ECM genes.

585

Whole mount *in situ* hybridization

586

587 N- and C-terminal fragments from a full-length *Nidogen* cDNA clone (DGRC cDNA

588 clone LP19846; GenBank: BT031149.1) were PCR-amplified and sub-cloned into the

589 pCRII-TOPO vector with the TOPO TA Dual Promoter Kit (Invitrogen). Primer

590 sequences for a 571 bp N-terminal fragment were

591 GGACCCATCCATATCCCGCCACAAT and GCAATCAGTGCCACCTGGAAGGTGT

592 while CGTGGCATTGCCGTGGATCCCT and GGTGCATCCTGTGGAGGCGCT were

593 employed to amplify a 549 bp C-terminal fragment. Templates for probe synthesis

594 were generated by PCR using M13 primers supplied with the TOPO TA Dual

595 Promoter Kit. Digoxygenin (DIG)-labeled *sense* and *antisense* probes were made by

596 SP6/T7 *in vitro* transcription with the DIG RNA Labeling Kit (Roche Applied Science).

597 *In situ* hybridization on *Drosophila* embryos was performed according to (Lecuyer et

598 al., 2008) with modifications adapted from (Pfeifer et al., 2012). Sheep anti-DIG-AP

599 Fab fragments (1:4.000, Roche Applied Science), biotinylated donkey anti-sheep IgG

600 (1:400, Dianova), the Vectastain ABC Standard Kit (Vector Laboratories) and the

601 TSA Amplification Renaissance Kit (PerkinElmer) were used for fluorescence *in situ*

602 hybridization (FISH) detection. Specificity of the probes was tested by *in situ*

603 hybridization on *white[1118]* and *Ndg* deficient *Df(2R)BSC281* embryos, whereupon we

604 detected no differences between the N- and the C-terminal probes (not shown).

605

Fly stocks and genetics

606

17

607   Flies were grown under standard conditions (Ashburner, 1989) and crosses were

608   performed at room temperature or at 25 °C. Staging of embryos was done according

609   to (Campos-Ortega and Hartenstein, 1997). The following mutations and fly stocks

610   were used in this study: as control or wild-type stocks we employed *white^1118* (*w^1118*),

611   *w*; Kr^lf-1/CyO P{Dfd-EYFP}2, Oregon-R* or balanced sibling embryos. We used

612   *Df(2R)BSC281* as a deficiency for *Ndg* (*Df Ndg*), *Df(2L)Exel7022* which deletes the

613   two adjacent *Drosophila* type IV Collagen genes *viking* (*vkg*) and *Col4a1* (previously

614   referred to as *Cg25C*), *Df(3L)Exel8101* as deficiency for *Laminin A*, *Df(2L)TE35B-2*

615   as deficiency for *Laminin wing blister* (Gubb et al., 1985), *Df(3L)Exel6114* as *Laminin*

616   *B2* deficiency (*Df LanB2*) and *Df(1)Exel6230* that removes the *trol* locus but also the

617   adjacent segmentation gene *giant*. *Mi{ET1}MB04184*, *P{hsILMiT}* and *P{Δ2-3}99B*

618   were used to generate the *Ndg* deletion alleles *Ndg^Δ0.4* and *Ndg^Δ1.4* (Sup. Fig. 2, see

619   below). The null alleles *LanB2^knod* (Wolfstetter and Holz, 2012) and *wb^HG10* were

620   used as well as *Lar^13.2* that encodes for a truncated form of the Leukocyte-antigen-

621   related-like receptor (Krueger et al., 1996). Protein trap lines were: *vkg::GFP* (Morin

622   et al., 2001), *trol::GFP* (Medioni and Noselli, 2005), and *Jupiter::GFP* (Karpova et al.,

623   2006).

624

Generation of *Nidogen* deletions

625

626   The *pMiET1* transposon insertion *Mi{ET1}MB04184* (Metaxakis et al., 2005) was

627   used in an imprecise excision screen. Lethality reported for this line was not

628   associated with the *Mi{ET1}* insertion and homozygous viable, isogenic stocks were

629   established after ~5 generations of free recombination over a wild-type chromosome.

630   The position of the *Mi{ET1}* insertion, initially revealed by flanking sequence recovery

631   ((Bellen et al., 2011), GenBank: ET201740.1), was confirmed in the isogenic stocks

632   by inverse PCR (Ochman et al., 1988). Thereby, we detected a 35 bp deletion 141 bp

633   upstream of the insertion site that was present in the flanking sequence recovery

634   data (ET201740.1) and in *w^1118* DNA samples but not in the 6th GenBank release of

635   the *D. melanogaster* genome annotation (Hoskins et al., 2015) or the corresponding

636   sequence derived from *Oregon-R* genomic DNA. Therefore, we considered this small

637   deletion as a naturally occurring sequence variation in the *Ndg* locus. Before serving

638   as Minos transposase source in the screen, the *P{hsILMiT}* insertion (Metaxakis et

639   al., 2005) was remobilized from the *SM6a* balancer and inserted onto a *w^1118* X-

640   chromosome employing *P{Δ2-3}99B* as transposase source. *pMiET1* remobilization

641   was induced in the germ line of 2nd and 3rd instar larvae by daily 1 h heat shocks at

642   37 °C in the presence of the w^{1118}, $P\{hsILMiT\}$ helper chromosome. A total of 301

643   single excision events were identified due to the absence of the $Mi\{ET1\}$-associated

644    $Mmus/Pax6$-GFP expression (Berghammer et al., 1999) and screened for deletions

645   by single fly PCR analysis with the following primer combination:

646   GCCAAGGAATGGGAGTGCTCTGGAT   and

647   GGAGCCATCCTCGAACTCGTACAATT. Deletions were detected in two viable

648    $Mi\{ET1\}MB04184$ excision lines (henceforth referred to as $Ndg^{\Delta0.4}$ and $Ndg^{\Delta1.4}$).

649   Sequencing the molecular lesions uncovered a 1401 bp deletion (2R:10312780-

650   10314180) and a P-Element remnant of 17 nucleotides (TGCCACGTAGCCGGAAT)

651   in $Ndg^{\Delta1.4}$. In the case of $Ndg^{\Delta0.4}$ we detected a 417 bp (2R:10313549-10313965)

652   deletion and an insertion of 19 nucleotides (CGAGCAAAATACAAAATAC) at the

653   former P-Element insertion site (Sup. Fig. 2).

654

655   **Dextran permeability assay**

656   Wandering 3rd instar larvae were picked and relaxed by briefly dipping them into 60

657   °C water. Larval filets were dissected and incubated in 50 µl of 25 µg/ml anionic

658   Texas Red-coupled Dextran (10.000 MW, Thermo Fischer Scientific) in PBS for 10

659   minutes. The dextran solution was removed and the larval filets were rinsed once

660   with 50 µl PBS. Larval filets were fixed for 10 min in a drop of 4 % formaldehyde in

661   PBS and rinsed with PBS before mounting in Fluoromount-G and analysis under a

662   Zeiss LSM 800 confocal microscope.

663

664   **Larval climbing and gravity assay**

665   Matching numbers of flies were placed on food-filled vials and allowed to lay eggs for

666   three days. Pupae were examined at the pharate adult stage (7-9 days after egg

667   laying at 25 °C). To assay larval climbing performance, the distance between the

668   food surface and the cotton plug was divided into four zones. Pupae formed in each

669   zone were counted. Pupae positioned on the border between two zones were

670   assigned to the next higher zone. Pupae were further assigned into three categories

671   (1. Upright = 0° "head up" or 180° "head down" +/- 22.5°, 2. Tilted = 45° or 225° +/-

672   22.5°, and 3. Flat = 90° or 270° +/- 22.5°) according to their orientation along the

673   gravity axis.

674

Scanning electron microscopy (SEM)

Wandering 3rd instar larvae were dissected in PBS. After 3 h fixation in 2.5 % glutaraldehyde in PBS, samples were rinsed several times in PBS and dehydrated in an ascending ethanol series (50 %, 70 %, 80 %, 90 %, and 2x 100 % ethanol for 10 min each). After critical-point-drying in a Balzers CPD 030 at 40 °C, samples were mounted on stubs using double-sided adhesive tape and sputter-coated with a thin layer of gold (Balzers SCD 004). Samples were analyzed under a Zeiss DSM982 scanning electron microscope. Acceleration voltage was set to 3 kV. Settings for tilt angle, spot size, scanning mode and magnifications were kept constant throughout image acquisition.

Osmotic stress assay

Wandering 3rd instar larvae of indicated genotypes were picked and rinsed with PBS. The larvae were cut in half and the anterior part was inverted in order to expose the wing imaginal discs. After removing the anterior part of the gut and fat body, the samples were transferred to distilled water and the time until bursting of the first wing imaginal disc was measured. Measurements were stopped after 10 min and wing imaginal discs that were found intact after this period were assigned to the 10 min group.

Lethality tests

Parental flies (3-7 days old) of the indicated genotype were shifted on grape juice agar plates for 8 days. Agar plates were changed every 2nd day and defined numbers of eggs were transferred to fresh agar plates supplemented with dried yeast for further comparative analysis. Undeveloped eggs, hatched larvae, pupal cases and enclosure were counted daily. All plates were incubated at 25 °C and constantly humidified.

Larval feeding assay

Fifty freshly hatched 1st instar larvae were transferred to apple juice agar plates with a central spot of food-dye-supplemented yeast paste. Larvae moving outside the food were counted 1 and 2 h after the transfer. Fresh yeast paste was added every day and individual larvae were checked for the uptake of the dyed food after 72 h. The assay was performed at 25 °C and 60 % humidity conditions.

709

Larval locomotion assay

710 Flies were allowed to lay eggs for 2 h on apple juice agar plates supplemented with

712 yeast paste. Fresh yeast paste was added every day and individual 2nd instar larvae

713 were picked after 72 h (at 25 °C and 60 % humidity) and rinsed with distilled water. A

714 single larva was placed in the middle of a $\varnothing$ 52.5 mm Petri dish prepared with a $\varnothing$ 35

715 mm central arena (1 % agar in PBS) surrounded by a ring of high salt agar (3 M NaCl

716 in PBS) as locomotion barrier. For every recording a fresh agar plate was used.

717 Larvae were allowed to move freely in the central arena for 2-5 min respectively and

718 locomotion was recorded in a square of 12,5x9,5 mm^2 using a Zeiss AxioZoom.V16

719 stereo zoom microscope equipped with LED ring light and an Axiocam 503 color

720 camera. Time series were recorded with 4 frames/second employing ZEN Blue

721 software and 25 s subsets were further analyzed and edited with the manual tracking

722 plugin MTrackJ and the Fiji distribution of ImageJ (Schindelin et al., 2012).

723

Larval crawling assay

724 Larval crawling analyses were performed as described by (Eschbach et al., 2011) for

726 responses to vibrational stimulation and measurement of body length and stride

727 frequency. For the trials, 3rd instar larvae were placed on 1 % agarose covered Petri

728 dishes (60 mm diameter) as a crawling stage. This stage was placed in a silencing

729 foam covered box (57x57x47 cm^3) and illuminated by a red darkroom lamp

730 (Pf712em, Philips, 15 W and 7 lm). Video recordings of the trials were performed via

731 an infrared CMOS-Color-Camera (CCD-651, Conrad Electronics). The analogue

732 video signals were grabbed by using an analogue/digital video converter (700-USB,

733 Pinnacle Systems) and recorded via a computer for further analysis (25 fps).

734 Measurements of crawling speed, stride frequency and body length of the larvae

735 were performed by using the video tracking software Cabrillo Tracker (v4.91, Douglas

736 Brown, Open Source Physics, 2015). To quantify mean velocity and stride frequency

737 for five larvae per trial, the terminal ends were video-tracked for 10-20 s. Only periods

738 of unrestricted and constant crawling behavior were used for analysis. The mean

739 velocity was calculated from 5 s of crawling and the stride frequency from the

740 duration of 5 complete strides in a row. The maximum crawling speed of a single

741 stride was defined as the starting point for each measurement. To determine body

742 length reduction due to vibrational stimuli, apical and terminal ends of four larvae per

743 trial were video-tracked. The vibrational stimuli were generated via the audio edition

744 software Audacity (v2.05, Audacity-Team, GNU GPLv2+, 2015) at a frequency of

745 100 Hz and delivered via a full-range speaker (BPSL 100/7, Isophon/Gauder Akustik,

746 60-20.000 Hz and 7 W) to the crawling stage. For each trial, three stimuli with the

747 duration of 1 s and an acceleration of 12 m/s^2 were applied (interpulse-interval 9 s).

748

Statistical analyses

750 Prism v6.0 and v7.02 (GraphPad Software Inc.) was used for all statistical analyses.

751

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759

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766

Author contributions

768 **GW:** Investigation, methodology, conceptualization, visualization, data curation, formal

769 analysis, supervision, validation, writing original draft, writing review and editing.

770 **ID:** Investigation, visualization, formal analysis, validation and writing original draft.

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774 **DP:** Investigation, visualization and writing original draft.

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779 funding acquisition, project administration, writing original draft, writing review and editing.

780

Competing interests

782 The authors declare that they have no competing interests.

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1066

1067 **Figure Legends**

1068 **Figure 1: *Ndg* transcript expression and protein localization during embryonic**

1069 **development.** Confocal stacks of *Drosophila white* (w^{1118}) embryos at

1070 developmental stage 12 **(A-C)**, stage 14 **(D, E)** and stage 16 **(F-I)**. *Ndg* mRNA

1071 (green) was visualized by fluorescence *in situ* hybridization (FISH), and antibody

1072 staining against NDG (red) reveals the secreted protein. Embryos are orientated in

1073 lateral views except for B, G and I which represent ventral views. AS: amnioserosa,

1074 CC: chordotonal cap cell, Cho: chordotonal organ, DMC: dorsal median cell, eVM:

1075 esophageal visceral mesoderm, CN: chordotonal neuron, sMB: somatic myoblast.

1076 Asterisk and arrowhead in **(A)** depict *Ndg* expression in the head region and the

1077 dorsal mesoderm respectively. Scale bars = 50 µm.

1078

1079 **Figure 2: NDG localization in the absence of core BM proteins.** Stage 16

1080 embryos in lateral orientation were stained for NDG protein (green). **(A)** Control

1081 sibling embryo with balancer-associated YFP expression (red) displays proper NDG

1082 localization to BMs with strong accumulation at the chordotonal organs. **(B)** Punctate

1083 NDG pattern in a *LanA$^{9-32}$/Df LanA* transheterozygous embryo. **(C)** NDG protein

1084 distribution appears unaffected in *wb^{HG10}/Df wb* embryos. **(D)** Disruption of the NDG

1085 pattern in *LanB2^{knod}/Df LanB2* embryos. **(E)** *Df trol* (loss of Perlecan) embryos

1086 display a segmentation phenotype due to the additional loss of the adjacent *giant*

1087 locus but no changes in NDG localization. **(F)** Regular NDG distribution in an embryo

1088 deficient for the two *Drosophila* type VI Collagen encoding loci *Col4a1* and *viking*

1089 (*vkg*). Scale bar = 50 µm.

1090

1091 **Figure 3: BM formation in the absence of *Ndg*. (A-D)** Distribution of LanA, LanWb,

1092 COLL IV and PCAN in control and transheterozygous *Ndg$^{Δ1.4}$*/*Df Ndg* embryos **(E-H)**

1093 at stage 16. Embryos appear in in lateral **(D)**, dorsolateral **(A, B, E, F, H)** or

1094 ventrolateral orientation **(C, G)**. Scale bar = 50 μm.

1095

1096 **Figure 4: *Ndg* is required for permeability, ultrastructure and mechanical**

1097 **stability of BMs. (A, B)** Confocal sections from somatic muscle preparations of

1098 control **(A)** and *Ndg$^{Δ1.4}$* **(B)** larvae incubated with low weight TexasRed-coupled

1099 Dextran (TexRed-Dextran, white) to reveal BM leakage. Insets are higher

1100 magnifications of the highlighted areas. Confocal images were acquired with identical

1101 channel settings. **(C-F)** Visceral BM ultrastructure of dissected *w^{1118}* control **(C)** 3rd

1102 instar larvae and homozygous **(D, E)** and transheterozygous **(F)** *Ndg* mutants

1103 revealed by SEM. LVM: Longitudinal visceral muscle. Scale bars = 20 μm **(A, B)**, 5

1104 μm **(C-F)**.

1105

1106 **Figure 5: Loss of NDG affects BM stability under osmotic stress conditions.**

1107 Quantification of osmotic stress applied to 3rd instar larval wing discs of the indicated

1108 genotypes. Means and SDs (whiskers) are shown. Compared to *w^{1118}* and *w*; Kr^{If-}*

1109 *^{1}*/*CyO* balancer controls, imaginal discs from *Ndg* mutant animals resist osmotic

1110 stress for a significantly reduced mean time (*Ndg$^{Δ0.4}$*/*CyO* = 472 s vs. *Ndg$^{Δ0.4}$* = 213

1111 s, *Ndg$^{Δ1.4}$*/*CyO* = 520 s vs. *Ndg$^{Δ1.4}$*, 239 s; and *Df Ndg*/*CyO* = 407 s vs. *Ndg$^{Δ1.4}$*/*Df*

1112 *Ndg*, 241 s, n ≥ 20 wing imaginal discs). 1-W anova indicated significant differences

1113 among means (F$_{(7,188)}$ = 11.8, p < 0.001) and unpaired, two-tailed t-tests were

1114 applied to compare heterozygous controls and homozygous samples (***p < 0.001,*p

1115 = 0.017).

1116

1117 **Figure 6: Altered crawling behavior and neuromuscular junction phenotypes of**

1118 ***Ndg* mutant larvae. (A)** Schematic example of the observed distribution of *w^{1118}* and

1119 *Ndg$^{Δ1.4}$* 1st instar larvae (black dots) on agar plates (depicted as circles) outside a

1120 central food source (red). **(B)** Quantification of the larval distribution assay. Mean of

1121 larvae outside the food: *w^{1118}* = 1.44, *Ndg$^{Δ0.4}$* = 2.31, *Ndg$^{Δ1.4}$* = 19.1, *Ndg$^{Δ0.4}$*/*Ndg$^{Δ1.4}$*

1122 = 8, *Ndg$^{Δ1.4}$*/*Df Ndg* = 19.3; 1-W anova p < 0.001, Tuckey's multiple comparisons test

1123 with adj. p-value: *w^{1118}* versus *Ndg$^{Δ0.4}$* = 0.947 non-significant (n.s.), *w^{1118}* versus

1124 *Ndg$^{Δ0.4}$*/*Ndg$^{Δ1.4}$* = *p < 0.01, *w^{1118}* vs. *Ndg$^{Δ1.4}$* ***p < 0.001, *w^{1118}* vs. *Ndg$^{Δ1.4}$*/*Df Ndg* =

29

1125   ***$p < 0.001$, n = 400 larvae for each genotype. **(C, D)** Snapshots taken from crawling

1126   recordings of w^{1118} and $Ndg^{\Delta1.4}$ 2nd instar larvae. **(E, F)** Representative tracking

1127   patterns of crawling w^{1118} and $Ndg^{\Delta1.4}$ 2nd instar larvae. Individually colored lines

1128   represent recordings of 25 s. Time points are denoted if the larva left the monitored

1129   area before the end of recording. Time intervals in brackets indicate absence from

1130   the recorded area. **(G)** Quantification of mean crawling velocity measured as 5 s of

1131   uniform crawling from recordings of w^{1118} and $Ndg^{\Delta1.4}$ L3 larvae. Mean: w^{1118} =

1132   1.265, $Ndg^{\Delta0.4}$ = 1.07, $Ndg^{\Delta1.4}$ = 0.907 mm/s) (K-W anova $p < 0.0001$, Dunn's-test

1133   with mult. adj. p-value: $Ndg^{\Delta0.4}$ = 0.0597, $Ndg^{\Delta1.4}$ ***$p < 0.0001$ versus w^{1118}. **(H)**

1134   Quantification of the stride frequency calculated from 5 strides in a row from

1135   recordings of w^{1118} and $Ndg^{\Delta1.4}$ 3rd instar larvae. Mean: w^{1118} = 73.32, $Ndg^{\Delta0.4}$ =

1136   62.25, $Ndg^{\Delta1.4}$ = 47.75 strides/min.; K-W anova $p < 0.0001$, Dunn's-test with mult. adj.

1137   p-value: $Ndg^{\Delta0.4}$ *$p = 0.0207$, $Ndg^{\Delta1.4}$ ***$p < 0.0001$ versus w^{1118}. Error bars represent

1138   SEM. N ≥ 23 larvae for each genotype **(I, J)** Neuromuscular junctions (NMJs,

1139   outlined by dashed boxes) innervating muscles 6/7 in the 2nd larval abdominal

1140   segment of w^{1118} and $Ndg^{\Delta1.4}$ 3rd instar larvae are stained with anti-Bruchpilot (BRP,

1141   green) and anti-HRP (red). Scale bars = 1 mm (C, E), 50 μm (I).

1142

1143   **Figure 7: Loss of *Ndg* affects response to vibrational stimuli and PNS**

1144   **morphology. (A)** Body length recordings of w^{1117} control and $Ndg^{\Delta0.4}$ and $Ndg^{\Delta1.4}$

1145   mutant larvae exposed to pulsed vibrational stimuli (indicated by black bars). **(B)**

1146   Quantification of body length reduction induced by vibrational stimulation in w^{1118}

1147   control and *Ndg* mutant larvae. The relative body length reduction (Δ body length) is

1148   calculated from the mean larval length before and during vibrational stimulation. Error

1149   bars represent SEM, n = 24, **$p < 0.01$ ***$p < 0.001$ versus w^{1118}. K-W anova $p <$

1150   0.0001 and Dunn's-test with mult. adj. p-value was applied for statistical analysis.

1151 $Ndg^{\Delta0.4}$ vs. w^{1118} $p = 0.0061$, $Ndg^{\Delta1.4}$ $p < 0.001$ vs. w^{1118}. **(C, D)** Morphology of the

1152   lateral pentascolopidial chordotonal organ (lch5) of control and $Ndg^{\Delta1.4}$ larvae

1153   revealed by Jupiter::GFP fusion protein (green) expression, NDG- (blue), and HRP-

1154   (red) antibody staining. Arrows highlight the sensory cilia whereas neuronal cell

1155   bodies are marked by arrowheads. **(E, F)** HRP labeling was employed to reveal the

1156   nervous system in one hemisegment of control and $Ndg^{\Delta1.4}$ mutant larvae. Structures

1157   of the peripheral nervous system (PNS) such as ddA neurons (surrounded by circles)

1158   transmitting nerves (arrow) and neuronal cell bodies (asterisks) are highlighted. Scale

1159   bars = 50 µm (C), 100 µm (E).

1160

1161   **Figure 8: Genetic interaction between *Ndg* and *Lar*.** NMJs of 3rd instar larva

1162   stained with anti-Bruchpilot (BRP, green) and anti-HRP (red). **(A)** Control NMJ of

1163   muscle 6/7 in the 2nd abdominal segment. **(B)** *Ndg* mutant (*Ndg$^{\Delta 1.4}$*/*Df Ndg*) larvae

1164   exhibit increased and premature synaptic branching as well as increased bouton

1165   number and clustering. **(C)** Quantification of bouton densities (bouton number per

1166   area) of control and *Ndg$^{\Delta}$* larvae. **(D)** Heterozygous *Ndg$^{\Delta 1.4}$*/*CyO* larvae display mild

1167   branching defects and increased bouton clustering. **(E)** Heterozygous *Lar$^{13.2}$*/*CyO*

1168   NMJ with reduced branching and boutons. **(F)** *Lar$^{13.2}$, Ndg$^{\Delta 1.4}$*/*CyO* larvae exhibit an

1169   intermediate phenotype when compared to the single mutations (D and E). Scale bar

1170   = 20 µm.

1171

1172   ## Supplementary Figure Legends

1173   **Supplementary Figure 1: Developmental Northern blot analysis of *Ndg***

1174   **expression**. Total *mRNA* from adult flies (lane 1), embryos from 0-6 h, 6-12 h, 12-18

1175   h (lanes 2-4), 3rd instar larvae (L3, lane 5) and pupae was extracted and detected by

1176   an *Ndg*-specific probe. No signal was detectable up to 6 h of embryonic development

1177   as well as in L3 larvae and adult flies. Later embryonic stages and pupae exhibit *Ndg*

1178   *mRNA* expression with the strongest intensity around 12 to 18 h of embryonic

1179   development. 19S rRNA was used as loading control.

1180

1181   **Supplementary Figure 2: Generation of *Ndg* mutants. (A)** Schematic

1182   representation (created with (Hulo et al., 2008; Rambaldi and Ciccarelli, 2009)) of the

1183   NDG protein domain organization (top) and the *Ndg* locus (below) including the

1184   positions of the mobilized P-element *Mi{ET1}MB04184* and the obtained genomic

1185   deletions *Ndg$^{\Delta 1.4}$* and *Ndg$^{\Delta 0.4}$*. Protein domains and parts of the locus that encode for

1186   these domains are highlighted in matching colors. The position of the epitope

1187   employed to generate the NDG antibody (Wolfstetter et al., 2009) is indicated by a

1188   red line. NIDO: NIDO domain, EGF: EGF-like domain, Nidogen G2: Nidogen G2 β-

1189   barrel domain, LY: LDL-receptor class B (LDLRB)/YWTD repeat **(B-D)** Confocal

1190   stacks from dorsal views of stage 16 *Drosophila* embryos stained for NDG (red). GFP

1191   (green) depicts balancer-associated reporter gene expression in a sibling control.

31

1192   DAPI and auto fluorescence of yolk droplets appears in one channel (white). **(B)**

1193   Strong NDG staining of BMs in control embryos. **(C)** Absence of detectable NDG

1194   protein in embryos homozygous for the $Ndg^{\Delta 1.4}$ deletion. **(D)** Decreased NDG protein

1195   levels in a homozygous $Ndg^{\Delta 0.4}$ embryo. Confocal images were acquired with

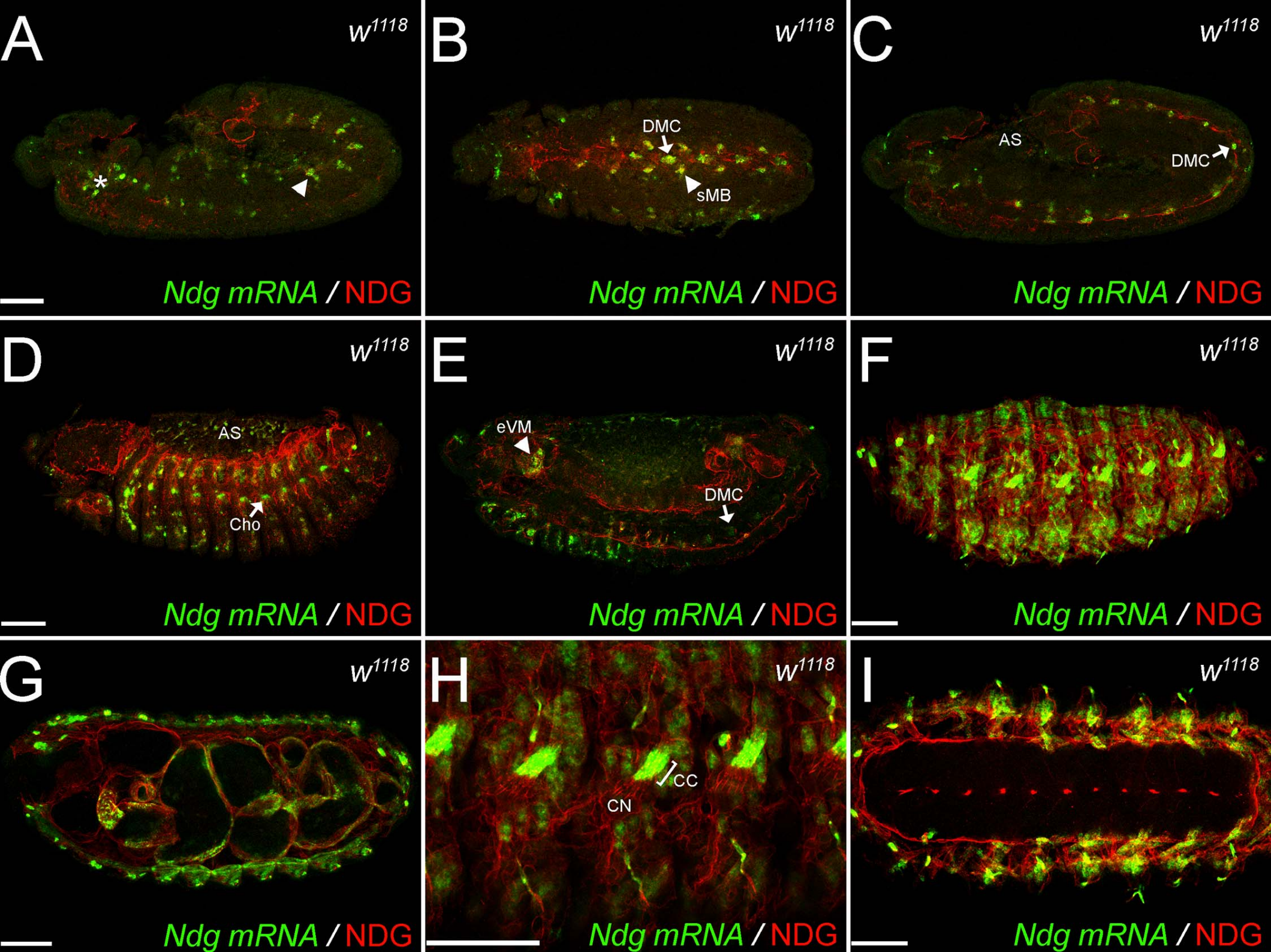1196   identical settings for the Cy3-detecting channel. Scale bar = 50 µm.

1197

1198   **Supplementary Figure 3: *Ndg* mutant larvae exhibit motility and gravity-sensing**

1199   **defects whereas *Ndg* mutant flies display decreased fecundity. (A)** Formation of

1200   pupal cases in vials of *white*^{1118} control and homozygous Ndg^{Δ} stocks (matching

1201   numbers of parental flies and breeding days at 25 °C). **(B)** Quantification of the

1202   climbing and orientation phenotypes. In comparison to the control, Ndg^{Δ} pupal cases

1203   are preferentially found in the lower half of the vials indicating impaired climbing

1204   performance (2-W anova and Tuckey's multiple comparisons test with adjusted p-

1205   values, *** = p < 0.001, **p = 0.005, non-significant (n.s.) = p > 0.05). In Ndg^{Δ} vials,

1206   the orientation of the pupal cases is significantly shifted towards the horizontal axis

1207   (2-W anova and Tuckey's multiple comparisons test with adjusted p-values, ***p <

1208   0.001, **p = 0.008, non-significant (n.s.) = p > 0.05). **(C)** Percentage of non-hatched

1209   eggs produced by 5 to 13 days old control and Ndg^{Δ} flies incubated at 25 °C. Ndg^{Δ}

1210   produce a significantly increasing number of non-hatched eggs over time, (2-W

1211   anova and Tuckey's multiple comparisons test with adjusted p-values, *** = p <

1212   0.001, ** = p < 0.01, * = p < 0.05 non-significant (n.s.) = p > 0.05). **(D)** Image of a

1213   control fly exhibiting properly inflated wings (n = 3492 animals). **(E)** Impaired wing

1214   inflation is observed at low penetrance in $Ndg^{\Delta 1.4}$ flies (140 of n = 2839 animals).

1215   Scale bar = 500 µm.

1216

1217   **Supplementary Figure 4: BM formation in the absence of *Ndg*. (A, B, C)** Antibody

1218   staining against LanKc, LanB1 or LanB2 labels BMs, and in the case of LanB1 and

1219   LanB2 additionally fat body and hemocytes of control and transheterozygous

1220 $Ndg^{\Delta 1.4}$/Df Ndg **(D, E, F)** embryos at stage 16. **(G, H, J, K)** A similar localization

1221   pattern was observed for *vkg::GFP* and SPARC in control and $Ndg^{\Delta 1.4}$/Df Ndg

1222   embryos. **(I, L)** *trol::GFP* localization in control and $Ndg^{\Delta 1.4}$/Df Ndg embryos.

1223   Embryos appear in lateral **(B, C, E, F, G, I, K, L)** or ventrolateral **(A, D, H, J)**

1224   orientation. Scale bar = 50 µm.

1225

1226 **Supplementary Figure 5: Heterozygous *Ndg^Δ* animals display a weak BM**

1227 **phenotype. (A-D)** BM ultrastructure of **(A)** *w**; *Kr^{If-1}/CyO* control 3rd instar larvae and

1228 **(B-D)** heterozygous *Ndg* mutants with indicated genotype revealed by SEM. LVM:

1229 Longitudinal visceral muscle. Scale bars = 5 µm.

1230

1231 **Supplementary Figure 6: Chordotonal organs display aberrant neuron**

1232 **morphology in the absence of NDG. (A)** Jupiter::GFP fusion protein (green)

1233 localizes to the microtubule network of the chordotonal organ in a *Jupiter-GFP* control

1234 larva. The chordotonal organ is surrounded by a NDG containing BM (blue) that also

1235 covers HRP-labelled neurons (red, arrowhead) and sensory cilia (arrows). **(B, C)** Two

1236 hemisegments of a *Jupiter-GFP; Ndg$^{Δ1.4}$* larva exhibit neuron detachment

1237 (arrowheads) and alignment defects of sensory cilia (arrow) in the lateral

1238 pentascolopidial chordotonal organ. Scale bar = 50 µm.

1239

1240 **Supplementary Movie:** Recordings of crawling patterns from *white^{1118}* control and

1241 *Ndg$^{Δ1.4}$* 1st instar larvae.

1242

Fig. 1
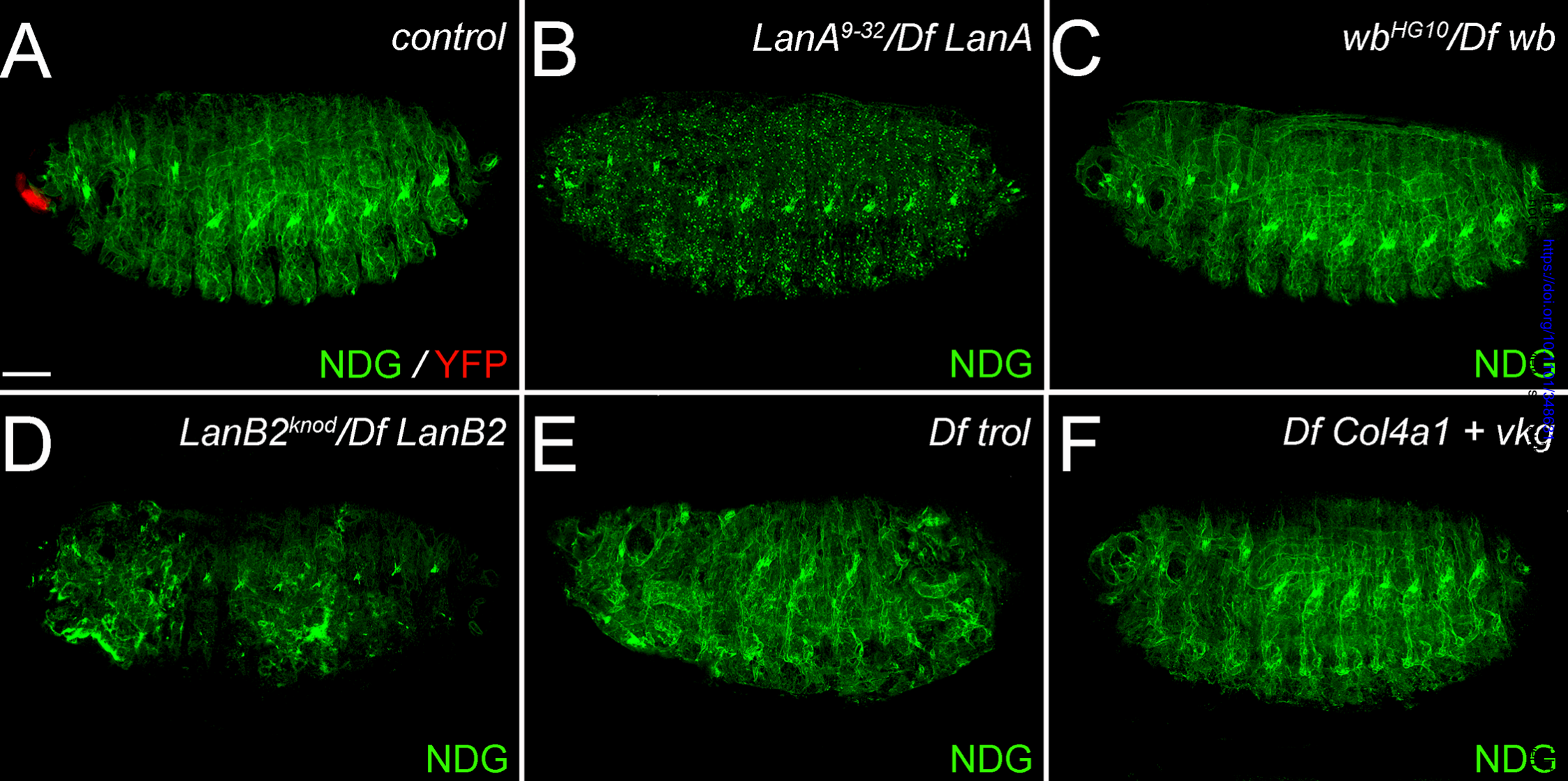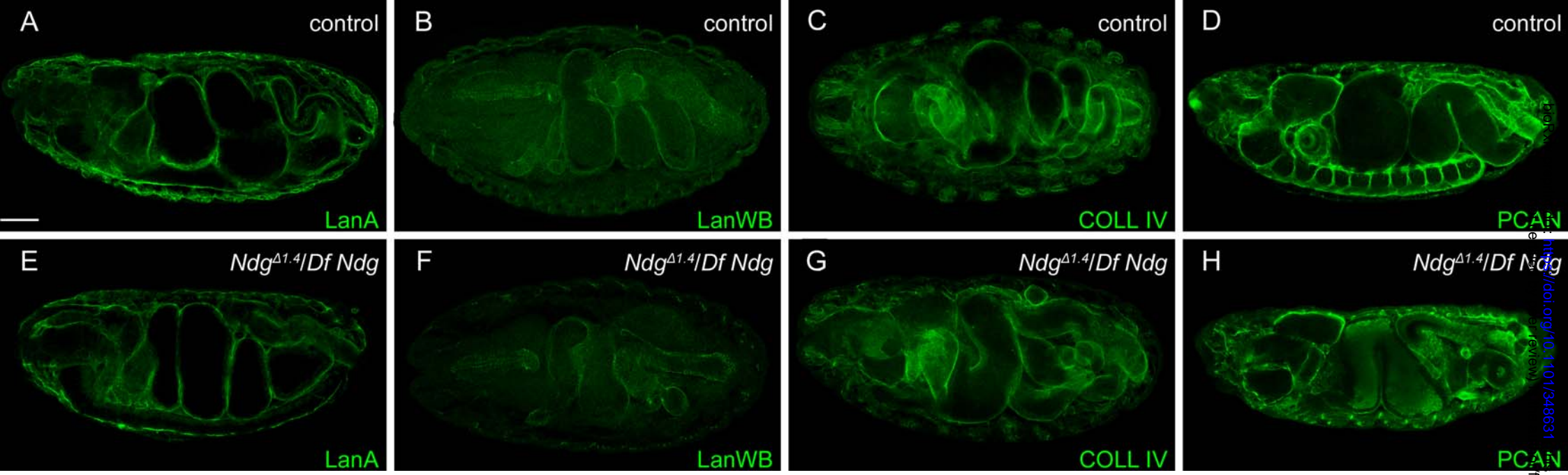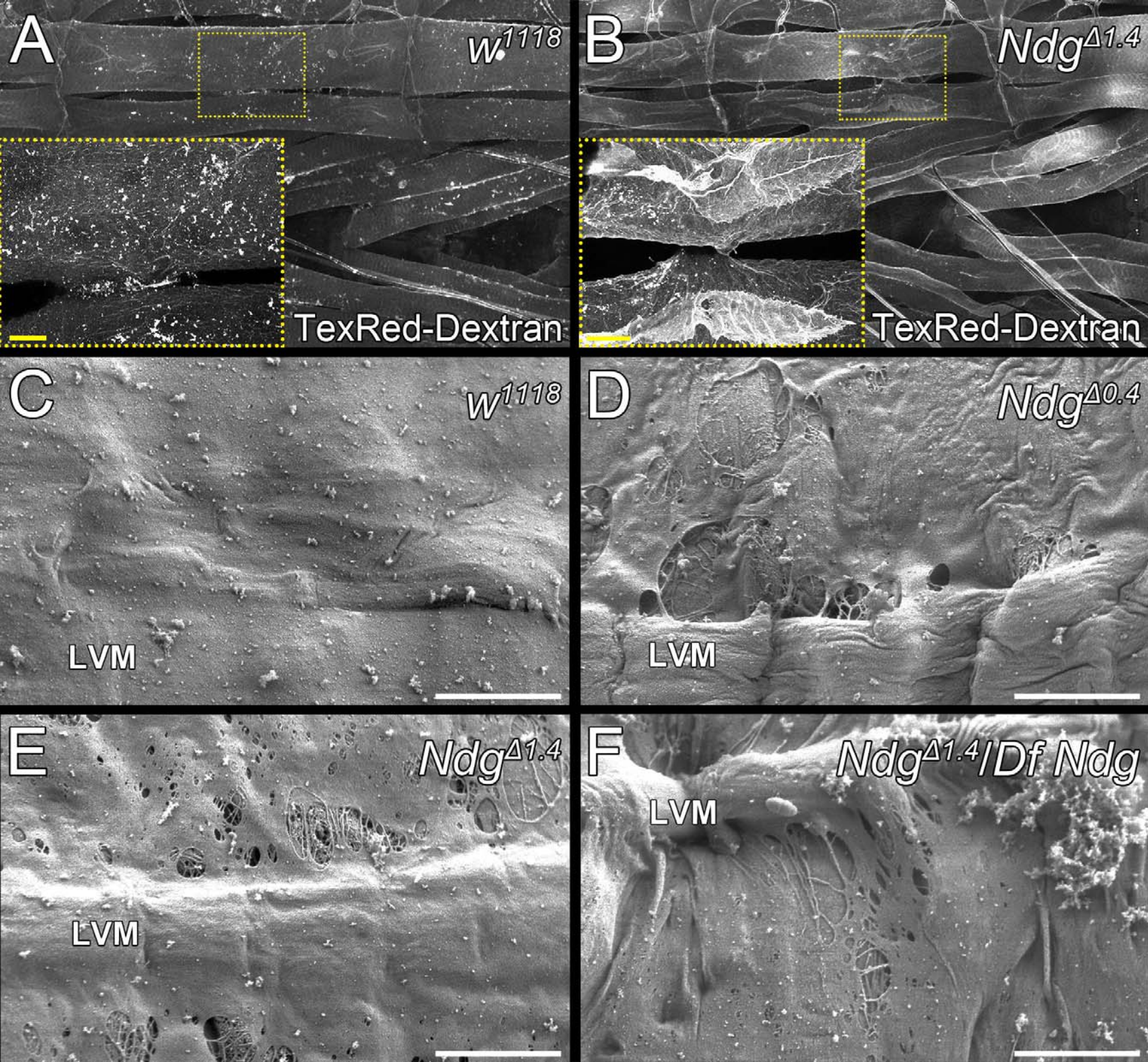
Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

BRP / HRP

A Pulse

w^{1118}

Ndg^{Δ0.4}

Ndg^{Δ1.4}

2 mm

10 s

B

Δ body length (%)

w^{1118} — *Ndg^{Δ0.4}* ** — *Ndg^{Δ1.4}* ***

C *Jupiter-GFP*

D *Ndg^{Δ1.4}; Jupiter-GFP*

GFP / HRP / NDG

E *w^{1118}*

F *Ndg^{Δ1.4}*

HRP

Figure 7

A — control — BRP / HRP

B — $Ndg^{\Delta 1.4}/Df\ Ndg$ — BRP / HRP

C

Boutons per 200 μm^2

*** ***

control (n=23) $Ndg^{\Delta 0.4}$(n=25) $Ndg^{\Delta 1.4}$(n=34)

D — $Ndg^{\Delta 1.4}/CyO$ — BRP / HRP

E — $Lar^{13.2}/CyO$ — BRP / HRP

F — $Lar^{13.2}, Ndg^{\Delta 1.4}/CyO$ — BRP / HRP

Figure 8

Figure S1

A

NIDO Nidogen G2 EGF EGF EGF EGF EGF EGF EGF LY LY LY LY

100 aa

Mi{ET1}MB04184

500 bp

Ndg$^{\Delta 1.4}$

Ndg$^{\Delta 0.4}$

B control

NDG / GFP / DAPI

C *Ndg*$^{\Delta 1.4}$

NDG / DAPI

D *Ndg*$^{\Delta 0.4}$

NDG / DAPI

Figure S2

A

B

C

D — control

E — $Ndg^{\Delta 1.4}$

Figure S3

A *w*; Kr^{lf-1}/CyO*

LVM

B *Ndg^{Δ0.4}/CyO*

LVM

C *Ndg^{Δ1.4}/CyO*

LVM

D *Df Ndg/CyO*

LVM

Figure S5

A — *Jupiter-GFP*

GFP / HRP / NDG

B — *Ndg[Δ1.4]; Jupiter-GFP*

C — *Ndg[Δ1.4]; Jupiter-GFP*

Figure S6