Article

Structural molecular components of Septate Junctions in cnidarians point to the origin of epithelial junctions in Eukaryotes

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Running title: SJs in Cnidarians

24 **Abstract**

25 Septate junctions (SJs) insure barrier properties and control paracellular diffusion of
26 solutes across epithelia in invertebrates. However, the origin and evolution of their molecular
27 constituents in Metazoa has not been firmly established. Here, we investigated the genomes of
28 early branching metazoan representatives to reconstruct the phylogeny of the molecular
29 components of SJs. Although Claudins and SJ cytoplasmic adaptor components appeared
30 successively throughout metazoan evolution, the structural components of SJs arose at the
31 time of Placozoa/Cnidaria/Bilateria radiation. We also show that in the scleractinian coral
32 *Stylophora pistillata*, the structural SJ component Neurexin IV (StpNrxIV) co-localizes with
33 the cortical actin network at the apical border of the cells, at the place of SJs. We propose a
34 model for SJ components in Cnidaria. Moreover, our study reveals an unanticipated diversity
35 of SJ structural component variants in cnidarians. This diversity correlates with gene specific
36 expression in calcifying and non-calcifying tissues, suggesting specific paracellular pathways
37 across the cell layers of these diploblastic animals.

38

39

Introduction

A unifying characteristic of metazoans evolution has been the building of joined layers of cells that form a physical barrier between the environment and the inner body, or between different compartments within the body. Desmosomes and adherens junctions insure mechanical binding between cells forming the epithelia, whereas occluding junctions seal and control paracellular transport across the epithelial layer. Two structurally different types of occluding junctions have been characterized, the Tight Junction (TJ) and the Septate Junction (SJ) (Banerjee, Sousa, et al. 2006; Magie and Martindale 2008).

Multiple studies have investigated ultrastructure and molecular composition of TJs and SJs in bilaterians. TJs appear restricted to chordates and form circular strands around the apical cell border joining together two adjacent plasma membranes (Shen, et al. 2011). In protostomes, SJs are the predominant occluding junctions typically arranged in a spiral manner around the cell lateral border forming large macromolecular complexes that span the extra-cellular space between two neighboring cells. In transmission electron microscope (TEM) cross-sections images, SJs display characteristic electron-dense ladder-like structures of 10-20 nm width called septa (Tepass, et al. 2001). SJs are also found in mammals, at the nodes of Ranviers where they form the paranodal junction between axons and myelinated glial cells (Hortsch and Margolis 2003; Poliak and Peles 2003; Nans, et al. 2011). In non-bilaterians, cell-cell junctions have been structurally investigated using different electron microscope techniques in the diverse phyla composing early branching metazoans, i.e. Cnidaria, Placozoa, Porifera, and Ctenophora. In cnidarians, both medusozoans and anthozoans possess belt junctions referred to as SJs that form a belt around the apical circumference of the cell, although the "*Hydra* type" (Hydrozoa) and the "Anthozoan type" (Actiniaria) of SJs were shown to slightly differ structurally (Wood 1959; Filshie and Flower 1977; Green and Flower 1980). In Placozoa, very little is known. In the ventral epithelium of *Trichoplax adhaerens* (*Trichoplax*), apical belt desmosomes with proximal "periodic connection" of intercellular material joining two adjacent cells have been noted (Ruthmann, et al. 1986). Although this is reminiscent of the SJ ladder structure, it awaits further clarification. Porifera encompass four distinct taxonomic classes, (Philippe, et al. 2009; Sperling, et al. 2009; Erwin, et al. 2011) with differences in junction depending on the class. No clear SJ were described in the more distant Hexactinellida or Demospongiae (Leys, et al. 2009). In Homoscleromorph, the presence of SJ is uncertain as the presence of septa is unclear (Leys, et al. 2009; Gazave, et al. 2010). In fact, the only clear report of SJ in Porifera was made in calcareous by Ledger (1975). In this study, the authors could show an electron dense ladder

74 between the spicule-secreting sclerocytes *Sycon ciliatum*, although electron dense junctions
75 with no visible septa were described in *Sycon coactum* (Eerkes-Medrano and Leys 2006).
76 Ctenophores share a very similar unique junctional structure, where epithelial cells are linked
77 with distinctive belt junction (see supplementary Figure S8). The junctional membranes are 2-
78 3 nm apart but they do not fuse nor are they linked by septa (Hernandez-Nicaise, et al. 1989;
79 Hernandez-Nicaise 1991).

80 At the molecular level, most functional studies rely on mammalian and insect model
81 organisms. Components of TJs and SJs can be sub-divided into inter-cellular structural and
82 cytosolic scaffolding/polarity proteins (see Table 1). While the structural transmembrane
83 proteins mediate cell-cell adhesion, the cytosolic junction plaque contains various types of
84 proteins that link the junction transmembrane proteins to the underlying cytoskeleton.

85 The structural components of *Drosophila* SJs and human axo-glial SJs consist of a
86 core complex of three cell-adhesion molecules (Hortsch and Margolis 2003). These are
87 Neurexin IV (NrxIV), Contactin (Cont), and Neuroglian (Nrg) in *Drosophila* corresponding
88 respectively to Caspr, Contactin, and Neurofascin in mammals. Loss of any one of these
89 proteins in either *Drosophila* or Mouse disrupts SJ formation and function, and cellular
90 trafficking of these proteins to SJs is interdependent (Baumgartner, et al. 1996; Boyle, et al.
91 2001; Genova and Fehon 2003; Faivre-Sarrailh, et al. 2004; Banerjee, Pillai, et al. 2006;
92 Bonnon, et al. 2007; Thaxton, et al. 2010; Tiklova, et al. 2010; Banerjee, et al. 2011). In both,
93 *Drosophila* and mammalian SJs, NrxIV/Caspr associates with Cont/Contactin in *cis* and with
94 Nrg/Neurofascin in *trans* (Hortsch and Margolis 2003). Further trans-membrane proteins have
95 been characterized to be required for SJ formation and/or function in *Drosophila.* Three
96 Claudin-like proteins, Megatrachea, Sinuous and Kune, are essential for SJ (see below) (Behr,
97 et al. 2003; Wu, et al. 2004; Nelson, et al. 2010). The Na+K+ ATPase subunits alpha and beta
98 are also necessary for SJs in a pump-independent function (Genova and Fehon 2003; Paul, et
99 al. 2003; Paul, et al. 2007; Krupinski and Beitel 2009). Other proteins have been characterized
100 as part of SJs, including Lachesin, Fasciclin III, Macroglobulin complement-related ((Woods,
101 et al. 1997; Llimargas, et al. 2004; Narasimha, et al. 2008; Batz, et al. 2014), see also (Hall, et
102 al. 2014) for a recent exhaustive listing).

103 Structural components of vertebrate TJs are members of the tetraspan family, i.e
104 Claudins, Occludin, and Tricellulin, and members of the immunoglobulin superfamily, i.e.
105 JAM1-3 (junctional adhesion molecules), ESAM (endothelial cell-selective adhesion
106 molecule), and CAR (coxsackie- and adenovirus receptor) (Furuse 2010). Claudins are the
107 focus of an abundant literature as they probably represent the principle TJ barrier-forming

108 proteins in vertebrates (Van Itallie and Anderson 2006; Shen, et al. 2011). The human
109 Claudins1-27, here referred to as Claudins *sensu stricto* (Claudins s.s.), represent a family of
110 small (20–27 kDa) highly specialized proteins predicted to have four transmembrane helices
111 (tetraspan), with two extracellular loops (EL1 and EL2). EL1 is characterized by a conserved
112 W-GLW-C-C amino acid (AA) motif. Claudins s.s. have been shown to interact with each
113 other in *cis* on the plasma membrane and in *trans* forming kissing complexes in the
114 paracellular space (Krause, et al. 2008). Moreover, several Claudin-like proteins sharing the
115 very same tetraspan topology and W-GLW-C-C signature motif have been identified,
116 although their function is much less understood. These further include human Claudin Like1-
117 2, lens fiber membrane intrinsic protein isoform 2 (Lim2), epithelial membrane proteins
118 (EMP1-3), and peripheral myelin protein 22 (PMP22), for which adhesive or barrier-forming
119 properties have been described (Van Itallie and Anderson 2006; Günzel and Alan 2013).
120 Additionally, more distantly related Claudin-like proteins with a similar structure, but with a
121 less conserved signature motif, have been identified. Among these are human Lipoma
122 HMGIC fusion partner (LHFP1-4) proteins, Clarins3, and uncharacterized protein C16orf52
123 (CPO52), for which a function remains to be fully clarified (Huang, et al. 2004; Geng, et al.
124 2009). In invertebrates, Claudin-like proteins with a similar tetraspan topology are also found,
125 although their relation to the vertebrate Claudins is unclear. In *Drosophila*, of the eight
126 Claudin-like proteins referenced in the genome, only three were shown to be required for the
127 formation and barrier function of SJs. These are Megatrachea, Sinuous, and Kune, all of
128 which carry the W-GLW-C-C signature. Furthermore, deletion of any of these genes impairs
129 SJ formation (Behr, et al. 2003; Wu, et al. 2004; Furuse and Tsukita 2006; Nelson, et al.
130 2010). Megatrachea co-immunoprecipitates with NrxIV, Nrg, and Cont, among others
131 (Jaspers, et al. 2012), showing that at least some of the invertebrate Claudin-like proteins
132 participate in SJs. Nonetheless, despite the identification of many Claudin-like proteins in
133 diverse invertebrate phyla, their phylogeny, association with SJs, and/or function remains to
134 be clarified.

135 Although SJs and TJs show striking differences in their respective structural
136 components, the cytosolic adaptor proteins responsible for their assembly and maintenance at
137 the plasma membrane appear to share, in part, similar machineries. MAGUK proteins are
138 evolutionary conserved scaffolding proteins that create and maintain multi-molecular
139 complexes, such as adherens and occluding junctions, at distinct subcellular sites like the
140 cytoplasmic surface of the plasma membrane for instance (Ikenouchi, et al. 2007; de
141 Mendoza, et al. 2010). In *Drosophila*, members of the MAGUK protein superfamily,

including Discs Large (Dlg), Zona Occludens (ZO), Varicose (Vari), and Stardust (Std) are necessary for epithelial polarity and scaffolding of SJs (Woods, et al. 1996; Bachmann, et al. 2001; Jung, et al. 2006; Moyer and Jacobs 2008). Similarly, the mammalian MAGUK proteins Discs Large (Dlg), Zona Occludens (ZO), MPP5 (Pals1), and MPP7 are also part of the cytosolic adaptors involved in TJ formation (Roh, et al. 2003; Van Itallie and Anderson 2006; Stucke, et al. 2007; Fanning, et al. 2012; Su, et al. 2012). ZO1 and ZO2 have been shown to bind several Claudins s.s. (Van Itallie and Anderson 2006). Likewise, the cytosolic FERM domain protein Coracle (Cora) binds to the cytoplasmic domain of NrxIV in *Drosophila* SJs, and its homologs in vertebrates (Band 4.1) participate in TJ formation (Fehon, et al. 1994; Lamb, et al. 1998; Ward, et al. 1998; Mattagajasingh, et al. 2000; Denisenko-Nehrbass, et al. 2003; Jensen and Westerfield 2004; Laprise, et al. 2009; Xia and Liang 2012). Finally, the Na^+/K^+ ATPase alpha- and beta-subunits (ATPalpha and Nervana2) have repeatedly been associated with both TJs and SJs although the specific function of this transporter in junctions is unclear (Paul, et al. 2003; Rajasekaran, et al. 2005; Laprise, et al. 2009; Vagin, et al. 2012).

Outside Bilateria, several studies have identified members of the structural and scaffolding SJ molecular components in early branching Metazoa. However, these data appear contradictory and incomplete (Chapman, et al. 2010; Fahey and Degnan 2010; Leys and Riesgo 2012). For example, Claudins appeared in Porifera (Leys and Riesgo 2012), in Cnidaria (Chapman, et al. 2010), or in Bilateria (Fahey and Degnan 2010). One homolog to NrxIV and Cont was present in both Porifera and Cnidaria (Chapman, et al. 2010) or absent in Porifera and noted as “aberrant” in Cnidaria (Fahey and Degnan 2010). In a more recent analysis by Suga and coworkers (Suga, et al. 2013), NrxIV was present in Trichoplax, cnidarians and bilaterians. In contrast Cont was specific to bilaterians, whereas Riesgo et al (2014) identified a Cont homolog in poriferans. Homology criteria may have been misaddressed and/or intermediate evolutionary precursor may have been missed. Thus, despite SJs having been structurally characterized already three decades ago, their gene complement, respective diversification, and evolution in early branching metazoans remains elusive. In other words, how and when body compartmentalization has arisen in Metazoa is still a controversial question.

In order to gain molecular insight into cnidarian SJs, we initiated the characterization of their principal molecular components in three different cnidarian representatives (i.e. the scleractinian coral *Stylophora pistillata*, the actiniarian *Nematostella vectensis*, and the medusozoan *Hydra magnipapillata*). We monitored expression and localization of key SJ

176 proteins in the coral *S. pistillata*, a tractable species for calcification studies. After having
177 defined the principal members of SJs in Cnidaria, we extended our genomic search to the
178 other representatives of the early branching metazoans (i.e. the placozoan *Trichoplax*
179 *adherens*, the homoscleromoph *Oscarella carmella*, the demosponge *Amphimedon*
180 *queenslandica*, and the ctenophore *Mneniopsis leidyi*) as well as the unicellular organisms
181 suspected to be at the origin of the metazoan lineage (i.e. the choanoflagellate *Monosiga*
182 *brevicollis* and the filasterean *Capsaspora owczarzaki*). Our analysis also includes
183 evolutionary related gene families (not necessarily functionally related) to apprehend SJ gene
184 evolution. The present study aims at providing a comprehensive repertoire of the components
185 involved in sealing epithelia of early metazoans as well as to reconstruct the stepwise
186 evolution of SJs in invertebrates that preceded the formation of TJs in chordates.
187

Results

Genomes representing several classes from the phylum Cnidaria are available, e.g. *Nematostella* (*N. vectensis*, Anthozoa, Actiniaria), *Acropora* (A. *digitifera*, Anthozoa, Scleractinia, complex clade), and *Hydra* (*H. magnipapillata*, Medusozoa, Hydrozoa) (Putnam, et al. 2007; Chapman, et al. 2010; Shinzato, et al. 2011). Additionally, several other cnidarian genome projects are ongoing, e.g. *Stylophora* (*S. pistillata*, Anthozoa, Scleractinia, robust clade), a reef building coral which benefits from numerous ecological and physiological studies (Allemand, et al. 2011; Tambutté, et al. 2011). The draft genome as well as the transcriptome (adult stage) of *Stylophora* is now completed (C.R.V. and M.A. personal communication, (Liew, et al. 2014)) and available for targeted gene identification and characterization. Starting from the protein set of the principal components of occluding junctions characterized in human (TJ) and *Drosophila* (SJ), we identified the complete set of genes encoding for occluding junction homologs in the cnidarian representatives (including *Stylophora*) as well as in other non-bilaterian representatives *Trichoplax* (*T. adherens*, Placozoa), *Amphimedon* (*A. queenslandica*, Demospongiae), *Oscarella* (*O. carmella*, Homoscleromorpha), and *Mnemiopsis* (*M. leidyi*, Ctenophore) plus the unicellular organisms *Monosiga* (*M. brevicollis*, Choanoflagellata) and *Capsaspora* (*C. owczarzaki*, Filasterea), all for which a complete genome is available (King, et al. 2008; Srivastava, et al. 2008; Srivastava, et al. 2010; Ryan, et al. 2013; Suga, et al. 2013).

Early branching metazoans encode SJ, but not TJ, components

Transcriptome and genome data mining was based on BLAST (bilaterian query sequences against non-bilaterian databases) and reciprocal BLAST (non-bilaterian candidate sequences against bilaterian databases) approaches (Supplementary Figure S3). The search was performed in an iterative manner, first targeting cnidarians and then extended to include the other phyla of interest. In addition to homology approaches, based on reverse BLAST against human and *Drosophila*, we used domain composition (SMART) and phylogenetic trees (PhyML and Bayesian) to identify and name homologs of known occluding junction components (our terminology followed the *Drosophila* nomenclature). Table 1 summarizes the presence/absence of homologs across non-bilaterians. None of the TJ structural components characteristic of chordates were found in non-bilaterians. However, all SJs components that we searched for were present in cnidarians, often in multiple copies. In the other phyla, the range of SJ component homologs was variable with a correlating trend of fewer homologs/copies and organismal simplicity.

222 *i) Claudins*

223 Human Claudin 1-27 (Claudins s.s.) homologs were not found. However, iterative
224 search with bilaterian Claudin-like sequences identified a variable number of Claudin-like
225 homologs in the different representative species of early branching metazoans as well as
226 protists (Table 1). Profile based search against the PFAM database confirmed that all
227 belonged to the PMP22_Claudin (PF00822), Claudin_2 (PF13903), or L_HGMIC_fpl
228 (PF10242) domain family, except for 3 of the Claudins identified in *Oscarella* (OcaClau4,5,8)
229 (Supplementary Table S1). Transmembrane domain prediction confirmed that all sequences
230 were tetraspan proteins (data not shown) with a larger EL1 (50.8 AA +/- 16.5) than EL2 (19.8
231 AA +/- 8.0) (Supplementary Table S1). The Claudin signature motif within EL1 appeared
232 slightly modified (i.e. W-G[LVI][WFYL]-C-C), except for a few cases. Bayesian and
233 Maximum Likelihood methods gave incongruent albeit comparable phylogenetic trees, i.e.
234 several well supported groups could be outlined using both methods (Figure 1, Supplementary
235 Figure S5a). Use of an alternative alignment method (MUSCLE) prior to phylogenetic
236 analyses supported the same groups (Supplementary Figure S5a). Group Ia contains
237 anthozoan Claudins AS1,2 with human Claudin domain-containing protein 2 (HsClauL2) and
238 lens fiber membrane intrinsic protein isoform 2 (HsLIM2), and group Ib contains *Hydra*
239 Claudins 4,7,8,10,12,14,15 with human epithelial membrane protein 1-3 (HsEMP1-3) and
240 peripheral myelin protein 22 (HsPMP22). Of note, the *Stylophora*, *Acropora*, and
241 *Nematostella* Claudin AS1 and AS2 proteins corresponded to two splice variants conserved in
242 anthozoans which vary in their first exon, and consequently in their first ~80 AA. This gave
243 rise to two Claudins differing in their EL1. Group II corresponds to homologs of the human
244 TMP211 and LHFP family of which some members have been involved in ear hair cell
245 formation (Xiong, et al. 2012). This large group contains Claudin members from *Drosophila*
246 (CG3770, CG12026), cnidarians (anthozoan Clau3-6, Hydra Clau4,5), homoscleromorph
247 (OcaClau1,2), and the placozoan and ctenophore Claudins (TriClau and MleClau1-4,
248 respectively). Group III (*Drosophila* CG14182, anthozoan Clau8-9, *Oscarella* Clau8,
249 *Monosiga* Clau8, *Capsaspora* Clau1), IV (anthozoan Clau2, *Oscarella* Clau3,4) and V
250 (anthozoan Clau7, *Oscarella* Clau5) comprise homologs of the human CPO52
251 (uncharacterized protein C16orf52), Clarin3 and TMP127, respectively, for which a function
252 has not yet been determined. Other Claudins sequences (for example MonoClauA,B,C or
253 amphiClau) could not be reliably positioned on the tree, inferring that the Claudin primary
254 sequences have considerably diverged during evolution. The human Claudins s.s. have been
255 recently subdivided into 5 subgroups (Gunzel and Fromm 2012). Two members from each

256 subgroup were randomly selected (HsCLDN1,2,3,8,11,12,16,18a,21,23) as representatives of
257 the human Claudins s.s. and included in our phylogenetic analysis. These human TJ specific
258 Claudins clustered as a single outgroup. Interestingly, the three *Drosophila* Claudin-like
259 proteins Megatrachea, Sinuous, and Kune, for which functional characterizations are
260 available, also clustered outside our 5 groups.

261 *ii) Neurexins*

262 The *Drosophila* Neurexin IV (NrxIV) and human Caspr family of proteins are closely
263 related extracellular ligands with parallel domain architecture (i.e. LamG, EGF and FBG).
264 One clear homolog of NrxIV/Caspr was found in cnidarians (NRX1), displaying the same
265 domain architecture, except for a missing NH2term FA58C domain (Figure 2A and
266 supplementary Figure S5b). In *Stylophora* NRX1, domain homology search using SMART
267 revealed a Band 4.1 binding motif. The presence of this motif indicates that StpNRX1
268 potentially binds to the putative Cora/Band 4.1 homolog as known from bilaterians. NRX1
269 was found to be duplicated in *Nematostella* (NvNRX1-2), *A. digitifera* (AdiNRX1-2), and
270 *Hydra* (HydNRX1-3). In addition, several extra copies for cnidarian NRX (StpNRX2-5,
271 AdiNRX3-5, NvNRX3-6, HydNRX4-5) were found, with missing domains and/or long
272 intracellular portions in comparison to the *bona fides* NRX1 homologs. Within the
273 phylogenetic tree, the position of these supernumerary homologs within non-bilaterians NRX
274 suggests duplication within the cnidarian lineage. StpNRX2 did not cluster with any
275 anthozoan homolog, suggesting that it may be either specific to *Stylophora* or the robust clade
276 of scleractinian corals, since it is found in *Acropora* (complex clade).

277 In *Trichoplax* we identified 5 potential NrxIV homologs, representing placozoan
278 specific duplications, showing variable domains composition, except for TriNRX1 which
279 harbor canonical Nrx domain composition. No NrxIV/Caspr homologs were found in the
280 remaining analyzed phyla. However, homologs of the more distant gene family, human
281 Neurexin 1-3 (HsNeu1-3) and *Drosophila* Neurexin 1 (DmNeu1) were present. Neurexins are
282 synaptic cell adhesion molecules in bilaterian composed of alternating LamG and EGF
283 domains (Bang and Owczarek 2013), similar to the NrxIV/Caspr, but without the FBG
284 domain (Figure 2A). One clear Neurexin1 homolog (see Supplementary Table S1 for reverse
285 BLAST hits) was found in *Oscarella* (but not in Amphimedon, supplementary Figure S6) and
286 in *Mnemiopsis*, with the same domain architecture as in bilaterians. Of note, omission of the
287 bilaterian Neurexin protein sequences placed the OcaNeu and MleNeu sequences at the base
288 of the cnidarian/placozoan NRX phylogeny (data not shown). Several conserved Neurexin
289 homologs were also found in cnidarians, although here, they were substantially shorter: 2

290 LamG and 1 EGF domain, instead of 6 lamG and 3 EGF domains in the canonical form.
291 Moreover, one putative Neurexin1 homolog was found in *Capsaspora* (also identified in
292 (Suga, et al. 2013)), which is composed of 6 LamG domains (Figure 2A) and positions in
293 between the Neu and Nrx families in the phylogenetic tree (see Radial representations in
294 Supplementary Figure S4), potentially representing the metazoan Neu-Nrx ancestor.

295 *iii) NRG, CONT, DSCAM*

296 The *Drosophila* Nrg and Cont, and the human Neurofascin and Contactin have closely
297 related domain structures, i.e. succession of Ig domains followed by FN3 domains. However
298 Nrg/Neurofascin has a Cterm transmembrane (TM) domain spanning the plasma membrane,
299 whereas Cont/Contactin is attached to the membrane via a GPI anchor. Both Nrg and Cont
300 have homologs in cnidarians displaying similar domain architecture as well as TM and GPI
301 anchor attachment, respectively (Figure 2B and Supplementary Figure S5b). In comparison to
302 *Nematostella*, the scleractinians *Stylophora* and *Acropora* have additional NRG copies
303 (StpNRG2, AdiNRG2,3). Their position within the phylogenetic tree indicates that they
304 represent scleractinian specific duplications. In *Trichoplax*, 4 NRG and 1 CONT homologs
305 were also found, likewise with placozoan-specific duplications (Figure 2B). Note that
306 differentiation between the *Trichoplax* NRGs and CONT was solely based on the TM/GPI
307 anchor prediction. In *Oscarella*, 1 potential NRG homolog (OcaNRGCAM) could be found.
308 However, based on the reverse BLAST hit approach, this protein could either be a NRG or a
309 DSCAM homolog. Indeed, NRG/CONT share a very similar domain composition with other
310 Ig/FN3 domain adhesion molecules such as Hemicentin, and DSCAM (Down Syndrome Cell
311 Adhesion Molecule), the latter being the closest relative of NRG/CONT. DSCAM are
312 extracellular ligands capable of homophilic associations and heterophilic interactions
313 involved in neural wiring in bilaterian as well as innate immunity in protostomes (Schmucker
314 and Chen 2009). We thus undertook a characterization of DSCAM proteins in the different
315 early branching metazoan to estimate the evolutionary convergence of the NRG/CONT and
316 DSCAM families. DSCAM homologs were identified in *Mnemiopsis* (1), *Oscarella* (2),
317 *Trichoplax* (1), *Hydra* (2) and anthozoans (2), but neither in *Amphimedon* nor in protists
318 (Figure 2B). With respect to domain architecture, the cnidarian DSCAM1 resembled the
319 bilaterian DSCAMs, whereas the cnidarian DSCAM2, TriDSCAM, OcaDSCAM and
320 MleDSCAM showed higher similarity to the NRG/CONT architecture, despite being closer to
321 DSCAM at the sequence level. In line with these finding, the OcaNRGCAM protein
322 represents an evolutionary intermediate between the two families (see supplementary Figure
323 S4).

324 *iv) MAGUK*

325 Members of the MAGUK super family share a central PDZ-SH3-GuKc domains
326 module. The various MAGUK members essentially differ by the addition of other domains,
327 commonly PDZ and L27 (Funke, et al. 2005). The phylogenetic analysis of MAGUK
328 members across early branching metazoans was based on the central module sequences
329 (Figure 3A and supplementary Figure S5c). This analysis complements the previous analysis
330 by de Mendoza et al. (2010). Both *Capsaspora* and *Monosiga* possess a MPP and Dlg
331 ancestor that gave rise to the phylogenetic diversity of the metazoan MAGUK family. We
332 show that MPP2-7 is split in 2 distinct groups, MPP2,6 (Varicose) with extended members in
333 all early branching metazoans, and MPP3,4,7 (Mena3) which is restricted to bilaterians and
334 cnidarians. MPP5 (Stardust) appears to have several related members (MPPb) in poriferans,
335 ctenophores and placozoan. However, we could not ascribe a *bona fides* Stardust homolog to
336 ctenophores. The ZO family is present in all early branching metazoans, except *Amphimedon*.

337 *v) Coracles*

338 Coracle, Yurt, and Nbl4 are structurally related FERM-FA domains proteins (Tepass
339 2009). Cnidarians possess a clear Coracle homolog (CORA) and 2 additional Coracle variants
340 that mainly differ by their COOH terminal moiety (Figure 3B and supplementary Figure S5c).
341 *Trichoplax*, *Oscarella*, *Amphimedon* and *Mnemiopsis* also harbor Coracle-like proteins,
342 structurally closer to the cnidarian Coracle variants than the canonical one. A Yurt homolog is
343 found in anthozoans, *Trichoplax* and *Oscarella* whereas the Nbl4 (Human 4.1-Like) appears
344 to have emerged at the time of Cnidarian/bilaterian radiation. Of note, OcaYurt clusters with
345 Nbl4 protein sequences in the Bayesian tree and Yurt protein sequences in the Maximum
346 Likelihood tree (Figure 3B and supplementary Figure S5c), and may therefore represent an
347 ancestor of Yurt-Nbl4 families. OcaYurt was ascribed as *Oscarella* Yurt homolog based on
348 BLAST results (Supplementary Table S1).

349 *vi) Phylogenetic conclusive remarks*

350 Taken together, cnidarians and placozoans appear to share the complete SJ
351 complement. Several gene duplications were observed in cnidarians (NRX, NRG, CORA),
352 some of which are likely specific to reef building corals. Dichotomy between *Hydra* and the
353 anthozoans was apparent in the gene phylogeny (e.g. Claudin-like, NRX, NRG), which
354 indicates class specific diversification with possible subsequent divergence in SJ structures. In
355 contrast, genes encoding for the structural components of SJs, i.e. NrxIV, Nrg and Cont, are
356 absent in the other early branching metazoan phyla analyzed here (Table 1), although
357 members of the scaffolding and polarity genes of SJs (MAGUK, Cora, Na+/K+ ATPase

358 exchanger, Supplementary Figure S5d) are present (despite noticeable losses in *Amphimedon)*
359 The absence of structural SJ proteins suggests that intercellular junctions in these taxa are
360 structurally different from those found in cnidarians and bilaterians.
361
362 *The diversified SJ components in anthozoans show distinct and tissue-specific gene*
363 *expression.*
364 Electron microscope investigation of *Stylophora* across the different tissue layers
365 clearly hallmarks the presence of SJs between the apical border of every ectodermal and
366 endodermal cell (Supplementary Figure S2) (Tambutté, et al. 2007). They are 0.2 to 1 μm
367 long, depending on the section, and display a characteristic ladder structure. On micrographs
368 where the two tissue layers are visible, SJs of the endoderm layer appear to show higher
369 electron density than those of the ectoderm layer. As the SJ complement in cnidarians appears
370 to have diversified, we next asked what the relative expression of different SJ components
371 was and whether differential expression between the oral (non-calcifying) and aboral
372 (calcifying) tissues could be observed in the adult coral. We developed a protocol to micro-
373 dissect the oral discs from the coral colony using the anesthetic drug MS222 and micro-
374 scissors (see Material and Methods). *Stylophora* total RNA and proteins were extracted from
375 a colony fragment (oral and aboral) or from the oral disc (oral only) and expression was
376 quantified by real-time PCR and western blotting for the genes described in Figure 4. qPCR
377 expression estimates were normalized arbitrarily to StpNRX1=1 as relative expression of the
378 SJ components was our primary focus and because NRX is a core-component of SJs in
379 bilaterians. StpNRX3, 4 and 5 showed relatively low expression in contrast to StpNRX2
380 (0.53-fold to NRX1) (Figure 4a). The two StpNRG copies were expressed at strikingly
381 different levels (StpNRG1=0.34-fold, StpNRG2=3.1-fold). Unexpectedly, StpCONT was
382 weakly expressed (0.047, see discussion). Claudin-like mRNAs were all expressed, although
383 at relatively low levels in comparison to StpNRX1. The SJ adaptor component StpCORA1
384 was expressed at a similar level as StpNRX1 and the different variants StpCORA2-4 and
385 StpYURT were also expressed, strongly arguing that these conserved anthozoan genes
386 represent functional rather than pseudo-genes. When assessing tissue specificity, three SJ
387 genes, namely StpNRX2, StpClaud3, and StpClaud6, were strongly down-regulated in the
388 oral disc as opposed to the total colony (oral and aboral) tissue, suggesting that these were
389 mainly expressed in the calcifying aboral tissue, similar to the TFZPD9 calicoblast control
390 (Figure 4b). Although to a lesser extent, StpNRG2 as well showed preferential expression in
391 the aboral tissue. Reversely, StpNRX3, StpCONT, and StpClaudAS2 showed high expression

392 in the oral disc, albeit displaying a lower colony-wide expression. In order to estimate the
393 relative expression between the endodermal and ectodermal tissue layers, we took advantage
394 of the large size of the sea anemone *Anemonia viridis* tentacles (oral tissue), where the
395 endodermal and ectodermal layer can be manually separated. A partial *A. viridis* cDNA
396 database is available (Sabourault, et al. 2009) and incomplete sequences corresponding to SJ
397 components homologs could be identified. Measurement of their relative tissue expression
398 show predominant tissue specificity for one gene among those tested, namely the duplicated
399 copy of NRX1 (AvNRX1b) (Supplementary Figure S7).
400 We generated antibodies against the StpNRX1 and StpClaud3 proteins. These
401 antibodies were specific as little to no cross-reactivity could be observed in Western blots
402 (Figure 4c). Similar to the Actin control, StpNRX1 was equally expressed in both the oral
403 disc and the total colony fractions and was present in the blot as a single band <150KDa. This
404 ascertained our qPCR results, namely, that StpNRX1 represents a central component in most
405 SJs. Conversely, StpClaud3 was mostly absent from the oral discs fraction but present in the
406 total colony. This Claudin-like protein is thus likely to be mainly expressed in the aboral
407 calcifying tissues of the coral. In conclusion, anthozoan specific gene diversification is
408 accompanied by differential tissue expression, suggesting the presence of multiple SJ
409 architectures and functions in the different cell layers comprising these diploblastic animals.
410
411 *Stylophora NRX1 is glycosylated and co-localizes with F-actin*
412 StpNRX1 has a predicted molecular weight of 126.5 KDa, which is in disagreement
413 with the molecular weight of 141 KDa determined by Western blotting (Figure 4c). In
414 humans, the Caspr1 protein is glycosylated (Bonnon, et al. 2003); we therefore examined
415 whether StpNRX1 also exhibits post-translational N-linked glycosylation that contributes to
416 the difference between the apparent and predicted molecular weight. Total protein extract was
417 treated with and without PGNaseF (which specifically cleaves between asparagine and N-
418 acetylglucosamines), and Western blotting showed a shift from 141 KDa to 128 KDa of
419 StpNRX1 after PGNase treatment (Figure 5f). StpNRX1 is conclusively N-glycosylated
420 similar to Caspr1 in human. We next addressed the cellular localization of StpNRX1 and
421 StpClaud3 in adult *Stylophora*. An immuno-localization protocol was therefore established.
422 Coral fragments were fixed, decalcified and cut into parts for investigation of the aboral
423 calcifying tissues (Figure 5a). Labeling performed on the basal discs (see Supplementary
424 Figure S1) with phalloidin identified the F-actin network framing every cell (Figure 5b). This
425 cortical F-actin is supposedly adjacent to SJs as SJs are linked to the cytoskeleton in

426 bilaterians, and anthozoan SJs display similar protein composition to bilaterian SJs. Immuno-
427 localization with phalloidin and anti-StpNRX1 showed overlapping signals for most of the F-
428 actin network (Figure 5c). Optical sectioning sagittal to the epidermal (calicoderm) tissue
429 layer showed that NRX1 and F-actin overlapped, albeit partially, on the apical face of the cell
430 layer (Figure 5d). In order to eliminate optical interference between the Alexa-conjugated
431 secondary antibody and potential endogenous autofluorescence (e.g. GFP), the rabbit anti-
432 NRX1 was detected simultaneously with anti-rabbit-Alexa488 and anti-rabbit-Alexa405. Both
433 channels showed identical labeling in the calicoderm layer (figure 5d1-2). Thus, StpNRX1
434 could be co-localized with, or very close to, the F-actin network at the apical border of the
435 calicoderm layer, strongly supporting that StpNRX1 is a core component of SJs in
436 *Stylophora*. Immuno-labeling of StpClaud3 showed a different pattern. First, labeling was
437 restricted to groups of calicodermal cells along the basal disc. In such groups, although
438 labeling juxtaposed the F-actin labeling, the overlap between Claud3 and F-actin was only
439 partial. In some cases, StpClaud3 encircled two or more cells (Figure 5e). Such a pattern
440 rather suggests that StpClaud3 has a supra-cellular function within the calicoderm layer for
441 yet-to-define specialized cells.
442
443 *Model of cnidarian SJ as a blueprint of bilaterian SJ*
444 Several lines of concordant evidences led us to propose a model for cnidarian SJs
445 (Figure 6), as inferred from bilaterian SJs (Laval, et al. 2008; Shimoda and Watanabe 2009):
446 i) protein sequence and domains conservation of the different SJ components, which suggest
447 common functionality; ii) congruent phylogeny of bilaterian and cnidarian SJ components,
448 which suggest evolutionary conserved function; iii) localization at the apical border of the
449 cells for StpNRX1; iv) conserved N-linked glycosylation between StpNRX1 and its
450 mammalian counterpart Caspr1; v) similar SJ ultrastructure in insects and anthozoans on
451 electron micrographs. The tripartite NRX-NRG-CONT complex forms the structural base
452 linking two adjacent cells. Coracle and Yurt proteins serve as intracellular scaffolds, possibly
453 attaching the intracellular part of the structural components. Members of the MAGUK
454 superfamily also serve in scaffolding and cellular polarity. Na^+/K^+ transporters in SJs have
455 been verified in various species. Our StpClaud3 labeling data substantiates Claudin-like
456 association with SJs, although the expression of this particular Claudin appears restricted to
457 specific cell types. Limitations of the above model include the low mRNA expression of
458 CONT, the absence of the diverse conserved variants of NRX, and the lack of evidence for
459 the presence of Claudin-like proteins as core-components of cnidarian SJs. However, the

model presented here accounts for both medusozoans and anthozoans, two cnidarian clades that diverged probably more than 540 million years ago (Chapman, et al. 2010): besides ultrastructural variation recognized in electron micrographs, the SJ components of the two clades are comparable and SJs should therefore be considered as structurally similar and evolutionary related.

467 **Discussion**

468 Data mining of representatives from the early branching metazoans using known
469 molecular components of bilaterian occluding junctions (TJs and SJs) has conclusively
470 identified SJs as the sole type of occluding junctions present in Cnidaria and Placozoa,
471 thereby asserting previous electron microscope investigations on these phyla. Although the
472 core components of SJ have not been definitively defined, Nrx, Nrg, Cont and Claudins are
473 likely to represent the structural core components and thus their expression in early metazoan
474 lineages is meaningful in determining the evolution of this occluding junction. In cnidarians,
475 the SJ gene repertoire is diversified, with differential tissue expression for variants of the
476 structural SJ components NRX and NRG, which suggests an unexpected complexity of SJs in
477 these diplobastic animals. Although epithelium sealing properties have been documented in
478 poriferans (Adams, et al. 2010), lack of SJ structural homologs in the poriferan Demospongiae
479 and Homoscleromorpha as well as in Ctenophora indicates that SJ arose in metazoans before
480 the placozoan/cnidarian/bilaterian radiation.

481 *Epitheliozoans as defined by acquisition of septate junctions*

482 The molecular phylogeny of the principal occluding junction components across the
483 metazoan lineages (restricted to representative organisms with complete genomes) allows
484 reconstructing a scenario of stepwise evolution for sealing epithelia, i.e. the emergence of
485 body compartments (Figure 7). However, the phylogeny of early branches is not settled
486 (Philippe, et al. 2011; Nosenko, et al. 2013). The tree presented in Figure 7 follows minimal
487 gene loss across metazoan evolution of the SJ complement. Ctenophores are positioned at the
488 base of the metazoan lineage, according to current studies (Ryan, et al. 2013; Moroz, et al.
489 2014); demosponges and homoscleromorphes are separated according to (Sperling, et al.
490 2009; Erwin, et al. 2011) although consensus on the mono *vs* paraphyly debate of poriferans
491 has not been reached (Worheide, et al. 2012). In the protists *Capsaspora* and *Monosiga*, we
492 identified the Na^+/K^+ ATPase exchanger (the Beta subunit appeared with *Monosiga*),
493 MAGUK ancestors (Dlg and MPP), and Claudin-like members, which prove that these were
494 already present in the metazoan ancestor lineage. The Na^+/K^+ ATPase transporter is an
495 integral part of occluding junctions (Krupinski and Beitel 2009). Although this exchanger is
496 required for SJ formation in insects, its function in SJs is pump-independent (Genova and
497 Fehon 2003; Paul, et al. 2007). Interestingly, the beta subunit of the Na^+/K^+ ATPase
498 transporter has been shown to create molecular bridges between two adjacent cells (Vagin, et
499 al. 2012). This moonlighting function of the Na^+/K^+ transporter might have represented a

500 potential building block for the further development of occluding junction in epithelia
501 (Krupinski and Beitel 2009). With multicellular animals, components of the cytosolic adaptor
502 plaque appeared successively. Homologs of the MAGUK members Varicose (MP2,6), ZO
503 and an ancestral form of MPP 3,4,5,7, as well as the FERM protein Coracle, arose in
504 ctenophores. These represent cytosolic components involved in cellular polarization (and
505 junction scaffolding) in bilaterians. Stardust (MPP5) appeared with demosponges but ZO was
506 absent. In homoscleromorphs, Yurt as well as a putative NRG ancestor (intermediate between
507 DSCAM and NRG) were identified. However, it is only with placozoans and cnidarians that
508 the structural components of SJs, i.e. NRX, NRG and CONT, emerged, hereby pointing to the
509 origin of SJs in metazoans. In bilaterians, SJs were kept as the principal type of occludin
510 junctions in protostomes, whereas vertebrates within the deuterostome lineage evolved a
511 specialized Claudin family (here referred as Claudins s.s.) and other structural proteins (JAM,
512 Marvels…) that permitted a novel type of junction, the Tight Junction.
513 Epitheliozoa, which includes the Bilateria, Cnidaria, and Placozoa, was originally proposed to
514 characterize animals with true epithelia defined as cell layers held together by belt
515 desmosomes (Ax 1996; Dohrmann and Worheide 2013). Our present study extends the
516 characteristics of the Epitheliozoa as animals with epithelia sealed by occluding junctions
517 (TJs and SJs). Importantly, the lack of structural SJ components in poriferans was not
518 assessed in calcareous sponges in this study, as no calcareous genome is available hitherto.
519 However, Ledger described potential SJs in the calcareous sponge *Sycon ciliatum* using TEM
520 experiments (Ledger 1975). Hence, genomic exploration of calcareous sponges is required
521 before a complete picture of SJ evolution can be drawn.
522
523 Are structural SJs components derived from neuronal junctions?
524 Poriferans and placozoan do not have recognized neurons contrary to ctenophores and
525 cnidarians which have well defined neurons and nerves (Moroz 2012). However candidate
526 neurosecretory cells have been found in both poriferans (flask cells (Renard, et al. 2009)) and
527 *Trichoplax* (fiber cells (Smith, et al. 2014)). Further, a set of protosynaptic genes have been
528 identified in poriferans (Sakarya, et al. 2007; Conaco, et al. 2012). Thus, irrespective of
529 whether or not Ctenophora represents the basal Metazoa, the genetic origin of the neural
530 system starts with animal multicellularity. Central to the organization of the bilaterian
531 neuronal network is the Neurexin-Neurolignin interaction (Bang and Owczarek 2013).
532 Neurexins are found at the synaptic membranes and bind to Neurolignin on the opposite
533 synaptic membrane across the 20 nm wide synaptic cleft (Sudhof 2008; Chen, et al. 2010). On

534 the cytosolic side, Neurexin binds to the MAGUK proteins Dlg (PSD-95) and CASK and the
535 FERM protein 4.1/Coracle (Hata, et al. 1996; Biederer and Sudhof 2001; Chen, et al. 2005;
536 Chen and Featherstone 2011). Thus, in addition to structural similarities (Bellen, et al. 1998),
537 the synaptic Neurexin (Neu) and the Septate junction Neurexin (Nrx) share common cytosolic
538 partners. Although the function of the Neurexin 1 homologs in homoscleromorphs (Nichols,
539 et al. 2006) and ctenophores (Moroz, et al. 2014) is not known, Neu arose before Nrx,
540 possibly originating from a *Capsaspora* ancestor (Figure 7 and Supplementary Figure S4). A
541 possible scenario implies, the primary addition of EGF domains to the *Capsaspora* ancestor in
542 the first multicellular animals, and subsequently, after a duplication event, the rearrangement
543 of central LamG-EGF domains by an FBG domain, that could have led to a
544 neofunctionalization of the Neu protein to the Nrx protein. Another molecular actor necessary
545 to neural wiring in bilaterians and structurally related to NRG is the DSCAM cell adhesion
546 molecule. DSCAM controls repulsion/attraction between two neurons via extracellular
547 homophilic recognition, (Schmucker and Chen 2009). Besides, DSCAM is part of the innate
548 immune response in arthropods via heterophilic binding to different pathogen molecules
549 (Cerenius and Soderhall 2013). Our investigation of non-bilaterian genomes shows that
550 DSCAM is an evolutionary conserved molecule with homologs in ctenophores, poriferans,
551 placozoan and cnidarians (Figure 2 and 7), although localization and function are unknown.
552 However, among the 2 DSCAM copies present in Homoscleromorpha, one (OcaDSCAM)
553 clusters with the DSCAM family whereas the other clusters with the NRG family, likely
554 pointing to the emergence of the NRG family. Thus, NRG could represent an evolution of
555 DSCAM, a molecule linked to neuronal development and immunity.
556
557 *Structural SJ components appeared and diversified in Cnidarians*
558 The SJ components of cnidarians and bilaterians are very similar at the protein level
559 and therefore, a common model for the SJ structure can be inferred from the characterization
560 of SJs both in insects and mammalian paranodal junctions (Charles, et al. 2002; Bonnon, et al.
561 2003; Faivre-Sarrailh, et al. 2004) (Figure 6). In particular, in *Stylophora*, StpNRX1 localizes
562 at the apical border of each cell (Figure 5), which is in strict correlation with the position of
563 SJs observed in TEM images (Supplementary Figure S2). StpNRX1 also co-localizes with the
564 F-actin network (Figure 5), strongly supporting a model where SJs are attached to the
565 cytoskeleton via cytoplasmic adaptor proteins. Finally, in human, Caspr proteins associate
566 with contactin during their biosynthesis, resulting in the expression of high-mannose
567 glycoforms of the two proteins at the cell surface (Bonnon, et al. 2003). In this study,

568 StpNRX1 has been shown to be N-Glycosylated as demonstrated by the apparent molecular
569 weight reduction after PGnaseF treatment on Western blot (Figure 5). StpNRX1 thus
570 performs as the faithful homolog of *Drosophila* NrxIV and human Caspr1. Moreover, several
571 other copies of NRX1 (StpNRX2-5) are also present in *Stylophora* (Figure 2). These
572 supernumerary anthozoan specific copies display similar domain architectures in their NH2
573 terminus but differ in their COOH terminus (all have a transmembrane signature). These
574 variations in domain architectures, conserved among anthozoans, may reflect functional
575 diversification, in conjunction with specific tissue expression. StpNRX2 (a *Stylophora*
576 specific NRX), which accounts for about half of StpNRX1 in the total fraction, is mostly
577 expressed in the aboral tissue (Figure 4). Conversely, StpNRX3 is dominantly expressed in
578 the oral tissue. This suggests that the aboral (calcifying) and oral (polyp) tissues harbor a
579 different set of structural components for their SJs. Indeed, one of the scleractinian specific
580 NRG copies shows preferential expression in the oral tissue. Although the cellular
581 localization and function of these additional NRX and NRG homologs remains to be
582 addressed, SJs with different composition may reflect structural differences and possibly
583 result in different paracellular properties between the different tissues layers or specialized
584 cell types. Along the same line of evidence, in the tentacle of *Anemonia viridis*, one of the two
585 copies of NRX1 (AvNRX1b) is found mostly expressed in the endoderm, unlike AvNRX1a
586 which is equally expressed in both tissue layers (Supplementary Figure S7). Thus, at least in
587 *A. viridis*, the structural composition of SJs between the ectoderm and the endoderm appears
588 to differ. Such discrepancy in NRX composition may be the cause of the differences observed
589 in TEM images of sea anemone SJs between the endoderm with double septum and the
590 ectoderm with single septum (Green and Flower 1980). Patchwork expression of different SJ
591 components in tissues/layers is substantiated by the differential expression of the additional
592 copies encoding for the cytoplasmic adaptor CORA in Corals. One surprising result in our
593 expression analysis was the very low mRNA expression level of StpCONT as compared to
594 StpNRX1 and StpNRG1,2, as these form a trimolecular complex in bilaterians. This result
595 was confirmed by other independent RNAseq approaches (data not shown). In addition, data
596 mining of other cnidarian EST databases (including *Nematostella* and *Hydra*) also showed
597 that putative ESTs homologous to CONT were scarce. Although thought provoking, our data
598 raise the possibility that CONT is not part of the tri-molecular core-complex that structures all
599 SJs in cnidarians. Alternatively, the turnover of the CONT mRNA and protein may be very
600 slow and therefore present in low copy numbers. Also, CONT may be required for specific

601 developmental stages or cell types. Hence, as the CONT mRNA is indeed expressed, we
602 included CONT in our cnidarian SJ model.
603
604 <u>*Conserved Claudin-like proteins*</u>
605 Claudins were first identified in vertebrate TJs and so far 27 members have been
606 identified in vertebrates (Gunzel and Fromm 2012). Beside these TJ specific Claudins
607 (Claudin s.s.), other Claudin-like proteins have been identified based on sequence and
608 tetraspan structure similarities both in human and invertebrates. In *Drosophila*, three Claudin-
609 like proteins were functionally associated with SJs. However, Claudin phylogeny is unclear as
610 these proteins loosely cluster in highly divergent clades (Simske and Hardin 2011). The
611 addition of Claudin-like proteins from early branching metazoans to the Claudin repertoire
612 highlights several clusters of evolutionarily conserved Claudin-like members. Claudins s.s.
613 are specific to TJs, which correlates with the fact that they form an outgroup to the other
614 Claudin-like sequences. Group Ia, Ib, II, and IV encompass human Claudin-like proteins
615 (LIM2, PMP22 EMP1-3, LHFPL1-4, Clarin-3) proposed to have cell-cell interaction
616 properties (reviewed in (Van Itallie and Anderson 2004; Simske and Hardin 2011; Cosgrove
617 and Zallocchi 2013). For example, LIM2 (Group Ia) and PMP22 (Group Ib) have been shown
618 to associate with TJ constituents and to display barrier properties (Notterpek, et al. 2001;
619 Grey, et al. 2003; Roux, et al. 2005), whereas a LHFP member (also called TMHS, group II)
620 was associated with hair-cell anchoring independently of TJs (Xiong, et al. 2012). What is the
621 role of analogous Claudin-like in invertebrates? The *Stylophora* StpClau3 (group II) clearly
622 localizes at the cell-cell border of specific cells (Figure 5), in agreement with specialized cell
623 interaction properties. In *Hydra*, the Claudin HydClau1 also localizes to the apical junctional
624 complexes (Bert Hobmayer, University of Innsbruck, personal communication). However,
625 outside the three *Drosophila* Claudin-like proteins associated with SJ formation, it would be
626 premature to involve any other invertebrate Claudin-like proteins with a particular function in
627 SJs. A junctional interaction in *trans* between two cells is highly improbable as the distance
628 separating adjacent plasma membranes is too large to allow kissing complexes in SJs. A
629 function in regulating the paracellular transport across an epithelium has only been described
630 for the TJ specific Claudin (Van Itallie and Anderson 2006). The ancient and diversified
631 Claudin repertoire may well represent diverse conserved functions, as part of macromolecular
632 complexes associated with the plasma membrane. Further biochemical characterization will
633 be needed to clarify the apparent discrepancies between the Claudin phylogeny presented here
634 and the function of Claudins inferred from vertebrates. Indeed, Claudin-like proteins are

635 present in the unicellular *Capsaspora* and *Monosiga* suggesting that tetraspan proteins had
636 ancestral functions besides promoting cell-cell interaction. Claudin-like Group III appears to
637 contain the most evolutionary conserved Claudin clade with Claudin-like members found in
638 vertebrates, *Drosophila*, cnidarians, poriferans, *Monosiga* and Capsaspora; yet functional data
639 is not available for any of them.
640
641 <u>*Functional implications*</u>
642 Occluding Junctions govern paracellular transport across epithelia. In invertebrates, SJs
643 control this paracellular pathway, as shown in insects using conductance experiments on
644 epithelia and by dextran injection after gene knock down (Pannabecker, et al. 1993; Lamb, et
645 al. 1998; Banerjee, Sousa, et al. 2006). Although classified as "leaky epithelium", as
646 compared to the vertebrates' "tight epithelia", epithelia in insects are nevertheless able to
647 show barrier properties comparable to vertebrates. For example, in female mosquitoes,
648 Malpighian tubes maintain very high [K^+] gradients and allow rapid paracellular transport of
649 Cl^- across the Malpighian epithelium after blood meals to maintain homeostasis (Beyenbach
650 and Piermarini 2011). In cnidarians, the epithelial layer also show different permselective
651 properties to Ca^{2+}, Na^+ and Cl^- (Bénazet-Tambutté, et al. 1996), suggesting that SJs
652 potentially control ion exchange across cnidarian tissue layers. In reef building corals, the oral
653 and aboral tissues have specialized roles in the process of biomineralization, and the transport
654 of ions from the surrounding sea water to the site of calcification is central to the
655 understanding of how the calcium carbonate skeleton is formed (Tambutté, et al. 2007;
656 Allemand, et al. 2011). Although the transcellular pathway is part of this ion transport (for
657 recent reviews see (Allemand, et al. 2011; Tambutté, et al. 2011), experiments have raised the
658 possibility that paracellular transport might also be involved (Tambutté, et al. 2012). Since
659 molecules such as calcein (molecular radius 6.5 A) are able to pass through the junction,
660 small ions such as calcium (molecular radius 1.8 A) should also, in principle, be able to pass
661 via the paracellular pathway (Tambutté, et al. 2011; Tambutté, et al. 2012). However, in
662 chordate epithelia, TJs not only regulate the flow of molecules based on the size, but also
663 based on the charge of the molecule/atom. Although in chordates it is generally accepted that
664 Claudins (Claudins s.s.) define the TJ permselective properties, almost nothing is known
665 about the mechanisms that govern the flow of molecules through SJs. In other words, the
666 respective roles of the Claudin-like, NRX, NRG and CONT proteins (or other molecules) in
667 regulating the paracellular transport still remain to be characterized, especially in regards to
668 tissue specific permselectivity. Further experiments using heterologous expression of SJ

669 components in conjunction with electrophysiological measurements will help to better
670 understand the role of these molecules in the permselective passage of ions. In addition to
671 shedding light onto the coral calcification process, determining the
672 permeability/permselectivity of SJs is also of major importance in the environmental context
673 of ocean acidification. Previous studies have shown that the decrease in pH in the oceans, due
674 to the increase in atmospheric CO_2 and its dissolution into seawater, negatively affects coral
675 calcification (Andersson and Gledhill 2013). One parameter that might explain this effect,
676 among others, is the degree to which the site of calcification is isolated from seawater. It has
677 been proposed that the sensitivity of corals to ocean acidification could readily be explained if
678 the paracellular route is the major supply of ions for calcification (Erez, et al. 2011). Different
679 studies have suggested a protective role of tissue layers against skeletal dissolution (Ries, et
680 al. 2009; Rodolfo-Metalpa, et al. 2011). However none of them has examined the potential
681 role of SJs in such a protection because no molecular data on junctions were hitherto
682 available. The results presented here lay the foundations for future studies that will allow to
683 monitor differential expression of genes involved in the formation of SJs and to determine
684 whether they play a role in the resistance to ocean acidification.
685

686 **Material and Methods**

687 Model organisms: Cnidaria comprise two major classes, Medusozoa (including Hydrozoa)
688 and Anthozoa (including Hexacorallia). Actiniaria and Scleractinia constitute two major
689 subclasses of Hexacorallia. Commonly, Actiniaria are represented by sea anemones such as
690 *Nematostella vectensis* (*Nematostella*) and Scleractinia are represented by reef building
691 corals, such as *Stylophora pistillata* and *Acropora digitifera* (Kayal, et al. 2013), which are
692 colonial polyps and have a specialized calcifying tissue layer (calicoderm, Supplementary
693 Figure S1 and S2).

694 Sequences: all human and *Drosophila melanogaster* reference protein sequences listed in
695 Supplementary Table S1 were retrieved from NCBI. Early branching metazoan sequences
696 were retrieved from the databases listed in Supplementary Table S2. The *Stylophora pistillata*
697 sequences were deduced from transcriptome and/or genome assemblies (C.R.V. and M.A.).
698 Note that some *Hydra* and *A. digitifera* protein families were omitted in our phylogenetic
699 analysis due to inconsistent sequences assemblies (gaps, Ns, misassemblies) of some
700 members.

701 Softwares and strategy used: BLAST (2.2.22) genome/transcriptome analysis was run locally,
702 at NCBI and JGI depending on organisms' database. An online version of MAFFT
703 (mafft.cbrc.jp/alignment/server/) was used with strategy L-INS-i default parameters. Prottest
704 (v2.4), PhyML (v3.0), MrBayes (v3.2.1) and FigTree (v1.3.1) (Huelsenbeck and Ronquist
705 2001; Abascal, et al. 2005; Rambaut and Drummond 2009; Guindon, et al. 2010) were run
706 locally. PFAM (pfam.sanger.ac.uk/ (and SMART (http://smart.embl-
707 heidelberg.de/smart/set_mode.cgi) were used to predict protein domains. Transmembrane
708 domains were predicted at www.cbs.dtu.dk/services/TMHMM/. GPI anchors were predicted
709 using the webtools "FragAnchor"
710 (http://navet.ics.hawaii.edu/~fraganchor/NNHMM/NNHMM.html), PredGPI
711 (http://gpcr2.biocomp.unibo.it/gpipe/pred.htm) and "GPI-anchored Protein Prediction"
712 (http://bolero.bi.a.u-tokyo.ac.jp:8201/GPI-Predictor/), which gave similar results.

713 Blast searches were run on the cnidarians databases to identify putative homologs using
714 *Drosophila* and Human protein sequences of the molecular SJ and TJ constituents
715 (Supplementary Figure S3). All newly identified protein sequences were added to the
716 previous pool of query sequences for iterative BLAST searches to identify novel potential
717 homologs. Selection criteria were based on reciprocal BLAST against the Human and

718 *Drosophila* RefSeq databases as well as domain homology. Once identified in cnidarians,
719 searches for homologs were further carried out in *Trichoplax*, then in *Amphimedon* and finally
720 in *Monosiga*, always using the entire pool of identified proteins as bait. When homologs were
721 missing in *Amphimedon*, BLAST searches were carried out against the whole NCBI sponge
722 database before deemed absent. For each family of SJ components identified, protein
723 sequences were aligned using MAFFT. Alignments were trimmed to the largest conserved
724 part of the proteins (Supplementary File S1) and then subjected to phylogenetic analyses. Best
725 substitution matrices and parameters were calculated using Prottest before running PhyML.
726 Alternatively, Bayesian analyses using MrBayes were run with default specification until
727 convergence reached standard deviation below 0.01, except for Claudins, which were stopped
728 after 7 million MCMC generations. Resulting trees were visualized using FigTree.

729 <u>Oral disc dissection</u>: fragments of the same *Stylophora pistillata* colony were used for both
730 RNA and protein extractions. Fragments were set to rest in a glass petri dish filled with sea
731 water until polyps were extended. 0.4% stock solution of Tricaine mesylate (MS-222, Sigma)
732 dissolved in sea water was added into the petri dish to a final concentration of 0.04% and left
733 to rest under dimmed light for 15 minutes. Subsequently, oral discs (the apparent portion of
734 the polyp, Supplementary Figure S1) were cut from the colony under binocular using 5 mm
735 blades micro-dissection scissors (Vannas). Batches of 10-15 oral discs were collected and
736 transferred into Trizol® or TNE solutions (see below). Dissections were stopped after a
737 maximum of 45 minutes of MS-222 incubation to elude any potential secondary effect of the
738 drug.

739 <u>RNA extraction</u>. Freshly dissected oral discs were put into Trizol® and homogenized for 1
740 minute using an electrical potter. Alternatively, entire fragments of colony were cryo-crushed
741 (Spex sampleprep® 6770) and the resulting powder was dissolved in Trizol®. RNA
742 extraction was carried out using a standard protocol (Moya, et al. 2008). Extracted RNAs
743 were treated with RNase-free DNaseI (Roche) and precipitate with NaAcetate/EtOH.
744 Concentrations were determined by spectrophotometry using an Epoch Microplate
745 Spectrophotometer (BioTek).

746 <u>Real-time qPCR</u>. cDNAs were synthesized using the Superscript®III kit (Invitrogen). qPCR
747 runs were performed on an ABi 7300 using “EXPRESS SYBR® GreenER™ qPCR Supermix
748 with Premixed ROX” (Lifetechnologies) for PCR amplification. Experimental procedures
749 were performed as in (Moya, et al. 2008). Data were either relative to stpNRX1 [dCt =

750 $(Ct_{gene_of_interest} - Ct_{NRX1})_{Total}]$ for the expression in the whole colony (Total), or normalized to
751 stpNRX1 and relative to Total [ddCt = $(Ct_{gene_of_interest} - Ct_{NRX1})_{Oral}$ - $(Ct_{gene_of_interest} -$
752 $Ct_{NRX1})_{Total}]$ for the expression in the oral disc (Oral) as compared to Total. Fold expressions
753 were further extrapolated using the 2^{-dCt} and 2^{-ddCt} function.

754 <u>Protein extraction protocol for enriched membrane proteins (all step on ice).</u> Freshly dissected
755 *Stylophora* oral discs were kept in cold TNE buffer [100 mM Tris_pH=7.2; 100 mM NaCl; 5
756 mM EDTA; 1X Protein Inhibitor Cocktail (Sigma)]. Alternatively, total tissue was extracted
757 from the skeleton in cold TNE using the air-pick (i.e. pressurized air through a pipet tip)
758 method on coral fragments. Batches of 1 ml crude extract were then dilacerated by repeatedly
759 passing them through a 21" syringe gauge until homogeneity was reached. Extracts were
760 centrifuged at 1000 g for 1 minute at 4°C. The supernatant (S1) was collected and kept on ice.
761 The pellet was resuspended in TNE, passed through a syringe and centrifuged again. The
762 supernatant (S2) was pooled with S1 and triton X-100 was added to a 1% final concentration.
763 The resulting intermediate extract was incubated for 1hr at 4°C on a rotating wheel and then
764 centrifuged at 15,000 g for 15 minutes at 4°C. The resulting supernatant was the final total
765 extract, now cleared of zooxanthellae but enriched in membrane proteins. Protein
766 concentrations were measured by comparison to a BSA standard curve. For the N-linked
767 glycosylation analysis, 250 µl of *Stylophora* total extract was divided into two reaction tubes;
768 half was incubated with 2 µl 2-mercaptoethanol and 8 µl PGNaseF (Roche) for 3H at 37 °C
769 and the other half was used as control.

770 <u>Custom made antibodies</u> (Eurogentec). Two antibodies were produced in rabbit using
771 synthetic peptides, one against the peptide KTNPYDPTSGRRTDDD (AA 1057-1073)
772 corresponding to the beginning of the extracellular part of StpNRX1 and the other one against
773 the peptide GRMASHGYYNQDTTTL (AA 220-236) corresponding to the COOH terminus
774 of the StpClaud3. For each antibody, ten rabbits were initially screened for non-cross-
775 reactivity with *Stylophora* proteins and two were selected for the Speedy program. Each
776 selected antibody was affinity purified before use.

777 <u>Western blotting</u>. Equal amounts of protein extracts were loaded onto 6% (NRX1), gradient
778 4-15% (actin) and 15% (Claud3) TGX precast gel (Bio-Rad). After electrophoresis, gels were
779 transferred onto PVDF membrane and blotted using SNAP i.d.® with anti-Stp_NRX1
780 (1:200), anti-StpClaud3 (1:200), anti-actin (mouse A4700 Sigma) (1:500) primary antibodies,
781 and HRP-coupled goat anti-rabbit (Sigma) (1:2000) or HRP-coupled goat anti-mouse (Sigma)

782 (1:2000) secondary antibody. ECL was conducted using Amersham ECL detection reagents
783 (GE Life Sciences). Imaging was carried out on a Fusion Fx7 (Peqlab)

784 Immunolocalization. One microcolony of *Stylophora* grown on a slide (Venn, et al. 2011) was
785 fixed in 25 ml chilled artificial-sea-water / paraformaldehyde (PAF) fixation buffer [425 mM
786 NaCl; 9 mM KCl; 9.3 mM CaCl2; 25.5 mM MgSO4; 23 mM MgCl2; 2 mM NaHCO3-;
787 100mM HEPES pH=7.9; 4.5% PAF] for 2H on ice. The microcolony was transferred into a
788 50 ml Falcon tube and decalcified in 50 ml [100 mM HEPES pH=7.9; 500 mM NaCl; 250
789 mM EDTA pH=8.0; 0.4% PAF] (renewed after 48h) at 4°C until dissolution of the skeleton
790 (3-5 days). The remaining soft tissue was transferred into in a small petri dish containing 10
791 ml decalcifying buffer/1X PBS (50/50 v/v). Here, basal discs were cut with 5 mm blades
792 micro-dissection scissors under a binocular. Basal discs were collected in 1X PBS and rinsed
793 3 times for 5 minutes. Samples were blocked in [1X PBS; 0.05% Tween_20 (PBST); 2%
794 BSA; 2% donkey serum; 0.1% Triton_X100] for 2H at 4°C. Samples were incubated in
795 antibody solution [PBST; 1% BSA] with either anti-Stp_NRX1 or anti-StpClaud3 (dilution
796 1:25) for 2 days at 4°C, then rinse 3 times 10 minutes in [PBST; 0.1% BSA] and further
797 incubated for 1 day at 4°C in antibody solution supplemented with 10 µl Phalloidin-Alexa568
798 and anti-rabbit-Alexa488 and anti-rabbit-Alexa405 (dilution 1:200 each). Finally, samples
799 were rinsed 3 times for 5 minutes in PBST, mounted in ProLong® Gold antifade reagent
800 (Molecular Probes) and left for 24h in darkness.

801 Imaging. Confocal imaging was performed using a Leica SP5 and the LAS AF lite software.
802 For imaging, each channel was acquired sequentially to ascertain lack of cross-emission.
803 Merging was achieved using the LAS AF lite tools option. Light microscope images were
804 acquired using a Leica Macroscope Z16 APO. Sample preparation and electron micrographs
805 obtained with a JEOL transmission microscope were described in (Tambutté, et al. 2007).
806 Image contrast and brightness were adjusted with the Photoshop levels tool.

807

808

809

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816

817

818 **Figure Legends**

819 Table1: Molecular components of occluding junctions in representative eukaryotes.

820 List of the major components of Tight Junctions (TJs) and Septate Junctions (SJs) in Human
821 and *Drosophila*, and the respective protein homologs in *S. pistillata*, *N. vectensis*, *H.*
822 *magnipapillata* (Cnidaria), *T. adherens* (Placozoa), *O. carmela* (Porifera,
823 Homoscleromorpha), *A. queenslandica* (Porifera, Demospongiae), *M. leidyi* (Ctenophora), *M.*
824 *brevicollis* (Choanoflagellata), and *C. owczarzaki* (Filasterea). Numbers in brackets refer to
825 the number of homologs found. "Not found" means absence of homologs whereas "nd" stands
826 for "non-determined" due to limitations in the assembly of the reference sequence (see
827 Material and methods). Claudins were arguably separated into two subgroups: Claudin s.s.
828 (Claudin *sensu stricto*) refers to the vertebrate Claudins (1-27) that are unique to the
829 vertebrate TJs, whereas Claudin-like proteins encompass other members of the tetraspan
830 family that are similar to Claudins in structure and also belong to the PFAM families
831 PF00822, PF13903, and PF10242 (see Table S1).

832

833 Figure 1: Phylogenetic trees of Claudins

834 Unrooted Bayesian phylogenetic tree (polar) of Claudins in holozoans. Five distinct families
835 (I-V) are well supported by both Bayesian and Maximum-Likelihood analysis. Hs: human;
836 Dm: *Drosophila*, Adi: *A. digitifera*; Stp: *S. pistillata*; Nv: *N. vectensis*; Hyd: *Hydra*; Tri:
837 *Trichoplax*; Oca: *Oscarella*; Amphi: *Amphimedon*; Pfi: *Petrosia ficiformis*; Cca: *Corticium*
838 *candelabrum*; Mle: *Mnemiopsis*; Mon: *Monosiga*; Cow: *Capsaspora*.

839

840 Figure 2: Phylogenetic trees of NRX, NRG-CONT

841

842 Unrooted Bayesian phylogenetic tree (rectangular) of holozoan Neurexins (A) and NRG,
843 CONT and DSCAM (B). The different taxa are color shaded. The domain arrangements of
844 each protein are schematized on the right hand side of the trees. Nrx and correspond to
845 Caspr/NrxIV and Neurexin1-3 homologs, respectively; NRG, CONT and DSCAM
846 correspond Neurofascin, Contactin and Down Syndrome Cell Adhesion Molecule homologs,
847 respectively. Hs: human; Dm: *Drosophila*, Adi: *A. digitifera*; Stp: *S. pistillata*; Nv: *N.*
848 *vectensis*; Hyd: *Hydra*; Tri: *Trichoplax*; Oca: *Oscarella*; Amphi: *Amphimedon*; Mle:
849 *Mnemiopsis*; Pba: *Pleurobrachia bachei*; Cow: *Capsaspora*.

850

851 Figure 3: Phylogenetic trees of MAGUKs and 4.1/Coracle-Yurt

852 Unrooted Bayesian phylogenetic tree (rectangular) of holozoan MAGUKs (A) and
853 4.1/Coracle-Yurt (B). The domain arrangements of each protein are schematized on the right
854 hand side of the trees. Vari, mena3, Stard, and Cora abbreviate Varicose, menage a trois,
855 Stardust and Coracle respectively. Hs: human; Dm: *Drosophila*, Adi: *A. digitifera*; Stp: *S.*
856 *pistillata*; Nv: *N. vectensis*; Tri: *Trichoplax*; Oca: *Oscarella*; Amphi: *Amphimedon*; Mle:
857 *Mnemiopsis*; Mon: *Monosiga*; Cow: *Capsaspora*.
858
859 Figure 4: Expression of the principal molecular components of Septate Junctions (SJs) in *S.*
860 *pistillata*.
861 a-b) Real-time PCR expression analysis of the SJ components NRX, NRG-CONT, Claudins,
862 Cora-Yurt, and the calicoblast specific three-fingered protein TFZPD9. The *S. pistillata* three
863 finger domain protein TFZPD9 is the nearest homolog of the *Pocillopora damicornis* Pdcyst-
864 rich protein specifically expressed in the calicoderm (Vidal-Dupiol, et al. 2009) and served as
865 a tissue positive control. Expressions were measured in (a) total tissues (whole coral
866 fragment), relative to NRX1, or in (b) oral disc versus total tissues, normalized to NRX1 and
867 relative to total. c) Western blotting of the total and oral discs protein extracts with anti-
868 Stp_NRX1, anti-StpClaud3 and anti-actin. Note that the pseudo-band around 75KDa (*) in
869 NRX1 corresponds to a compression of middle to low MW non-specific bands apparent in
870 this 6% polyacrylamide gel that are barely detectable on gradient 4-15% gels.
871
872 Figure 5: Immunolocalization of Stp_NRX1 and StpClaud3 and N-linked glycosylation of
873 Stp_NRX1
874 a) Bright-field microscope image of the calcifying tissue including basal discs, after
875 decalcification. b) Phalloidin-Alexa 568 staining of a basal disc showing the F-actin network
876 framing each cell (Z-stack projection). c1-3) Portion of a basal disc showing the near
877 complete superimposition of the NRX1 (c1) and the F-actin (c2) labeling (Z-stack projection).
878 d1-4) Optical cross-section through the calicoblastic epithelium showing the co-localization
879 of NRX1 (d1,2) with F-actin (d3). e1-4) Detail of a group of cells where Claud3 labeling is
880 visible. Claud3 (e1,2) juxtaposes with F-actin (e3) although the overlap is partial (e4) (Z-stack
881 projection). Scale bars are in µm. f) Western blot of *S. pistillata* total protein extract after
882 PGNaseF treatment with anti-Stp_NRX1. After removal of N-linked glycosylation, the
883 original 141 KDa NRX1 has a reduced molecular weight of 125 KDa (as estimated with
884 Fusion software tools).
885

886 Figure 6: Heuristic model for Septate Junctions (SJ) in Cnidaria

887 The relative arrangement of the different SJ components in cnidarians is inferred from the
888 models of SJs in *Drosophila* and human paranodes. We distinguished the structural
889 components NRX, NRG and CONT from the cytosolic adaptor protein families
890 CORA/YURT and MAGUK shown to be involved in SJ scaffolding in bilaterians. Although
891 Claudins are involved the permselectivity of Tight Junctions, a similar role in SJs has not yet
892 been demonstrated.

893

894 Figure 7: Proposed evolution of occluding junctions.

895 Model of the evolutionary emergence of occluding junctions based on the presence/absence of
896 the different occluding junction (TJ+SJ) components in diverse representative taxa of
897 Holozoa. See text for details..

898

899

900 **Supplementary Information**

901 Figure S1-8: SJ_cnidarian_Supplementary_information.pdf

902 *Figure S1: Coral anatomy.*

903 *Figure S2: Transmission electron microscopy of the oral and aboral tissue.*

904 *Figure S3: Analysis pipeline*

905 *Figure S4: Radial tree representation of the NRX, NRG-CONT, MAGUK, and CORA-YURT*

906 *Figure S5a-d: PhyML and/or MrBayes Trees of a) Claudins; b) NRX, NRG-CONT; c)*
907 *MAGUKs and CORA-YURT; d) Na+/K+ ATPase transporter alpha and beta*

908 *Figure S6: Domain structure of the best NRX, NRG-CONT homologs in demosponges.*

909 *Figure S7: Relative Ectoderm-Endoderm expression of several SJ components in Anemonia*
910 *viridis*

911 *Figure S8: The ctenophore apical belt junction*

912 File S1: Alignments (Nexus) used to compute Trees relative to SJ components:
913 Nexus_align_seq.txt

914 Table S1: Sequences used in this analysis: Occluding_Junction_sequences.xls

915 Table S2: Web site location of the different databases used for the phylogeny: databases.xls

916 Table S3: Primer used for PCR amplification: Primers.xls

917

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919

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