Structural Molecular Components of Septate Junctions in Cnidarians Point to the Origin of Epithelial Junctions in Eukaryotes

Philippe Ganot,*1 Didier Zoccola,1 Eric Tambutté,1 Christian R. Voolstra,2 Manuel Aranda,2 Denis Allemand,1 and Sylvie Tambutté1

1Marine Biology Department, Centre Scientifique de Monaco, Quai Antoine Premier, Monaco
2Red Sea Research Center, King Abdullah University of Science and Technology (KAUST), Thuwal, Saudi Arabia

*Corresponding author: E-mail: pganot@centrescientifique.mc.
Associate editor: John True

Abstract

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

Key words: Epitheliozoa, Claudin, Neurexin, Contactin, Neuroglian, Coracle, MAGUK, Na+/K+ ATPase transporter, DSCAM, Nbl4, para-cellular pathway, permselectivity, corals, ctenophores, poriferans, Monosiga, Capspaspora.

Introduction

A unifying characteristic of metazoa 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 extracellular 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 (TePas et al. 2001). SJs are also found in mammals, at the nodes of Ranvier 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 nonbilaterians, cell–cell junctions have been structurally investigated using different electron microscope techniques in the diverse phyla composing early branching metazoa, that is, 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 connections” 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 was described in the more distant Hexactinellida or Demospongiae (Leys et al. 2009). In Homoscleromorpha, the presence of SJs is uncertain as the presence of septa is unclear (Leys et al. 2009; Leys et al. 2009; Gazave et al. 2010). In fact, the only clear report of SJs in Porifera was made in calcareous sponges by Ledger (1975). In this study, the authors could show an electron dense ladder between the spicule-secreting sclerocytes of Sycon ciliatum, although electron dense junctions with no visible septa were described in Sy. coactum (Eerkes-Medrano and Leys 2006).
Ctenophores share a very similar unique junctional structure, where epithelial cells are linked with distinctive belt junction (see supplementary fig. S8, Supplementary Material online). The junctional membranes are 2–3 nm apart but they do not fuse nor are they linked by septa (Hernandez-Nicaise et al. 1989; Hernandez-Nicaise 1991).

At the molecular level, most functional studies rely on mammalian and insect model organisms. Components of TJs and SJs can be subdivided into intercellular structural and cytosolic scaffolding/polarity proteins (see table 1).

Although the structural transmembrane (TM) proteins mediate cell–cell adhesion, the cytosolic junction plaque contains various types of proteins that link the junction TM proteins to the underlying cytoskeleton.

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

Although SJs and TJs show striking differences in their respective structural components, the cytosolic adaptor proteins responsible for their assembly and maintenance at the plasma membrane appear to share, in part, similar machineries. MAGUK proteins are evolutionarily conserved scaffolding proteins that create and maintain multicomplexes, such as adherens and occluding junctions, at distinct subcellular sites like the cytoplasmic surface of the plasma membrane for instance (Ikenouchi et al. 2007; de Mendoza et al. 2010). In Drosophila, members of the MAGUK protein superfamily, including Disc Large (Dlg), Zona Occludens (ZO), Varicose (Var) and Stardust (Std), are necessary for epithelial polarity and scaffolding of SJs (Wood et al. 1996; Bachmann et al. 2001; Jung et al. 2006; Moyer and Jacobs 2008). Similarly, the mammalian MAGUK proteins Dlg, 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...
Table 1. Molecular Components of Occluding Junctions in Representative Eukaryotes.

<table>
<thead>
<tr>
<th>Eukaryote Type</th>
<th>Bilateria</th>
<th>Nematoda</th>
<th>Nematoda</th>
<th>Nematoda</th>
<th>Nematoda</th>
<th>Nematoda</th>
<th>Nematoda</th>
<th>Nematoda</th>
<th>Nematoda</th>
<th>Nematoda</th>
<th>Nematoda</th>
<th>Nematoda</th>
<th>Nematoda</th>
<th>Nematoda</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Structural</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TJ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MarvelD1 (Occludin)</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
</tr>
<tr>
<td></td>
<td>MarvelD2 (Tricellulin)</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
</tr>
<tr>
<td></td>
<td>MarvelD3</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
</tr>
<tr>
<td></td>
<td>JAM1–3</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
</tr>
<tr>
<td></td>
<td>CAR</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
</tr>
<tr>
<td></td>
<td>ESAM</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
</tr>
<tr>
<td></td>
<td>Claudin (s.s.) 1–27</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
</tr>
<tr>
<td></td>
<td>SJ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Claudin like</td>
<td>Sinu,Mega,Kune, + (8)</td>
<td>Claudin (9)</td>
<td>Claudin (10)</td>
<td>Claudin (14)</td>
<td>Claudin (1)</td>
<td>Claudin (6)</td>
<td>Claudin (1)</td>
<td>Claudin (4)</td>
<td>Claudin (4)</td>
<td>Claudin (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurofascin/NF155</td>
<td>Nrg (neuroglin)</td>
<td>NRG (2)</td>
<td>NRG (1)</td>
<td>NRG (1)</td>
<td>NRG (4)</td>
<td>NRG/DSCAM</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
</tr>
<tr>
<td>Contactin/F3</td>
<td>Cont (Contactin)</td>
<td>CONT (1)</td>
<td>CONT (1)</td>
<td>CONT (1)</td>
<td>CONT (1)</td>
<td>CONT (1)</td>
<td>CONT (1)</td>
<td>CONT (1)</td>
<td>CONT (1)</td>
<td>CONT (1)</td>
<td>CONT (1)</td>
<td>CONT (1)</td>
<td>CONT (1)</td>
<td>CONT (1)</td>
</tr>
<tr>
<td>Caspr/NCP1/Paranodine</td>
<td>NrxF (neurexin IV)</td>
<td>NRX (5)</td>
<td>NRX (6)</td>
<td>NRX (5)</td>
<td>NRX (1)</td>
<td>NRX (1)</td>
<td>NRX (1)</td>
<td>NRX (1)</td>
<td>NRX (1)</td>
<td>NRX (1)</td>
<td>NRX (1)</td>
<td>NRX (1)</td>
<td>NRX (1)</td>
<td>NRX (1)</td>
</tr>
<tr>
<td>Scaffold</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SJ+TJ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaKATPase_Alpha</td>
<td>NaKATPase_Alpha</td>
<td>NaK (2)</td>
<td>NaK (2)</td>
<td>NaK (5)</td>
<td>NaK (1)</td>
<td>NaK (1)</td>
<td>NaK (1)</td>
<td>NaK (1)</td>
<td>NaK (1)</td>
<td>NaK (1)</td>
<td>NaK (1)</td>
<td>NaK (1)</td>
<td>NaK (1)</td>
<td>NaK (1)</td>
</tr>
<tr>
<td>Band 4.1</td>
<td>Cora</td>
<td>Cora (3)</td>
<td>Cora (3)</td>
<td>nd</td>
<td>Cora (1)</td>
<td>Cora (2)</td>
<td>Cora (2)</td>
<td>Cora (2)</td>
<td>Cora (2)</td>
<td>Cora (2)</td>
<td>Cora (2)</td>
<td>Cora (2)</td>
<td>Cora (2)</td>
<td>Cora (2)</td>
</tr>
<tr>
<td>Mo/E PB41L5</td>
<td>Yurt</td>
<td>Yurt (1)</td>
<td>Yurt (1)</td>
<td>nd</td>
<td>Yurt (1)</td>
<td>Yurt (1)</td>
<td>Yurt (1)</td>
<td>Yurt (1)</td>
<td>Yurt (1)</td>
<td>Yurt (1)</td>
<td>Yurt (1)</td>
<td>Yurt (1)</td>
<td>Yurt (1)</td>
<td>Yurt (1)</td>
</tr>
<tr>
<td>MAGUK</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPP2,6</td>
<td>Vari</td>
<td>Vari (1)</td>
<td>Vari (1)</td>
<td>nd</td>
<td>Vari (1)</td>
<td>Vari (1)</td>
<td>Vari (1)</td>
<td>Vari (1)</td>
<td>Vari (1)</td>
<td>Vari (1)</td>
<td>Vari (1)</td>
<td>Vari (1)</td>
<td>Vari (1)</td>
<td>Vari (1)</td>
</tr>
<tr>
<td>MPP5</td>
<td>Std</td>
<td>Std (1)</td>
<td>Std (1)</td>
<td>Std (1)</td>
<td>Std (1)</td>
<td>Std (1)</td>
<td>Std (1)</td>
<td>Std (1)</td>
<td>Std (1)</td>
<td>Std (1)</td>
<td>Std (1)</td>
<td>Std (1)</td>
<td>Std (1)</td>
<td>Std (1)</td>
</tr>
<tr>
<td>MPP3,4,7</td>
<td>Menage a 3 (Metro)</td>
<td>Mena3 (1)</td>
<td>Mena3 (1)</td>
<td>Mena3 (1)</td>
<td>Mena3 (1)</td>
<td>Mena3 (1)</td>
<td>Mena3 (1)</td>
<td>Mena3 (1)</td>
<td>Mena3 (1)</td>
<td>Mena3 (1)</td>
<td>Mena3 (1)</td>
<td>Mena3 (1)</td>
<td>Mena3 (1)</td>
<td>Mena3 (1)</td>
</tr>
<tr>
<td>ZO1,2,3</td>
<td>ZO/polychaetoid</td>
<td>ZO (1)</td>
<td>ZO (1)</td>
<td>ZO (1)</td>
<td>ZO (1)</td>
<td>ZO (1)</td>
<td>ZO (1)</td>
<td>ZO (1)</td>
<td>ZO (1)</td>
<td>ZO (1)</td>
<td>ZO (1)</td>
<td>ZO (1)</td>
<td>ZO (1)</td>
<td>ZO (1)</td>
</tr>
<tr>
<td>Dlg1–4</td>
<td>Dlg</td>
<td>Dlg (1)</td>
<td>Dlg (1)</td>
<td>Dlg (1)</td>
<td>Dlg (1)</td>
<td>Dlg (1)</td>
<td>Dlg (1)</td>
<td>Dlg (1)</td>
<td>Dlg (1)</td>
<td>Dlg (1)</td>
<td>Dlg (1)</td>
<td>Dlg (1)</td>
<td>Dlg (1)</td>
<td>Dlg (1)</td>
</tr>
</tbody>
</table>

Note.—List of the major components of TJs and SJs in human and Drosophila, and the respective protein homologs in St. pistillata, N. vectensis, H. magnipapillata (Cnidaria), T. adhesens (Placozoa), O. carmela (Porifera, Homoscleromorpha), Am. queenslandica (Porifera, Demospongiae), Mn. leidyi (Choanoflagellata), and C. owczarzaki (Filasterea). Numbers in brackets refer to the number of homologs found. “Not found” means absence of homologs whereas “nd” stands for “nondetermined” due to limitations in the assembly of the reference sequence (see Materials and Methods). Claudins were arguably separated into two subgroups: Claudin s.s. refers to the vertebrate Claudins (1–27) that are unique to the vertebrate TJs, whereas Claudin-like proteins encompass other members of the tetraspan family that are similar to Claudins in structure and also belong to the PFAM families PF00822, PF13903, and PF10242 (see supplementary table S1, Supplementary Material online).
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 NrxC 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 2012). For example, Claudins from the demosponge *Stylophora pistillata* (Chapman et al. 2010), or in Bilateria (Fahey and Degnan 2010). One homolog to NrxC 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 et al. (2013), NrxC was present in *Trichoplax*, cnidarians, and bilaterians. In contrast Cont was specific to bilaterians, whereas Riesgo et al. (2014) identified a Cont homolog in porifers. Homology criteria may have been missed 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 remain 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 proteins in the coral *St. pistillata*, a tractable species for calcification studies. After having defined the principal members of SJs in Cnidaria, we extended our genomic search to the other representatives of the early branching metazoans (i.e., the placozoan *Trichoplax adherens*, the homoscleromorph *Oscarella carmella*, the demosponge *Amphimedon queenslandica*, and the ctenophore *Mnemiopsis leidyi*) as well as in other phyla, the range of SJ component homologs was variable with a correlating trend of fewer homologs/copies and organismal simplicity.

### Claudins

Human Claudin 1–27 (Claudins s.s.) homologs were not found. However, iterative search with bilateral Claudin-like sequences identified a variable number of Claudin-like homologs in the different representative species of early branching metazoans as well as protists (table 1). Profile-based search against the Pfam database confirmed that all belonged to the PMP22_Claudin (PF00822), Claudin_2 (PF13903), or L_HGMIC_fpl (PF10242) domain family, except for 3 of the Claudins identified in *Oscarella* (OcaClau4,5,8) (supplementary table S1). TM domain prediction confirmed that all sequences were tetraspan proteins (data not shown) with a larger EL1 (50.8 AA ± 16.5) than EL2 (19.8 AA ± 8.0) (supplementary table S1). The Claudin signature motif within EL1 which benefits from numerous ecological and physiological studies (Allemand et al. 2011; Tambutte 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 nonbilateral representatives. 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 nonbilaterians. None of the TJ structural components characteristic of chordates was found in nonbilaterians. 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.

### Results

Genomes representing several classes from the phylum Cnidaria are available: *Nematostella* (*N. vectensis*, Anthozoa, Actiniaria), *Acropora* (*Ac. 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 cnidian genome projects are ongoing, for example, *Stylophora* (*St. pistillata*, Anthozoa, Scleractinia, robust clade), a reef building coral which benefits from numerous ecological and physiological studies (Allemand et al. 2011; Tambutte 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 nonbilateral representatives. 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 nonbilaterians. None of the TJ structural components characteristic of chordates was found in nonbilaterians. 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.

Claudins

Human Claudin 1–27 (Claudins s.s.) homologs were not found. However, iterative search with bilateral Claudin-like sequences identified a variable number of Claudin-like homologs in the different representative species of early branching metazoans as well as protists (table 1). Profile-based search against the Pfam database confirmed that all belonged to the PMP22_Claudin (PF00822), Claudin_2 (PF13903), or L_HGMIC_fpl (PF10242) domain family, except for 3 of the Claudins identified in *Oscarella* (OcaClau4,5,8) (supplementary table S1). TM domain prediction confirmed that all sequences were tetraspan proteins (data not shown) with a larger EL1 (50.8 AA ± 16.5) than EL2 (19.8 AA ± 8.0) (supplementary table S1). The Claudin signature motif within EL1
appeared slightly modified (i.e., W-G[LVI][WFYL]-C-C), except for a few cases. Bayesian and maximum-likelihood methods gave incongruent albeit comparable phylogenetic trees, that is, several well-supported groups could be outlined using both methods (fig. 1 and supplementary fig. S5a, Supplementary Material online). Use of an alternative alignment method (MUSCLE) prior to phylogenetic analyses supported the same groups (supplementary fig. S5a, Supplementary Material online). Group Ia contains anthozoan Claudins AS1,2 with human Claudin domain-containing protein 2 (HsClauL2) and lens fiber membrane intrinsic protein isoform 2 (HsLIM2), and group Ib contains Hydra Claudins 4,7,8,10,12,14,15 with human epithelial membrane protein 1-3 (HsEMP1-3) and peripheral myelin protein 22 (HsPMP22). Of note, the Stylophora, Acropora, and Nematostella Claudin AS1 and AS2 proteins corresponded to two splice variants conserved in anthozoans which vary in their first exon, and consequently in their first approximately 80 AA. This gave rise to two Claudins differing in their EL1. Group II corresponds to homologs of the human TMP211 and LHFP family of which some members have been involved in ear hair cell formation (Xiong et al. 2012). This large group contains Claudin members from Drosophila (CG3770, CG12026), cnidarians (anthozoan Clau3–6, Hydra Clau4,5), homoscleromorph (OcaClau1,2), and the placozoan and ctenophore Claudins (TriClau and MleClau1–4, respectively). Group III (Drosophila CG14182, anthozoan Clau8–9, Oscarella Clau8, Monosiga Clau8, Capsaspora Clau1), IV (anthozoan Clau2, Oscarella Clau3,4) and V (anthozoan Clau7, Oscarella Clau5) comprise homologs of the human CPO52 (uncharacterized protein C16orf52), Clarin3 and TMP127, respectively, for which a function has not yet been determined. Other Claudins sequences (e.g., MonoClauA,B,C or amphiClau) could not be reliably positioned on the tree, inferring that the Claudin primary sequences have considerably diverged during evolution. The human Claudins s.s. have been recently subdivided into five subgroups (Gunzel and Fromm 2012). Two members from each subgroup were randomly selected (HsCLDN1,2,3,8,11,12,16,18a,21,23) as representatives of the human Claudins s.s. and included in our phylogenetic analysis. These human TJ-specific Claudins clustered as a single outgroup. Interestingly, the three Drosophila Claudin-like proteins Megastracea, Sinuous, and Kune, for which functional characterizations are available, also clustered outside our five groups.
Neurexins

The Drosophila NrxIV and human Caspr families of proteins are closely related extracellular ligands with parallel domain architecture (i.e., LamG, EGF, and FBG domains). One clear homolog of NrxIV/Caspr was found in cnidarians (NRX1), displaying the same domain architecture, except for a missing NH2 term FA58C domain (fig. 2A and supplementary fig. S5b, Supplementary Material online). In Stylophora NRX1 (StpNRX1), domain homology search using SMART revealed a Band 4.1 binding motif. The presence of this motif indicates that StpNRX1 potentially binds to the putative Cora/Band 4.1 homolog as known from bilaterians. NRX1 was found to be duplicated in Nematostella (NvNRX1–2), Acropora digitifera (AdiNRX1–2), and Hydra (HydNRX1–3). In addition, several extra copies for cnidarian NRX (StpNRX2–5, AdiNRX3–5, NvNRX3–6, and HydNRX4–5) were found, with missing domains and/or long intracellular portions in comparison to the bona fide NRX1 homologs. Within the phylogenetic tree, the position of these supernumerary homologs within nonbilaterians NRX suggests duplication within the cnidarian lineage. StpNRX2 did not cluster with any anthozoan homolog, suggesting that it may be either specific to Stylophora or the robust clade of scleractinian corals, as it is not found in Acropora (complex clade).

In Trichoplax we identified five potential NrxIV homologs, representing placozoan-specific duplications, showing variable domains composition, except for TriNRX1 which harbor canonical Nrx domain composition. No NrxIV/Caspr homologs were found in the remaining analyzed phyla. However, homologs of the more distant gene family, human Neurexin 1–3 (HsNeu1–3), and Drosophila Neurexin 1 (DmNeu1) were present. Neurexins are synaptic cell adhesion molecules in bilaterian composed of alternating LamG and EGF domains (Bang and Owczarek 2013), similar to the NrxIV/Caspr, but without the FBG domain (fig. 2A). One clear Neurexin1 homolog (see supplementary table S1, Supplementary Material online, for reverse BLAST hits) was found in Oscarella (but not in Amphimedon, supplementary fig. S6, Supplementary Material online) and in Mnemiopsis, with the same domain architecture as in bilaterians. Of note, omission of the bilaterian Neurexin protein sequences placed the OcaNeu and MleNeu sequences at the base of the cnidarian/placozoan NRX phylogeny (data not shown). Several conserved Neurexin homologs were also found in cnidarians, although here, they were substantially...
shorter: two LamG and one EGF domain, instead of six LamG and three EGF domains in the canonical form. Moreover, one putative Neurexin1 homolog was found in Capsaspora (also identified in Suga et al. 2013), which is composed of six LamG domains (fig. 2A) and positions in between the Neu and Nrx families in the phylogenetic tree (see radial representations in supplementary fig. S4, Supplementary Material online), potentially representing the metazoan Neu–Nrx ancestor.

NRG, CONT, and Down Syndrome Cell Adhesion Molecule

The Drosophila Nrg and Cont, and the human Neurofascin and Contactin have closely related domain structures, that is, succession of Ig domains followed by FN3 domains. However Nrg/Neurofascin has a C-term TM domain spanning the plasma membrane, whereas Cont/Contactin is attached to the membrane through a GPI anchor. Both Nrg and Cont have homologs in cnidarians displaying similar domain architecture as well as TM and GPI anchor attachment, respectively (fig. 2B and supplementary fig. S5b, Supplementary Material online). In comparison to Nemastostella, the scleritarians Stylophora and Acropora have additional NRG copies (StpNrg2 and AdiNRG2,3). Their position within the phylogenetic tree indicates that they represent scleritarian-specific duplications. In Trichoplax, four NRG and one CONT homologs were also found, likewise with placozoan-specific duplications (fig. 2B). Note that differentiation between the Trichoplax NRGs and CONT was solely based on the TM/GPI anchor prediction. In Oscarella, one potential NRG homolog (OcaNRGcam) could be found. However, based on the reverse BLAST hit approach, this protein could either be an NRG or a Down Syndrome Cell Adhesion Molecule (DSCAM) homolog. Indeed, NRG/CONT shares a very similar domain composition with other Ig/FN3 domain adhesion molecules such as Hemicentin, and DSCAM, the latter being the closest relative of NRG/CONT. DSCAM are extracellular ligands capable of homophilic associations and heterophilic interactions involved in neural wiring in bilaterian as well as innate immunity in protostomes (Schmucker and Chen 2009). We thus undertook a characterization of DSCAM proteins in the different early branching metazoan to estimate the evolutionary convergence of the NRG/CONT and DSCAM families. DSCAM homologs were identified in Mnemiopsis (1), Oscarella (2), Trichoplax (1), Hydra (2) and anthozoans (2), but neither in Amphimedon nor in proists (fig. 2B). With respect to domain architecture, the cnidarian DSCAM1 resembled the bilaterian DSCAMs, whereas the cnidarian DSCAM2, TriDSCAM, OcaDSCAM and MleDSCAM showed higher similarity to the NRG/CONT architecture, despite being closer to DSCAM at the sequence level. In line with these finding, the OcaNRGcam protein represents an evolutionary intermediate between the two families (see supplementary fig. S4, Supplementary Material online).

MAGUK

Members of the MAGUK super family share a central PDZ–SH3–GuKc domains module. The various MAGUK members essentially differ by the addition of other domains, commonly PDZ and L27 (Funke et al. 2005). The phylogenetic analysis of MAGUK members across early branching metazoans was based on the central module sequences (fig. 3A and supplementary fig. S5c, Supplementary Material online). This analysis complements the previous analysis by de Mendoza et al. (2010). Both Capsaspora and Monosiga possess an MPP and Dlg ancestor that gave rise to the phylogenetic diversity of the metazoan MAGUK family. We show that MPP2–7 is split into two distinct groups, MPP2,6 (Vari) with extended members in all early branching metazoans, and MPP3,4,7 (Mena3) which is restricted to bilaterians and cnidarians. MPP5 (Std) appears...
to have some related members (MPPb) in poriferans, ctenophores, and placozoans. However, we could not ascribe a bona fide Std homolog to ctenophores. The ZO family is present in all early branching metazoans, except *Amphimedon*.

**Coracles**

Cora, Yurt, and Nbl4 are structurally related FERM-FA domains proteins (*Tepass* 2009). Cnidarians possess a clear Cora homolog (CORA) and two additional Cora variants that mainly differ by their COOH terminal moiety (fig. 3B and supplementary fig. S5c, Supplementary Material online). *Trichoplax*, *Oscarella*, *Amphimedon* and *Mnemiopsis* also harbor Cora-like proteins, structurally closer to the cnidian Cora variants than the canonical one. A Yurt homolog is found in anthozoans, *Trichoplax* and *Oscarella*, whereas the Nbl4 (human 4.1-Like) appears to have emerged at the time of cnidian/bilaterian radiation. Of note, OcaYurt clusters with NbI4 protein sequences in the Bayesian tree and Yurt protein sequences in the maximum-likelihood tree (fig. 3B and supplementary fig. S5c, Supplementary Material online), and may therefore represent an ancestor of Yurt–Nbl4 families. OcaYurt was ascribed as Oscarella Yurt homolog based on BLAST results (supplementary table S1, Supplementary Material online).

**Phylogenetic Conclusive Remarks**

Taken together, cnidarians and placozoans appear to share the complete SJ complement. Several gene duplications were observed in cnidarians (NRX, NRG, and CORA), some of which are likely specific to reef building corals. Dichotomy between *Hydra* and the anthozoans was apparent in the gene phylogeny (e.g., Claudin-like, NRX, NRG), which indicates class specific diversification with possible subsequent divergence in SJ structures. In contrast, genes encoding for the structural components of SJs, that is, NrxIV, Nrg, and Cont, are absent in the other early branching metazoan phyla analyzed here (table 1), although members of the scaffolding and polarity families. OcaYurt was ascribed as Oscarella Yurt homolog based on BLAST results (supplementary table S1, Supplementary Material online).

**The Diversified SJ Components in Anthozoans Show Distinct and Tissue-Specific Gene Expression**

Electron microscope investigation of *Stylophora* across the different tissue layers clearly hallmark the presence of SJs between the apical border of every ectodermal and endodermal cell (supplementary fig. S2, Supplementary Material online) (*Tambutté et al.* 2007). They are 0.2–1 μm long, depending on the section, and display a characteristic ladder structure. On micrographs where the two tissue layers are visible, SJs of the endodermal layer appear to show higher electron density than those of the ectoderm layer. As the SJ complement in cnidarians appears to have diversified, we next asked what the relative expression of different SJ components was and whether differential expression between the oral (noncalcifying) and aboral (calcifying) tissues could be observed. We developed a protocol to microdissect the oral discs from the coral colony using the anesthetic drug MS222 and microscissors (see Material and Methods). *Stylophora* total RNA and proteins were extracted from a colony fragment (oral and aboral) or from the oral disc (oral only) and expression was quantified by real-time polymerase chain reaction (PCR) and western blotting for the genes described in figure 4. Quantitative PCR (qPCR) expression estimates were normalized arbitrarily to StpNRX1 = 1 as relative expression of the SJ components was our primary focus and because NRX is a core-component of SJs in bilaterians. StpNRX3–5 showed relatively low expression in contrast to StpNRX2 (0.53-fold to NRX1) (fig. 4A). The two StpNRG copies were expressed at strikingly different levels (StpNRG1 = 0.34-fold, StpNRG2 = 3.1-fold). Unexpectedly, StpCONT was weakly expressed (0.047, see Discussion). Claudin-like mRNAs were all expressed, although at relatively low levels in comparison to StpNRX1. The SJ adaptor component StpCORA1 was expressed at a similar level as StpNRX1 and the different variants StpCORA2, 3 and StpYURT were also expressed, strongly arguing that these conserved anthozoan genes represent functional rather than pseudogenes. When assessing tissue specificity, three SJ genes, namely StpNRX2, StpClaud3, and StpClaud6, were strongly downregulated in the oral disc as opposed to the total colony (oral and aboral) tissue, suggesting that these were mainly expressed in the calcifying aboral tissue, similar to the TFZPD9 calicoblast control (fig. 4B). Although to a lesser extent, StpNRG2 as well showed preferential expression in the aboral tissue. Reversely, StpNRX3, StpCONT, and StpClaudAS2 showed high expression in the oral disc, albeit displaying a lower colony-wide expression. In order to estimate the relative expression between the endodermal and the ectodermal tissue layers, we took advantage of the large size of the sea anemone *Anemone viridis* tentacles (oral tissue), where the endodermal and ectodermal layers can be manually separated. A partial *A. viridis* cDNA database is available (*Salourault et al.* 2009) and incomplete sequences corresponding to SJ components homologs could be identified. Measurement of their relative tissue expression shows predominant tissue specificity for one gene among those tested, namely the duplicated copy of NRX1 (*AvNRX1b*) (supplementary fig. S7, Supplementary Material online).

We generated antibodies against the StpNRX1 and StpClaud3 proteins. These antibodies were specific as little to no cross-reactivity could be observed in Western blots (fig. 4C). Similar to the Actin control, StpNRX1 was equally expressed in both the oral disc and the total colony fractions and was present in the blot as a single band <150 kDa. This ascertained our qPCR results, namely, that StpNRX1 represents a central component in most SJs. Conversely, StpClaud3 was mostly absent from the oral discs fraction but present in the total colony. This Claudin-like protein is thus likely to be mainly expressed in the aboral calcifying tissues of the coral. In conclusion, anthozoan-specific gene diversification is accompanied by differential tissue expression, suggesting the presence of multiple SJ architectures.
and functions in the different cell layers comprising these diploblastic animals.

**StpNRX1 Is Glycosylated and Colocalizes with F-Actin**

StpNRX1 has a predicted molecular weight of 126.5 kDa, which is in disagreement with the molecular weight of 141 kDa determined by Western blotting (fig. 4C). In humans, the Caspr1 protein is glycosylated (Bonnon et al. 2003); we therefore examined whether StpNRX1 also exhibits posttranslational N-linked glycosylation that contributes to the difference between the apparent and predicted molecular weight. Total protein extract was treated with and without PGNaseF (which specifically cleaves between asparagine and N-acetylglucosamines), and Western blotting showed a shift from 141 to 128 kDa of StpNRX1 after PGNase treatment (fig. 5f). StpNRX1 is conclusively N-glycosylated similar to Caspr1 in human. We next addressed the cellular localization of StpNRX1 and StpClaud3 in adult _Stylophora_. An immunolocalization protocol was therefore established. Coral fragments were fixed, decalcified, and cut into parts for investigation of the aboral calcifying tissues (fig. 5a). Labeling performed on the basal discs (see supplementary fig. S1, Supplementary Material online) with phalloidin identified the F-actin network framing every cell (fig. 5b). This cortical F-actin is supposedly adjacent to SJs as SJs are linked to the cytoskeleton in bilaterians, and anthozoan SJs display similar protein composition to bilaterian SJs. Immunolocalization with phalloidin and anti-StpNRX1 showed overlapping signals for most of the F-actin network (fig. 5c). Optical sectioning sagittal to the epidermal (calicoderm) tissue layer showed that NRX1 and F-actin overlapped, albeit partially, on the apical face of the cell layer (fig. 5d). In order to eliminate optical interference between the Alexa-conjugated secondary antibody and potential endogenous autofluorescence (e.g., GFP), the rabbit anti-NRX1 was detected simultaneously with antirabbit-Alexa488 and antirabbit-Alexa405. Both channels showed identical labeling in the calicoderm layer (fig. 5d1 and d2). Thus, StpNRX1 could
be colocalized with, or very close to, the F-actin network at the apical border of the calicoderm layer, strongly supporting that StpNRX1 is a core component of SJs in *Stylophora*. Immunolabeling of StpClaud3 showed a different pattern. First, labeling was restricted to groups of calicodermal cells along the basal disc. In such groups, although labeling juxtaposed the F-actin labeling, the overlap between Claud3 and F-actin was only partial. In some cases, StpClaud3 encircled two or more cells (fig. 5e). Such a pattern rather suggests that StpClaud3 has a supracellular function within the calicoderm layer for yet-to-define specialized cells.

**Model of Cnidarian SJ as a Blueprint of Bilaterian SJ**

Several lines of concordant evidence has led us to propose a model for cnidarian SJs (fig. 6), as inferred from bilaterian SJs (Laval et al. 2008; Shimoda and Watanabe 2009): 1) Protein sequence and domains conservation of the different SJ components, which suggest common functionality; 2) congruent phylogeny of bilaterian and cnidarian SJ components, which suggest evolutionary conserved function; 3) localization at the apical border of the cells for StpNRX1; 4) conserved N-linked glycosylation between StpNRX1 and its mammalian counterpart Caspr1; and 5) similar SJ ultrastructure in insects and anthozoans on electron micrographs. The tripartite NRX–NRG–CONT complex forms the structural base linking two adjacent cells. Cora and Yurt proteins serve as...
intracellular scaffolds, possibly attaching the intracellular part of the structural components. Members of the MAGUK superfamily also serve in scaffolding and cellular polarity. \(\text{Na}^+ / \text{K}^+\) transporters in SJs have been verified in various species. Our StpClaud3 labeling data substantiate Claudin-like association with SJs, although the expression of this particular Claudin appears restricted to specific cell types. Limitations of the above model include the low mRNA expression of \(\text{CONT}\), the absence of the diverse conserved variants of \(\text{NRX}\), and the lack of evidence for the presence of Claudin-like proteins as core-components of cnidian SJs. However, the model presented here accounts for both medusozoans and anthozoans, two cnidian clades that diverged probably more than 540 Ma (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.

**Discussion**

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

**Epitheliozoans as Defined by Acquisition of SJs**

The molecular phylogeny of the principal occluding junction components across the metazoan lineages (restricted to representative organisms with complete genomes) allows reconstructing a scenario of stepwise evolution for sealing epithelia, that is, the emergence of body compartments (fig. 7). However, the phylogeny of early branches is not settled (Philippe et al. 2011; Nosenko et al. 2013). The tree presented in figure 7 follows minimal gene loss across metazoan evolution of the SJ complement. Ctenophora are positioned at the base of the metazoan lineage, according to current studies (Ryan et al. 2013; Moroz et al. 2014); demosponges and homoscleromorphs are separated according to Sperling et al. (2009) and Erwin et al. (2011) although consensus on the mono versus paraphyly of poriferans has not been reached (Worheide et al. 2012). In the protists *Capsaspora* and *Monosiga*, we identified the \(\text{Na}^+ / \text{K}^+\) ATPase exchanger (the Beta subunit appeared with *Monosiga*), MAGUK ancestors (\(\text{Dg}\) and \(\text{MPP}\)), and Claudin-like members, which prove that these were already present in the metazoan ancestor lineage. The \(\text{Na}^+ / \text{K}^+\) ATPase transporter is an integral part of occluding junctions (Krupinski and Beitel 2009). Although this exchanger is required for SJ formation in insects, its function in SJs is pump-independent (Genova and Felon 2003; Paul et al. 2007). Interestingly, the beta subunit of the \(\text{Na}^+ / \text{K}^+\) ATPase transporter has been shown to create molecular bridges between two adjacent cells (Vagin et al. 2012). This moonlighting function of the \(\text{Na}^+ / \text{K}^+\) transporter might have represented a potential building block for the further development of occluding junction in epithelia (Krupinski and Beitel 2009). With multicellular animals, components of the cytosolic adaptor plaque appeared successively. Homologs of the MAGUK members \(\text{Vari}\) (\(\text{MP2,6}\)), \(\text{ZO}\), and an ancestral form of \(\text{MPP}\) \(3,4,5,7\), as well as the FERM protein \(\text{Cora}\), arose in cnenophores. These represent cytosolic components involved in cellular polarization (and junction scaffolding) in bilaterians. \(\text{Std}\) (\(\text{MPP5}\)) appeared with demosponges but ZO was absent. In homoscleromorphs, Yurt as well as a putative NRG ancestor (intermediate between \(\text{DSCAM}\) and \(\text{NRG}\)) were identified. However, it is only with placozoans and cnidarians that the structural components of SJs, that is, \(\text{NRX}, \text{NRG}, \text{CONT}\), emerged, hereby pointing to the origin of SJs in metazoans. In bilaterians, SJs were kept as the principal type of occludin junctions in protostomes, whereas vertebrates within the deuterostome lineage evolved a specialized Claudin family (here referred as Claudins s.s.) and other structural proteins (JAM, Marvels, etc.) that permitted a novel type of junction, the TJ.

**Epitheliozoa**, which includes the Bilateria, Cnidaria, and Placozoa, was originally proposed to characterize animals with true epithelia defined as cell layers held together by belt desmosomes (Ax 1996; Dohrmann and Worheide 2013). Our present study extends the characteristics of the **Epitheliozoa** as animals with epithelia sealed by occluding junctions (TJs and SJs). Importantly, the lack of structural SJ components in poriferans was not assessed in calcareous sponges in this study, as no calcareous genome is available hitherto. However, Ledger described potential SJs in the calcareous sponge *Sy. ciliatum* using TEM experiments (Ledger 1975). Hence, genomic exploration of calcareous sponges is required before a complete picture of SJ evolution can be drawn.

**Are Structural SJs Components Derived from Neuronal Junctions?**

Poriferans and placozoans do not have recognized neurons contrary to ctenophores and cnidarians which have well-defined neurons and nerves (Moroz 2012). However, candidate neurosecretory cells have been found in both poriferans (flask cells [Renard et al. 2009]) and *Trichoplax* (fiber cells [Smith et al. 2014]). Further, a set of protosynaptic genes have been identified in poriferans (Sakarya et al. 2007; Conaco et al. 2012). Thus, irrespective of whether or not Ctenophora represents the basal Metazoa, the genetic origin of the neural...
system starts with animal multicellularity. Central to the organization of the bilaterian neuronal network is the Neurexin–Neuroligrin interaction (Bang and Owczarek 2013). Neurexins are found at the synaptic membranes and bind to Neuroligin on the opposite synaptic membrane across the 20-nm-wide synaptic cleft (Sudhof 2008; Chen et al. 2010). On the cytosolic side, Neurexin binds to the MAGUK proteins Dlg (PSD-95) and CASK and the FERM protein 4.1/Cora (Hata et al. 1996; Biederer and Sudhof 2001; Chen et al. 2005; Chen and Featherstone 2011). Thus, in addition to structural similarities (Bellen et al. 1998), the synaptic Neurexin (Neu) and the SJ Neurexin (Nrx) share common cytosolic partners. Although the function of the Neurexin 1 homologs in homoscleromorphs (Nichols et al. 2006) and ctenophores (Moroz et al. 2014) is not known, Neu arose before Nrx, possibly originating from a Capsaspora ancestor (fig. 7 and supplementary fig. S4, Supplementary Material online). A possible scenario implies the primary addition of EGF domains to the Capsaspora ancestor (fig. 7 and supplementary fig. S4, Supplementary Material online). A possible scenario implies the primary addition of EGF domains to the Capsaspora ancestor (fig. 7 and supplementary fig. S4, Supplementary Material online). A possible scenario implies the primary addition of EGF domains to the Capsaspora ancestor (fig. 7 and supplementary fig. S4, Supplementary Material online). A possible scenario implies the primary addition of EGF domains to the Capsaspora ancestor (fig. 7 and supplementary fig. S4, Supplementary Material online).

Structural SJ Components Appeared and Diversified in Cnidarians

The SJ components of cnidarians and bilaterians are very similar at the protein level and therefore, a common model for the SJ structure can be inferred from the characterization of SJs in both insects and mammalian paranodal junctions (Charles et al. 2002; Bonnon et al. 2003; Faire-Sarrailh et al. 2004) (fig. 6). In particular, in Styllophora, StpNRX1 localizes at the apical border of each cell (fig. 5), which is in strict correlation with the position of SJs observed in TEM images (supplementary fig. S2, Supplementary Material online). StpNRX1 also colocalizes with the F-actin network (fig. 5), strongly supporting a model where SJs are attached to the cytoskeleton through cytoplasmic adaptor proteins. Finally, in human, Caspr proteins associate with Contactin during their biosynthesis, resulting in the expression of high-mannose glycoforms of the two proteins at the cell surface (Bonnon et al. 2003). In this study, StpNRX1 has been shown to be N-glycosylated as demonstrated by the apparent molecular weight reduction after PGnaseF treatment on Western blot (fig. 5). StpNRX1 thus performs as the faithful homolog of Drosophila NrxIV and human Caspr1. Moreover, several other copies of NRX1 (StpNRX2–5) are also present in Stylophora (fig. 2). These supernumerary anthozoan-specific copies display similar domain architectures in their NH2 terminus but differ in their COOH terminus (all have a TM signature). These variations in domain architectures, conserved among anthozoans, may reflect functional diversification, in conjunction with specific tissue expression. StpNRX2 (a Stylophora-specific NRX), which accounts for about half of StpNRX1 in the total fraction, is mostly expressed in the aboral tissue (fig. 4). Conversely, StpNRX3 is dominantly expressed in the oral
tissue. This suggests that the aboral (calcifying) and oral (polyp) tissues harbor a different set of structural components for their SJs. Indeed, one of the scleractinian-specific NRG copies shows preferential expression in the oral tissue. Although the cellular localization and function of these additional NRX and NRG homologs remain to be addressed, SJs with different composition may reflect structural differences and possibly result in different paracellular properties between the different tissue layers or specialized cell types. Along the same line of evidence, in the tentacle of An. viridis, one of the two copies of NRX1 (AvNRX1b) is found mostly expressed in the endoderm, unlike AvNRX1a which is equally expressed in both tissue layers (supplementary fig. S7, Supplementary Material online). Thus, at least in An. viridis, the structural composition of SJs between the ectoderm and the endoderm appears to differ. Such discrepancy in NRX composition may be the cause of the differences observed in TEM images of sea anemone SJs between the endoderm with double septum and the ectoderm with single septum (Green and Flower 1980). Patchwork expression of different SJ components in tissues/layers is substantiated by the differential expression of the additional copies encoding for the cytoplasmic adaptor CORA in Corals. One surprising result of our expression analysis was the very low mRNA expression of StpCONT as compared with StpNRX1 and StpNRG1,2, as these form a trimolecular complex in bilaterians. Our data raise the possibility that CONT is not part of these complexes in cnidarians. This result was confirmed by other independent RNAseq approaches (data not shown). In addition, data mining of other cnidian expressed sequence tag (EST) databases (including Nemastostella and Hydra) also showed that putative ESTs homologous to CONT were scarce. Although thought provoking, our data raise the possibility that CONT is not part of the trimolecular core-complex that structures all SJs in cnidarians. Alternatively, the turnover of the CONT mRNA and protein may be very slow and therefore present in low copy numbers. Also, CONT may be required for specific developmental stages or cell types. Hence, as the CONT mRNA is indeed expressed, we included CONT in our cnidian SJ model.

Conserved Claudin-Like Proteins

Claudins were first identified in vertebrate TJs and so far 27 members have been identified in vertebrates (Gunzel and Fromm 2012). Beside these TJ-specific Claudins (Claudin s.s.), other Claudin-like proteins have been identified based on sequence and tetraspan structure similarities both in human and in invertebrates. In Drosophila, three Claudin-like proteins were functionally associated with SJs. However, Claudin phylogeny is unclear as these proteins loosely cluster in highly divergent clades (Simske and Hardin 2011). The addition of Claudin-like proteins from early branching metazoans to the Claudin repertoire highlights several clusters of evolutionarily conserved Claudin-like members. Claudin s.s. are specific to TJs, which correlates with the fact that they form an outgroup to the other Claudin-like sequences. Groups Ia, Ib, II, and IV encompass human Claudin-like proteins (LIM2, PMP22, EMP1–3, LHFPL1–4, and Clarin-3) proposed to have cell–cell interaction properties (reviewed in Van Itallie and Anderson 2004; Simske and Hardin 2011; Cosgrove and Zalocchi 2014). For example, LIM2 (group Ia) and PMP22 (group Ib) have been shown to associate with TJ constituents and to display barrier properties (Notterpek et al. 2001; Grey et al. 2003; Roux et al. 2005), whereas an LHFP member (also called TMHS, group II) was associated with hair-cell anchoring independently of TJs (Xiong et al. 2012). What is the role of analogous Claudin-like in invertebrates? The Stylophora StpClau3 (group II) clearly localizes at the cell–cell boundary of specific cells (fig. 5), in agreement with specialized cell interaction properties. In Hydra, the Claudin HydClau1 also localizes to the apical junctional complexes (Hombmayer B, personal communication). However, outside the three Drosophila Claudin-like proteins associated with SJ formation, it would be premature to involve any other invertebrate Claudin-like proteins with a particular function in SJs. A junctional interaction in trans between two cells is highly improbable as the distance separating adjacent plasma membranes is too large to allow kissing complexes in SJs. A function in regulating the paracellular transport across an epithelium has only been described for the TJ-specific Claudin (Van Itallie and Anderson 2006). The ancient and diversified Claudin repertoire may well represent diverse conserved functions, as part of macromolecular complexes associated with the plasma membrane. Further biochemical characterization will be needed to clarify the apparent discrepancies between the Claudin phylogeny presented here and the function of Claudins inferred from vertebrates. Indeed, Claudin-like proteins are present in the unicellular Capaspora and Monosiga suggesting that tetraspan proteins had ancestral functions besides promoting cell–cell interaction. Claudin-like group III appears to contain the most evolutionarily conserved Claudin clade with Claudin-like members found in vertebrates, Drosophila, cnidarians, poriferans, Monosiga, and Capaspora; yet functional data are not available for any of them.

Functional Implications

Occluding junctions govern paracellular transport across epithelia. In invertebrates, SJs control this paracellular pathway, as shown in insects using conductance experiments on epithelia and by dextran injection after gene knock down (Pannabecker et al. 1993; Lamb et al. 1998; Banerjee, Sousa, et al. 2006). Although classified as “leaky epithelium,” as compared with the vertebrates’ “tight epithelium,” epithelia in insects are nevertheless able to show barrier properties comparable to vertebrates. For example, in female mosquitoes, Malpighian tubes maintain very high [K+] gradients and allow rapid paracellular transport of Cl− across the Malpighian epithelium after blood meals to maintain homeostasis (Beyenbach and Piermarini 2011). In cnidarians, the epithelial layer also show different permselective properties to Ca2+, Na+, and Cl− (Bénazet-Tambutté et al. 1996), suggesting that SJs potentially control ion exchange across cnidian tissue layers. In reef-building corals, the oral and aboral tissues have specialized roles in the process of
biomineralization, and the transport of ions from the surrounding sea water to the site of calcification is central to the understanding of how the calcium carbonate skeleton is formed (Tambutté et al. 2007; Allemend et al. 2011). Although the transcellular pathway is part of this ion transport (for recent reviews, see [Allemend et al. 2011; Tambutté et al. 2011]), experiments have raised the possibility that paracellular transport might also be involved (Tambutté et al. 2012). As molecules such as calcine (molecular radius 6.5 Å) are able to pass through the junction, small ions such as calcium (molecular radius 1.8 Å) should also, in principle, be able to pass through the paracellular pathway (Tambutté et al. 2011, 2012). However, in chordate epithelia, TJs regulate the flow of molecules not only based on the size but also based on the charge of the molecule/atom. Although in chordates it is generally accepted that Claudins (Claudins s.s.) define the TJ permselective properties, almost nothing is known about the mechanisms that govern the flow of molecules through SJs. In other words, the respective roles of the Claudin-like, NRX, NRG, and CONT proteins (or other molecules) in regulating the paracellular transport still remain to be characterized, especially in regards to tissue-specific permselectivity. Further experiments using heterologous expression of SJ components in conjunction with electrophysiological measurements will help to better understand the role of these molecules in the permselective passage of ions. In addition to shedding light onto the coral calcification process, determining the permeability/permselectivity of SJs is also of major importance in the environmental context of ocean acidification. Previous studies have shown that the decrease in pH in the oceans, due to the increase in atmospheric CO2 and its dissolution into seawater, negatively affects coral calcification (Andersson and Gledhill 2013). One parameter that might explain this effect, among others, is the degree to which the site of calcification is isolated from seawater. It has been proposed that the sensitivity of corals to ocean acidification could readily be explained if the paracellular route is the major supply of ions for calcification (Erez et al. 2011). Different studies have suggested a protective role of tissue layers against skeletal dissolution (Ries et al. 2009; Rodolfo-Metalpa et al. 2011). However none of them has examined the potential role of SJs in such a protection because no molecular data on junctions were hitherto available. The results presented here lay the foundations for future studies that will allow to monitor differential expression of genes involved in the formation of SJs and to determine whether they play a role in resistance to ocean acidification.

### Materials and Methods

#### Model Organisms

Cnidaria comprise two major classes, Medusozoa (including Hydrozoa) and Anthozoa (including Hexacorallia). Actiniaria and Scleractinia constitute two major subclasses of Hexacorallia. Commonly, Actiniaria are represented by sea anemones such as *N. vectensis* (*Nematostella*) and Scleractinia are represented by reef-building corals, such as *St. pistillata* and *Ac. digitifera* (Kayal et al. 2013), which are colonial polyps and have a specialized calcifying tissue layer (calicoderm; supplementary figs. S1 and S2, Supplementary Material online).

#### Sequences

All human and *Drosophila melanogaster* reference protein sequences listed in supplementary table S1, Supplementary Material online, were retrieved from National Center for Biotechnology Information (NCBI). Early branching metazoan sequences were retrieved from the databases listed in supplementary table S2, Supplementary Material online. The *St. pistillata* sequences were deduced from transcriptome and/or genome assemblies (C.R.V. and M.A.). Note that some Hydra and Ac. digitifera protein families were omitted in our phylogenetic analysis due to inconsistent sequences assemblies (gaps, Ns, and misassemblies) of some members.

### Software and Strategy Employed

BLAST (2.2.22) genome/transcriptome analysis was run locally, at NCBI and JGI depending on organisms’ database. An online version of MAFFT (mafft.cbrc.jp/alignment/server/) was used with strategy L-INS-i default parameters. Prottest (v2.4), PhyML (v3.0), MrBayes (v3.2.1), and FigTree (v1.3.1) (Huelenbeck and Ronquist 2001; Abascal et al. 2005; Rambaut and Drummond 2009; Guindon et al. 2010) were run locally. PFAM (http://pfam.xfam.org/) and SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi) were used to predict protein domains. TM domains were predicted at www.cbs.dtu.dk/services/TMHMM/, GPI anchors were predicted using the webtools “Frag/Anchor” (http://navetics.hawaii.edu/~fraganchor/NNHMM/NNHMM.html), PredGPI (http://gpcr2.biocomp.unibo.it/gpipe/pred.htm), and “GPI-anchored Protein Prediction” (http://mendel.imp.ac.at/sat/gpi/gpi_prediction.html), which gave similar results.

BLAST searches were run on the cnidarians databases to identify putative homologs using *Drosophila* and human protein sequences of the molecular SJ and TJ constituents (supplementary fig. S3, Supplementary Material online). All newly identified protein sequences were added to the previous pool of query sequences for iterative BLAST searches to identify novel potential homologs. Selection criteria were based on reciprocal BLAST against the human and *Drosophila* RefSeq databases as well as domain homology. Once identified in cnidarians, searches for homologs were further carried out in *Trichoplax*, then in *Amphimedon* and finally in *Monosiga*, always using the entire pool of identified proteins as bait. When homologs were missing in *Amphimedon*, BLAST searches were carried out against the whole NCBI sponge database before deemed absent. For each family of SJ components identified, protein sequences were aligned using MAFFT. Alignments were trimmed to the largest conserved part of the proteins (supplementary file S1, Supplementary Material online) and then subjected to phylogenetic analyses. Best substitution matrices and parameters were calculated using Prottest before running PhyML. Alternatively, Bayesian analyses using MrBayes were run with default specification until convergence reached standard deviation.
below 0.01, except for Claudins, which were stopped after 7 million Markov chain Monte Carlo generations. Resulting trees were visualized using FigTree.

**Oral Disc Dissection**

Fragmented samples of the same *St. pistillata* colony were used for both RNA and protein extractions. Fragments were set to rest in a glass petri dish filled with sea water until polyps were extended. 0.4% stock solution of Tricaine mesylate (MS-222; Sigma) dissolved in sea water was added into the petri dish to a final concentration of 0.04% and left to rest under dimmed light for 15 min. Subsequently, oral discs (the apparent portion of the polyp; supplementary fig. S1, Supplementary Material online) were cut from the colony under binocular using 5-mm blade microdissection scissors (Vannas). Batches of 10–15 oral discs were collected and transferred into Trizol or TNE solutions (see below). Dissections were stopped after a maximum of 45 min of MS-222 incubation to elude any potential secondary effect of the drug.

**RNA Extraction**

Freshly dissected oral discs were put into Trizol and homogenized for 1 min using an electrical potter. Alternatively, entire fragments of colony were cryo-crushed (SpeX sampleprep 6770) and the resulting powder was dissolved in Trizol. RNA extraction was carried out using a standard protocol (Moya et al. 2008). Extracted RNAs were treated with RNase-free DNasel (Roche) and precipitate with NaAcetate/EtOH. Concentrations were determined by spectrophotometry using an Epoch Microplate Spectrophotometer (BioTek).

**Real-Time qPCR**

cDNAs were synthesized using the SuperscriptIII kit (Invitrogen). qPCR runs were performed on an ABI 7300 using “EXPRESS SYBR GreenER qPCR Supermix with Premixed ROX” (Lifetechnologies) for PCR amplification. Experimental procedures were performed as in Moya et al. (2008). Data were either relative to StpNRX1 

\[ \frac{Ct_{gene\ of\ interest} - Ct_{NRX1\ Total}}{Ct_{Total}} \]

for the expression in the oral disc (Oral) as compared with Total. Fold expressions were further extrapolated using the \(2^{-\Delta\Delta Ct}\) and \(2^{-\Delta Ct}\) functions.

**Protein Extraction Protocol for Enriched Membrane Proteins (All Step on Ice)**

Freshly dissected *Stylophora* oral discs were kept in cold TNE buffer (100 mM Tris–pH 7.2, 100 mM NaCl, 5 mM ethylenediaminetetraacetic acid [EDTA], 1× Protein Inhibitor Cocktail [Sigma]). Alternatively, total tissue was extracted from the skeleton in cold TNE using the air-pick (i.e., pressurized air through a pipet tip) method on coral fragments. Batches of 1 ml crude extract were then dialyzed by repeatedly passing them through a 21" syringe gauge until homogeneity was reached. Extracts were centrifuged at 1,000 × g for 1 min at 4 °C.

The supernatant (S1) was collected and kept on ice. The pellet was resuspended in TNE, passed through a syringe, and centrifuged again. The supernatant (S2) was pooled with S1 and triton X-100 was added to a 1% final concentration. The resulting intermediate extract was incubated for 1 h at 4 °C on a rotating wheel and then centrifuged at 15,000 × g for 15 min at 4 °C. The resulting supernatant was the final total extract, now cleared of zooxanthellae but enriched in membrane proteins. Protein concentrations were measured by comparison to a BSA standard curve. For the N-linked glycosylation analysis, 250 μl of *Stylophora* total extract was divided into two reaction tubes; half was incubated with 2 μl 2-mercaptoethanol and 8 μl PGNaseF (Roche) for 3 h at 37 °C and the other half was used as control.

**Custom-Made Antibodies (Eurogentec)**

Two antibodies were produced in rabbit using synthetic peptides, one against the peptide KTNPYDPDTSGRRTDDD (AA 1057–1073) corresponding to the beginning of the extracellular part of StpNRX1 and the other one against the peptide GRMASHGYYNQDTTTL (AA 220–236) corresponding to the COOH terminus of the StpClaud3. For each antibody, ten rabbits were initially screened for noncrossreactivity with *Stylophora* proteins and two were selected for the Speedy program. Each selected antibody was affinity purified before use.

**Western Blotting**

Equal amounts of protein extracts were loaded onto 6% (NRX1), gradient 4–15% (actin), and 15% (Claud3) TGX precast gel (Bio-Rad). After electrophoresis, gels were transferred onto PVDF membrane and blotted using SNAP i.d. with anti-Stp_NRX1 (1:200), anti-StpClaud3 (1:200), antiactin (mouse A4700 Sigma) (1:500) primary antibodies, and HRP-coupled goat antirabbit (Sigma) (1:2000) or HRP-coupled goat antimouse (Sigma) (1:2000) secondary antibody. ECL was conducted using Amersham ECL detection reagents (GE Life Sciences). Imaging was carried out on a Fusion Fx7 (Peqlab).

**Immunolocalization**

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

**Imaging**

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

**Supplementary Material**

Supplementary file S1, figures S1–S8, and tables S1–S3 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

**Acknowledgments**

The authors thank Natacha Segonds and Nathalie Techer for technical assistance and Dominique Desgré for coral maintenance. They are very grateful to M.L. Hernandez-Nicaise (University Nice Sophia-Antipolis) for discussion and photography on intercellular junction in Ctenophora. This work was supported by the Centre Scientifique de Monaco research program, funded by the Government of the Principality of Monaco. This project was partially funded by KAUST baseline funds to C.R.V. and M.A.

**References**


Adams ED, Goss GG, Leys SP. 2010. Freshwater sponges have functional, sealing epithelia with high transepithelial resistance and negative transepithelial potential. Plas One 5:e15040.


