β-catenin has both conserved and novel functions in the sponge *Ephydatia muelleri*

Authors:
Klaske J. Schippers[1,2] and Scott A. Nichols[1]

Affiliations:
1. Biological Sciences, University of Denver, 2101 E. Wesley Ave, Denver, CO 80208, USA
2. Current address: EMBL, Meyerhofstrasse 1, 67117, Heidelberg, Germany

Corresponding author:
Scott. A. Nichols (scott.nichols@du.edu)

ABSTRACT

β-catenin acts as a transcriptional co-activator in the Wnt/β-catenin signaling pathway and a cytoplasmic effector in cadherin-based cell adhesion. These functions are ancient within animals, but the earliest steps in β-catenin evolution remain unresolved due to limited data from key lineages -- sponges, ctenophores and placozoans. Previous studies in sponges have characterized β-catenin expression dynamics and have ectopically activated the Wnt pathway through pharmacological inhibition of GSK3β, a negative regulator of β-catenin. However, both approaches rely upon untested assumptions about the conservation of β-catenin function and regulation in sponges. Here, we test these assumptions using a custom antibody raised against β-catenin from the sponge *Ephydatia muelleri*. We find that cadherin-complex genes co-precipitate with Emβcatenin from endogenous cell lysates, but that Wnt pathway components do not. However, through immunostaining we detect both cell boundary and nuclear populations, and we find evidence that Emβcatenin is a conserved substrate of GSK3β. Collectively, these data support the conservation of Emβcatenin in adhesion and signaling. In addition to its conserved functions shared with bilaterians, we also find evidence for an entirely novel Emβcatenin function. Emβcatenin localizes to the distal ends of F-actin stress fibers in focal adhesion-like structures (typically integrin-based adhesions) in the substrate-attachment epithelium. This finding suggests a fundamental difference in β-catenin function and in the cell adhesion mechanisms operating in sponge versus bilaterian tissues.

INTRODUCTION

A well-known pattern in the fossil record is the appearance of most animal lineages over a relatively short time interval (between 541-485 Mya) during the Cambrian. Many of the characteristics that define and distinguish modern animals from each other had already evolved by the time that these first body fossils appear, leaving few clues about either the process or sequence of animal body plan diversification. As a result, our understanding of the earliest events in animal evolution rests largely upon the comparative study of extant organisms. The rationale for this approach is that body plan differences between living organisms reflect accumulated evolutionary changes to their underlying cell and developmental biology.

For practical reasons, large research communities have established around a handful of biomedical research models such as mouse, fruit fly, roundworm and zebrafish. Data from these few organisms largely form the basis for textbook generalizations about animal biology, and ultimately have contributed to a bilaterian-biased perspective on animal evolution. However, to

study the deepest periods of animal ancestry, it will be critical to incorporate data from non-bilaterian animals such as sponges and ctenophores (Dunn, et al. 2015). Non-bilaterians are more phylogenetically divergent from each other, and from other animals, than are any of the biomedical research models listed above (Dohrmann and Wörheide 2017; Schuster, et al. unpublished data) but a lack of detailed mechanistic knowledge about their cell and developmental biology is currently limiting. However, progress in this area has accelerated due to advances in comparative genomics, with the initially surprising result that starkly different organisms share highly conserved developmental regulatory genes (McGinnis, et al. 1984; Kusserow, et al. 2005; Technau, et al. 2005; Nichols, et al. 2006). An important part of understanding animal body plan evolution will be to explain how conserved bilaterian regulatory genes/pathways function in non-bilaterian animals, how they may have functioned ancestrally, and how changes to their functions may have contributed to morphological evolution.

To begin to address these questions, the pleiotropic gene β-catenin is of considerable interest because it has pivotal roles in developmentally important processes ranging from cell adhesion to transcriptional regulation in the Wnt/β-catenin signaling pathway. In cell adhesion, β-catenin interacts with the cytoplasmic tail of cadherin receptors and with α-catenin, an unrelated protein that links the adhesion complex to the actin cytoskeleton (Huber and Weis 2001; Shapiro 2009). This molecular complex forms the foundation of adherens junctions (AJs), and functions as the primary cell-cell adhesion mechanism in all studied bilaterian tissues, including epithelial tissues where AJs also serve as a spatial cue for the establishment of tissue polarity (Capaldo and Macara 2007). In Wnt/β-catenin signaling, β-catenin is a downstream effector that translocates to the nucleus in response to Wnt signals, where it forms a complex with the transcription factor TCF/Lef. Wnt/β-catenin signaling regulates hundreds of downstream target genes and is involved in developmental processes ranging from stem cell maintenance and renewal to axial patterning and gastrulation (Clevers and Nusse 2012).

An early study of β-catenin evolution in the sea anemone *Nematostella vectensis* predicted that the dual signaling and adhesion functions of β-catenin were ancient, and already established in early animal ancestors (Schneider, et al. 2003). Consistent with this view, it has since been established that not only β-catenin, but most components of the cadherin/catenin adhesion complex and the Wnt/β-catenin pathway are conserved in other non-bilaterian lineages as well. Moreover, expression studies suggest roles for Wnt/β-catenin in developmental patterning [reviewed in (Holstein 2012)] in cnidarians (Hobmayer, et al. 2000; Wikramanayake, et al. 2003;

Lee, et al. 2006; Momose, et al. 2008), ctenophores (Pang, et al. 2010; Jager, et al. 2013) and sponges (Adamska, et al. 2010; Leininger, et al. 2014), and based upon immunological and biochemical data, the cadherin/catenin adhesion complex appears to have conserved roles in epithelial cell adhesion in *N. vectensis* (Clarke, et al. 2016).

Among non-bilaterian animals, studies of β-catenin in sponges are particularly of interest because their body plan differs significantly from other animals. With respect to the possibility of a conserved Wnt/β-catenin signaling pathway and how it might function in development, the adult sponge body plan lacks any clear axial polarity and it is contentious as to whether they undergo any developmental process with homology to gastrulation during embryogenesis (Nakanishi, et al. 2014); both axial patterning and gastrulation are hypothesized to be ancestral roles of Wnt/β-catenin signaling. With respect to a possible role for β-catenin in cadherin-based adhesion in sponges, ultrastructural studies suggest their tissues generally lack prominent and widespread AJs (Leys, et al. 2009). Instead, sponge cell adhesion (at least in demosponges) has long been attributed to a secreted glycoprotein complex called the Aggregation Factor (Henkart, et al. 1973; Müller and Zahn 1973; Fernàndez-Busquets and Burger 1997; Grice, et al. 2017), contributing to the view that sponge tissues are fundamentally different from other animal epithelia.

Several studies have begun to address the function of β-catenin in sponges. In both the demosponge *Amphimedon queenslandica* and in the calcareous sponge *Sycon ciliatum*, β-catenin and other Wnt pathway components are dynamically expressed during embryonic development, suggesting a conserved role in developmental patterning (Adamska, et al. 2010; Leininger, et al. 2014). In adult tissues of *S. ciliatum*, β-catenin is also expressed in the choanoderm (feeding epithelium) and in a ring of migratory cells around the osculum (exhalant canal).

Other studies have taken a pharmacological approach to study the function of Wnt/β-catenin signaling by treating the sponges with Glycogen Synthase Kinase 3 Beta (GSK3β) inhibitors. GSK3β is a negative regulator of Wnt signaling that functions by phosphorylating free cytosolic β-catenin, causing its ubiquitination and degradation by the proteasome. When GSK3β is inhibited (i.e., in the presence of Wnt ligands), cytosolic β-catenin accumulates, translocates to the nucleus and activates TCF/Lef and the transcription of Wnt target genes. A study by Lapébie and colleagues (2009) reported that GSK3β inhibitors caused the homoscleromorph

sponge *Oscarella lobularis* to develop ectopic ostia (incurrent water pores) and caused morphological changes to the exopinacoderm (outer epithelium), suggesting a role for Wnt/β-catenin signaling in sponge epithelial morphogenesis. Another study by Windsor and Leys (2010) reported that treating the freshwater demosponge *Ephydatia muelleri* with GSK3β inhibitors resulted in the formation of ectopic oscula (exhalant water canals) and malformed choanocyte chambers (filter feeding epithelia), suggesting that the Wnt/β-catenin pathway plays a role in establishing the aquiferous system in sponges.

With respect to the possible role of β-catenin in cadherin-based adhesion in sponges, all AJ components have been identified (Srivastava, et al. 2010; Nichols, et al. 2012; Riesgo, et al. 2014), but experimental evidence for their function is limited. Sequence analyses and structural predictions indicate that sponge homologs of β-catenin and classical cadherins are sufficiently similar to their bilaterian counterparts to suggest conserved protein interactions. From an experimental perspective, a single study of the homoloscleromorph sponge *Oscarella pearsei* [previously called *O. carmela* (Ereskovsky, et al. unpublished data)] detected the interaction between β-catenin and a classical cadherin by yeast two-hybrid screen (Nichols, et al. 2012).

In general, hypotheses about early animal evolution rest heavily on assumptions made about developmental regulatory genes in organisms where their function and interactions have been insufficiently tested. In the case of β-catenin, reported gene expression patterns may reflect the conservation of one or more bilaterian functions, or entirely alternative functions; it is not possible to distinguish between these possibilities from *in situ* hybridization data alone. Likewise, phenotypes produced by pharmacological inhibition of GSK3β may or may not reflect perturbations to the Wnt/β-catenin pathway; GSK3β is pleiotropic, and β-catenin is not a confirmed GSK3β substrate in sponges. Here, we examine β-catenin function in the freshwater sponge, *E. muelleri*. Using an antibody raised against *E. muelleri* β-catenin (Emβcatenin), we 1) identify endogenous Emβcatenin binding partners, 2) characterize tissue-specific Emβcatenin populations at the cell membrane (adhesion), the nucleus (signaling), and at probable focal adhesions in the attachment epithelium (novel function), and 3) find that Emβcatenin is a probable substrate of GSK3β.

RESULTS

Adherens Junction (AJ) and Wnt pathway components in the *E. muelleri* transcriptome

We searched the published *E. muelleri* transcriptome (Peña, et al. 2016) and detected a single ortholog of β-catenin. The *Emβcatenin* transcript has a predicted 2,685 bp coding sequence, corresponding to an 895aa predicted peptide. Furthermore, as reported from multiple other sponge species (Nichols, et al. 2006; Adamska, et al. 2010; Fahey and Degnan 2010; Leininger, et al. 2014; Riesgo, et al. 2014), we detected conserved homologs of AJ and Wnt signaling pathway components (Supplement Table S1).

Emβcatenin has 53% sequence identity to mouse β-catenin, 50% identity to fly, and between 30-93% identity with β-catenin from other sponge species. The core of the Emβcatenin protein contains 12 predicted armadillo (Arm) repeats flanked by less conserved and presumably unstructured N- and C-terminal regions. In bilaterians, the Arm repeat region is known to serve as the binding interface for interacting with APC, Axin and TCF/Lef which are involved in the Wnt signaling pathway, but also classical cadherins, a component of AJs (Valenta, et al. 2012). By aligning Emβcatenin to structurally and functionally characterized orthologs in bilaterians, we found the conservation of residues Lys-312 and Lys-435 which are required for binding of mouse β-catenin to E-cadherin (Huber and Weis 2001) (see Fig1 and Supplement Fig S1). Also, Emβcatenin has conserved GSK3β phosphorylation sites in the N-terminal domain (Ser33,37 and Thr41 in mouse), which are required for initiating the “destruction complex” in the Wnt signaling pathway (see Fig1 and Supplement Fig S1). The putative binding site for α-catenin (a constitutive AJ component) in the N-terminal domain was found to be conserved to a lesser extent (35% sequence identity with mouse) and only 2 of 5 important binding residues (Miller, et al. 2013) were conserved in Emβcatenin (Supplement Fig S1).

Immunoprecipitation (IP) to identify endogenous Emβcatenin binding partners

To identify endogenous Emβcatenin binding-partners, we raised an antibody against a 24 kDa recombinant protein corresponding to the N-terminal region. The resulting antibody was affinity purified and its specificity tested by Western Blot against whole-cell lysates; a ~110 kDa band was detected (lower MW bands were also detected, but were lower intensity and therefore interpreted as probable degradation products) (Fig 2A).

To further validate the antibody we performed IP (Fig 2B) coupled with mass spectrometry to confirm specificity to Emβcatenin and to detect endogenous protein interactions. As

summarized in Table 1 (full results available in Supplement), the most abundantly detected peptides in the precipitate corresponded to Emβcatenin. Two co-precipitates were α-catenin (Emα-catenin) and a classical cadherin (EmCDH2, see (Peña, et al. 2016)); both are homologs of AJ components. Another co-precipitate was coiled-coil domain-containing protein 91 (ccdc91, also known as p56). The only available studies of this protein in animals reveal that it is a trans-Golgi accessory protein involved in vesicle transport between the Golgi and the lysosome (Mardones, et al. 2007). There is no precedent in the literature for interactions between ccdc91 and β-catenin.

We found no evidence for conserved interactions with Wnt pathway components, such as TCF/Lef, Axin or APC. However, the antibody appeared to be inefficient for use in IP experiments, as we were unable to visualize discrete bands when precipitates were run on an SDS-PAGE gel and analyzed by coomassie staining. β-catenin itself could only be detected within the precipitate by Western blotting (Fig 2B) or mass spectrometry. Furthermore, our experiments were conducted exclusively on somatic tissues from juvenile sponges, whereas, Wnt/β-catenin signaling plays an important role in embryonic patterning, and there is evidence that components of this signaling pathway are developmentally expressed in sponges (Adamska, et al. 2010; Leininger, et al. 2014). The relationship between genetic mechanisms that regulate development from a zygote versus development from a gemmule are unknown.

Immunostaining to test for adhesion (cell boundary) versus signaling (nuclear) Emβcatenin populations

The apical endopinacoderm (AEP) is an epithelium cored by tracts of F-actin that form dense plaques at points of alignment between neighboring cells (Elliot and Leys, 2007). It has been proposed that these F-actin plaques resemble desmosomes (but they are more likely AJs, as desmosomes are phylogenetically restricted to vertebrates). By immunostaining with the validated β-catenin antibody, we found that Emβcatenin indeed co-localizes with F-actin plaques in the AEP (Fig 3). This result is consistent with detected interactions between Emβcatenin with Emα-catenin and EmCDH2, and indicates a conserved role for cadherin-mediated cell-cell adhesion within the AEP.

In the attachment epithelium - a cellular monolayer at the interface with the substrate - we detected continuous cell-boundary staining of Emβcatenin, consistent with the presence of belt-AJs. Albeit, this staining was relatively low intensity (see Fig 4). More intense Emβcatenin

staining was evident in the cytosol, at the ends of F-actin stress fibers that closely resemble Focal Adhesions (FAs) (see Fig4). These stress fibers differ from actin tracts of the AEP in that they were not regularly oriented in the cell, they were not aligned between neighboring cells, and did not extend to the full diameter of any individual cell. This result is surprising, because FAs are integrin-based junctions that mediate interactions between the cell and the extracellular matrix (ECM). At this point, it is not possible to test for homology to FAs, but this Emβcatenin population is not associated with cell-cell contacts, and almost certainly corresponds to cell-substrate contacts. We might also expect to find FA-like structures in highly migratory cells of the mesohyl, such as archeocytes. However, we find no evidence for FA-like actin populations in archeocytes, nor are there filopodia-associated Emβcatenin populations in these cells (Fig 5). Solitary cell migration may involve different mechanisms than collective cell migration.

Another distinctive, polarized epithelium in sponges is the choanoderm, which consists of spherical chambers lined with collar cells (i.e. choanocytes), which have an apical ring of microvilli that surround a central flagellum. These cells pump water through internal canals and phagocytose bacterial prey. As in the attachment epithelium, in newly developing choanocyte chambers (see Fig 6A), we detected continuous cell boundary staining of Emβcatenin. However, in mature choanocyte chambers (see Fig 6B and C), this staining pattern was less evident, and instead Emβcatenin was enriched at points of contact between three adjacent cells (Fig 6C).

Whereas we did not find evidence of conserved interactions between Emβcatenin and other Wnt pathway component by IP, we further examined the possible signaling roles of Emβcatenin by immunostaining to test for nuclear populations. We detected nuclear Emβcatenin in the attachment epithelium (see Fig 7) as well as in migratory cells in the mesohyl (see Fig 5), which is consistent with a conserved role for Emβcatenin as a transcriptional coactivator of TCF/Lef. We did not detect nuclear Emβcatenin in choanocytes (see Fig 6).

Is Emβcatenin a conserved substrate of GSK3β?

GSK3β is a negative regulator of β-catenin in the Wnt/β-catenin signaling pathway, and previous studies have shown that treating sponges with multiple, independent GSK3β inhibitors has reproducible phenotypic effects. To more directly test whether the effects of GSK3β are related to Wnt/β-catenin signaling, we sought to examine whether GSK3β inhibitors affect endogenous Emβcatenin phosphorylation states. We treated juvenile *E. muelleri* sponges with

the GSK3β inhibitors Alsterpaullone and BIO, and monitored their effects by Western Blot (see Fig 8). We detected an increase in electrophoretic mobility of Emβcatenin in lysates from GSK3β inhibited sponges, which we interpreted as a change in the Emβcatenin phosphorylation state (dephosphorylated Emβcatenin was expected to migrate faster in the gel). As confirmation, the electrophoretic migration rate of Emβcatenin was also monitored in sponge lysates that were variously treated with phosphatases, phosphatase inhibitors, both, or neither. We found that phosphatase treatment increased the electrophoretic mobility of Emβcatenin, whereas phosphatase inhibition decreased electrophoretic mobility (Supplement Fig S3).

Through titration of the GSK3β inhibitors we determined that treatment with ~ 0.1 µM AP or ~1 µM BIO resulted in a complete shift from phosphorylated to dephosphorylated states of Emβcatenin and was therefore considered the minimal effective dosage (Fig 8). Sponges treated with this dosage lacked canals and the choanoderm was either absent or malformed. Thus, GSK3β inhibition at the minimal effective dosage disrupts the development of the aquiferous system, including differentiation of the choanoderm (Fig 9).

Efforts were also made to directly disrupt Emβcatenin function using RNAi and Vivo-morpholinos. These techniques caused non-specific phenotypic effects (which varied between individuals collected from different localities), but no measurable change in Emβcatenin levels as measured by Western Blot (Supplement Fig S4).

DISCUSSION

β-catenin is thought to have played a critical role in animal body plan evolution due to its pleiotropic roles in development signaling (Wnt signaling pathway) and in cadherin-based cell-cell adhesion. These roles are broadly conserved in all studied animals, but functional data are available from only cnidarians and bilaterians. Earlier diverging animal lineages -- including sponges -- have conserved homologs of Wnt pathway and cell adhesion genes, but there is limited experimental evidence pertaining to their function. Thus, hypotheses about the role of Wnt signaling and cadherin-based cell adhesion during the earliest periods of animal evolution depend partly upon improved understanding of β-catenin function in these understudied lineages.

Looking outside of animals, there is evidence for a possible β-catenin homolog (*Aardvark*) in the social amoeba *Dictyostelium discoideum* that interacts with an α-catenin-like protein to regulate

the polarity of the tip epithelium of the fruiting body (Dickinson, et al. 2011). Nevertheless, *D. discoideum* lacks both cadherins and Wnt pathway components. If we consider lineages that are more closely related to animals, cadherins have been detected in several unicellular opisthokonts, but they lack β-catenin interacting regions, they lack conserved β-catenin orthologs, and there is no evidence for other conserved Wnt pathway components (Abedin and King 2008; Nichols, et al. 2012). Collectively, these data suggest that the pleiotropic functions of β-catenin in cell adhesion and Wnt signaling are an early innovation in the animal stem lineage.

Adherens junctions (AJs) in sponges

The presence of AJs has been predicted in sponges based upon genome and transcriptome data from diverse species (Nichols, et al. 2006; Srivastava, et al. 2010; Nichols, et al. 2012; Riesgo, et al. 2014), and upon limited ultrastructural evidence [reviewed in (Leys, et al. 2009)]. Our Emβcatenin Co-IP data provide new experimental support for endogenous interactions between AJ components in *E. muelleri*. Notably, we detected an interaction between Emβcatenin with EmCDH2 and Emα-catenin, even though the residues required for the latter interaction are incompletely conserved in *E. muelleri* (Miller, et al. 2013). This result underscores the limitations of functional predictions made using exclusively bioinformatic approaches, and parallels the example of Axin in the sponge *O. pearsei*, which lacks the expected binding motif for β-catenin but was identified as a binding partner via yeast two-hybrid screen (Nichols, et al. 2012). A possible explanation for why EmCDH2 and not EmCDH1 was detected as a candidate Emβcatenin binding partner is that EmCDH1 may be expressed at a different developmental stage than the juvenile tissues examined. In bilaterians, classical cadherins often exhibit tissue-specific expression. For example, in mammals, E-cadherin is expressed in epithelial tissues, N-cadherin expressed in the nervous system, and P-cadherin in the placenta. As sponges appear to be largely epithelial organisms it is intriguing to consider that they may have different adhesion receptors expressed in different contexts. Spatiotemporal differences in expression of cadherin paralogs may reveal differences in the evolutionary history and organization of different sponge tissues.

Immunostaining of Emβcatenin further supports the existence of AJs in sponge tissues. Continuous cell boundary staining is evident in the attachment epithelium (Fig 4) as well as in newly developing choanocyte chambers (Fig 6A). This staining pattern resembles the organization of AJs in cnidarian and bilaterian epithelial tissues, which form a continuous ‘belt’ around the periphery of the cell, thereby dividing the plasma membrane into apical and

basolateral domains. Together with occluding junctions (tight junctions in vertebrates and septate junctions in invertebrates), AJs can help create a barrier that regulates paracellular transport, effectively separating the environment outside of the tissue from the interior environment -- a fundamental feature of multicellular organization (Banerjee, et al. 2006; Hartsock and Nelson 2008; Zihni, et al. 2016). There is evidence that freshwater sponges have "sealed" epithelia (Adams, et al. 2010), but it is not yet established which molecules are involved.

Instead of continuous cell-boundary staining, mature choanoderm tissue (Fig 6C) and the AEP (Fig 3) were found to have probable adhesion-associated Emβcatenin populations with a much more restricted distribution. In mature choanocyte chambers, Emβcatenin was largely enriched at lateral membranes where three adjacent cells adjoin; this localization pattern superficially resembles tricellular junctions of *Drosophila*, although they have a different molecular composition than AJs. In the AEP, Emβcatenin co-localizes with F-actin plaques that form where F-actin stress fibers are aligned between neighboring cells. This is consistent with the view that the AEP is a contractile epithelium (Elliott and Leys 2007; Nickel, et al. 2011) and that the plaques represent AJs that form at focal points of tissue stress.

A possibly novel cell-substrate adhesion role for Emβcatenin

Detected interactions of Emβcatenin with Emα-catenin and EmCDH2, and its presence at cell-cell contacts, support predictions about its possible role in AJs in sponge tissues. A less expected result is the detection of Emβcatenin at FA-like structures in the attachment epithelium (Fig 4). FAs are well-characterized cell junctions that utilize integrins rather than cadherins as adhesion receptors to mediate interactions between cells and the ECM. Despite this staining pattern, we found no evidence that FA components co-precipitate with Emβcatenin, and in bilaterians β-catenin is not a typical FA component. However, Langhe and colleagues (2016) have shown that *Xenopus* β-catenin and cadherin 11 co-localize with FA components when overexpressed in HeLa cells and Kuo and colleagues (2011) found β-catenin associated with FAs isolated from HFF1 cells. The functional significance of these studies is unknown, and thus the implications for our finding of Emβcatenin at FA-like structures in *E. muelleri* is unclear.

It is possible that classical cadherins mediate both cell-cell and cell-matrix adhesion in *E. muelleri*. Like classical cadherins in *Drosophila* and other non-chordate animals, EmCDH1 and EmCDH2 have membrane-proximal epithelial growth factor (EGF) and Laminin G (LamG)

domains (Supplement Fig S2). In *Drosophila* this region has been implicated in trafficking to the plasma membrane (Oda and Tsukita 1999), but in an unusual classical cadherin-like protein (BbC) in the lancelet *Branchiostoma*, this region is sufficient to mediate epithelial cell adhesion (BbC naturally lacks cadherin repeats; (Oda, et al. 2002; Oda, et al. 2004)). In general, EGF and LamG are involved in extracellular protein interactions and are common in both cell surface receptors and secreted components of the ECM. In the future, it will be critical to determine the expression dynamics and subcellular localization of candidate sponge adhesion receptors, themselves; both integrins and classical cadherins.

Evidence for a functional Wnt/β-catenin pathway in sponges

Activation of the Wnt/ β-catenin signaling pathway results in the stabilization of cytosolic β-catenin and its subsequent translocation to the nucleus. In *E. muelleri*, we find no evidence for conserved interactions of Emβcatenin with Wnt pathway components (such as TCF/Lef, Axin or APC) by Co-IP, but by immunostaining we detect a nuclear population of Emβcatenin in cells of the attachment epithelium and in archeocytes (migratory cells within of the mesohyl) where it presumably functions as a transcriptional coactivator. Additionally, we find evidence that Emβcatenin is a substrate of GSK3β in *E. muelleri*, as inhibition of GSK3β leads to a shift in endogenous Emβcatenin phosphorylation states. This resembles the condition in bilaterians, where GSK3β functions as a negative regulator of Wnt signaling by phosphorylating free cytosolic β-catenin, leading to its ubiquitination and degradation via the proteasome.

Archeocytes are hypothesized to be pluripotent cells. Part of the evidence for this perspective is that they express stem cell markers such as *Musashi* and *Piwi* (Alié, et al. 2015), and disruption of archeocyte cell division inhibits differentiation of the choanoderm (Peña, et al. 2016). Thus, detection of nuclear Emβcatenin in archeocytes is consistent with the known role of Wnt/β-catenin signaling in bilaterian stem-cell maintenance and renewal. Wnt/β-catenin pathway genes expression has also been reported in archeocytes of the sponges *A. queenslandica* (Adamska, et al. 2010) and *S. ciliatum* (Leininger, et al. 2014). If β-catenin has a conserved role in maintaining archeocyte pluripotency in *E. muelleri*, it may explain aspects of the aquiferous system phenotypes observed with GSK3β inhibition. Specifically, sustained activation of the Wnt/β-catenin pathway may inhibit differentiation of archeocytes into choanocytes.

A nuclear population of Emβcatenin is also present in cells of the attachment epithelium. One possible explanation of this finding comes from a study implicating Wnt/β-catenin signaling as

an upstream regulator of collective cell migration in the migrating zebrafish lateral line primordium, particularly in cells of the leading edge of migration (Aman and Piotrowski 2008). It is well established that sponge are capable of entire-colony mobility, and in juveniles of *E. muelleri* this behavior is the result of collective migration of the attachment epithelium (Bond and Harris 1988; Bond 1992). Furthermore, FAs are well-known to be dynamically regulated during bilaterian cell migration (particularly when migrating on a 2D surface), and FA-like structures are present in the attachment epithelium (Sastry and Burridge 2000; Stehbens and Wittmann 2012; Kim and Wirtz 2013).

A limitation of our study is that we were restricted to working with gemmule-hatched juvenile tissues, whereas Wnt/β-catenin has previously been implicated in embryonic development in other sponge species. Specifically, in *A. queenslandica* (Adamska, et al. 2010) and *S. ciliatum* (Leininger, et al. 2014), Wnt/β-catenin pathway genes were found to exhibit posterior/anterior expression gradients during embryogenesis, consistent with a conserved role in axial patterning. These data are difficult to relate to our findings in *E. muelleri* because embryogenesis is a fundamentally different process than gemmule hatching, and the regulatory similarities between these disparate developmental processes have never been examined. Nonetheless, Windsor and Leys (Windsor and Leys 2010) also studied gemmule-hatched *E. muelleri* juveniles and reported that GSK3β inhibition disrupted development of the aquiferous system and caused the formation of ectopic oscula. From these data, they hypothesized that the adult body axis in sponges may be defined by the unidirectional aquiferous system. Whereas our data do not bear on this question directly, they do support the underlying assumption that Emβcatenin is a substrate of GSK3β in *E. muelleri*, and confirm that the minimal effective dosages of GSK3β inhibition of β-catenin phosphorylation recapitulate the reported knockdown phenotypes.

Finally, a gene expression study in *S. ciliatum* has also implicated Wnt/β-catenin signaling in endomesoderm specification, and revealed β-catenin expression in the embryonic micromeres (precursors of choanocytes) as well as mature choanocytes and mesohyl cells (Leininger, et al. 2014). Based upon this study, it was hypothesized that sponges have germ layers homologous to those of bilaterians and that the choanoderm derives from endoderm. However, contradictory data from lineage-tracing experiments in *A. queenslandica* indicate that cell layers in sponges do no not undergo progressive fate determination and are therefore not homologous to bilaterian germ layers (Nakanishi, et al. 2014). Our data bear on this question only in that we detect a cell-adhesion-associated population of Emβcatenin in the choanoderm, but not a

nuclear population. It is therefore possible that the β-catenin expression detected in the choanoderm of *S. ciliatum* relates to its roles in cell adhesion in this tissue rather than Wnt signaling. However, it is notable that the modern sponge lineages to which *E. muelleri* and *S. ciliatum* belong, diverged ~750-850 Ma, during the Neoproterozoic (Dohrmann and Wörheide 2017), and they have quite divergent developmental features. Furthermore, Wnt pathway components other than β-catenin are also expressed in the choanoderm of *S. ciliatum*.

SUMMARY AND CONCLUSIONS

Here, we study β-catenin in the freshwater sponge *E. muelleri* and find 1) that AJ components (α-catenin and classical cadherin), but no Wnt pathway components, co-precipitate with endogenous Emβcatenin from whole-cell lysates, 2) there are cell boundary (adhesion-related) and nuclear (signaling-related) populations of Emβcatenin, 3) that previously reported phenotypic effects of GSK3β inhibitors on aquiferous system development can be replicated and are associated with shifts in Emβcatenin phosphorylation state, and 4) there is an Emβcatenin population associated with FA-like structures in the attachment epithelium, reflecting a potentially novel function.

The evidence presented here supports the underlying assumptions of previous studies: that β-catenin has conserved functions in Wnt signaling and cadherin-based cell adhesion in sponges. However, the discovery of a β-catenin population associated with FA-like, cell-substrate adhesion structures underscores that functional conservation should not be uncritically accepted based upon bioinformatics and gene expression studies alone. Particularly, in the case of pleiotropic genes, the interpretation of gene expression patterns is potentially fraught without more explicit functional and biochemical support. To this end, future progress will depend largely upon the development of experimental approaches to manipulate gene function directly, *in vivo*, without the ambiguities that accompany pharmacological approaches. Moreover, comparative research should continue in multiple sponge species, as modern sponges represent many anciently divergent lineages.

MATERIAL AND METHODS

Collection and growing of sponges

Adult specimens of *E. muelleri* containing gemmules were collected in October 2013 from upper Red Rock Lake, Colorado, USA. The gemmule-bearing sponges were stored in autoclaved lake water in the dark at 4 °C and water was periodically refreshed. Gemmules were removed from the adult by gentle rubbing of the sponge tissue or by picking gemmules from the tissue with forceps. To clean the gemmules, they were washed several times with autoclaved lakewater.

For obtaining sponge cell lysate for Co-IP, ~100 sponges were cultivated in a petridish (90 mm diameter) containing 25 ml of autoclaved lake water or sterile M-medium (1 mM $CaCl_2 \cdot 6H_2O$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM $NaHCO_3$, 0.05 mM KCl, 0.25 mM Na_2SiO_3; (Funayama, et al. 2005)) in the dark at room temperature (20-22 °C). Typically sponges hatched 4-5 days after plating. After hatching, the lakewater or M-medium was refreshed every other day. Three to four days after hatching the sponges were fully developed and were harvested to make cell lysates.

To culture sponges for immunostaining and the pharmacological studies, #1.5 22mmx22mm glass coverslips were placed into 6-well plate format and each well would contain 5 ml of autoclaved lakewater or M-medium. The individual gemmules were placed on the coverslips in the dark at room temperature (20-22 °C). Again, 3-4 days after hatching the sponges were fixed for immunostaining or harvested for western blot analysis.

cDNA library construction

Material for constructing the cDNA library was obtained from juvenile sponges that had developed from hatched gemmules. All gemmules were collected from one adult specimen of *E. muelleri*. Total RNA (~ 1 mg) was isolated using TRIzol Reagent (ThermoFischer Scientific #15596026) following manufacturer's instructions. To purify mRNA from total RNA we used Oligotex Suspension (Qiagen #79000) and followed the protocol "Purification of Poly A+ mRNA from Total RNA using a Batch Procedure" from the Oligotex handbook (pg. 23-25). The purified mRNA was then concentrated using Corning Spin-X UF 500 µl Concentrators with a molecular weight cut-off of 10,000 (Corning #431478). To construct the cDNA library we used the CloneMiner II cDNA Library Construction Kit (ThermoFischer Scientific #A11180) according to the manufacturer's instructions.

Protein expression and purification

The N-terminal part of *E. muelleri* β-catenin (EmβcatN amino acids 1-221) was amplified by PCR from *E.muelleri* cDNA with the following primers:

EmβcatN forward:

1) tacttccaatccaatgcaATGGAGGTGGACAGATCATACTAC, which contains an LIC adapter (lower case)

EmβcatN reverse

2) ttatccacttccaatgttatta<u>CTA</u>GAGCTCGGAGGAACCCTGAT, which contains an LIC adapter (lower case) and a STOP codon (underlined).

The amplified fragment was cloned into the pET His6 Sumo TEV LIC cloning vector (1S) (Addgene plasmid # 29659). This vector generates proteins that are N-terminally tagged with a TEV-cleavable His6-Sumo fusion tag. The construct was transformed into *Escherichia coli* Rosetta (DE3) competent cells (Novagen). Recombinant protein expression was induced at OD600 ~0.4 and was conducted at room temperature for 6 hours (0.25 mM IPTG). Cells were spun down and resuspended in cell resuspension buffer (1x PBS, 2M NaCl, 5% glycerol, 0.1 mM PMSF, pH 8.0) with lysozyme (1mg/ml) and incubated at room temperature for 10 min. Cells were sonicated by 4 x 30sec pulses and bacterial debris was removed by centrifugation at 20,000xg for 30 min at 4 °C. To purify the recombinant protein, the supernatant was incubated with HisPur Cobalt Resin (ThermoFisher # 89964) overnight at 4 °C. The beads were washed with washing buffer (1xPBS, 5% glycerol, pH 8.0) and the His-tagged proteins were eluted with elution buffer (150 mM imidazole, 1 x PBS, 5% glycerol, pH 8.0). To cleave the His6-Sumo fusion tag, recombinant proteins were incubated with AcTEV protease (ThermoFischer # 12575015) overnight at 4 °C, using 1 U for ~40 µg of recombinant protein. To remove imidazole and DTT, a buffer exchange was performed using PD-10 Desalting columns (GE Healthcare # 17-0851-01) and the protein samples were equilibrated in 1x PBS (pH 8.0) with 5% glycerol. Subsequently HisPur Cobalt resin was used to remove the His6-Sumo tag and AcTEV protease. The eluate contained the EmβcatN recombinant protein (without His6-Sumo fusion tag), which was used to inject in rabbits.

Antibody Production

Polyclonal antibodies were raised in rabbits against recombinant EmβcatN antigen (Syd labs, Natick, USA) and the anti-EmβcatN antibodies were affinity purified from the rabbit serum using

recombinant EmβcatN protein with the AminoLink Plus Immobilization Kit (ThermoFischer, #44894) according to the manufacturer's instructions. Two Amino-link resin columns were prepared, one with EmβcatN recombinant protein and one with *E. coli* proteins. First the rabbit serum was incubated with the *E.coli* protein-column by running it 10x over the column to remove possible IgG's against bacterial proteins. Next, the eluate was incubated with the EmβcatN protein-column by running it 10x over the column and the column was washed extensively with 1x PBS. Finally, the affinity-purified antibody was eluted from the column with Elution buffer (0.1 M Glycine, pH 2.5) and neutralized with Neutralization buffer (0.75M Tris, pH 8.8). The fractions with the highest amount of antibodies were combined, and buffer was exchanged with PD-10 Desalting columns and the affinity purified antibody was equilibrated in 1x PBS with 0.05% sodium azide. The final concentration of antibody was ~ 1 mg/ml.

Co-IP and Mass Spectrometry

Two independent Emβcatenin Co-IP experiments were performed using Dynabeads Protein G for Immunoprecipitation (ThermoFischer Scientific, # 10004D) according to the manufacturer's instructions. For each experiment, we coupled 5 µg of EmβcatN-antibody to 50 µl (=1.5 mg) of Dynabeads protein G. As a negative we control, we also coupled normal rabbit IgG (Santa Cruz Biotechnology, #sc-2027) to Dynabeads. To crosslink the antibody to the beads, the antibody-coupled beads were incubated with 350 µl of 0.75 mM BS3 crosslinker (ThermoFischer Scientific, #21580) in conjugation buffer (20mM Sodium Phosphate, 0.15M NaCl, pH 8.1) for 30 min at room temperature, crosslinking reaction was stopped by adding 17.5 µl Quenching buffer (1M Tris-HCl, pH 7.5), and the beads were washed with PBST (0.1% Tween-20).

Whole cell lysates were derived from ~1 week old gemmule-hatched juveniles. At this stage, all somatic tissues had developed. The lysate was prepared by dissolving the juvenile sponges in lysis buffer (20 mM Hepes, pH 7.6, 150 mM NaCl, 1 mM EDTA, 10% Glycerol, 1% Triton X-100, 1 mM DTT, 1 mM PMSF, 1x phosphatase inhibitor cocktail A and B (Biotool, #B15001), 1x protease inhibitor cocktail (Biotool, #B14011, EDTA free)). The crude lysate was clarified by centrifugation at 14,000xg for 10min at 4 °C. From the crude lysate we took 600 µg of soluble sponge proteins (measured with Bradford Protein Assay) and diluted this 2x with binding/washing buffer (same as lysis buffer, but without glycerol and DTT). The lysate was incubated with the antibody-coupled beads for 1.5 hr at 4 °C, and was washed with binding/washing buffer and proteins were eluted with 50 µl of 0.2 M Glycine pH 2.5.

The samples were digested, desalted and analyzed with mass spectrometry by EPFL-Plateforme technologique de proteomique using Orbitrap Elite (4x short LC-MS/MS gradient). The raw data was searched against the *E. muelleri* protein database using Scaffold software with a protein threshold of 99% and a peptide threshold of 95%. We compared the results with the control IP (rabbit IgG) to identify non-specific interactions of sponge proteins with IgG-coupled Dynabeads. We selected proteins that had at least 5 unique peptide matches and no hits in the control-IP (see Table 1. For a complete list of all identified proteins, see Supplement Table S2).

Immunostaining

Sponges that grew on the coverslips in 6-wells format plates were fixed with ice-cold EtOH with 4% formaldehyde for 1 h at 4 °C. To remove the fixative, the sponges were washed 3x with PBST (0.1% Tween-20). The coverslips containing the coverslips were transferred to parafilm in a humid chamber. First sponges were incubated with blocking reagent (3% BSA in PBST (0.1% Tween-20)) for 1 h at room temperature. Next, they were incubated with the primary antibody (anti-EmβcatN 1:500 in blocking reagent) for 2 hrs at room temperature or overnight at 4 °C. After incubating with the primary antibody, sponges were washed 3x with PBST and were incubated with the secondary antibody (1:300, goat anti-rabbit Alexa Fluor 488, ThermoFischer #A11034) together with Alexa Fluor 568 Phalloidin (1:40, ThermoFischer #A12380) and Hoechst33342 (1 µg/ml) in blocking reagent for 45 min at room temperature. Sponges were washed 3x with PBS before they were mounted with mounting medium (90% glycerol, 1x PBS, 0.1 M propyl gallate) on a microscopic glass slide with clay feet and sealed into place with heated VALAP (vaseline, lanolin and paraffin, 1:1:1). The negative controls were incubated with secondary antibody only. Images were taken with a 60x and 100x oil immersion objective on an Olympus Fluoview 1000 inverted confocal microscope, and images were processed using ImageJ (z-stack: maximal intensity projection).

Pharmacological treatment (AP and BIO) and Western blot analysis

All treatments were done in sterile M-medium with different concentrations of AP (Alsterpaullone, Sigma-Aldrich #A4847) and BIO (6-bromoindirubin-3′-oxime, Sigma-Aldrich #B1686). The tested concentrations for AP were 0.025 µM, 0.05 µM, 0.1 µM and 0.2 µM and for BIO 0.125 µM, 0.25 µM, 0.5 µM and 0.1 µM. Since AP and BIO were initially dissolved in DMSO (10 mM stocks), the control treatments consisted of 0.02% DMSO in M-medium. Sponges were treated for the duration of the experiment and were imaged during day 5, 6 and 7 using a Leica

MZ 16FA stereoscope. At day 7, sponges were harvested for Western Blot analysis. To prepare samples for western blot, sponges were dissolved in 1x Lysis buffer and samples were boiled together with Laemmli loading buffer for 5 min, followed by a quick spin. The protein samples (~2 sponges per lane) were separated by SDS-PAGE on an 8% gel and the proteins were transferred to a PVDF membrane (Bio-Rad #1620177). Membranes were blocked for 1 h at room temperature in blocking solution (5% non-fat dry milk in 1x PBST (0.1% Tween-20)), and then incubated with anti-EmβcatN antibody (1:10.000) in blocking solution overnight at 4 °C Membranes were washed extensively with PBST and incubated with the secondary antibody (goat anti-rabbit IgG antibody, horseradish peroxidase conjugate, Promega #W401B 1:5000) in blocking solution for 1h at room temperature. Again membranes were washed extensively in PBST and were developed using Western Lightning Plus Enhanced Chemiluminescence Substrate (PerkinElmer #NEL104001EA). To visualize the loading control, the membrane was stripped and reprobed. Stripping was done by incubating the membrane for 10 min at room temperature in mild stripping buffer (1.5% w/v glycine, 0.1% w/v SDS and 0.1% w/v Tween-20) and washing extensively in PBS and PBST. Reprobing procedure was similar as described above, but using mouse anti-alpha tubulin (1:10.000, Sigma Aldrich #T5168) as a primary antibody and goat anti-mouse rabbit IgG antibody, horseradish peroxidase conjugate (1:5000 Promega #W402B) as a secondary antibody.

ACKNOWLEDGEMENTS

The authors thank Brigitte Galliot for providing labspace, reagents and administrative support; Detlev Arendt for providing support to finalize the project; and Jennyfer Mora Mitchell for help with field collection of *E. muelleri.* This work was supported by a Marie-Curie Fellowship (FP7-PEOPLE-2012-IOF- 327684 Sponge Signaling) to K.J.S. and the University of Denver Faculty Research Fund to S.A.N.

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FIGURE LEGENDS

Figure 1. **Domain architecture of Emβcatenin.** The core contains 12 predicted Arm repeats flanked by N- and C-terminal regions. The N-terminal has conserved GSK3β and CKI phosphorylation residues and a putative α-catenin binding site. Also critical cadherin binding residues are conserved.

Figure 2. **Emβcatenin antibody specificity**. (A) Western Blot of *E. muelleri* whole-cell lysate. (B) Western blot of precipitate and unbound fractions from IP with anti-EmβcatN compared to IP with IgG control.

Figure 3. **Emβcatenin co-localizes with plaques of F-actin at cell contacts in the apical endopinacoderm (AEP).** Composite images (A/B) with DNA (A'/B') in blue, anti-EmβcatN (A"/B") in green, and F-actin (A'''/B''') in red. Arrowheads indicate Emβcatenin enrichment at sites of cell-cell contact. Scale bar: 10 µm. (C) Illustrative cross section of *E. muelleri* with AEP in red.

Figure 4. **Emβcatenin localization in the attachment epithelium at cell boundaries and at FAs**. Composite images (A) with DNA (A') in blue, anti-EmβcatN (A") in green, and F-actin (A'''') in red. Arrow indicates Emβcatenin enrichment at cell boundaries and open arrowhead indicates Emβcatenin enrichment at FA. Scale bar: 10 µm. (B) Illustrative cross section of *E. muelleri* with attachment epithelium in red.

Figure 5. Nuclear localization of Emβcatenin in migratory cells in the mesohyl. Composite images (A) with DNA (A') in blue, anti-EmβcatN (A") in green, and F-actin (A'''') in red. Scale bar: 10 µm. (B) Illustrative cross section of *E. muelleri* with migratory cells in mesohyl in red.

Figure 6. Emβcatenin co-localizes at cell boundaries in the choanoderm. Composite images (A/B/C) with DNA (A'/B'/C') in blue, anti-EmβcatN (A"/B"/C''') in green, and F-actin (A'''/B'''C''') in red. Arrows indicate Emβcatenin enrichment at cell boundaries. Scale bar: 10 µm. (D) Illustrative cross section of *E. muelleri* with choanoderm in red.

Figure 7. **Nuclear localization of Emβcatenin in cells of the leading edge of the attachment epithelium.** Composite images (A/B) with DNA (A'/B') in blue, anti-Emβcatenin (A"/B") in green, and F-actin (A'''/B''') in red. Scale bar: 10 µm. (C) Illustrative cross section of *E. muelleri* with leading edge of the attachment epithelium in red.

Figure 8. Western blot showing effect of AP and BIO on phosphorylated Emβ-catenin protein levels from sponge cell lysate. Open arrowhead: GSK3β - phosphorylated Emβcatenin; closed arrowhead: active Emβcatenin (i.e. unphosphorylated); asterisk: minimal effective dosage of AP and BIO which results in a complete shift from phosphorylated to active Emβ-catenin.

Figure 9. The effect on sponge morphology after treating the sponges with the minimal effective dose of the drugs AP (0.1 µM) and BIO (1 µM). Canals and choanoderm did not develop.

TABLES

Table 1. Mass spectrometry results of Co-IP

Identity	T-PEP ID	Summed # unique peptides	Summed # total spectrum	MW (kDa)
Emβcatenin	m.48575	32	104	98
ccdc-91/p56	m.42889	14	22	79
EmCDH2	m.5230 / m.46898	10	12	587
Emα-catenin	m.75406	5	11	102

FIGURES

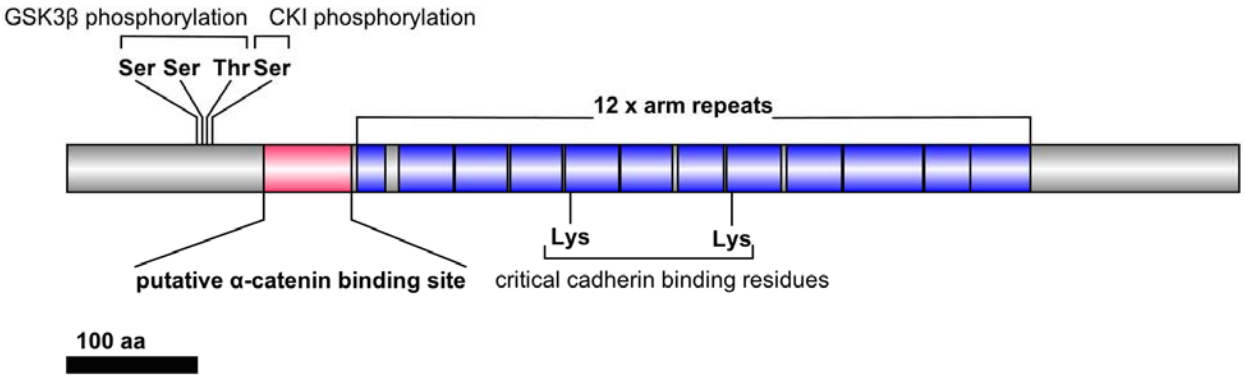

Figure 1

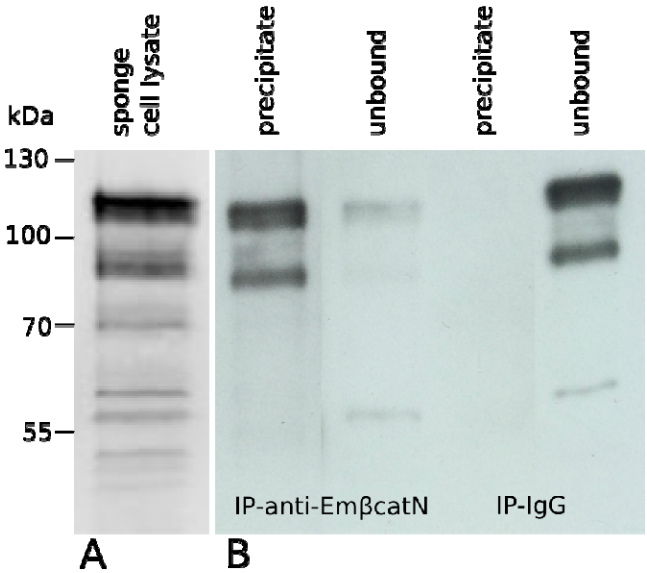

Figure 2

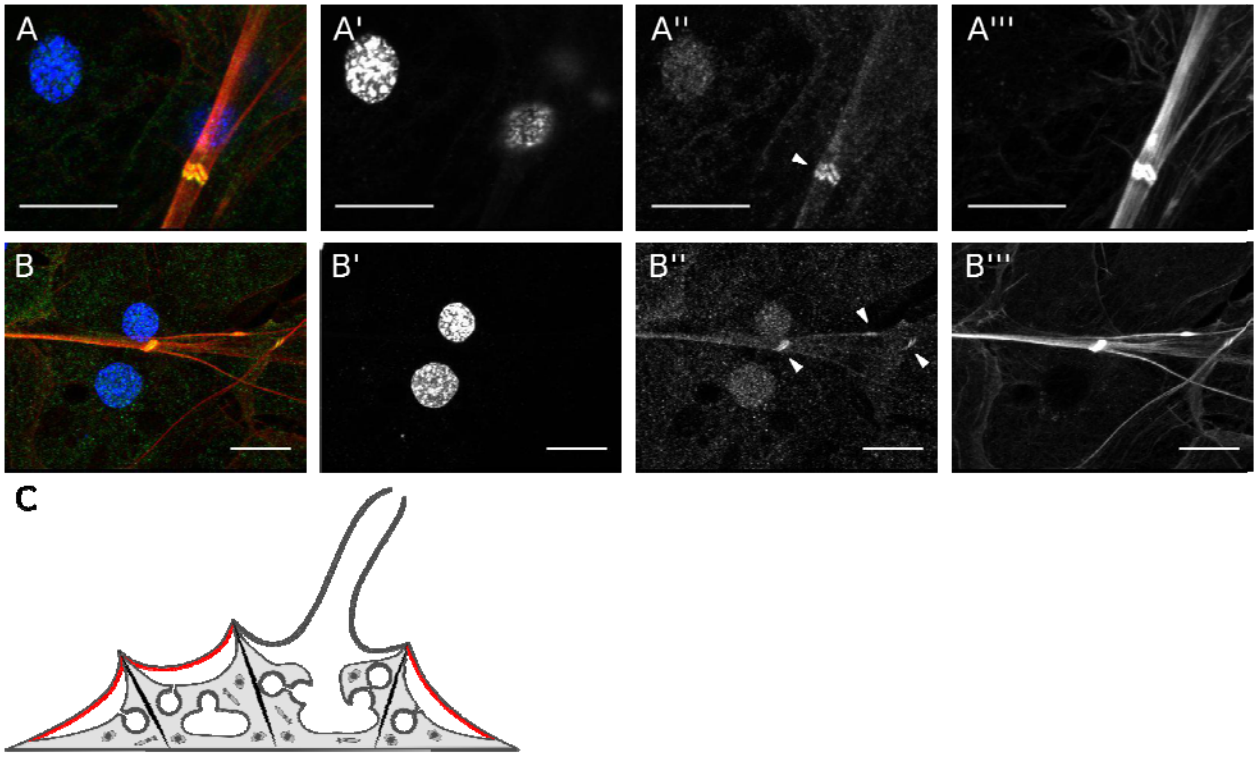

Figure 3

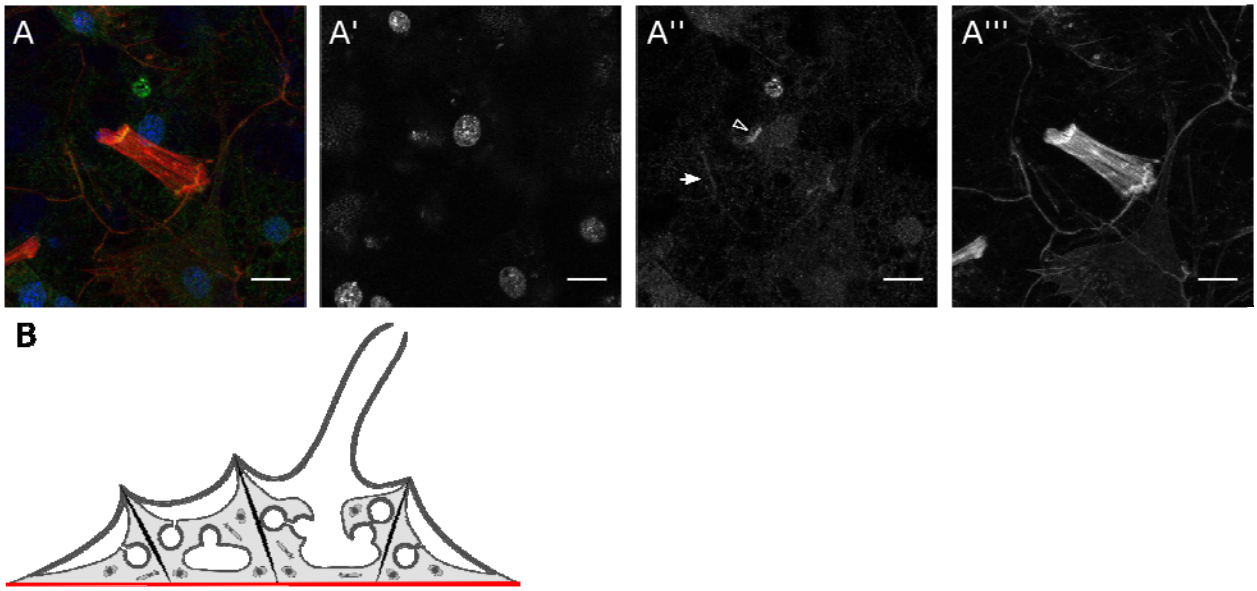

Figure 4

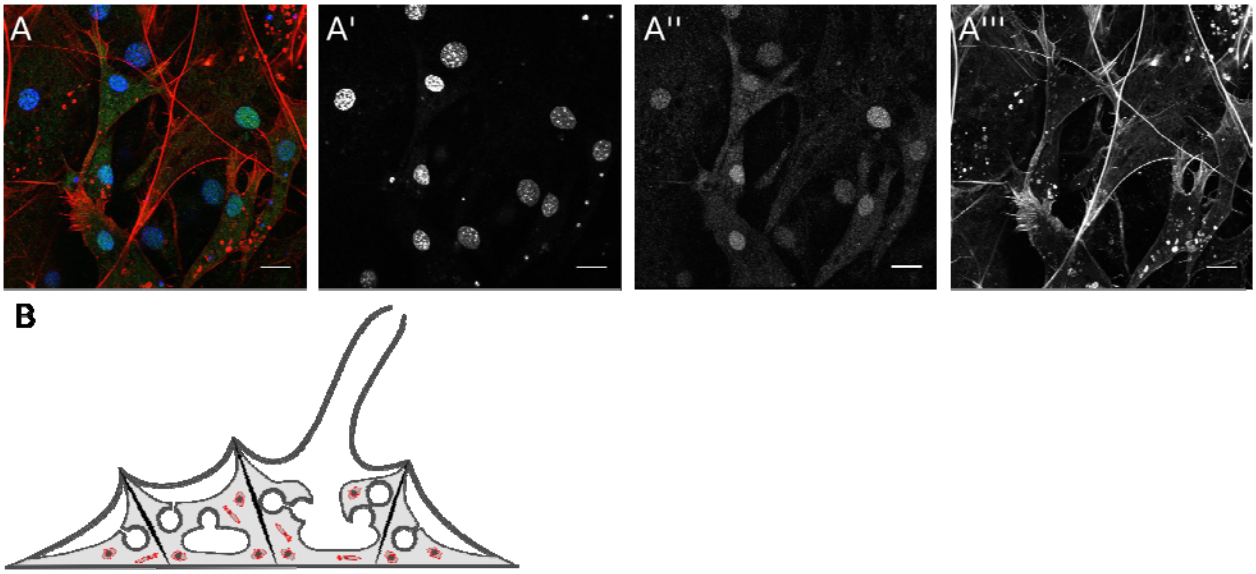

Figure 5

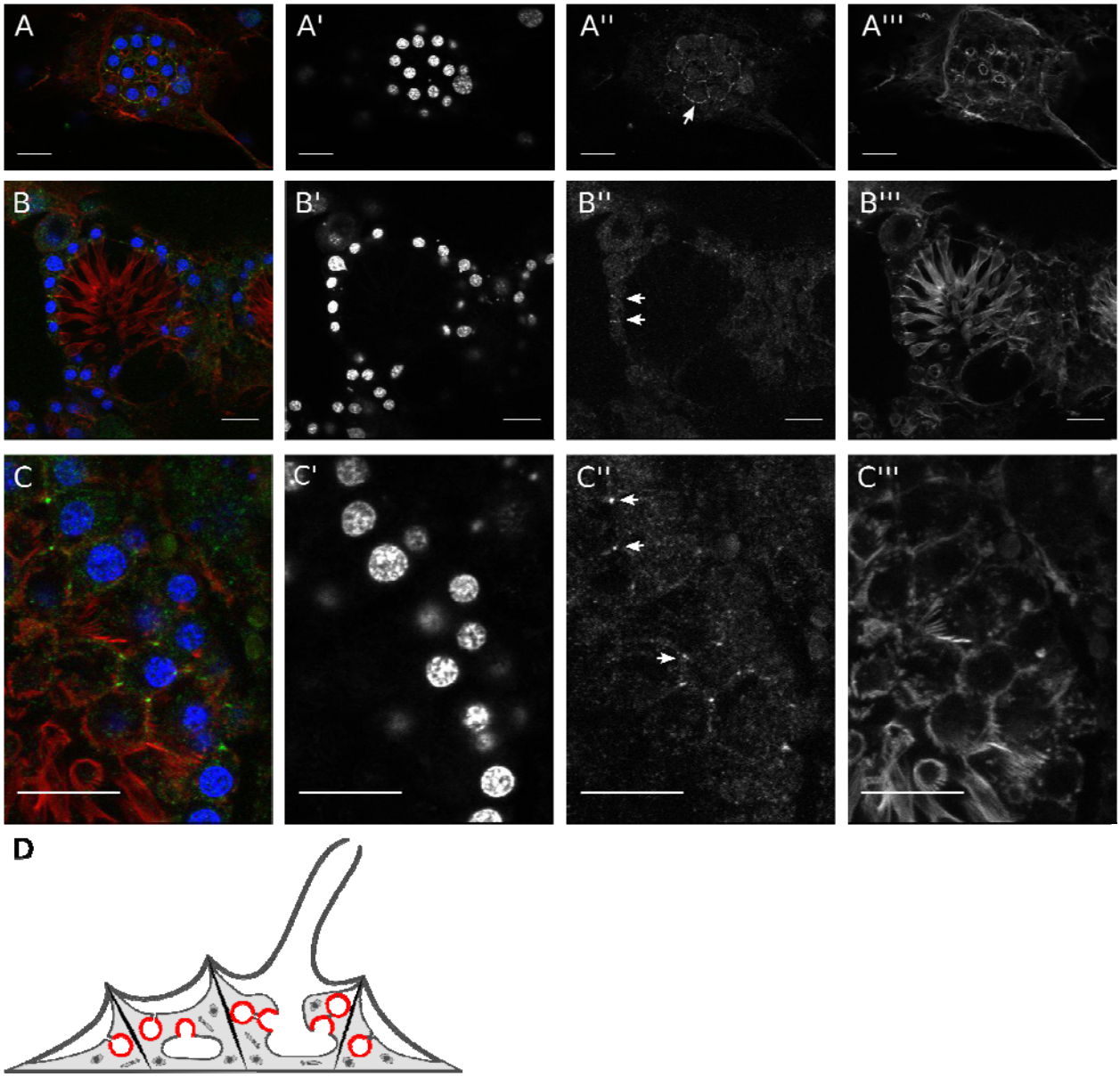

Figure 6

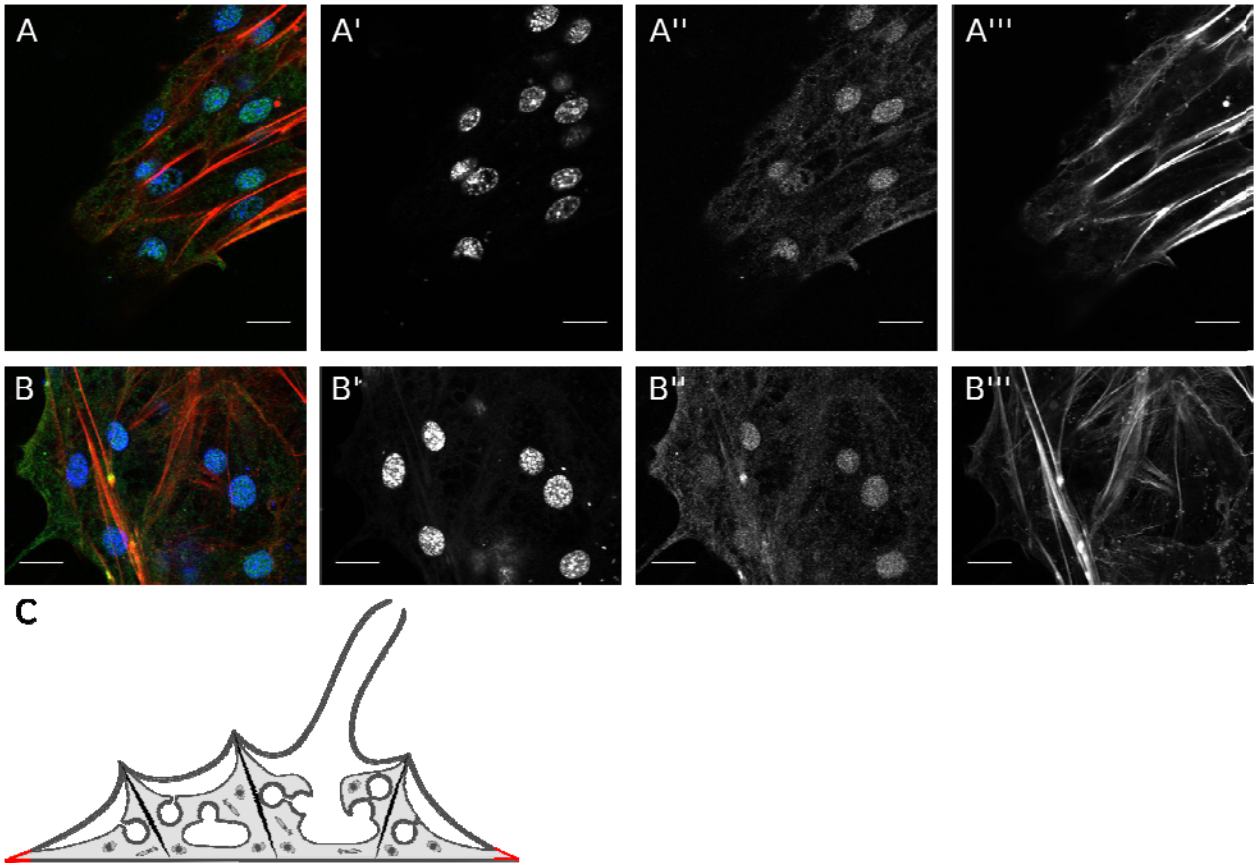

Figure 7

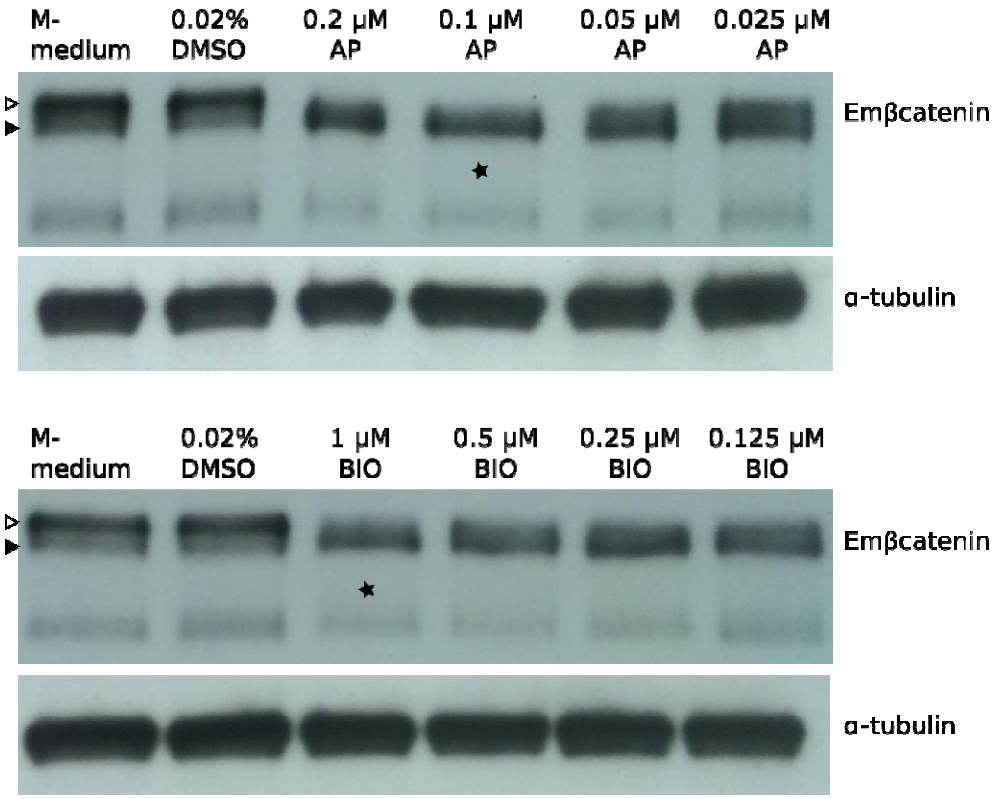
M-medium
0.02% DMSO
0.2 µM AP
0.1 µM AP
0.05 µM AP
0.025 µM AP
Emβcatenin
α-tubulin
M-medium
0.02% DMSO
1 µM BIO
0.5 µM BIO
0.25 µM BIO
0.125 µM BIO
Emβcatenin
α-tubulin

Figure 8

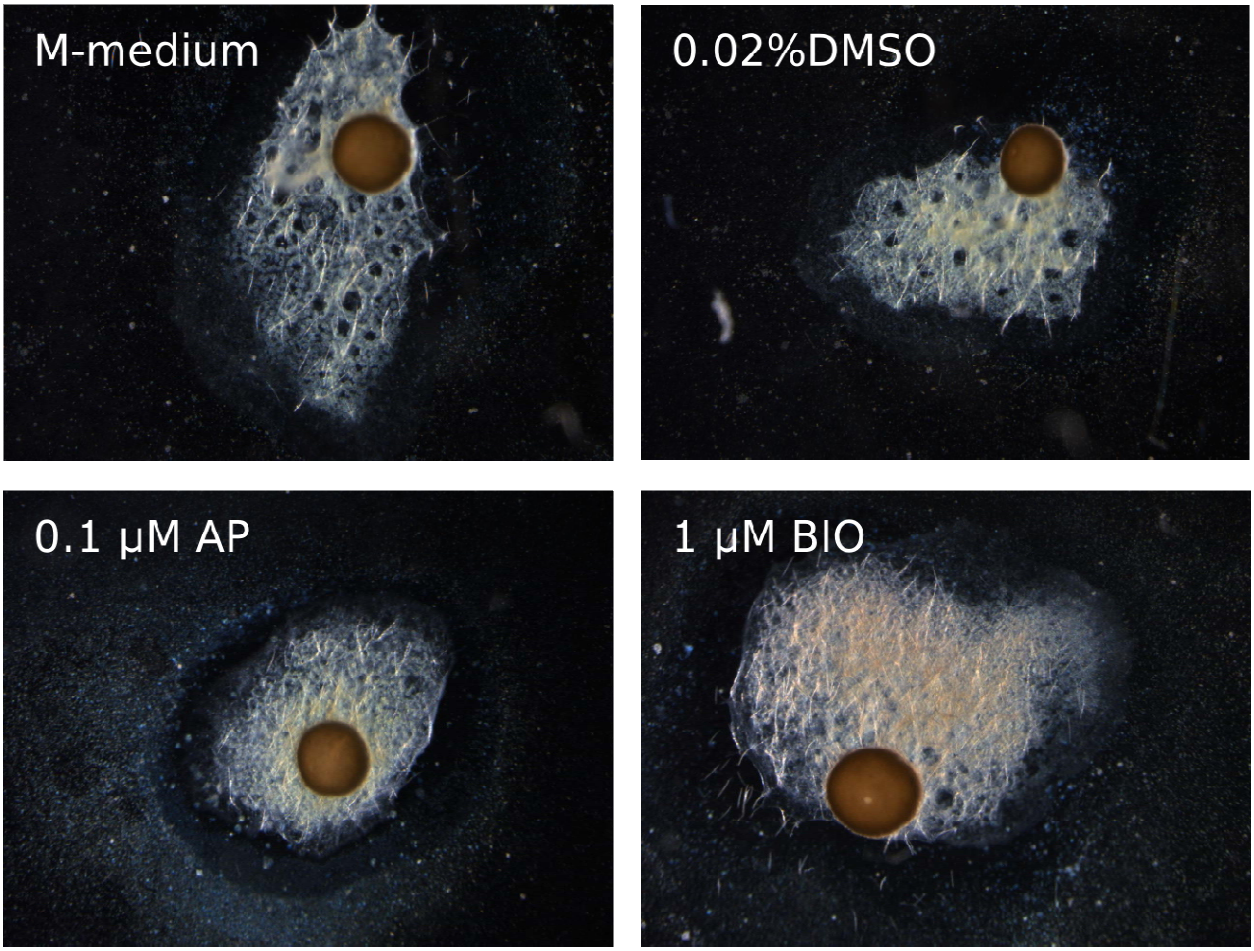

Figure 9