Looking beyond the Wnt pathway for the deep nature of β -catenin

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After two decades of stardom, one would think that β -catenin has revealed all of its most intimate details. Yet the essence of its duality has remained mysterious—how can a single protein both be the core link between cadherins and the cytoskeleton, and the nuclear messenger for Wnt signalling? On the basis of the available evidence and on molecular and evolutionary considerations, I propose that β -catenin was a born nuclear transport receptor, which by interacting with adhesion molecules acquired the property to coordinate nuclear functions with cell–cell adhesion. While Wnt signalling diverted this activity, the original pathway might still function in modern eukaryotes.

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See the Glossary for abbreviations used in this article.

Introduction

The canonical Wnt pathway states that free β -catenin is captured by the Axin–APC complex, phosphorylated by casein kinase 1 and GSK3, ubiquitinated by the SCF– β -TRCP complex and, thereby, targeted for proteasomal degradation. On Wnt binding to the co-receptors Frizzled and LRP5/6, the Axin complex is inactivated by not yet fully elucidated events. Proposed mechanisms include heterodimerization of Dishevelled and Axin, GSK3 inhibition by LRP5/6 and Dishevelled, direct Axin recruitment by LRP5/6, complex dissociation and Axin degradation [1,2]. This results in the appearance of a stable pool of soluble β -catenin. β -catenin can enter the nucleus, bind to TCF/LEF1 transcription factors and function as a co-activator [1,2]. β -catenin also associates with cadherin cell adhesion molecules at the plasma membrane, forming a link between cadherins and the actin cytoskeleton through α -catenin and other actin-binding proteins. Within this adhesive complex, β -catenin is protected, however as discussed below, the complex might be rather dynamic. Binding to cadherins is classically considered to antagonize Wnt signalling by sequestering the free cytosolic pool of β -catenin [3,4].

The actual picture is probably much more complex, as β -catenin levels and their localization and function can be

regulated by a multitude of processes. β -catenin interactions with Axin, APC, cadherins, α -catenin and TCF are regulatable in different ways. β -catenin activity also seems to be affected by alternative routes, for instance, inhibition of GSK3 by pathways other than Wnt, degradation of β -catenin by β -TRCP-independent mechanisms and stabilization by part proteolytic cleavage (see [1,2,4] for reviews). This complexity is certainly expected when one considers the key role of β -catenin in cell proliferation—long-term animal evolution and short-term evolution of cancer cells might have explored all possible ways to exploit β -catenin activity, and reciprocally, fences must have been superimposed to prevent uncontrolled activation.

This abundance of information tends to obscure the picture of β -catenin function. In particular, we lack a sense of the relative impact of each of these multiple interactions—quantitative information is largely missing, and particular mechanisms are often studied in one specific model, generally cancer cell lines, which are all heavily genetically modified. On the other hand, some mechanisms that seem today merely anecdotal could be of general significance for all cells, but the conditions under which they are fully active might not yet have been discovered. It might, therefore, be useful to take a step away from the details and try to extract some of the general properties of this protein. I propose here to contribute to this attempt by combining mechanistic and evolutionary information. I start by summarizing knowledge about the origins of β -catenin and its relationship with other proteins, comment on the mechanisms controlling β -catenin subcellular localization, explore similarities with import receptors, and end by considering the regulation of cadherin– β -catenin interaction and the potential direct link with β -catenin signalling.

Cytoplasmic/nuclear localization of Arm proteins

β -catenin is the founding member of the Armadillo family [5,6]. Its core region is made of a continuous row of 12 repeats, called Arm repeats. This type of repeat is found in a variety of proteins [7,8]. I consider here only those proteins similar to β -catenin, which are largely made of one extended domain of Arm repeats, defined here as ‘Arm proteins’.

At first sight the Arm proteins seem functionally disparate—apart from cadherin-associated proteins such as β -catenin, δ -catenin and plakophilins, one also finds for instance SMG-GDS, a GTPase-nucleotide exchange factor, constituents of the flagellum (PF16/SPAG6) and the nuclear import receptor importin- α

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Table 1 | Arm proteins with documented nuclear localization

Arm protein	Cytoplasmic function	Nuclear function and partners	Conserved classical NLS	Import	Export	Kingdoms	References
β -catenin	Adhesion, cytoskeleton (actin, microtubules)	Transcriptional regulator TCF (HMG TF), Sox, FOXO, NF κ B	No	Arm 1–8/10–12	Arm 10–12	Animals	2,28–30,32, 43–45
Plakoglobin	Adhesion, cytoskeleton (actin, IF)	Transcriptional regulator TCF (HMG TF), Sox	No	Arm		Animals	98,99
δ -catenin/p120ctn/ARVCF	Adhesion, cytoskeleton (actin)	Transcriptional regulator Kaiso (BTB/POZ TF)	Yes, but not essential	Arm (3+5)	Arm 8	Animals	33
Plakophilins 1–4	Adhesion, cytoskeleton (IF)	RNA polymerase III, RNA-binding proteins Fct?	No	Arm		Animals	100
CTNNB1	?	Component of the spliceosome	No			Most	101
RCD1-related proteins	?	Transcriptional regulator TFs	No			All	102–104
Smg-GDS	Rho nucleotide exchange (actin)	?	No	Rac1 co-transport?		Animals	105–107
PF16/SPAG6*	Flagellum (microtubules)	?	No			All	108
Importin- α	Cytoskeleton (actin)	Transport receptor Transcriptional regulator	IBB	Through importin- β	Through CAS	All	9–10
Kap3*	Cytoskeleton (microtubules), signalling	?	No			Most	107
ArmC8	Protein degradation, cytoskeleton?	?	NO			Most	110
ARO1	Cytoskeleton (actin)	?	NO			Plants	111
ARIA	Ubiquitination?	Transcriptional regulator ABE2 (BTB/POZ TF)	NO			Plants	21

Only 'Arm proteins'—proteins mainly composed of Arm repeats—were included. *Nuclear localization not reported explicitly, inferred from published immunofluorescent images. Classical (monopartite and bipartite) NLS sequences were searched in sequences from human, *Xenopus*, *Drosophila*, *Ciona* and *Nematostella* for animals, and *Arabidopsis*, *Oryza*, *Vitis*, *Picea* for plants. A conserved NLS consensus is only found in the p120-catenin subfamily, and in this case it was shown to be dispensable for nuclear transport [33]. ABE2, ABRE-binding bZIP factor; ARIA, ARM repeat protein interacting with ABE2; ArmC8, Armadillo repeat-containing 8; ARO1, Armadillo repeat only 1 protein; ARVCF, Armadillo repeat protein deleted in velo-cardio-facial syndrome homologue; BTB/POZ, BR-C, TTL and BAB/Pox virus and Zinc finger; CAS, cellular apoptosis susceptibility protein homologue; CTNNB1, β -catenin-like 1; FOXO, forkhead box proteins, O subclass; HMG, high mobility group; IBB, importin- β -binding domain; IF, intermediate filament; Kap3, kinesin-associated protein 3; NF κ B, nuclear factor kappa B; Rac1, Ras-related C3 botulinum toxin substrate 1; RCD1, required cell differentiation 1; Smg-GDS, small GTPase GDP dissociation stimulator; Sox, Sry-related HMG box proteins; SPAG6, sperm-associated antigen 6; TCF, T-cell factor; TF, transcription factor; ?, unknown.

(Table 1). Yet, some marked similarities can be observed: first, virtually all of those for which information is available were detected in the nucleus (Table 1). For some of them, the main or only known function is nuclear—for example, CTNNB1 is a component of the spliceosome. Others have at least two distinct activities, one in the nucleus and one in the cytoplasm—all catenins. In some cases, such as with SMG-GDS and PF16, the nuclear function is not yet known. None of these Arm proteins seems to have a classical functional NLS, implying that they must enter the nucleus through a non-classical pathway, as I discuss below. Another recurrent theme is a connection with the cytoskeleton (Table 1), which despite the diversity of interactions (actin, microtubules and intermediate filaments) points to an intimate link with structural cytoplasmic functions. One might extrapolate from this comparison that Arm proteins might be characterized by a dual role in the cytoplasm and in the nucleus. Even importin- α , in addition to its central role in nuclear import, can interact with the actin cytoskeleton [9], and has been found to act as a transcription factor [10].

These similarities could certainly be fortuitous—after all, dozens of other proteins interact with the cytoskeleton and hundreds can localize to the nucleus—and Arm repeats could simply constitute one among many other protein–protein interaction modules. I argue on the contrary that these functional analogies reflect unique properties of Arm proteins that have been evolutionarily conserved. Unfortunately, phylogenetic information is insufficient on its own to address effectively this question. The invention of Arm repeats is remote, probably coinciding with the appearance of the early eukaryotes, and few Arm proteins are conserved in all modern eukaryotes (Table 1; [8]). The β -catenin sequence is remarkably conserved in all animals, and its characteristic features can be found even in the ancient plakozoans and sponges, including its ability to bind to cadherins, the amino-terminal casein kinase 1 and GSK3 consensus motif required for its regulation by the Wnt pathway, and the carboxy-terminal transactivation domain CTA ([11]; Sidebar A). β -catenin partners are similarly conserved—for instance, even sponges have a classical cadherin, which binds to β -catenin [12], as well as the central constituents of the Wnt pathway [2,12,13].

Glossary

β -TRCP	β -transducin repeat protein 3
ARIA	ARM repeat protein interacting with ABF2
APC	adenomatous polyposis coli protein
CAS/exportin 2	cellular apoptosis susceptibility protein homologue
CTA	carboxy-terminal transactivation domain
CTNNBL1	β -catenin-like protein 1
FOXO	forkhead box proteins, O subclass
GSK3	glycogen synthase kinase 3
HEAT	Huntingtin, elongation factor 3, protein phosphatase 2A, TOR1
HGF	hepatocyte growth factor
IBB	importin- β -binding domain
LEF1	lymphoid enhancer-binding factor 1
LMB	leptomycin B
LRP5/6	low-density lipoprotein receptor-related protein 5/6
MDCK	Madine–Darby canine kidney
NES	nuclear export signal
NF κ B	nuclear factor kappa B
NLS	nuclear localization signal
Nup	nucleoporin
PF16/SPAG6	<i>Chlamydomonas</i> gene PF16/human sperm antigen 6
Rac1	Ras-related C3 botulinum toxin substrate 1
RanBP3	Ran-binding protein 3
SCF	Skp, Cullin, F-box
SMG-GDS	small GTPase GDP dissociation stimulator 1
Sox	Sry-related HMG box proteins
TCF	T-cell factor
Wnt	combination of Wingless and mammalian proto-oncogene int1

Thus, both the adhesive and the signalling function of β -catenin seem to have been present in the metazoan ancestors. There is however no trace of β -catenin in non-metazoan holozoans, and although several cadherins are found in choanoflagellates, they all lack a typical β -catenin-binding domain [14]. The scarcity of live representatives for other branches of holozoans makes it unlikely that one will be able to reconstitute the birth of classical cadherin- β -catenin adhesion and of the Wnt pathway. Interestingly, a cousin of β -catenin stands out in a remote type of organism—the slime mould *Dictyostelium* belongs to a completely different kingdom (Amoeboae), but its ability to form a temporary multicellular structure, the fruiting body, is reminiscent of metazoans. The fruiting body is an epithelial-like tissue held together by cellular junctions. Formation of these junctions requires two proteins, one of which is Aardvark, the closest β -catenin relative in *Dictyostelium* [15], and the second a homologue of α -catenin [16]. As with metazoans, Aardvark binds to α -catenin, and both regulate local actin structures [16,17]. As with β -catenin, Aardvark also has a signalling function that involves GSK3 [18]. However, Aardvark is probably not directly regulated by GSK3, as no GSK3 consensus motif can be found in its sequence. Important questions regarding the relationship between β -catenin and Aardvark remain to be answered—Aardvark localizes at cell junctions, but whether it interacts directly with a cell surface protein is not known. Adhesion molecules have been identified in the mould, which would be obvious candidates [19]. For the signalling function, one would predict that Aardvark enters the nucleus and that its signalling partners are nuclear proteins. The region potentially equivalent to β -catenin CTA shows weak similarity (Sidebar A), but the evolutionary distance is too large to make functional predictions.

Sidebar A | Evolution of the β -catenin carboxy-terminal transactivation domain

Vert SEDKPDYKKRLSVELTSSLFRx -xM WNE ADLG--LDLGAQGEFL-GYR
 Arth SEDKPDYKKRLSIELTNSLFR -xN WN-- DLG GPDLDMLGP EGYx
 Moll SEDKPDYKKRLSMELTSSLYRGD-NMxWS P--PG-FEDVG --LPEESRD
 Cnid SEDKSODYKKRLSVELTSSLFRDD--MPWE-PG-NTEMADILTSQSYQDELx
 *Pori SEDKSODYKRLSVELSSSLARTDSGGQWSQADV--FGRQLPGHSYQPAPP
 **Amoe LKNLSCNPKNKLTIARSGGSLME-IACINHPYSYSNRFLELMRILOSALEEA

**Oscarella lobularis*
 **Protein Aardvark

This characteristic region of β -catenin marks the end of the Arm repeat domain and is crucial for recruitment of transcription factors. Each aligned sequence represents the consensus for a phylum and subphylum, built from representative and available species. The corresponding *Dictyostelium* Aardvark sequence is included. Empty coloured boxes indicate variable amino acids with conserved properties (grey, hydrophobic; blue, acidic). Note a core conserved sequence followed by short motives, some of which seem to be characteristic of either higher or of lower metazoans. β -catenin homologues with highly divergent sequences found in several Phyla, including Ctenophora and various worms (such as Platyhelminthes, Nematoda and Annelida) are not shown.

Actin regulation at the membrane and nuclear signalling are also intimately connected in another branch of the Arm protein family, the γ -catenin-p120-catenin-plakophilins subclass. These proteins evolved independently of β -catenin from a pre-metazoan Arm protein [20], yet have striking functional similarities. They also interact with cadherins, although with a different region of their tail, regulate the actin cytoskeleton and interact with transcription factors (Table 1). One intriguing observation suggests an even earlier origin of the nuclear function of Arm proteins: plants express an Arm protein, called ARIA, which binds to transcription factors that are evolutionarily related to those binding δ -catenin-type proteins (Table 1; [21]).

Taken together, these considerations support the hypothesis that β -catenin-like Arm proteins might have been present in ancient eukaryotes, within which they already might have been organizing the actin cytoskeleton as well as sensing cytoplasmic and cell surface cues, and transmitting this information to the nucleus. The comparison between moulds and metazoans raises the possibility that β -catenin-type Arm proteins might have been instrumental in multicellularization. Adhesion molecules can be found in many unicellular organisms, including bacteria, but they are not expected to build any stable multicellular structure without synchronized changes in cell shape, and thus reorganization of the actin cytoskeleton. One can imagine how β -catenin-like Arm proteins—with a role in the regulation of the actin cytoskeleton in unicellular organisms—might have been captured by adhesion molecules, establishing immediately a coordinated link between extracellular adhesion and cell structure. Considering the early link between Arm proteins and nuclear activities—as for example in plants and *Dictyostelium*—it is possible that a cross-talk between the cytoskeleton and the nucleus existed even before the link to adhesion molecules.

Assuming this hypothesis is correct, can one detect whether modern β -catenin shows any sign of such an original dual function? Could β -catenin signalling occur independently of the Wnt pathway? Is this situation likely to occur in metazoans, including humans? To address these questions, we need first to compare the mechanisms of

nuclear transport of β -catenin with the well-characterized properties of its remote cousins, importins and exportins. We must also consider some basic properties that control β -catenin subcellular localization and ultimately function.

Classical nuclear transport at a glance

Nuclear import and export are mediated by soluble transporters, importins and exportins, also generically called karyopherins [22,23]. Except for importin- α , they all belong to the same importin- β family. The core mechanism of nuclear translocation relies on the unique coat of these proteins and its patches of hydrophobic residues. Binding of these patches to the filamentous hydrophobic repeats of the Nups allows importins and exportins to enter and exit the nucleopores freely, alone and with cargo (Fig 1A,B; [23]). The loading and unloading of cargo is controlled allosterically by the small GTPase Ran—RanGTP bound to importins causes the release of cargo and it promotes binding of cargo to exportins, whilst RanGDP has a complementary role. The concentrations of RanGTP and RanGDP are higher in the nucleus and in the cytoplasm, respectively, thus creating net directional transport. Different members of the importin- β family, either alone or in combination, import and export specific groups of cargos [23]. Cargos carrying the generic monopartite or bipartite NLS are imported by a similar mechanism, in which the NLS binds to importin- α , which in turn binds to importin- β [22].

Structural similarities between Arm and HEAT repeats

Importin- β -type transporters are built as long compact rows of HEAT repeats. Although HEAT repeats, as with Arm repeats, are found in functionally heterogeneous groups of proteins, the importin- β family is quite homogeneous, and all its members are essentially only made of HEAT repeats [7]. Arm and HEAT repeats have striking similarities. They belong to the group of α -solenoids, which are defined by an array of α -helices that form a curved structure [7,24]. Although not fully superimposable, Arm and HEAT proteins have a similar overall structure (Fig 1A,B; [7,24]). In both cases, a concave surface lines the whole repeat region, which is highly charged and represents the main interface for protein–protein interactions (Fig 1). For example, the groove of the β -catenin Arm domain binds to cadherins, Axin, APC, TCF and various other regulators and transcription factors [2]. Similarly, the concave surface of HEAT importins and exportins is the site within which cargos dock through various nuclear localization or export sequences. By contrast, the convex surface of these proteins is exceptionally rich in hydrophobic amino acids compared with other cytosolic proteins [25]. In the case of importins and exportins, this surface has the unique property to bind to Nups. Whilst the origin of these ancient structures cannot be tracked with certainty, Arm and HEAT repeats have so many common features that it is reasonable to invoke an evolutionary relationship [7].

β -catenin nucleo-cytoplasmic transport

The mode of β -catenin nuclear transport has been a matter of debate. β -catenin does not contain any classical NLS or NES, yet it can shuttle rapidly between both compartments. It has been proposed that APC, which contains multiple NLS and NES, carries β -catenin through the pore by a piggyback mechanism (discussed in [26]). Yet, APC nuclear localization does not seem to

be essential for Wnt signalling [27]. We and others have demonstrated that β -catenin can enter and exit the nucleus independently of APC and of any nuclear transport receptor [26,28–30]. Virtually all aspects of β -catenin import and export rather fit with free diffusion, similarly to translocation of the importins and exportins— β -catenin transport is insensitive to inhibition of the Ran GTPase cycle, does not require energy and is bidirectional [28–30]. Furthermore, as with exportin 1, β -catenin export is stimulated by direct interaction with RanBP3 [31].

All these observations are in agreement with the structural similarity between β -catenin and importins and exportins. On the basis of the properties of the latter, we had proposed that β -catenin interacts with Nups [28]. This has been demonstrated in a report by Henderson and colleagues [32]. Their biochemical and functional data show that specific Arm repeat–Nup interactions are required for the import and export of β -catenin. β -catenin is so far the only Arm protein for which nuclear transport has been studied in some detail, yet many other members of the family can localize to the nucleus, despite the absence of classical NLS (Table 1). In the case of p120-catenin, it was confirmed that the Arm repeats are responsible for nuclear transport [33]. Thus, I propose that binding to Nups might be a core property of Arm and HEAT proteins. The case of importin- α is consistent with this hypothesis; although it requires binding to importin- β 1 for import and to CAS for export [22,23], it has the basic transport properties of Arm and HEAT repeat proteins. It can bind directly to Nups [34], and a mutant lacking the short NLS-like IBB can cross the nucleopore on its own [35]. Importin- α shuttling on its own seems to be simply blocked by the short but heavily charged IBB.

Does the fact that Arm proteins diffuse freely through the pore imply that they will distribute homogeneously throughout the cell? Is a process similar to the Ran-dependent mechanism that controls classical nuclear transport the only way to create an asymmetrical nuclear and cytoplasmic distribution? I discuss how one could conceive regulation of nuclear transport without Ran, which I presume might have been the situation for the first importins, and how similar principles might still apply to control the localization of Arm proteins.

Nuclear transport without Ran?

As mentioned before, Ran is not required for nuclear translocation itself but for the association and release of cargo, thereby driving directional transport [22]. In the absence of Ran function, cargo-transport receptor complexes can still translocate but will distribute equally between cytoplasmic and nuclear compartments, based on the principle of diffusion [36,37]. However, directional transport could also be achieved by any other mechanism that influences the concentration of cargo on either side of the pore. One could easily conceive a primitive import mechanism for DNA-binding proteins, within which binding to a ‘proto-importin’ would allow them to cross the nucleopore, and their higher affinity for DNA would be sufficient for direct delivery to and retention at the chromatin (Fig 1C). Conversely, any cytoplasmic process that would serve as a sink for the cargo would similarly create directional export. Such a sink could be produced by sequestration of the cargo to an immobile fraction—for example, the cytoskeleton or membrane—or by degradation (Fig 1C). The latter mechanism is used efficiently to deplete nuclear proteins that shuttle through the classical import–export pathway [38,39].

Fig 1 | Structural and functional similarities between importin and exportin and β -catenin. (A) Diagram of the basic structure of an importin and exportin. The HEAT repeats form two surfaces—a convex outer surface rich in hydrophobic residues, which interacts with FG-repeats of the Nups, and a groove that binds to transport cargos (red). (A') Three-dimensional structure of importin- β . (B) Summary diagram of β -catenin showing the components involved in nuclear transport, gene regulation and cell–cell adhesion. Direct binding of Arm repeats to Nups is responsible for rapid nuclear import and export. The groove formed by the Arm repeats (blue) can bind to TCF/LEF1 and several other nuclear factors, as well as the scaffold proteins Axin and APC, which constitute the β -catenin degradation complex. These interactions are mutually exclusive with cadherin binding. The carboxy-terminal activation domain (CTA) binds to several components involved in transcription and chromatin remodelling and is essential for classical Wnt signalling. (B') 3D structures of β -catenin. (C) General diagram of classical Ran-dependent import and export, and of a hypothetical nuclear transport that is based on simple diffusion, in which directionality would be dictated by the compartmentalization of a sink for cargos. Sinks might bind to chromatin, membrane or cytoplasmic proteins, or they might be due to protein degradation. (D) Potential mechanisms of transport and transcriptional regulation by β -catenin. The original pathway might have involved passive shuttling of the β -catenin–cargo complex and delivery to chromatin. Modern β -catenin can de-repress target genes simply by competing out co-repressors (Groucho in the case of TCF/LEF1). In addition, β -catenin can directly recruit transcription factors (as well as histone deacetylases and components of chromatin remodelling complexes, not shown) and positively regulate transcription. Last, β -catenin could mediate export of nuclear factors. TCF–DNA interactions can be released by TCF phosphorylation, and similar reactions could regulate other DNA-binding partners of β -catenin. Degradation in the cytoplasm could serve as a sink for the diffusion reaction. APC, adenomatous polyposis coli protein; CTA, carboxy-terminal transactivation domain; HEAT, Huntingtin, elongation factor 3, protein phosphatase 2A, TOR1; NF κ B, nuclear factor kappa B; Nup, nucleoporin; Sox, Sry-related HMG box proteins; TCF/LEF, T-cell factor/ lymphoid enhancer-binding factor 1.

The same considerations apply to the distribution of the Arm and HEAT proteins themselves. Beside multiple potential sinks, such as the cytoskeleton, the plasma membrane and the chromatin, any interaction or modification that would interfere with

binding to the hydrophobic repeats would obviously cause accumulation in one of the compartments. Control by Ran improves general importin and exportin-dependent nuclear transport, allowing fast loading and unloading of different cargos without the need

for direct competition with nuclear or cytoplasmic interacting partners. However, alternative Ran-free modes of regulation might have been exploited by β -catenin and other Arm proteins.

β -catenin subcellular pools

The consensus for regulation of β -catenin levels is that soluble β -catenin is efficiently captured by the Axin-based destruction complex. It can, however, be protected from this immediate destruction when associated with other cellular components. The main stable pool of β -catenin is found in the adhesion complexes. When bound to cadherins, β -catenin is not accessible to Axin, APC or TCF, because the cadherin cytoplasmic tail occupies virtually the whole length of the β -catenin groove [40]. Other partners might also provide stability, including APC and fascin, which have roles in microtubule and actin filament organization, respectively [41,42]. The other main event that stabilizes β -catenin is inhibition of the Axin complex by activation of the Wnt pathway. In this case, β -catenin accumulates in the cytoplasm and is considered active for signalling, as it can cross the nucleopore and bind to specific promoter sequences through association with TCF/LEF1 [1,2] and other transcription factors such as Sox, FOXO and NF κ B, [43–45]. Apart from the degradation of the cytosolic pool through the Axin complex, we know little about the regulation of other β -catenin pools.

Despite thousands of reports, even the simple relative distribution of β -catenin between the nucleus and the cytoplasm is poorly characterized. The idea that Wnt signalling leads to nuclear accumulation of β -catenin is a common misconception that has biased the analysis of β -catenin localization. Nuclear levels of β -catenin do not necessarily exceed cytoplasmic levels [46], and a more accurate account should state that nuclear levels are increased in stimulated cells. One complication when evaluating endogenous β -catenin localization comes from intrinsic limitations of immunofluorescence techniques, for example the preferential leakage of the cytosol during fixation might lead to apparent nuclear enrichments [47]. This trivial but too often ignored artefact probably explains at least partly the diverse β -catenin nuclear-to-cytoplasmic ratios observed in different systems.

It remains nevertheless probable that at least part of the nuclear β -catenin accumulation observed in various cell types and tissues is real and due to active regulation.

Krieghoff and colleagues have convincingly shown that the rate of β -catenin nuclear transport can be reduced in the presence of its main partners, including TCF, Axin and APC [48]. The relative β -catenin distribution probably varies according to the levels and distribution of binding proteins, and to mechanisms that regulate these associations. For instance, B-cell lymphoma 9 might sequester β -catenin in the nucleus, in cancer cells cleavage of cadherins by Presenilin 1 releases β -catenin from the plasma membrane increasing the cytosolic pool, and in neurons cytosolic β -catenin can be stabilized by cleavage of its N-terminus by calpain (reviewed in [1,2,4]).

Phosphorylation is another mechanism that could affect binding of β -catenin to Nups and modulate retention in one of the two compartments [32]. Apart from the N-terminal GSK3/casein kinase 1 phosphorylation that targets β -catenin for degradation, the Arm repeat region is also phosphorylated at several residues. There is evidence that Wnt stimulates β -catenin phosphorylation through a Rac1–Jun kinase 2 pathway [49,50]. This seems to increase

nuclear accumulation, which was interpreted as stimulation of nuclear transport [50]. Thus, β -catenin might not only be stabilized but also actively modified. This would be consistent with the observation that endogenously activated β -catenin seems to signal more efficiently than overexpressed β -catenin [51].

Unfortunately, our knowledge about the distribution of β -catenin is confused by unconfirmed claims that are based on changes in steady-state distribution, without examination of import and export kinetics or consideration of immobilization and sequestration. Similar hasty conclusions were drawn from β -catenin nuclear accumulation on treatment with the export inhibitor LMB. Such treatment, especially when carried on for several hours, causes the nuclear sequestration of many components, including APC and Axin [26,52,53], which increases the number of binding sites for β -catenin in the nucleus. Nuclear transport is a rapid process; its accurate study requires measurements of transport rates [32,48] and support from *in vitro* reconstitution assays [28,29,52]. There is clearly a need to re-evaluate systematically the effect of binding proteins and other mechanism on the cellular distribution of β -catenin.

Is β -catenin a transport receptor?

The role of β -catenin as transcriptional co-activator is firmly established [2], and the fact that the CTA domain is well conserved in all metazoans including sponges ([11]; Sidebar A) indicates that it has an ancient and fundamental relationship with transcriptional regulators. The striking structural and functional similarities with importins and exportins suggest, however, that β -catenin might have also or originally been a nuclear transport receptor. This transport activity might still be present in modern β -catenin, which would constitute a completely different and ignored aspect of the signalling function of β -catenin. I comment here on evidence demonstrating that β -catenin has the capacity to transport a cargo [32], and on the conditions under which this capacity might be physiologically relevant.

I should first make clear that there is no evidence for a cargo being transported by β -catenin under physiological conditions. Sharma *et al* have shown that β -catenin can export TCF/LEF1, but these experiments required a trick, because full-length LEF1 binds strongly to chromatin and is not readily available for transport. Thus, the experiments were carried out with a LEF1 deletion construct lacking the DNA-binding sequence. Despite this artificial setting, the result remains important, as it demonstrates that β -catenin has transport activity. On the basis of what we know of the nucleopores and of the classical nuclear transport, carrying LEF1 through a pore is not a small deed—it does not only imply binding of Nups, but probably also hiding the numerous charges of the cargo (more than 20% of the LEF1 sequence) in the Arm repeat groove.

Thus there is little doubt that β -catenin has maintained the properties of a genuine transport receptor. What remains fully speculative at the moment is the identity of its physiological cargos. Among its known nuclear partners, one can probably eliminate all the general transcriptional regulators, which clearly function in many processes that are independent of β -catenin and rather look for more specific factors. Members of the TCF/LEF1 family are the most obvious candidates. Although they have a classical NLS and can clearly enter the nucleus independently of β -catenin, they do not harbour any obvious NES and

how they export is unclear. It has been reported that TCF is carried by the 14-3-3 scaffold protein, which itself uses the classical exportin 1, LMB-sensitive pathway [54]. 14-3-3 proteins are known to shuttle between the cytoplasm and the nucleus and were proposed, among many other functions, to serve as adaptors for transport [55]. However, a direct involvement of 14-3-3 in export of TCF, or any other interacting partner, has not yet been formally demonstrated. The observed accumulation of TCF and other cargos on inhibition of the exportin 1 pathway could also be explained by nuclear sequestration due to the accumulation of 14-3-3. The same caveat had led to the claim that APC or Axin functioned in β -catenin export [53,56]. 14-3-3s might mainly mediate retention in the cytoplasm, by masking the NLS of interacting partners [57,58]. TCF export was also only partly inhibited by exportin 1 depletion, suggesting the existence of an alternative export pathway [54]. There is clearly a need to re-examine TCF transport, which might reveal a new role of β -catenin. Two conditions are already fulfilled for potential directional export: TCF/LEF1 can be released from DNA by phosphorylation [59–61], thus generating a soluble pool, whilst TCF/LEF1 ubiquitination and degradation in the cytoplasm [62] would provide a sink (Fig 1D).

Among other nuclear partners, Sox proteins, which have NLS and NES sequences [43], do not seem to require β -catenin for nuclear transport. Sox proteins provide an excellent example for the complexity of nuclear transport—despite the presence of a classical NLS sequence, importin- α -dependent import of Sox2 is the weakest of three distinct import pathways [63]. Many other cargos might similarly use two or more import or export mechanisms. The relative importance of a particular pathway might vary under different conditions. In the case of a hypothetical transport of TCF and Sox by β -catenin, one would expect it to depend on the availability of soluble β -catenin and thus on Wnt signalling, as well as on more specific regulations, such as cargo- β -catenin or cargo-DNA interactions. It is conceivable that protein binding to or phosphorylation of the groove might trigger a change in β -catenin conformation to regulate cargo loading and unloading, similarly to the function of RanGTP for importins and exportins [23].

Cadherin- β -catenin association—more than a sink?

Since the discovery of β -catenin and its duality, attempts have been made to connect the cadherin-bound pool with the soluble signalling pool. Two complementary links have been proposed: first, Wnt stimulation increases not only soluble β -catenin levels, but also the membrane pool, although to a lesser extent. These early observations suggested that Wnt might stimulate cell–cell adhesion [46,64]. Despite some experimental support [64–67], this attractive hypothesis has not been much further explored and potential physiological implications remain to be revealed. One of the obstacles that might have hampered progress on this issue is the potent transcriptional regulation of cell adhesive properties by β -catenin signalling [4], making it difficult to distinguish direct from indirect effects.

Second, cadherins have been viewed as a sink, which by sequestering β -catenin moderate its transcriptional activity. This model has received more extensive experimental validation—cadherin overexpression competes with Wnt signalling [3,68–70], whilst β -catenin transcriptional activity can be upregulated on loss of cadherin [71–74]. Cadherin downregulation is a trademark of epithelial-to-mesenchymal transition (EMT), and it has been suggested to

cause the concomitant activation of β -catenin signalling (reviewed in [2,4]). However, the presence or absence of cadherins does not always affect Wnt- β -catenin signalling [74–76].

Both models imply that cadherins at the membrane have the capacity to absorb cytosolic β -catenin. Yet, there is strong evidence that cadherins are at all times saturated with β -catenin. Cadherins and β -catenin are present at the membrane as 1:1 complexes [77,78], the association of which is immediately formed after cadherin synthesis [79,80] and is required for cadherin localization and stability [80–82]. Under saturating conditions, additional β -catenin produced by the Wnt pathway could not be sequestered, and should not have any influence on cadherin adhesion. The two views can be reconciled considering that cadherin complexes expressed at the cell surface have a high turnover and that the cadherin- β -catenin association might be much more dynamic *in vivo* than suggested by the strong binding *in vitro* [83]. In other words, one might only be able to detect a 1:1 complex because cadherins and β -catenin might be rapidly degraded on their dissociation. Cadherin and β -catenin levels could influence each other by controlling the lifespan of the cell membrane complexes [80,84]. Alternatively, a cadherin sub-pool might be maintained at the membrane without constant association with β -catenin. Cadherin stability is also controlled by the δ -catenin-p120-catenin family [85], which could bypass the requirement for β -catenin.

Both models still consider Wnt signalling and adhesion as two parallel activities of β -catenin, and they only draw lateral connections between the two pathways. They do not address the more fundamental question of the coexistence of these two functions. It is difficult to imagine how both activities have simultaneously but independently arisen. On the basis of the functional and evolutionary information available, I propose that the adhesive function of β -catenin came before the appearance of Wnts. The interaction with the actin cytoskeleton and the capacity to enter the nucleus, thus the signalling function, might be even more ancient than the adhesive function of β -catenin.

Presumably, β -catenin might have served as an integrator of actin cytoskeleton dynamics through α -catenin and nuclear functions. Primitive multicellular assemblies might have added a β -catenin-binding sequence to cadherins, using it as a simple and direct way to link adhesion to cytoskeleton dynamics simultaneously and sense the status of cell–cell contacts (Fig 2). Originally, the mechanism could have been entirely passive—for instance, cadherin- β -catenin binding and inversely soluble β -catenin might have varied with the degree of cadherin clustering (Fig 3), the linkage to the cytoskeleton and cadherin stability at the surface. It is easy to conceive subsequent improvements, including regulated association and dissociation by phosphorylation and dephosphorylation (Fig 3). Evolution of multicellular organisms might have demanded a tighter regulation of β -catenin activity, starting with a lower cytoplasmic noise, thus the appearance of the destruction complex. Wnts might have exploited the cadherin- β -catenin signalling pathway—inhibition of the destruction complex might have been a straightforward trick to create an abnormally large cytoplasmic pool of β -catenin and short-circuit the control by cadherins.

Assuming that the cadherin-chromatin connection is the fundamental axis of β -catenin signalling offers a more coherent context than centring β -catenin activity on the Wnt pathway. For instance, several alternative regulations of β -catenin signalling, independently

Fig 2 | Hypothetical evolutionary and functional relationship between β -catenin and nuclear transport receptors. Importins and exportins on one side and β -catenin and other Arm proteins on the other side might have evolved from a common transporter prototype, which could shuttle DNA-binding proteins, delivering them to chromatin and/or retrieving them back to the cytoplasm. Such an ancient importin probably had a groove enriched with acidic residues (red) selective for DNA-binding proteins. An important evolution of HEAT-repeat-containing importins and exportins might have been the acquisition of Ran binding, which regulates cargo assembly and release on either side of the nuclear pore and thus mediates efficient unidirectional transport. Along the branch of the Arm proteins, importin- α might have lost its ability to translocate on its own and become dependent on importin- β and CAS for nuclear import and export, respectively. The importin- α - β pair became the main pathway for import of cargos carrying a classical NLS sequence composed of one or two small clusters of basic residues. β -catenin (and other related Arm proteins) might have been specialized carriers for a subset of cargos, and in addition, might have acquired the ability to interact with adhesion molecules, thus establishing a primitive signalling pathway between the cell surface and the nucleus. Note that a physiological role of β -catenin in nuclear transport has not yet been demonstrated and putative cargos have not yet been identified (question mark). This putative basal mechanism, based on competition between cargos and membrane anchors, might have been refined by eliminating excess free β -catenin through the Axin-based degradation complex. β -catenin might also have acquired transcriptional co-activation properties by bridging its DNA-binding partners (such as TCF) with transcription factors (TFs). The cadherin- β -catenin signalling pathway might have been high-jacked by Wnts and their receptors LRP5/6 and Frizzled (Fz)—by inhibiting the degradation complex, this pathway builds a large pool of cytosolic β -catenin and thus might bypass the basal control by cell adhesion. Note that several proteins harbouring HEAT or Arm repeats might have lost the property to cross the nuclear pore and have evolved different functions. These side branches of the two families are omitted in the diagram. CAS, cellular apoptosis susceptibility protein homologue; HEAT, Huntingtin, elongation factor 3, protein phosphatase 2A, TOR1; LRP5/6, low-density lipoprotein receptor-related protein 5/6; NLS, nuclear localization signal; TCF, T-cell factor; Wnt, combination of Wingless and mammalian proto-oncogene int1

of Wnts, GSK3 and the Axin complex, have been reported [86–90]. The Wnt pathway might have been a particularly successful mechanism among others, which has appropriated β -catenin and high-jacked the original mechanism.

Is there any trace of this putative cadherin-based pathway in modern β -catenin? Such a pathway would minimally require dynamic association and dissociation of β -catenin from the adhesion complexes. Studies on cell–cell adhesion have shown that

Fig 3 | Cell-adhesion-dependent β -catenin signalling. We propose that the state of adhesion controls specific phosphorylation reactions. The simplest mechanism could involve increased/decreased accessibility to kinases and phosphatases depending on the degree of cadherin clustering. Under conditions of strong adhesion, β -catenin is stable—it is protected within the cadherin complex, both by the tight clustering of the cadherins, and by the recruitment of actin-binding proteins (ABPs) and actin filaments. When adhesion is loose or absent, cadherins are de-clustered and connections with the cytoskeleton are dissolved. The cadherin– β -catenin bond is then exposed to various regulators. Both cadherin and β -catenin undergo multiple modifications, in particular serine/threonine and tyrosine phosphorylation (pS and pY). Cadherin serine/threonine phosphorylation tends to stabilize its interaction with β -catenin, whilst phosphorylation of specific tyrosine residues destabilizes it [2]. Phosphorylation of β -catenin decreases its interaction with α -catenin, and perhaps also with cadherins [2]. Note that multiple regulators, including kinases and phosphatases, can directly associate with cadherin and β -catenin [91]. Endocytosis of the cadherin complex (not represented) is probably also implicated in β -catenin signalling [95]. The pool of β -catenin released from the cadherin complex could be biochemically marked by intramolecular interaction [97], phosphorylation and interaction with other components. This activated β -catenin would signal more efficiently, either due to protection from degradation and rebinding to cadherins, increased nuclear transport, stronger binding to TCF or higher transactivation capabilities.

cadherin– β -catenin interaction can be regulated by phosphorylation of either of the two partners (reviewed in [2,91]). Whether cell adhesion is regulated directly—by association and dissociation of the cadherin complex—or indirectly is unclear [92]. However, the fact that phosphorylation can change the affinity of β -catenin for cadherin by two orders of magnitude [93], and that kinases and phosphatases are intimately associated with cadherin complexes (Fig 3; [91]), suggests

Sidebar B | In need of answers

- (i) Does β -catenin transport nuclear proteins under physiological conditions, and if so what is the functional significance for modern metazoan cells? TCF would be an obvious candidate, but direct observation of β -catenin-mediated transport in isolation from other parameters such as retention (for example, by FRAP) will probably only be possible in situations where a large pool of TCF is being dissociated from chromatin.
- (ii) If β -catenin can indeed transport cargos, how is this process regulated? Is β -catenin conformation affected by phosphorylation or protein interaction, and does this change favour cargo association and release?
- (iii) Are other Arm proteins also directly diffusing through the nuclear pore? This is the easiest experiment—it might only require testing recombinant proteins in the classical nuclear transport assay in semi-permeabilized cells.
- (iv) Wnt-independent signalling—is cadherin really required for β -catenin signalling during EMT? Formal demonstration would involve, in particular, the use of cadherin variants lacking specifically β -catenin binding. Are there other circumstances in which a putative Wnt-independent, cadherin-dependent signalling can be observed?
- (v) How similar is Aardvark to β -catenin? What is the membrane protein partner for Aardvark? Is it also an adhesion molecule? What is the nuclear activity of Aardvark? Is it interacting with a transcription factor? What is the function of the nuclear pool for the other Arm proteins? Transcription? Transport?

that β -catenin might be released from membranes in a regulated way. That a membrane pool could effectively supply β -catenin for signalling was originally shown in the early *Xenopus* embryo [94], and was more recently confirmed in cultured mammalian cells, in which β -catenin was found to be released from endocytosed cadherins [95].

Furthermore, an article by Howard *et al* [96] has provided evidence for a positive role of the cadherin– β -catenin association in β -catenin signalling. The study analyses the regulation of β -catenin activity during HGF-induced EMT of MDCK cells, a process during which β -catenin signalling increases in parallel with E-cadherin downregulation. As mentioned earlier, the common interpretation of this phenomenon had been that E-cadherin loss equates to the removal of a sink for β -catenin. Howard and colleagues propose a different explanation— β -catenin signalling is not enhanced due to cadherin depletion, but rather due to the process of cadherin endocytosis. They argue that the presence of cadherins and the ability of β -catenin to bind to them are in fact required for β -catenin signalling during EMT. Moreover, the presence of cadherins also increases the responsiveness of cells undergoing EMT to exogenous Wnt stimulation. However, this interesting model requires further confirmation. The positive role of cadherins on β -catenin signalling might be indirect, because E-cadherin also interacts with the HGF receptor, and cadherin levels might therefore affect HGF-induced EMT and only secondarily responsiveness to Wnts. Expression of a cadherin variant specifically defective in β -catenin-binding would strengthen the case. Despite these caveats and the lack of mechanistic insight, these data provocatively suggest that interaction with cadherins could provide a positive instructive signal to β -catenin. That this putative pathway was discovered during EMT is probably not anecdotal—apart from cancer, EMT and its reverse process, mesenchymal-to-epithelial transition, are basic morphogenetic processes and represent the most drastic physiological changes in cell–cell adhesion. During animal evolution, the same ability to switch reversibly from free roaming cells to clusters might have been at the origin of multicellularity, and might have involved a β -catenin ancestor.

Although still fragmentary, a putative cadherin-regulated β -catenin signalling pathway seems to emerge. What would be the conditions for this pathway to work effectively in metazoan cells? Besides the regulated release of β -catenin from cadherins discussed above, one might expect some degree of protection from degradation by the Axin complex. Any modification that would decrease binding to Axin and APC is expected to fulfil this function—for example phosphorylation and dephosphorylation in the groove of the Arm domain, or intramolecular interaction between the C-terminal tail and the Arm repeats [97]. As mentioned above, additional mechanisms that affect nuclear transport or the levels of soluble β -catenin in the cytosol and nucleus could further boost or moderate this pathway.

Clearly there is still much to be discovered about β -catenin (Sidebar B). One of the difficulties is the strongly dominant effect of Wnts and their rampant activity present in most cell lines, which by creating a stable cytoplasmic β -catenin pool might mask other regulations. The putative cadherin-based signal might be low and relatively systemic, and a modest regulation of transcriptional activity might be sufficient for sustaining proper tissue homeostasis. Yet, it is also possible that changes in adhesive conditions might lead to acute peaks of β -catenin activity, which would certainly be easier to study. Howard *et al* might have identified such a system, which could be further exploited to unravel the putative ancient function of β -catenin. The fact that the reported events occur during EMT, and that cadherin-dependent β -catenin signalling might participate directly in this crucial transition towards cancer malignancy, presages another era of fame for the cadherin– β -catenin pair.

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CONFLICT OF INTEREST

The author declares that he has no conflict of interest.

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