



## Hypothesis

## Stochastic machines as a colocalization mechanism for scaffold protein function



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## ABSTRACT

**The axis inhibition (Axin) scaffold protein colocalizes  $\beta$ -catenin, casein kinase I $\alpha$ , and glycogen synthetase kinase 3 $\beta$  by their binding to Axin's long intrinsically disordered region, thereby yielding structured domains with flexible linkers. This complex leads to the phosphorylation of  $\beta$ -catenin, marking it for destruction. Fusing proteins with flexible linkers vastly accelerates chemical interactions between them by their colocalization. Here we propose that the complex works by random movements of a "stochastic machine," not by coordinated conformational changes. This non-covalent, modular assembly process allows the various molecular machine components to be used in multiple processes.**

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### 1. Introduction

The Axin [1] scaffold protein [2] colocalizes adenomatous polyposis coli protein (APC),  $\beta$ -catenin, casein kinase I $\alpha$  (CKI- $\alpha$ ), and glycogen synthetase kinase 3 $\beta$  (GSK3 $\beta$ ) [3]. The kinases phosphorylate  $\beta$ -catenin, thus signaling  $\beta$ -catenin's ubiquitination and proteasomal destruction [4,5], important steps in the Wnt signaling pathway [2]. The two kinases and the  $\beta$ -catenin bind to Axin's long intrinsically disordered region [4–6], yielding structured domains connected by flexible linkers. Stochastic, uncoordinated movements of the linkers and their bound proteins are proposed to

enable productive kinase-substrate collisions leading to phosphorylation. Thus, the complex works by random movements of a "stochastic machine," not by cooperative conformational changes. Unlike typical machines, the different parts of the device are loosely connected, with random movements bringing components together.

Proteins with flexible linkers accelerate their chemical interactions by colocalization [7]. Here such colocalization is proposed to result from proteins binding onto a single intrinsically disordered scaffold protein, with this modular assembly allowing the components to be used in multiple processes. Colocalization with flexibility and modular assembly are proposed to underlie the common occurrence of intrinsic disorder in scaffold proteins [8,9].

To give more detail regarding the Wnt pathway [2], CKI- $\alpha$  carries out the initial phosphorylation of  $\beta$ -catenin [3], followed by GSK-3 $\beta$  phosphorylation of three other sites on  $\beta$ -catenin [10]. The tetra-phosphorylation of  $\beta$ -catenin then signals its destruction. By binding to its cell surface receptor, the Wnt protein induces the inactivation of the complex,  $\beta$ -catenin levels then elevate and activate a number of genes when it migrates into the nucleus.

*Abbreviations:* APC, adenomatous polyposis coli protein; Axin, axis inhibition protein; CKI- $\alpha$ , casein kinase I $\alpha$ ; DIX, disheveled and Axin (domain); GSK3 $\beta$ , glycogen synthetase kinase 3 $\beta$ ; HSQC, heteronuclear single quantum coherence; MBP, multiple binding partners; MBPAS, multiple binding partners and alternative splicing isoforms; RGS, regulator of G-protein signaling (domain); TGF- $\beta$ , transforming growth factor  $\beta$  pathway

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The conformational changes underlying the phosphorylation of  $\beta$ -catenin by the complex remain unclear mainly due to the absence of a structural model. Below we present the evidence for intrinsic disorder and binding site locations in the various proteins in the complex, then we put this information together to develop a model for the complex. This model leads to the mechanism proposed herein.

## 2. Materials and methods

The sequential, structural, and functional information of the Wnt pathway proteins were extracted from Swissprot, and PDB. Intrinsically disordered residues and regions are assigned from experiments through missing electron density in X-ray crystallography, regions sensitive to proteolysis, or to regions indicated to lack stable structure by NMR. Disorder predictions were made using PONDR<sup>®</sup>VSL2 [11]. Binding regions within disordered segments were predicted using the MoRFpred predictor [12].

## 3. Results and discussion

### 3.1. Intrinsic disorder in the $\beta$ -catenin destruction complex

The first step is to identify the regions of order and disorder in the Axin-scaffolded complex. An ordered (or structured) protein exhibits a stable conformation, whereas an intrinsically disordered protein or region does not [13]. Ordered proteins yield 3D structures by X-ray crystallography or NMR spectroscopy while disordered proteins do not. Instead disorder is identified by the absence of electron density in X-ray structures, by collapsed NMR spectra, or by various other biophysical methods [13]. Also, disorder predictors, currently above 75% accuracy, can also be used to indicate which proteins and protein regions lack stable structure [14]. Concurrent use of prediction and experiment provides a useful overview.

With regard to the locations of ordered and disordered regions for 4 of the 5 proteins in the Axin-scaffolded complex (APC is omitted because its length of >2700 residues complicates the figure, and because the complex functions in APC's absence [15]), the experimental data and the predictions [16] show reasonable agreement (Fig. 1). Axin and the omitted APC are both predominantly disordered by prediction, with <21% of the amino acids being structured [16]. The  $\beta$ -catenin protein is mostly structured but with disordered termini. GSK3 $\beta$  and CK1 $\alpha$  have less than 16% disorder as is often observed for enzymes.

CK1 $\alpha$ ,  $\beta$ -catenin, and GSK3 $\beta$  all bind to single stretch of disorder in Axin (Fig. 1), located between the regulator of G-protein signalling (RGS) domain and the dishevelled and axin (DIX) domain. Both  $\beta$ -catenin and GSK3 $\beta$  dock onto short segments of Axin. Crystal structures show that the Axin segments both become helices [5,6]. Similar conversion from disorder into helix upon binding has been inferred for many other protein complexes [17].

Among all the proteins involved in the Wnt-pathway, Axin plays the central role in increasing the local concentrations of the various players. In various other pathways, Axin binds ~30 other proteins through its ~500 residue-long flexible region [8]. The Stokes radius of a polypeptide chain with such a length in a globular compact conformation is ~30 Å. However, for a flexible pre-molten globule-like chain (which is the most likely conformation of this fragment in solution) the Stokes radius can be close to ~50 Å (see Supplementary material). The increased hydrodynamic dimensions augment the prospects of binding to other molecules through the increase in the protein capture radius, which is commonly suggested to increase the binding rates for disordered

regions. Simulations suggest that the increased capture radius of IDPs is counter balanced by their slower diffusion [18] with a typo correction in [19]. On the other hand, the reduced structural constraints of IDPs has been suggested to lead to a higher rate of productive collisions, thus increasing IDP binding rates by this alternative mechanism [18,19]. Note however, that the key events proposed herein occur after binding via a colocalization mechanism, so whether the high binding rates for IDPs result from an increased capture radius or from a higher fraction of productive collisions is not crucial to the hypothesis being developed here.

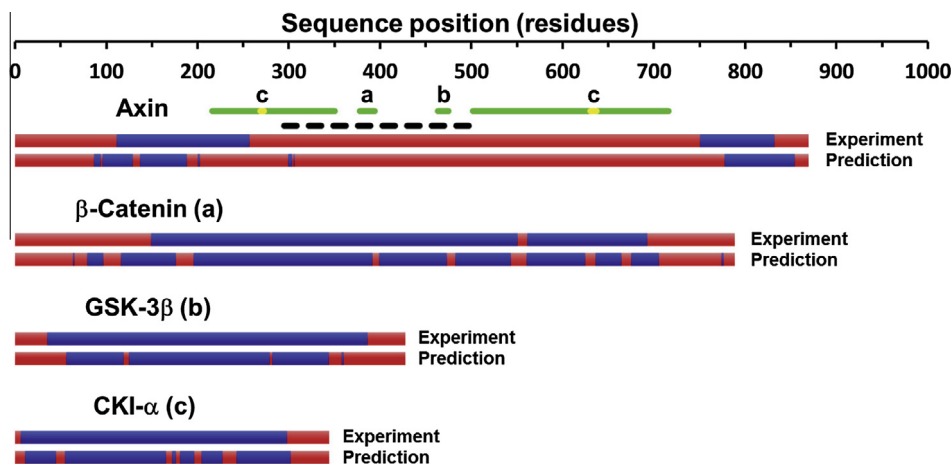
The structure of CK1 $\alpha$  bound to Axin has not yet been determined. Pull-down experiments indicate that the binding between Axin and CK1 $\alpha$  involves two well separated Axin regions [4]. Many complexes with more than one binding site separated by regions of disorder have been resolved by crystallography with the disordered connecting loops remaining intact. Further study on one of these, phosphatase 1 complexed with inhibitor 2, shows that the protein crystal contains large holes to accommodate the unstructured loops [20]. The requirement for large holes to accommodate the regions disorder is likely a barrier to crystallization and might help explain why the CK1 $\alpha$ -Axin complex has resisted structure-determination efforts, especially given the long distance between the two binding sites (Fig. 1).

A possible loop in the Axin-CK1 $\alpha$  complex is interesting for several reasons. First, several docking proteins (similar to scaffold proteins in many respects [9]) were shown to contain structured domains at their amino termini followed by long disordered tails at their amino termini, with the disordered regions binding to the structured domains multiple times. The end result is likely a structured core surrounded by multiple loops [16]. Such loops were suggested to be protective by leading to structural condensation, and different partners binding to different loops were suggested to functionally partition collections of partner proteins [21,22]. In addition, loop formation would lead to a further decrease the volume of the complex as compared to binding to a non-looped disordered region, thus increasing the local concentrations. For these reasons, it would be interesting to determine whether indeed the Axin-CK1 $\alpha$  interaction involves loop formation.

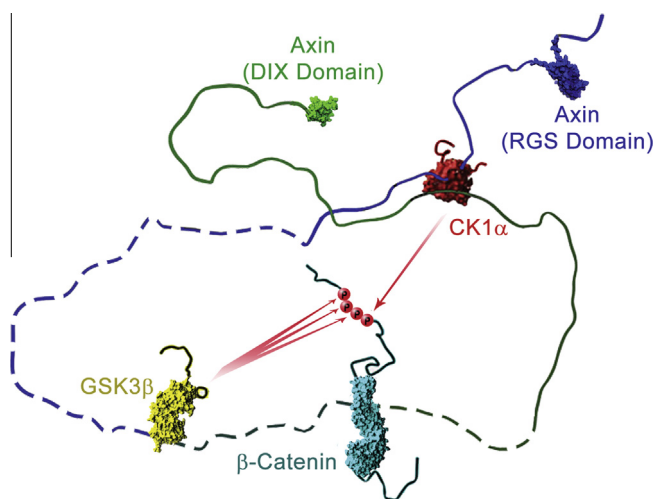
### 3.2. The stochastic machine mechanism

Using the intrinsic disorder and binding sites information described in Fig. 1, a plausible model for the  $\beta$ -catenin destruction complex was constructed (Fig. 2). As for Fig. 1, APC was omitted for simplification, and as also for Fig. 1, the G295-A500 fragment is indicated by a dashed line. This figure is drawn to scale using the structures from the PDB and a value of 3.8 Å/residue to estimate the lengths of the intrinsically disordered regions. The estimated distance of 3.8 Å/residue corresponds to the peak value of a histogram of distance/residue for several hundred long irregular regions in protein crystal structures.

How do we know for sure that attachment of kinases and  $\beta$ -catenin to a region of disorder would increase the rate of phosphorylation? To test this possibility, in a previously published experiment [23], we isolated an Axin fragment containing the binding sites for  $\beta$ -catenin and GSK3 $\beta$ , namely the fragment from G295-A500 shown in both Figs. 1 and 2, and we demonstrated that this segment lacks structure by four separate biophysical methods, namely: (1) Its lack of unfolding during heating as monitored by fluorescence; (2) Its extended structure as shown by size exclusion chromatography; (3) Its random coil conformation as shown by circular dichroism; (4) Its high internal mobility as shown by the <sup>1</sup>H-<sup>15</sup>N HSQC NMR spectrum [23]. When mixed with  $\beta$ -catenin and GSK3 $\beta$ , this disordered fragment greatly accelerates the phosphorylation rate of  $\beta$ -catenin as estimated by <sup>32</sup>P incorporation as reported previously [23].



**Fig. 1.** Structural/sequence information for Axin, CKI- $\alpha$ , GSK-3 $\beta$ , and  $\beta$ -catenin. The bars represent the proteins' full sequences, with blue corresponding to structure and red to disordered regions. The top bar for each protein shows the experimentally identified order/disorder distribution (see Supplementary Table S1 [17]), whereas the bottom bar depicts the predicted order/disorder. The green lines above Axin's bar depict the partner proteins' binding sites [8]: (a) GSK-3 $\beta$ , (b)  $\beta$ -catenin, and (c) CKI- $\alpha$ 's (two segments). The CKI- $\alpha$  binding regions were estimated using rather long fragments [8]. Possible binding site locations within these fragments were estimated by the MoRFpred algorithm [12] shown in yellow. The dashed line above Axin's diagram corresponds marks the G295-A500 disordered segment [24].



**Fig. 2.** The stochastic machine mechanism. This figure shows a possible configuration for the complex involving Axin,  $\beta$ -catenin, GSK-3 $\beta$ , and CKI- $\alpha$ . Axin is shown with colour variation to make its pathway easier to follow. The dashed line corresponds approximately to the location of the G295-A500 disordered segment [24]. Axin binds to CKI- $\alpha$  (at two separate sites), to GSK-3 $\beta$ , and also to  $\beta$ -catenin. Since the  $\beta$ -catenin binding site of Axin is located between the GSK-3 $\beta$  and CKI- $\alpha$  interaction sites, and since the two binding sites with CKI- $\alpha$  may lead to the formation of a loop,  $\beta$ -catenin becomes close to both kinases. Hence, the formation of this  $\beta$ -catenin destruction complex pulls all the proteins together, and substantially raises their local concentrations. Because the phosphorylation sites are in a disordered region of  $\beta$ -catenin and because the various binding sites are all in a long disordered region in Axin, random motions of these flexible regions can readily bring about the substrate-enzyme collisions needed for function.

To test for possible allosteric activation of the substrate or kinase by Axin binding with the  $\beta$ -catenin but not with the GSK3 $\beta$  and vice versa, fragments with one binding site but not the other were added to mixtures of  $\beta$ -catenin and GSK3 $\beta$ . Very little phosphorylation was observed if the fragment contained just one binding site [23], with the rates essentially the same as observed for comparable mixtures of  $\beta$ -catenin and GSK3 $\beta$  without fragment addition [23]. This experiment rules out the possibility of allosteric activation of the phosphorylation by Axin binding to either the kinase or to its substrate.

The previously published experiments just described in [23] show that joint attachment of both  $\beta$ -catenin and the GSK3 $\beta$  kinase to a disordered segment increases the phosphorylation rate, but what is the mechanism by which this rate increase occurs? Given the flexibility described above for the G295-A500 segment and likely for the entire disordered region shown in Fig. 2, the mechanism very likely involves random motions that lead to collisions between the kinases and their phosphorylation sites at  $\beta$ -catenin's Ser45, Thr41, Ser37, and Ser33. By attaching the two proteins to a common region of disorder, their time-averaged concentrations relative to each other would become greatly raised, thus increasing the reaction rates. Basically, the region of disorder functions by colocalizing the substrate and the kinase into a small volume, while the flexibility of the disorder allows movements to bring about the same collisions that would occur in the unbound state but at a much greater rate due to their higher local concentrations. Also complex formation creates an entropically favourable situation, whereby an inefficient intermolecular interaction is transformed into a more efficient intramolecular interaction. This effect is further augmented via Axin oligomerization through the DIX domain [24], thus increasing the phosphorylation of  $\beta$ -catenin bound to one Axin molecule by a kinase bound to another by the colocalization of the complexes.

Colocalization is a fundamentally important, general biological principle [7]. Several mechanisms for colocalization have been suggested, including the connection of two proteins via a flexible linker, thus accounting for the observed acceleration of phosphorylation when the G250-A500 fragment of Axin is added to mixtures of  $\beta$ -catenin and GSK3 $\beta$  [23].

Flexible-linker-based colocalization was suggested previously to arise from gene fusion [7]. Indeed, connecting structured protein domains by flexible linkers via gene fusion has been long recognized [25]. Assembling domains by binding onto regions of disorder provides an alternative to gene fusion, but with the added benefit that the same components could be reused in different assemblies. Indeed, Axin acts as the scaffold protein for at least three other pathways: the transforming growth factor  $\beta$  (TGF- $\beta$ ) pathway [26], the c-Jun NH2-terminal/stress activated protein kinase (JNK) pathway [27], and the p53 signaling pathway [28]. Likewise,  $\beta$ -catenin and the two kinases discussed here are also used for several other biochemical processes [29].

Note also that phosphorylation of serine or threonine [30,31] and ubiquitination of lysine [32] both show strong preferences for regions of intrinsic disorder. In addition poly-ubiquitin-triggered proteasomal digestion is accelerated by an appropriately located region of disorder [33]. Thus, the disordered amino terminus of  $\beta$ -catenin is important for the degradation process [13].

Rather than forming a compact machine, which would require shape complementarity over large regions of sequence, the interactions between the disordered scaffold and each of its partners depends on only a few residues for each partner of each interaction. The small number of residues needed to form each complex facilitates their evolvability [34]. Finally the conformational changes underlying the phosphorylations are suggested to be stochastic movements of disordered regions, not the coordinated movements of a structured machine.

### 3.3. Possible alternative mechanism and experimental test

The formation of the various binary interactions, including the interactions with ATP, could lead to new surface features that result in additional protein–protein interactions as has been reported previously [35], thereby leading to higher-order structures that accelerate the phosphorylation steps via directed rather than random conformational changes. A second relevant example was observed when the binding of calmodulin to its site in a long disordered region in calcineurin was found to lead to an additional, unexpected interaction between calcineurin's long disordered region and the exterior of the calmodulin complex [36]. To distinguish our model from such alternative models involving higher order structures, one could test for additional sites of protein–protein interactions using protection against amide hydrogen exchange and/or by atomic force microscopy as has been done previously [35–37].

## 4. Summary

Here we have suggested that intrinsically disordered protein can provide flexible linkers between structured domains, and by concentrating them, accelerate their interactions, not through specific conformational changes, but through random collisions via stochastic movements. The stochastic machine mechanism could also apply to structured domains connected by flexible linkers has have been described previously, and the disordered regions need not be particularly long as in the case of Axin. Thus, it is likely that stochastic machines are very common, especially within the disorder-rich eukaryotic cell [38]. Thus, stochastic machines based on random movements within intrinsically disordered proteins are likely to be exceedingly common, thus providing support for viewing cells as being composed of collections of molecular machines [39].

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.febslet.2013.04.006>.

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