Intrinsically disordered regions (IDRs) are protein regions that lack persistent secondary or tertiary structure under native conditions. IDRs represent >40% of the eukaryotic proteome and play a crucial role in protein–protein interactions. The classical approach for identification of these interaction interfaces is based on mutagenesis combined with biochemical techniques such as coimmunoprecipitation or yeast two-hybrid screening. This approach either provides information of low resolution (large deletions) or very laboriously tries to precisely define the binding epitope via single amino acid substitutions. Here, we report the use of a peptide microarray based on the human scaffold protein AXIN1 for high-throughput and -resolution mapping of binding sites for several AXIN1 interaction partners in vitro. For each of the AXIN1-binding partners tested, i.e. casein kinase 1 ε (CK1ε); c-Myc; peptidyl-prolyl cis/trans isomerase, NIMA-interacting 1 (Pin1); and p53, we found at least three different epitopes, predominantly in the central IDR of AXIN1. We functionally validated the specific AXIN1–CK1ε interaction identified here with epitope-mimicking peptides and with AXIN1 variants having deletions of short binding epitopes. On the basis of these results, we propose a model in which AXIN1 competes with dishevelled (DVL) for CK1ε and regulates CK1ε-induced phosphorylation of DVL and activation of Wnt/β-catenin signaling.

Proper spatial and temporal organization of molecules acting in the cell signaling pathways is crucial for specific and efficient information transfer. Scaffold proteins coordinate the action of the signaling components by bringing them together into multiprotein complexes, thereby increasing their proximity and effective concentration (1). To bind multiple partners, scaffold proteins usually contain several modular structured domains, often in combination with regions that do not adopt secondary structures under native conditions, termed intrinsically disordered regions (IDRs) (2). IDRs significantly increase binding capacity of the scaffold due to their flexibility and ability to mediate protein–protein interactions via relatively short linear peptide motifs (3, 4).

Tumor suppressor AXIN1 is a representative of intrinsically disordered scaffold proteins. It consists of N-terminal RGS and C-terminal DIX (Dishevelled, Axin) structured domains that are separated by the large “central” IDR (5). Besides its central role in Wnt signaling, AXIN1 has been identified as a component of several different pathways, including p53, c-Myc, transforming growth factor β, and c-Jun N-terminal kinase (for reviews, see Refs. 3 and 6). To gain mechanistic insight into the formation of the signaling complexes (i.e. which proteins can bind simultaneously/exclusively), it is essential to map binding epitopes for the interaction partners on the scaffold at high resolution, which is complicated to achieve with commonly used biochemical methods such as coimmunoprecipitation or yeast two-hybrid screening.

A high-throughput screening technique derived from oligonucleotide microarray (7, 8), in which peptides were immobilized via a linker on a solid surface, has been used recently to achieve this goal and to profile the structural requirements of the binding of protein domains (9, 10). For systematic mapping of protein-binding epitopes, IDRs of protein of interest are chopped into small (overlapping) peptides, which are immobilized via a linker on a solid surface, usually a glass or plastic chip. Posttranslational modifications, such as phosphorylation, can be incorporated in a site-specific manner by organic synthesis

3 The abbreviations used are: IDR, intrinsically disordered region; CK1, casein kinase 1; DVL, dishevelled; RGS, regulator of G protein signaling; aa, amino acid(s); Pin1, peptidyl-prolyl cis/trans isomerase, NIMA-interacting 1; HEK, human embryonic kidney; APC, adenomatous polyposis coli protein; EGFP, enhanced GFP; TCF, T-cell factor; TBS, Tris-buffered saline; LU, light units; DMEM, Dulbecco’s modified Eagle’s medium; CM, conditioned medium; NP-40, Nonidet P-40; WB, Western blotting.
procedures into the peptides involved in the array. For the detection of protein–peptide interactions, the array can be incubated either with a purified recombinant protein or a crude cell lysate that can serve as the source of protein (11). Finally, the protein partner bound to the array can be easily detected using an immunofluorescence technique. The array format allows a high-throughput approach for determination of short peptide motifs within the (scaffold) protein of interest that are responsible for docking of the protein partners involved in the complex.

In this study, we present the use of a peptide microarray for detailed in vitro mapping of interaction epitopes within the IDRs of the scaffold protein AXIN1. Using this approach, we identified putative binding interfaces in the IDRs of human AXIN1 with several interaction partners (casein kinase 1ε (CK1ε); c-Myc; p53; and peptidyl-prolyl cis/trans isomerase, NIMA-interacting 1 (Pin1)) at high resolution. Subsequent validation of these findings allowed us to propose a model for AXIN1-dependent regulation of Wnt/β-catenin signaling by CK1ε via dishevelled (DVL).

**Results**

**AXIN1 contains several binding epitopes for CK1ε, c-Myc, p53, and Pin1**

All Axin proteins have a conserved structure with two well-defined domains: an N-terminally located RGS domain (aa 88–211 in human AXIN1) and a C-terminally located DIX domain (aa 780–862 in human AXIN1). There are also two IDRs in the rest of protein: (i) the N-terminal IDR (aa 1–87) and (ii) the central IDR (aa 212–779) (Fig. 1A, top).

The binding regions for biologically very important AXIN1 interaction partners, namely CK1ε, c-Myc, p53, and Pin1, have been mapped earlier by deletion mutagenesis (for schematics see Fig. 1A, bottom). Not surprisingly, these proteins regulating distinct biochemical processes bind to the central IDR of AXIN1.
AXIN1. It is obvious, just simply for steric reasons, that only a very limited number of proteins can be associated with the AXIN1 scaffold at the same time, and it is likely that one AXIN1 complex is dedicated to only one biological function. To understand better how AXIN1 achieves this, we decided to map binding epitopes within the IDR of AXIN1 with high resolution. We designed a peptide microarray containing 13-mer peptides overlapping by 10 residues covering the entire human AXIN1 sequence (Fig. S1) that were immobilized via a linker molecule on a microscopic slide. To perform a single peptide microarray experiment, the glass slide with immobilized peptides was incubated with the recombinant protein of interest followed by antibody staining and visualization using a fluorescence marker. The signal was subsequently detected by a high-resolution fluorescence scanner and analyzed using spot-recognition software (schematized in Fig. 1B).

To identify the peptide epitopes required for direct interaction with AXIN1, the following candidates were used for a peptide microarray experiment: CK1ε, c-Myc, p53, and Pin1. These candidates were chosen as the representative AXIN1-binding partners from individual signaling pathways (Fig. 1A, bottom). Microarray screening analysis (Fig. 2; for an example of raw data, see Fig. S2) revealed that each interaction partner of AXIN1 can efficiently interact with at least three linear peptide motifs. All tested proteins showed interaction with at least one of two linear peptides forming solvent-exposed α-helix (aa 133–152 and aa 154–175 in human AXIN1) from the RGS domain; here, however, the interpretation is not straightforward because the linear peptides may not truly recapitulate the situation in the folded RGS domain. However, this information can be still relevant because the epitopes identified within the RGS domain were located on the α-helices and exposed to the solvent. Peptides mapping to the DIX domain were not considered as putative binding sites of c-Myc (see Fig. 2B) as they were located predominantly neither on the surface nor on the “linear” α-helix (see “Experimental procedures” for more details about quantification). All the remaining binding sites of the aforementioned candidate proteins were located in the central IDR of AXIN1 (Fig. 2, A–D).

The identified epitopes narrow down the regions involved in the interaction from several hundred aa reported in the literature to the ~20-aa-long motifs. Specifically, for CK1ε, two binding sites (located in the central IDR) previously mapped to (i) the N-terminal part of IDR, aa 198–353 in mouse AXIN1 (corresponding to aa 198–353 in human AXIN1) (12), and (ii) the C-terminal part of IDR in different studies attributed to aa 509–832 in mouse AXIN1 (corresponding to aa 511–862 in human AXIN1) (12), aa 475–676 in human AXIN1 (13), or aa 495–684 in mouse AXIN1 (corresponding to aa 495–683 in human AXIN1) (14) could now be mapped with high resolution to (i) aa 274–295 and (ii) aa 592–616 (Fig. 2A). For c-Myc, the previously reported large region aa 331–777 in human AXIN1 (15) was narrowed down to aa 724–743, and an additional epitope, aa 280–304, in the central IDR was discovered (Fig. 2B). For p53, the initially reported interaction region aa 210–337 in mouse AXIN1 (corresponding to aa 210–337 in human AXIN1) (16) was reduced to aa 274–304, and one novel binding epitope, aa 364–382, in the C-terminal part of the central IDR was identified (Fig. 2C). Although Pin1 is known to interact with AXIN1, no information on binding interface has been available thus far. The microarray screening analysis indicated two binding sites for Pin1 in the C-terminal part of the central IDR of AXIN1 (Fig. 2D).

Despite that each analyzed interaction partner (with notable exception of Myc) of AXIN1 showed a unique interaction motif within the central IDR, aa 592–616 for CK1ε, aa 637–658 for Pin1, and aa 364–382 for p53, there was a significant overlap in the remaining sites. Namely, CK1ε, c-Myc, and p53 partially overlapped in aa 274–304, whereas c-Myc and Pin1 overlapped in aa 721–742. These results provide a rationale for the molecular mechanism that ensures mutually exclusive recruitment of AXIN1 to the distinct molecular complexes involved in Wnt signaling, c-Myc degradation, or p53-mediated transcription.

The binding epitopes aa 154–175, 274–295, and 592–616 are required for the recruitment of CK1ε to AXIN1 complex

The AXIN1 epitopes identified with the help of peptide arrays agreed very well with the previously reported interaction regions of AXIN1 obtained by deletion mapping. This suggests that our results are reliable and can be used as a basis for future studies and for the design of an intervention in a particular binding event. To provide a proof of principle that it is indeed the case, we decided to functionally validate the importance of epitopes involved in the interaction of AXIN1 with CK1ε, one of the key kinases in the Wnt/β-catenin pathway (17).

With the peptide microarray screen, we identified three CK1ε-binding epitopes in AXIN1 comprising aa residues 154–175, 274–295, and 592–616, herein referred to as epitopes “1,” “2,” and “3” (Figs. 2A and 3A). To verify the results of the AXIN1–CK1ε interaction interfaces from the peptide microarray assay, we prepared seven AXIN1 deletion mutants: three variants, each lacking one of the CK1ε epitopes; three variants lacking two of the three CK1ε epitopes; and a variant lacking all three epitopes (Fig. 3B). Mutations of the epitopes within the central IDR of AXIN1 were designed as precise in-frame deletions of aa 274–295 and 592–616 (Δ2 and Δ3 constructs). Because the deletion of a peptide motif within the RGS domain might compromise its three-dimensional structure, the RGS domain was removed completely to obtain mutant Δ1, which lacks the binding site aa 154–175 (Fig. 3B).

The AXIN1 constructs were expressed as N-terminally Myc-tagged fusion proteins in transiently transfected HEK293 cells, and recruitment of endogenous CK1ε to the complexes formed on mutant AXIN1 scaffold was tested using coimmunoprecipitation. This approach revealed that deletion of binding sites gradually reduced interaction between AXIN1 and CK1ε but also showed that only the loss of all three epitopes abolished the binding completely (Fig. 3C). These results confirm the functional importance of the AXIN1 epitopes identified by peptide arrays for CK1ε binding and provide validation to the results from the AXIN1 peptide array.

**AXIN1-derived binding epitope peptides inhibit interaction of CK1ε and AXIN1**

Identification of the interaction interfaces at high resolution in principle provides the possibility to use an approach based on
competitive blocking of the interaction using epitope-mimicking peptides. We synthesized three peptides corresponding to the CK1ε-binding sites on AXIN1. We named these peptides corresponding to aa 154–175, 274–295, and 592–616 in human AXIN1 as 1, 2, and 3 (Fig. 4A). To investigate whether these peptide reagents can be used to study the functional importance of CK1ε–AXIN1 interaction, we aimed to analyze the capacity of these peptide “competitors” to interfere with the CK1ε–AXIN1 binding.

First, we investigated whether the peptide competitors impair CK1ε kinase activity. Not surprisingly, in vitro kinase assays showed that, in the presence of AXIN1-derived peptides, CK1ε was still able to phosphorylate its substrate, casein, as well as undergo autophosphorylation (Fig. 4B).

Second, we tested the capacity of AXIN1-derived peptides to block the interaction of CK1ε and AXIN1. Binding of CK1ε and AXIN1 in HEK293 cell lysates was tested by a modified coimmunoprecipitation approach where a mixture of the three AXIN1 peptides, 1+2+3, stabilized by PEGylation at their C termini was added to the fresh HEK293 cell lysate (Fig. 4C). Indeed, addition of the peptides decreased the amount of endogenous AXIN1 coprecipitated with either endogenous or overexpressed CK1ε from the cell lysate (Fig. 4D). These results demonstrate that the peptide blockers can compete with AXIN1 for CK1ε binding and that they are able to disrupt AXIN1–CK1ε interaction in cell lysates.

Dissociation of CK1ε from AXIN1 complex promotes its binding to DVL

CK1ε is known to interact with several other cytoplasmic components of the Wnt pathway. Among them, DVL protein, the key Wnt signal mediator, is probably the best defined CK1ε target (18, 19). Interestingly, AXIN1 and DVL exhibit opposite functions in the Wnt/β-catenin pathway: AXIN1 serves as a key component of the β-catenin destruction complex, whereas DVL mediates dissolution/inhibition of the destruction complex upon pathway activation.

We thus hypothesized that release of CK1ε from AXIN1 may be accompanied by changes in its interaction with DVL and decided to test how the presence of WT AXIN1 or AXIN1 Δ2+3 variant, with decreased capacity to bind CK1ε, affects the interaction of CK1ε with DVL. The RGS domain in AXIN1 was kept intact to prevent the interaction with another component of the destruction complex, APC, that binds via this domain (20). In HEK293 cells, EGFP-tagged AXIN1 constructs were coexpressed either with FLAG-tagged DVL3 or FLAG-tagged Shp2, an unrelated protein, as a control. The endogenous CK1ε was precipitated from the lysates with anti-CK1ε antibody, and the interacting DVL3 and AXIN1 were detected on an immunoblot using anti-FLAG and anti-GFP antibodies, respectively (Fig. 5A). The analysis confirmed the previous observation that CK1ε binds to AXIN1 Δ2+3 construct with lower affinity than to full-length AXIN1. Moreover, upon coexpression of FLAG-DVL3, overall binding of CK1ε to AXIN1 constructs decreased, which suggests that indeed DVL and AXIN1 compete for CK1ε. However, at the same time, the analysis revealed that decreased binding of CK1ε to AXIN1 Δ2+3 was accompanied with increased CK1ε binding to DVL3 (Fig. 5A). Similar data, i.e. increased binding of CK1ε to DVL3, were reproduced when we interfered with the CK1ε–AXIN1 interaction in HEK293 cell lysates by the AXIN1 peptide competitors 1+2+3 (Fig. 5B).

These data suggest that AXIN1 sequesters CK1ε and limits its availability for DVL, but the functional consequences of this competition are unclear. To address this question, we decided to test how CK1ε binding-compromised AXIN1 Δ2+3 variant differs from WT AXIN1. For example, can the presence of AXIN1 affect the phosphorylation of DVL3 by CK1ε? We took advantage of the phosphospecific antibody against pSer643-DVL3 that serves as a good readout of DVL3 phosphorylated by CK1ε (21). When we coexpressed CK1ε with AXIN1 WT and Δ2+3 variant, pSer643-DVL3 phosphorylation signal was decreased by AXIN1 WT but not by AXIN1 Δ2+3 variant (Fig. 5C). This is in line with the observations in Fig. 4, A and B, and suggests that AXIN1 can, via control of CK1ε availability, regulate phosphorylation of DVL and activation of the Wnt/β-catenin pathway.

To further validate this assumption, we compared AXIN1 WT and AXIN1 Δ2+3 mutant behavior in the Dual-Luciferase TopFlash/Renilla reporter gene assay (21, 22) that monitors the β-catenin/TCF-dependent transcription. In these assays, AXIN1 Δ2+3 variant behaved differently than AXIN1 and (i) promoted β-catenin/TCF-dependent transcription even in the absence of the external stimuli (Fig. 5D, left) and (ii) behaved as a less potent inhibitor of Wnt-3a–driven transcription when compared with AXIN1 WT (Fig. 5D, right). These events relate to the cytoplasmic Wnt pathway complexes because neither WT AXIN1 nor AXIN1 Δ2+3 mutant was able to interfere with the TopFlash signal triggered by constitutively activated β-catenin S33A (Fig. 5E). In summary, our data demonstrate that AXIN1 sequesters CK1ε and via this mechanism controls its capacity to stimulate the Wnt pathway via DVL.

Discussion

In this study, we applied peptide microarrays to identify the interaction epitopes for AXIN1-binding partners CK1ε, c-Myc, p53, and Pin1. Our peptide microarray–based results revealed that many of the binding sites overlap: at least one “common” binding interface was found to be shared, and one or more binding interfaces were unique for each binding partner. This observation opens the possibility that AXIN1 exploits just few dedicated regions in its sequence for the binding to other protein partners. After binding of a single partner protein/protein

Figure 2. AXIN1-binding epitopes for CK1ε, c-Myc, Pin1, and p53 identified by peptide array approach. The microarray slides with AXIN1 peptide library were incubated with the following purified recombinant proteins: CK1ε (A), c-Myc (B), p53 (C), and Pin1 (D). The charts show normalized signal values from single peptide spots, and the dashed line represents the threshold signal intensity. AXIN1 is schematically depicted above the charts with the numbers and sequences of amino acids defining the particular binding sites. The signals from control glass (antibodies only) were subtracted from the experimental values, and the four strongest signals of at least four consecutive (i.e. neighboring and overlapping) peptides higher than 10,000 LUs were considered as candidate binding sites; see “Experimental procedures” and main text for more details. For each protein of interest, the average from two to three replicates is shown. hAXIN1, human AXIN1.
**Binding interfaces of AXIN1**

### A

| aa [hAXIN1] | hAXIN1 |
|--------------|--------|
| 88           | RGS    |
| 211          |        |
| 780          | DIX    |

CK1ε binding sites: 1, 2, 3

- **human AXIN1**
- **mouse Axin1**
- **rat Axin1**
- **chick Axin1**
- **danio Axin1**
- **xenopus Axin1**

### B

| aa [hAXIN1] | human AXIN1 |
|--------------|-------------|
| 154          | GIVSRDKPTKS  |
| 274          | ETAAPRSRRESE |
| 592          | LHSGKVGVACN  |

- **human AXIN1**
- **mouse Axin1**
- **rat Axin1**
- **chick Axin1**
- **danio Axin1**
- **xenopus Axin1**

### C

| myc-hAXIN1 | human AXIN1 |
|------------|-------------|
| 257        | DSSHCAGN    |
| 273        | RRIRLSDEER  |

**Figure 3. AXIN1 contains three epitopes for direct CK1ε binding whose deletions lead to a loss of AXIN1–CK1ε interaction.** A, schematic depiction of the defined regions (arbitrarily named 1 for aa 154–175, 2 for aa 274–295, and 3 for aa 592–616) in human AXIN1 (hAXIN1) identified as CK1ε-binding epitopes in the peptide microarray screen. The multiple sequence alignment of AXIN1 shows their conservation throughout vertebrate species. Residues with >80% similarity are highlighted. Linear α-helices in epitope 1 from the RGS domain are depicted as purple coils above. B, schematic depiction of the AXIN1 deletion constructs used for coimmunoprecipitation experiments. The numbers indicate regions of deleted aa in human AXIN1. C, HEK293 cells were transfected with the indicated N-terminally Myc-tagged AXIN1 constructs and empty vector as a negative control. Proteins from the cell lysates were immunoprecipitated (IP) with specific mouse anti-Myc antibody. Coimmunoprecipitated CK1ε was detected using goat anti-CK1ε antibody. Input panels represent 10% of the initial material. Numbers on the right correspond to the molecular mass marker in kDa. TCL, total cell lysate.

**Discussion**

AXIN1 is the increased recruitment of CK1ε to DVL3. It is an important observation because the phosphorylation of DVL proteins induced by Wnt ligands and mediated via action of CK1ε is the key event that mediates signal transduction in the Wnt/β-catenin pathway (19, 21, 23). Local availability of CK1ε thus represents an important regulatory mechanism that can control activation of the Wnt pathway.

The biological importance of our observations remains to be defined. The binding epitopes identified on AXIN1 and subsequently deleted in AXIN1 Δ2+3 variant, which has lowered ability to bind CK1ε, do not obviously interfere with other known functions of AXIN1 in the Wnt signaling pathway, such as associations with APC (mediated via the RGS domain (20)), β-catenin, or GSK3 that were mapped to the central IDR of AXIN1 (aa 438–508 in human AXIN1 for the binding of β-catenin (24) and aa 383–401 in human AXIN1 for the binding of GSK3β (25, 26)). The deleted regions are also distinct from the parts of AXIN1 that were reported to be involved in the formation of “closed” and “open” conformations of AXIN1 (27). Open/closed dynamics of AXIN1 is mediated via interaction of the DIX domain with the β-catenin–binding domain (aa 435–498 in human AXIN1 (24) and regulated through phosphorylation of four conserved serine residues (including Ser497 and Ser500 in mouse Axin1, which correspond to Ser593 and Ser196 in human AXIN1). Phosphorylation induced by GSK3β promotes the open (i.e. active) AXIN1 conformation (27). The three identified CK1ε-binding epitopes in AXIN1 are distinct.

Nevertheless, AXIN1 Δ2+3 variant (compared with WT AXIN1) was able to induce Wnt/β-catenin–dependent transcription but failed to reduce phosphorylation of Ser643 in DVL3 (21) or to efficiently inhibit Wnt-3a–induced transcription (Fig. 5C). This suggests that interaction of AXIN1 and CK1ε is required for the efficient regulation of the inhibitory complex, likely to appear by combination of simultaneous interaction with three binding interfaces to the scaffold, the structure of scaffold protein (here human AXIN1) can adopt the unique 3D fold leading to one particular “cell signaling message.” This explanation would help in understanding how AXIN1 distinctively influences multiple signaling pathways and events in a cell.

For CK1ε, we validated the results from peptide microarray screen using deletion mutagenesis and small peptide competitors. These data provide a proof of principle that relatively minor deletions in AXIN1 can generate mutants that will be biased or even restricted in function for a certain signaling cascade. The same outcome can be achieved, at least in cell lysates, with the combination of small peptides corresponding to the binding epitopes that, in a specific manner, interact with AXIN1-binding partners and decrease their abundance in the AXIN1-based complexes.

We observed that the functional consequence of weakening the interaction between CK1ε and AXIN1 is the increased recruitment of CK1ε to DVL3. It is an important observation because the phosphorylation of DVL proteins induced by Wnt ligands and mediated via action of CK1ε is the key event that mediates signal transduction in the Wnt/β-catenin pathway (19, 21, 23). Local availability of CK1ε thus represents an important regulatory mechanism that can control activation of the Wnt pathway.
activity of AXIN1 in the Wnt/β-catenin pathway. This may seem surprising given the previous reports that described formation of a trimeric AXIN1–DVL–CK1ε complex (18) and recruitment of endogenous AXIN1 to DVL3 upon CK1ε overexpression (19). These data can be reconciled by the existence of (at least) two distinct complexes containing both AXIN1 and CK1ε. Direct interaction of AXIN1 with CK1ε can be important for the proper function and regulation of the destruction complex but is dispensable for recruitment of AXIN1 to the DVL-based "signalosome" complex. CK1ε binds DVL directly (18), and CK1ε-mediated phosphorylation of DVL results in the more efficient recruitment of AXIN1 to the signalosome (19). Recruitment of AXIN1 to the signalosome, connected with the inhibition/inactivation of the destruction complex, is mediated and critically dependent on the direct interaction between DIX domains of AXIN1 and DVL (28), and as such, AXIN1 in the signalosome does not need to directly bind CK1. Future work must elucidate whether the proposed mechanism is unique to CK1ε and the closely related CK1β (connected primarily with DVL phosphorylation) or whether it applies also to CK1α that seems to a principal CK1 component in the destruction complex in vivo (29).

Taken together, our AXIN1 peptide microarray–based results (specifically those from CK1ε–AXIN1 interaction) open up the possibility to design and generate AXIN1 variants and specific peptide competitors that can direct signaling activities of AXIN1. Using this approach, we describe a novel regulatory mechanism limiting the availability of CK1 for DVL phosphorylation mediated by direct interaction of AXIN1 and CK1ε.

**Experimental procedures**

**Peptide array**

*Generation of the peptide arrays*—The peptide library comprised 13-mer peptides overlapping with 10 residues that cover the whole sequence of human AXIN1 (Fig. S1). The designed peptide library was generated by JPT Peptide Technologies GmbH (Berlin, Germany) in an array format wherein the peptides were immobilized on a glass slide (25 × 75-mm slides). Briefly, all peptides were synthesized in a stepwise manner (SPOT-synthesis) on a cellulose membrane with a JPT-developed fully automated robotic system, resulting in a defined arrangement (3 × 284 peptide spots per slide). By coupling a reactivity tag (tag + linker-Ttds-linker molecule, where Ttds stands for trioxatridecan-succinamic acid) on the N terminus of the peptides (truncated side products are capped by acetylation steps), all target peptides could be immobilized chemoselectively and purified by reaction of the peptides with the modified glass surface. An N-terminal reactivity tag for immobilizing the peptides onto microarray slides ensures that only full-length peptides are bound to the final chip after cleavage from the cellulose membrane. The peptides were transferred onto the slide by a contact printing technique. The resulting information of a covalent bond between the target peptide and the chip surface allows removal of all truncated (and acetylated) sequences by subsequent washing steps. The peptide microarray was printed in three identical subarrays. This enables efficient intrachip-reproducibility tests. Each peptide subarray was printed in individual blocks. Each spot in the microarray repre-
Binding interfaces of AXIN1

Figure 5. Reduced CK1ε-AXIN1 interaction increases binding of CK1ε to DVL3 and subsequent Wnt/β-catenin downstream signaling. A, DVL3 and AXIN1 variants were overexpressed in HEK293 cells as indicated. Interaction of DVL3 and AXIN1 with endogenous CK1ε was assessed by coimmunoprecipitation and subsequent Western blotting. AXIN1 variants were overexpressed in HEK293 cells as indicated. Interaction of DVL3 and AXIN1 with endogenous CK1ε significantly promoted interaction of endogenous CK1ε with DVL3. Bottom, quantification of FLAG/CK1ε signal (IP fraction) from three independent experiments is shown. Data in B are normalized to the strongest signal and shown as mean ± S.D. (error bars); differences were assessed by one-way analysis of variance test (*, p = 0.05; **, p = 0.01; ns, not significant, p > 0.05). C, HEK293 cells were transfected as indicated. WB analysis of DVL3 phosphorylation status using the anti-phospho-Ser643 antibody showed that only WT, but not AXIN1 Δ2+3, can decrease DVL3 phosphorylation at Ser643. D and E, HEK293 cells treated with LGR974 (Porcupine) inhibitor were transfected as indicated and treated with control (ctrl) or Wnt-3a CM. TCF/LEF–dependent transcription was monitored by the Dual-Luciferase TopFlash/Renilla reporter assay. Data in D and E are shown as mean ± S.D. (error bars); differences were analyzed by two-tailed Student’s t test (*, p ≤ 0.05; ns, not significant, p > 0.05). In E, data are normalized to the condition with β-catenin S33A only. Numbers on the right in A, B, and C correspond to molecular mass marker in kDa. IP, immunoprecipitation; TCL, total cell lysate; hAXIN1, human AXIN1.

Subjects and Methods

**Screening of the peptide arrays for binding to protein of interest**—In each experiment, two glass slides were used: control, incubated with antibodies only, and experimental, incubated with the recombinant protein of interest. Each individual slide was assembled in a sandwich-like format with a dummy/blank slide and two plastic spacers to create a simple incubation chamber with a reaction volume of ~300 µl. The slides were first blocked with 300 µl of SmartBlock solution (CANDOR Bioscience, 113125) for 1 h at 30°C. Then the experimental slide was incubated overnight with 10 µg/ml recombinant protein, CK1ε (OriGene Technologies, TP302436), p53 (OriGene Technologies, TP300003) Pin1 (OriGene Technologies, TP302543), or c-Myc (Abnova, H00004609-P01), in SmartBlock solution (final volume, 300 µl) at 4°C. The next day, both slides were washed in 300 µl of TBS buffer (4 × 10 min, room temperature) and then incubated with 1 µg/ml primary antibody in SmartBlock solution (final volume, 300 µl) for 1 h at 30°C. Then the slides were washed again in 300 µl of TBS buffer (4 × 10 min, room temperature) followed by incubation with 1 µg/ml secondary fluorescent antibodies in SmartBlock solution (final volume, 300 µl) for 45 min at 30°C and subsequently washed in 300 µl of TBS buffer (4 × 10 min, room temperature) and in 300 µl of water (4 × 10 min, room temperature). To remove excess water, the slides were dried by centrifugation (1200 relative centrifugal force for 2 min at room temperature with slides placed in 50-ml Falcon tubes). The bound recombinant protein was detected by reading the fluorescence intensity of the peptide spots. In these experiments, the following primary antibodies were used (1 µg of antibody/ml): anti-CSNK1E (Abnova, 2276), anti-p53 (Abcam, 17869), anti-Pin1 (Abnova, 1080), and anti-glutathione S-transferase (Amersham Biosci-
ties, PA92002). For the secondary antibody (1 µg of antibody/ml), DyLight 649 anti-mouse-IgG (Pierce, 35515) was used. The slide data were analyzed using PepSlide Analyzer software (Sicasys). Representative images of the peptide array are in Fig. S2.

Quantification of the signal

Intensities of corresponding spots on the control glass (i.e. incubated with antibodies only) were subtracted from the experimental values. The experiment was repeated three times (two to three technical replicates each), and averages of the signal values were plotted in the graph (light units (LU) on y axis).

There are no strictly given criteria that define the binding epitope in a peptide array. Our parameters included (i) three to four consecutive peptides with signal exceeding 10,000 and (ii) a maximum of four of the strongest sites per protein. These parameters were set arbitrarily, and the final decision, which site to consider as a binding epitope for further testing, was made upon visual inspection of the data. The peptide array approach in principle enables searching for binding epitopes within linear (intrinsically disordered) protein regions. Therefore, the signals within structured domains were not taken into consideration except for the two sites in the RGS domain that have been mapped to the linear α-helices exposed to the solvent.

The following ad hoc exceptions in the central IDR were made. CK1ε-binding site aa 274–295 in human AXIN1 (although possessing one central peptide below 10,000 LU) was considered as putative, according to previously published results (21). c-Myc-binding site aa 724–743 in human AXIN1 was also considered as putative, according to previously published results (15).

Cell culture, transfection, treatments, and mutagenesis

HEK293 WT cells were grown at 37 °C and 5% (v/v) CO2 in Dulbecco’s modified Eagle’s medium (DMEM), 10% (v/v) fetal bovine serum (Gibco, 10270), 2 mM l-glutamine (Life Technologies, 25030024), and 1% (v/v) antibiotics (penicillin/streptomycin; HyClone, SV30010). For Western blotting and the Dual-Luciferase TopFlash/Renilla reporter assay, cells were seeded (60,000/well in 24-well plate 24 h after seeding. For performing this assay, the Promega Dual-Luciferase assay kit (Promega, E1910) was used according to the manufacturer’s instructions. Luminescence was measured by a Hidex Bioscan Plate Chameleon luminometer. Results are depicted as the ratio of TopFlash and Renilla signal (TopFlash -fold induction), which was normalized in Fig. 5E to the control column with β-catenin S33A only (normalized TopFlash -fold induction). Data were analyzed by MS Excel 2007 and GraphPad Prism 6, and results are shown as means ± S.D. (number of experiments indicated ad hoc).

Coimmunoprecipitation

The coimmunoprecipitation protocol was used as described previously in Bryja and co-workers (21) The antibodies used for immunoprecipitation were as follows (1.0 µg of antibody/sample): goat anti-CK1ε (Santa Cruz Biotechnology, sc-6471) and mouse anti-Myc (Santa Cruz Biotechnology, sc-40).

Modified coimmunoprecipitation with the competitor peptides was performed as follows. DMEM was removed 24 h after transfection, cells were washed with PBS, and 1 ml of cold NP-40–based lysis buffer supplemented with 1 × protease inhibitors (from 1000× concentrated original stock; Roche Applied Science, 11836145001) and 1 × phosphatase inhibitors (from 200× concentrated original stock; Calbiochem, 524625) was used per 10-cm dish. Lysate was collected after 15 min of lysis at 4 °C and cleared by centrifugation at 16.1 relative centrifugal force for 30 min. 60 µl of supernatant were then acquired and mixed with 15 µl of 5 × Laemmli buffer, representing total cell lysate samples. The remaining supernatant was then divided into separate Eppendorf tubes (300 µl/sample) and incubated at room temperature for 30 min on a carou-

Plasmid preparation

Full-length AXIN1 coding sequence was amplified from human cDNA using forward primer 5’-CACCAATGAATATC-CAAAGAGCAGG-3’ and reverse primer 5’-TCAGTCCACCT-TCTCCACTT-3’ (where bold type represents the start codon in the forward primer and the stop codon in the reverse primer). For the truncated version of AXIN1 lacking the RGS domain, forward primer 5’-CACGGGAGAGCCTGTCC-CCC-3’ was used. The PCR products were inserted into pENTR/TEV/D-TOPO vector. For in-frame deletions of the CK1ε-binding sites within the AXIN1 IDR, a PCR-based mutagenesis approach was used with the following pairs of primers: 5’-CTCCCTCTAGAAGCTGTCTCTTGCGCGGAG-GCCAGTCA-3’ and 5’-TGACTGCTTCCGACAGAGTAGCAGCAGTTGG-GGC-3’ for site 2 and 5’-GGCCCAACGCCAGTACCGAGTTGCGAGCTCTTCTAGAGGAG-3’ for site 3. For expression in mammalian cells, the AXIN1 constructs were inserted from pENTR/TEV/D-TOPO vector into pDEST-Myc and pDEST-EGFP vectors using the Gateway cloning system. All mutations described in this study were verified by sequencing.

Dual-Luciferase TopFlash/Renilla reporter assay

Cells (seeded at 60,000/well in 24-well plates a day before; 500 µl of DMEM/well) were transfected with 0.1 µg of Super8X TopFlash construct and 0.1 µg of Renilla luciferase construct/well in a 24-well plate 24 h after seeding. For performing this assay, the Promega Dual-Luciferase assay kit (Promega, E1910) was used according to the manufacturer’s instructions. Luminescence was measured by a Hidex Bioscan Plate Chameleon luminometer. Results are depicted as the ratio of TopFlash and Renilla signal (TopFlash -fold induction), which was normalized in Fig. 5E to the control column with β-catenin S33A only (normalized TopFlash -fold induction). Data were analyzed by MS Excel 2007 and GraphPad Prism 6, and results are shown as means ± S.D. (number of experiments indicated ad hoc).
Western blotting and sample preparation

Samples containing 1 body/sample) for 1 h at 4 °C on a carousel. Then 30 μl of protein G-Sepharose beads (GE Healthcare, 17–0618-05) equilibrated in BSA-free complete NP-40–based lysis buffer were added to each sample (final volume, ~300 + (3 × 1) + 1 + 30 = 350 μl). After overnight incubation on a carousel (4 °C), samples were washed four times in NP-40–based lysis buffer (800 μl/wash), and 40 μl of 2 × Laemmli buffer were added. All samples were boiled and processed for Western blotting (WB).

Peptide reagents

The AXIN1 peptides mimicking the CK1ε-binding sites (aa sequences GIVSRQTKPATSKFIKGCMIKQ, ETAAPRVSRRYSEGREFRYG, and LAHSKGVKVACKRNAKKAESGKSAS) were used for in vitro experiments were synthesized by PSL Peptide Speciality Laboratories GmbH, Germany. The peptides stabilized by C-terminal PEGylation (GIVSRQTKPATSKFIKGCMIKQ-Lys-PEG49, ETAAPRVSRRYSEGREFRYG-Lys-PEG49, and LAHSKGVKVACKRNAKKAESGKSAS-Lys-PEG49) for use in cell lysates were synthesized at JPT Peptide Technologies GmbH. For detailed information, see Fig. S3.

Western blotting and sample preparation

WB and sample preparation were performed as described previously (32) and developed using a chemiluminescence documentation system, FusionSL (Vilber-Lourmat) or Odyssey® Fc (LI-COR Biosciences). The following primary antibodies were used (all at a dilution of 1:1000 from the original stock): anti-AXIN1 (Cell Signaling Technology, sc-789), and anti-DVL3 phospho-Ser643 (Fitzgerald, 20R-GR-011), anti-Myc (Santa Cruz Biotechnology, sc-9045), anti-AXIN1 (Cell Signaling Technology, 2087), anti-FLAG M2 (Sigma-Aldrich, F1804), anti-CK1ε (BD Biosciences, 610445), anti-CK1ε (Santa Cruz Biotechnology, sc-6471), anti-GFP (Fitzgerald, 20R-GR-011), anti-Myc (Santa Cruz Biotechnology, sc-789), and anti-DVL3-phospho-Ser443 (21). The following secondary antibodies were used (all at a dilution of 1:5000 from the original stock): anti-goat IgG (Sigma-Aldrich, A4174), anti-mouse IgG (Sigma-Aldrich, A4416), and anti-rabbit IgG (Sigma-Aldrich, A0545). Quantification of the WB signal was performed by ImageJ software (v1.47).

In vitro kinase assay

2 μg of recombinant CK1ε (OriGene Technologies) were mixed with 2 μg of dephosphorylated cascin in reaction buffer containing 1× magnesium/ATP mixture (Millipore, 20-113), 50 mM NaCl, 0.5 mM DTT, 100 μg/ml BSA, 30 mM HEPES, pH 7.5, and [γ-32P]ATP (final activity, 5 μCi) either in presence or absence of 4 μM each AXIN1 peptide (1+2+3; aa sequences GIVSRQTKPATSKFIKGCMIKQ, ETAAPRVSRRYSEGREFRYG, and LAHSKGVKVACKRNAKKAESGKSAS). After 60-min incubation at 37 °C, the reaction (final volume, 20 μl) was stopped by addition of SDS sample buffer (5 μl of 5 × SDS buffer) and heating at 95 °C for 5 min. The samples were loaded on a 12% polyacrylamide gel and separated by SDS-PAGE. The gel was fixed and stained with Coomassie Blue, and phosphorylation was visualized using autoradiography.

Multiple sequence alignment

The multiple sequence alignment of selected sequences was performed using the ClustalW algorithm. The output alignment was refined manually using the BioEdit v7.0.1 sequence editor.

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