Snapshots of Protein Dynamics and Post-translational Modifications In One Experiment—β-Catenin and Its Functions*

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β-catenin plays multiple roles in the canonical Wnt signaling pathway and in cell-cell adhesion complexes. In addition, β-catenin is a proto-oncogene and activating β-catenin mutations are relevant in the genesis of colorectal, hepatocellular and other common cancers. Different functions of β-catenin as transcriptional co-activator or cell adhesion molecule are orchestrated by changes in concentration and phosphorylation as well as its ability to complex with proteins such as cadherins or transcription factors. Detailed quantitative and time-resolved analysis of β-catenin, based on the evaluation of the changes in the Wnt pathway, enable greater insights into health and disease-related β-catenin function. The present paper describes a novel suspension bead array assay panel for β-catenin, which requires minimal amounts of sample and is able to relatively quantify total β-catenin, the extent of phosphorylation at multiple sites and the ratio of complexed and free β-catenin. This is the first study to combine three biochemical methods—sandwich immunoassay, co-immunoprecipitation, and protein-protein interaction assay—in one suspension bead assay panel. The assay was used to measure changes in the concentration of eight different β-catenin forms in HEK293 cells in a time-resolved manner. In contrast to the general consensus, our study demonstrates an increase in β-catenin phosphorylated at Ser-45 upon treatment of cells with rWnt3a or a GSK3 inhibition; we also link C-terminal phosphorylation of β-catenin on Ser-552 and Ser-675 with canonical Wnt signaling. Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.007377, 1–10, 2011.

The Wnt signaling pathway is involved in the regulation of physiological processes, embryonic development, and tissue maintenance (1). Dysregulation of the Wnt pathway is associated with various types of cancer, including colorectal and hepatocellular carcinoma (2, 3). β-Catenin is a key player in the Wnt pathway and its different cellular functions are orchestrated by changes in its concentration, phosphorylation state, and the extent of binding to proteins such as cadherins and transcription factors (4). In the absence of Wnt growth factors, β-catenin is mainly found at the membrane where it forms a complex in which E-cadherin is linked to the actin cytoskeleton (5, 6) and functions as a cell-adhesion molecule (7, 8). Newly synthesized β-catenin, which is not sequestered in cell-adhesion complexes, is fed into a phosphorylation-dependent degradation process. In the destruction complex composed of GSK3β, CK1α, Axin, and APC, β-catenin is sequentially targeted for degradation by CK1α-mediated phosphorylation at Ser-45 and GSK3β-mediated phosphorylation at Ser-33, Ser-37, and Thr-41 (9, 10), followed by ubiquitination and proteasomal degradation. Upon the activation of Wnt receptors, the destruction complex dissociates, β-catenin starts to accumulate and then translocates into the nucleus where it promotes expression of Wnt-response genes (http://www.stanford.edu/~russe/wntwindow.html). This is achieved by forming a complex with either of the TCF/LEF family of transcription factors (T-cell-specific transcription factor/lymphoid enhancer-binding factor 1) (11, 12). β-catenin function can also be regulated through post-translational modifications that are not Wnt receptor triggered: for example, the phosphorylation of β-catenin at Ser-675 through protein kinase A (PKA) enhances transcriptional activity (13, 14). Wnt-dependent and Wnt-independent modifications are likely to compete or cooperate in the regulation of β-catenin function depending on cell state (15).

To gain detailed insight into the function of β-catenin, methods are required that allow a time-resolved quantification of β-catenin states involving post-translational modifications, binding to other proteins and transcriptional activity. Accomplishing this with traditional methods such as Western blots,

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co-immunoprecipitation, and reporter assays would be tedious for systematic analysis of pathway activity in different conditions and cell types. Moreover, relatively large numbers of cells are needed because different assays often require different sample preparation. In contrast, multiplexed bead-based immunoassays are ideal for multiparametric protein analysis (16–19) and reduce the need for separately conducted experiments. As described previously, a further characteristic of miniaturized immunoassays is that they work under ambient analyte conditions (20, 21). When very small amounts of capture antibody are immobilized on a microspot or a microbead, so little of the target analyte is captured that the analyte concentration in the remaining solution is virtually unchanged. As such, multiple post-translational modifications from the same protein in the same sample can be measured in parallel without the fear of competition (22).

The present paper describes a new miniaturized multiplex assay, which combines protein-protein interaction, co-immunoprecipitation and sandwich immunoassay in a single suspension bead array panel. The new bead-based assay allows the quantification of different forms of one protein in the same sample. The use of a sandwich immunoassay format allows the simultaneous measurement of the (i) total amount of β-catenin, (ii) four phosphorylated β-catenin forms and the detection of (iii) E-cadherin-complexed β-catenin in a miniaturized co-immunoprecipitation. As a novelty, a miniaturized fishing assay was introduced to monitor the concentration of (iv) transcriptionally active β-catenin (see Fig. 1). The Wnt signaling pathway was analyzed in HEK293 cells treated either with recombinant Wnt3a (rWnt3a) ligand or the GSK3 β protein for 100,000 beads) was incubated in 50 μl PBS for 1 h at room temperature. Each of the GST-tagged bait proteins (1 μg/ml, Jackson Immunoresearch) containing 1% donkey serum (Sigma-Aldrich) was added and applied for 45 min at room temperature. Following the final washing step, the microspheres were transferred into 100 μl assay buffer containing 0.1% Tween (Merck KGaA) in the concentration as described below.

The beads were transferred from the incubation plate to a wash plate containing phosphate-buffered saline (PBS) 0.1% Tween. The microspheres were washed twice following each of the following steps. β-catenin-specific antibody (1 μg/ml, BD Biosciences, San Diego, CA) was incubated with the microspheres for 1 h at room temperature before goat anti-mouse phycoerythrin-conjugated antibody (2.5 μg/ml, Jackson ImmunoResearch) containing 1% donkey serum (Sigma-Aldrich) was added and applied for 45 min at room temperature. Following the final washing step, the microspheres were transferred into 100 μl assay buffer containing 0.1% Tween and analyzed with a Luminex 100 IS system (Luminex Corp., Austin, TX). Mean fluorescence intensities of at least 100 microspheres per assay were recorded for each sample. Standard deviation was calculated based on biological triplicates for the time course experiments. For calculation of intra- and inter-assay variation four technical replicates performed on the same day or four technical replicates performed on four different days were used.

Cell Culture and Sample Preparation for Bead Array Analysis—Human 293 embryonic kidney (HEK293) cells (ATCC # CRL-1573) were cultured in DMEM (PAAR Laboratories, Pasching, Austria) containing 2 mM L-glutamine (PAAR Laboratories GmbH), 1% penicillin/streptomycin (PAAR Laboratories GmbH), and 10% fetal bovine serum (PAAR Laboratories GmbH) at 37 °C and 5% CO₂. Cells were plated on 24-well plates, starved in serum-free medium for 24 h prior to the experiment and then treated with 200 ng/ml recombinant human Wnt 3a (R&D Systems) or 20 μM GSK3 inhibitor SB216763 (Sigma-Aldrich). The cells were washed twice with PBS and lysed using 100 μl of lysis buffer (1% Triton X-100 (Sigma-Aldrich) 150 mM NaCl (Sigma-Aldrich), 50 mM Tris pH 7.4 (Sigma-Aldrich), 1× complete protease inhibitor (Roche Applied Science), and 1× phosphatase inhibitor I and II (Sigma-Aldrich)) for 30 min at 4 °C under shaking. Cell debris was removed by centrifugation at 15,000 × g at 4 °C for 30 min and the protein concentration of the supernatant was determined (DC protein assay, Bio-Rad, Hercules, CA). The samples were stored at −78 °C until further analysis.

Transfection Experiments and Reporter Genes—Human 293 embryonic kidney (HEK293) cells (ATCC # CRL-1573) were cultured as previously described (24). Reporter gene experiments involved 1 × 10⁵ cells per well (24-well plates). The cells were transfected with 100 ng of the Super8xTOP-flash reporter (25) and 20 ng of the Renilla luciferase expression vector pRL-TK (Promega, Madison, WI) for normalization purposes using the FuGENE6 (Roche Applied Science)
Fig. 1. Overview of β-catenin suspension bead array based assay panel. The figure summarizes biological function, localization, and posttranslational modification of the different forms of β-catenin investigated. The appropriate miniaturized assay set-up is shown for each detected form of β-catenin. HEK293 cells were starved for 24 h and treated with 200 ng/ml rWnt3a or 20 μM SB216763 for 3 h. Control cells received PBS or DMSO as solvent control. Twenty-five micrograms cell lysate of each sample (biological triplicates) were analyzed with the β-catenin bead array panel. Signal intensities are displayed as ratios treated/untreated. Results are indicated in white and hatched for the rWnt3a and inhibitor treated cells, respectively.

Expression and Purification of Glutathione S-Transferase Fusion Proteins—Expression vectors for glutathione S-transferase (GST) and GST fusion proteins with β-catenin residues 536–781, β-catenin residues 1–150 fused to 577–781 and the cytoplasmic tail of E-cadherin (GST-ECT) were previously described (27–29). The GST-ECT fusion protein contains amino acids 1–80 from human TCF4. The fusion protein was prepared by inserting a cDNA fragment derived from pcDNA3+TCEF4 (24) into the EcoRI and NotI restriction sites of pGEX2TK (GE Healthcare). GST fusion proteins were expressed in E. coli BL21 or Top10F′ cells and purified on glutathione Sepharose as described previously (27).

Preparation of Cell Lysates, Fishing of β-Catenin and Western Blot—Protein extracts were prepared by lysing the cells in ice-cold IPN150 buffer (50 mM Tris-HCl pH 7.6, 5 mM MgCl₂, 1% Nonidet P-40, 150 mM NaCl, 0.1 mM Na-vanadate, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, phosphatase inhibitor mixture I and II (Sigma Aldrich) and complete protease inhibitor (Roche Applied Science)) for 30 min on ice with occasional mixing. Following cell lysis, the samples were centrifuged at 20,000 × g for 10 min and the protein concentration of cleared supernatant was determined (DC protein assay, Bio-Rad). To analyze total β-catenin concentrations, fractions of cellular lysates containing 20 μg total protein were separated by SDS-PAGE on 10% gels and analyzed by Western blot as described previously (27).

RESULTS

Multiplexed Monitoring of Different β-Catenin Characteristics—For a relative quantification of β-catenin states involving post-translational modifications, binding to other proteins, and transcriptional activity a suspension bead array panel was developed involving the following sandwich immunoassays (Fig. 1). One antibody pan-specific for β-catenin and antibodies specific for different β-catenin phosphorylation sites were used to determine the total and modified β-catenin levels. The post-translational modifications include the phosphorylation of Ser-33/Ser-37/Thr-41 and Ser-45 residues at the N terminus as well as Ser-675 and Ser-552 at the C terminus of β-catenin. Levels of E-cadherin/β-catenin complexes were determined by micro-co-immunoprecipitation (30) using an antibody/antigen complexes were visualized by chemiluminescence (ECL system, GE Healthcare).
E-cadherin-specific capture antibody. A miniaturized protein-protein interaction assay was integrated following previously proposed pulldown assays (31, 32) for the detection of free β-catenin. This μ-fishing enables the measurement of free β-catenin, which reflects the activated status of the canonical Wnt pathway. Recombinantly expressed binding partners—E-cadherin cytosolic tail (ECT) and TCF—were used as capture molecules (bait) for the analysis of freely accessible β-catenin (prey). Pan-specific and phospho-specific antibodies and recombinant ECT and TCF were immobilized on different color-coded microspheres (Luminex 100™) and combined for the multiplex assay panel. The microspheres were incubated with differently treated HEK293 cell lysates. Captured analytes were visualized in a Luminex 100 system (Luminex Corp., Austin, TX) using one β-catenin-specific detection antibody and a fluorescently labeled reporter molecule.

In order to demonstrate the capability of this multiplexed assay for the detailed analysis of the different β-catenin functions, HEK293 cells were treated with Wnt pathway-activating components for 3 h: rWnt3a was used to activate the pathway via the Wnt receptor and a GSK3 inhibitor (SB216763) (33) was used to induce the accumulation of free β-catenin at the level of the destruction complex. Each cell culture experiment was performed in biological triplicates. Cell lysates were analyzed for the different functions of β-catenin using the described suspension bead array. As shown in Fig. 1, following the addition of rWnt3a and SB216763 the relative amount of β-catenin increased two- to threefold, respectively, compared with nonactivated cells. Second, elevated amounts of all phosphorylated β-catenin forms were observed following Wnt pathway activation. The application of the GSK3 inhibitor led to an increase of at Ser-675, Ser-552 and Ser-45 phosphorylated β-catenin, whereas β-catenin phosphorylated at Ser-33/Ser-37/Thr-41 decreased at the respective time point. Third, the E-cadherin/β-catenin complex did not show any significant changes following the activation of the Wnt signaling pathway. Finally, β-catenin able to bind to ECT and TCF was present at a low basal level in nonactivated cells and did increase upon treatment of cells with rWnt3a and GSK3β inhibitor. Overall, these data demonstrate that a multiplex bead-based assay is able to perform parallel analysis of multiple β-catenin forms from a relatively small sample (25 μg lysate corresponding to extract from 10⁵ cells).

**Specificity of the Sandwich Immunoassays for Phosphorylated β-Catenin**—The specificity of the established sandwich immunoassays targeting phosphorylated β-catenin was tested by analyzing 10 ng recombinant nonphosphorylated GST-β-catenin. No or only very weak signals over background were detectable suggesting high specificity for the phosphorylated protein sequences (supplemental Fig. 1).

**Cross-validation of μ-fishing Data Sets Using Conventional Biochemical Methods**—To demonstrate the selectivity and sensitivity of the data obtained by bead-based μ-fishing, HEK293 cell lysates were analyzed using conventional biochemical methods, including quantitative Western blots, a GST pulldown assay (β-catenin fishing) and Super8xTOPFlash luciferase reporter assays. The total amount of β-catenin that was present in activated and nonactivated HEK293 cells was quantified by Western blot (Fig. 2A). GST-ECT was used as bait molecule in the pulldown assay to capture free β-catenin from the cell extracts (32, 34). β-catenin captured in the pulldown assay was visualized in a Western blot (Fig. 2A). Relative concentrations of β-catenin were determined by signal normalization using a recombinant β-catenin as calibration standard (data not shown). Using both miniaturized and conventional pulldown assays, elevated levels of β-catenin were detected in cells exposed to rWnt3a or GSK3 inhibitor SB216763 (Figs. 2A and 2B). In parallel, a Super8xTOPFlash luciferase reporter assay was used to visualize β-catenin/TCF-mediated gene expression (Fig. 2C). Because elevated levels of free β-catenin in cells exposed to rWnt3a or SB216763 were expected to promote luciferase expression, the detected increase in luciferase activity confirms that the free β-catenin monitored using conventional and miniaturized assays is transcriptionally active.

In summary, the changes in free β-catenin levels induced by exposure to rWnt3a and SB216763 measured using bead-based μ-fishing assay were similar in magnitude to those measured using fishing assay in combination with quantitative Western blotting (Fig. 2D).

**Technical Reproducibility of the Multiplex β-Catenin Assay**—The reproducibility of the assay was demonstrated by performing technical replicates from one protein extract prepared from a GSK3 inhibitor (SB216763) treated cell culture. Four technical replicates were performed to determine the intra-assay variability and the assay was repeated independently on four different days to show the inter-assay variability. Table I lists the intra- and inter-assay variability for all eight analytes separately. Intra-assay variation was below 15%, whereas an average inter-assay variation of 20% was calculated.

**Time-Resolved Analysis of Wnt Signaling**—Comprehensive information on the dynamics of cell signaling is a prerequisite for detailed analysis and computational modeling. With regard to the Wnt signaling pathway, time-resolved data are required to create new or to verify existing models. Time-course experiments were therefore performed by treating HEK293 cells with either rWnt3a or SB216763 in biological triplicates. Cell lysates were collected at specific time points between 0–10 h in order to visualize changes that occurred immediately following the addition of rWnt3a or SB216763 (5 min) and monitor the effects of long-term treatment (10 h). Stimulation of the HEK293 cells with rWnt3a led to an increase in total β-catenin (Fig. 3A) as well as β-catenin phosphorylated at Ser-675 (Fig. 3B), Ser-552 (Fig. 3C) and Ser-45 (Fig. 3E), starting 60 min after treatment and reach-
ing a peak at \(t = 180\) min (Ser-675 and Ser-552) or 300 min (Ser-45), respectively. \(\beta\)-catenin phosphorylated at Ser-33, Ser-37 and Thr-41 exhibited different kinetics: starting from a relatively high signal level, it decreased threefold, reaching a minimum at \(t = 60\) min, subsequently returning to its basal level (Fig. 3D). Free \(\beta\)-catenin, a measure of activated Wnt signaling, accumulated transiently following exposure of the cells to rWnt3a (Figs. 3G and 3H); whereas the amount of membrane-associated \(\beta\)-catenin did not change (Fig. 3F). In addition to the analysis based on protein-protein interaction, a time-resolved measurement of Wnt pathway activity was performed on gene expression level using a Super8xTOP-flash luciferase reporter assay (Fig. 3I). A steady increase was monitored starting 3 h following ligand treatment.

Exposure of cells to SB216763 led to a steady increase (starting at \(t = 60\) min) in total \(\beta\)-catenin levels (Fig. 4A) as well as to an increase in \(\beta\)-catenin phosphorylated at Ser-675 (Fig. 4B), Ser-552 (Fig. 4C), and Ser-45 (Fig. 4E). The inhibition of GSK3 also resulted in a strong decrease in the amount of \(\beta\)-catenin phosphorylated at Ser-33, Ser-37, and Thr-41 by \(t = 5\) min; this rapid decrease is expected because \(\beta\)-catenin Ser-33, Ser-37, and Thr-41 are direct substrates of GSK3. By 10 h, the level of phosphorylation returned to basal levels. In comparison what was observed in rWnt3a-treated cells, the amount of free \(\beta\)-catenin increased steadily following \(t = 60\) min (Figs. 4G and 4H). The levels of membrane-associated protein did not change either during rWnt3a treatment (Fig. 3F) or SB216763 treatment (Fig. 4F). In line with the ligand-mediated activation, time-resolved Wnt activity following SB216763 exposure was measured on gene expression level using a Super8xTOP-flash luciferase reporter assay (Fig. 4I). Again, a steadily increased reporter gene expression was detected starting 3 h following GSK3 inhibition.

In summary, both treatments led to changes in the modification status and the accumulation of free \(\beta\)-catenin consistent with Wnt pathway activation, but the detailed dynamics differed in the two cases. As expected a time-delay of the transcriptional activity detected by the reporter assay com-

Fig. 2. Cross-validation of miniaturized fishing assay. HEK293 cells were starved for 24 h and treated with 200 ng/ml rWnt3a or 20 \(\mu\)M SB216763 for 3 h. Control cells received PBS or DMSO as solvent control. Samples were analyzed using either a \(\beta\)-catenin fishing and Western blot analysis (A) or the miniaturized \(\beta\)-catenin fishing assay (B). Super8xTOP-flash luciferase reporter measuring of \(\beta\)-catenin activity was done in separate experiments (C). A, Upon harvest and preparation of cell lysates, the free, signaling-competent pool of \(\beta\)-catenin was recovered by supplementing samples of each cell lysate with glutathione Sepharose beads and the GST-ECT fusion protein as indicated. Following the binding reaction and extensive washing, proteins bound to the glutathione Sepharose matrix were eluted with SDS-PAGE loading buffer. Samples were separated by SDS-PAGE and analyzed by Western blot with an antibody recognizing \(\beta\)-catenin. As input control, a fraction of each cell lysate was analyzed in parallel. Cellular \(\beta\)-catenin was detected by Western blot with an anti-\(\beta\)-catenin antibody. B, Twenty-five micrograms cell lysate of each sample (biological triplicates) were analyzed. Bead-bound GST-ECT was transferred with the Wnt/\(\beta\)-catenin-inducible Super8xTOP-flash luciferase reporter and the constitutively active Renilla luciferase reporter pRL-CMV, which was used for normalization purposes. Values shown represent average activities and the corresponding standard deviations derived from at least three independent transfection experiments. D, Table compares results (A–C) from the three methods. MFI, median fluorescence intensities; AU, arbitrary units.
pared with the protein-protein interaction assay was observed during ligand and inhibitor treatment.

DISCUSSION

β-catennin is one of the central molecules in Wnt signaling. Its diverse cellular roles are determined by its state of phosphorylation, intracellular localization, and extent of binding to proteins such as cadherins. The concentration of free β-catennin responsible for transcriptional activity is triggered by GSK3β and CK1α-dependent phosphorylation occurring in a multiprotein complex—called the destruction complex. Following phosphorylation, β-catennin is ubiquitinated and subsequently degraded by the proteasome. Wnt stimulation results in increasing β-catennin concentration by impairing this destruction machinery. Thus, determining the role played by β-catennin activity in cell adhesion or transcription requires a multifactorial analysis of its biochemical state. This is usually accomplished using a combination of Western blots, transcription reporter assays, and immunohistochemistry. The present paper describes a multiplex assay capable of investigating the sophisticated phosphorylation pattern of β-catennin, the interaction with E-cadherin and the uncomplexed free pool of β-catennin from a minimal amount of sample within the same experiment. The detection of transcriptionally active β-catennin was made possible by the adaptation of previously described protein-protein interaction assays (31, 32) to the miniaturized bead-based format using recombinant GST-TCF or GST-ECT as capture molecules. The technical innovation in this work, relative to other suspension bead array-based assays described previously, is the integration of three distinct types of affinity capture: (i) capture of β-catennin with pan- and phospho specific antibodies (ii) capture of a protein-complex using antibodies against E-cadherin, and (iii) capture of unbound β-catennin using two biologically relevant binding partners.

The multiplex nature of this assay eliminates the need to carry out separate cell culture experiments to prepare material for different analytical procedures. Using conventional methods, multiple separate cell cultures or large scale cultures are needed in order to carry out a Western blot, a reporter assay and a co-immunoprecipitation assay. This leads to the potential for great variability with respect to culturing, the different sample preparation methods and the read-out system used. These potential sources of error are eliminated in our assay set-up resulting in comparable data free from biological variability because all parameters are measured within the same sample. Measurements using the presented suspension bead array assay panel were highly reproducible (Table I). The measurement of three independent cell culture experiments led to a variability that was below 30%, which includes the variability because of technical and biological issues and sample preparation. Moreover, low sample consumption (10^5 cells) and low hands-on time increased sample throughput compared with standard methods. Multiple time points, stimuli, or inhibitor concentrations can be examined using cells cultured in 24-well or even 96-well plates. Applied as a screening tool for chemical compounds perturbing the Wnt pathway, for example, the proposed assay could minimize the work load compared with the standard reporter assays and Western blots as previously performed.

| Table I: Inter- and intra-assay variances |
|------------------------------------------|
| **Inter-assay variances**                |
| Control                                 |
| Mean [AU] (n = 4)                        | CV [%]     |
| Total                                   | 753       | 12 |
| pSer-33/pSer-37/pThr-41                 | 174       | 8  |
| pSer-45                                 | 224       | 6  |
| pSer-552                                | 80        | 11 |
| pSer-675                                | 450       | 11 |
| E-cadherin/β-catennin                   | 4481      | 3  |
| Free/ECT                                | 26        | 17 |
| Free/TCF                                | 45        | 27 |
| Solvent control                         |
| Mean [AU] (n = 4)                        | CV [%]     |
| Total                                   | 722       | 17 |
| pSer-33/pSer-37/pThr-41                 | 149       | 5  |
| pSer-45                                 | 198       | 13 |
| pSer-552                                | 78        | 13 |
| pSer-675                                | 411       | 19 |
| E-cadherin/β-catennin                   | 4245      | 6  |
| Free/ECT                                | 23        | 19 |
| Free/TCF                                | 37        | 33 |
| Inhibitor                               |
| Mean [AU] (n = 4)                        | CV [%]     |
| Total                                   | 1307      | 12 |
| pSer-33/pSer-37/pThr-41                 | 195       | 7  |
| pSer-45                                 | 744       | 11 |
| pSer-552                                | 163       | 11 |
| pSer-675                                | 814       | 14 |
| E-cadherin/β-catennin                   | 4527      | 6  |
| Free/ECT                                | 231       | 9  |
| Free/TCF                                | 421       | 30 |

| **Intra-assay variances**                |
| Control                                 |
| Mean [AU] (n = 4)                        | CV [%]     |
| Total                                   | 644       | 3  |
| pSer-33/pSer-37/pThr-41                 | 169       | 4  |
| pSer-45                                 | 216       | 5  |
| pSer-552                                | 70        | 5  |
| pSer-675                                | 392       | 3  |
| E-cadherin/β-catennin                   | 4370      | 1  |
| Free/ECT                                | 26        | 10 |
| Free/TCF                                | 47        | 5  |
| Solvent control                         |
| Mean [AU] (n = 4)                        | CV [%]     |
| Total                                   | 568       | 4  |
| pSer-33/pSer-37/pThr-41                 | 138       | 2  |
| pSer-45                                 | 177       | 1  |
| pSer-552                                | 64        | 4  |
| pSer-675                                | 320       | 4  |
| E-cadherin/β-catennin                   | 3929      | 1  |
| Free/ECT                                | 22        | 15 |
| Free/TCF                                | 28        | 8  |
| Inhibitor                               |
| Mean [AU] (n = 4)                        | CV [%]     |
| Total                                   | 1118      | 5  |
| pSer-33/pSer-37/pThr-41                 | 182       | 2  |
| pSer-45                                 | 706       | 3  |
| pSer-552                                | 686       | 7  |
| pSer-675                                | 4248      | 2  |
| E-cadherin/β-catennin                   | 219       | 11 |
| Free/ECT                                | 379       | 1  |
by Huang et al. (35). A limitation of the assay panel described in this paper could be the presence of unknown interactions. All assays described in this work require precipitation of the protein of interest using, either an antibody or a bait protein as capture molecule. The influence of unknown interaction partners, which may influence or even prevent capture or detection, cannot be excluded. Phosphorylated protein sites, can be hidden or masked by interaction partners for instance. Nevertheless, changes in the concentration of e.g. the E-cadherin/β-catenin complex or other complexes can be cross-validated by exchanging the capture and detection molecule (30).

The analysis of time-dependent Wnt stimulation and GSK3 inhibition in HEK293 cells using the proposed multiplex assay revealed novel insights into the dynamics of β-catenin. Although the detailed mechanism of β-catenin phosphorylation inhibition is still unclear (36), our data confirm that receptor activation and inhibition of β-catenin destruction machinery leads to an increase in the total amount of β-catenin in accordance with literature. Both rWnt3a exposure and GSK3 inhibition transiently reduced the amount of Ser-33/Ser-37/Thr-41 phosphorylated β-catenin (Fig. 3D and 4D). However, the two stimuli induced a different time-dependent response, resulting in more efficient and enduring GSK inhibition by the drug than by rWnt3a itself. The strongly decreased amount of Ser-33/Ser-37/Thr-41 phosphorylated β-catenin triggered by the activation of the Wnt pathway at the receptor level or by GSK3 inhibition fits perfectly into the postulated models of the destruction machinery, because GSK3β mediates the key steps that target β-catenin for proteasomal degradation (9, 37).

Of greater interest is the discovery that levels of β-catenin phosphorylated at Ser-45, Ser-552, and Ser-675 increased concomitantly with a rise in total β-catenin and free β-catenin.
levels during both GSK3 inhibitor and Wnt treatment. Ser-45 phosphorylation by CK1 is considered to be a priming event that enhances the affinity of GSK3 for binding to β-catenin (9, 38). Ser-675 and Ser-552 -catenin were recently described as substrates of PKA and AKT, respectively (13, 14). Further regulatory functions and roles of Ser-45 phosphorylation aside from its priming activity have recently been discussed (39–42). Regardless, the phospho-acceptor sites Ser-45, Ser-552, and Ser-675 in -catenin are less well characterized and further investigations are needed to precisely determine their regulatory functions and to clarify whether or not they occur in the context of the destruction complex.

In contrast to the alterations detected at the post-translational modification level, the cell adhesion complex remained unaffected by the treatments over time. These data suggest that membrane-associated β-catenin is not released from the membrane to function as a transcription factor. To exclude assay saturation effects, various amounts of HEK293 protein extracts were analyzed (supplemental Fig. 2). Finally, free β-catenin, which correlates with transcriptionally active β-catenin, accumulated transiently or linearly over time depending on treatment with either rWnt 3a or the GSK3 inhibitor, respectively. In addition to the changes on protein level, steadily increasing β-catenin/TCF-mediated reporter gene expression was monitored following both ligand and inhibitor induced activation of the Wnt pathway (Figs. 3I and 4I). The data confirms the capability of the -fishing assay to monitor transcriptional activity of β-catenin on protein level as an indicator for Wnt activity and otherwise to discriminate between the different extents of activation as observed using ligand or inhibitor.

Differences in the kinetics were observed when comparing the time-resolved responses to inhibitor and natural ligand. Following 3 h of inhibitor treatment, the level of free β-catenin...
was still increasing whereas for the natural ligand, a steady state had already been reached and the levels were on the decline (Figs. 3 and 4). These findings suggest that there are fundamental differences between the mechanisms that control β-catenin levels and the dynamics in response to rWnt3a and the GSK3 inhibitor. In order to exclude the possibility that Wnt ligand degradation caused the transient, but not steady activation, HEK293 cells were treated every 2 h with freshly prepared rWnt3a. The result revealed comparable activation profiles (supplemental Fig. 3) as observed in the pulse stimulation (Fig. 3).

Taken together our findings support that Wnt-pathway activity is under negative feedback control. The pulse as well as the interval stimulation revealed a plateau of the detected free β-catenin. Moreover the different response intensity toward inhibitor and ligand corroborate the model that feedback control occurs on the destruction complex level (43–45). An Axin2-based feedback loop might be responsible for the differences between the dynamic changes caused by the small molecule inhibitor and rWnt3a. In the case of Wnt3a stimulation, up-regulation of Axin2 may restore activity of the destruction complex and trigger a return to the initial equilibrium of β-catenin turnover. In contrast, in the presence of the small molecule inhibitor, an Axin-containing destruction complex would still be functionally impaired because GSK3 activity remains blocked. Accordingly, negative feedback by Axin2 would have less influence, if any, on β-catenin degradation as experimentally observed.

Monitoring β-catenin and its modifications in the same sample within a single experiment enabled very frequent analysis of Wnt pathway status. For instance, opposing time-dependent responses of free and phosphorylated β-catenin Ser-33/Ser-37/Thr-41 to a GSK3 inhibitor or Wnt ligand were captured using the ECT and TCF bait molecules. The study revealed a potential link between β-catenin’s C-terminal phosphorylation sites Ser-552 and Ser-675 and canonical Wnt signaling. Moreover the increase in phosphorylated β-catenin at Ser-45 upon rWnt3a or GSK3 inhibitor treatment was observed for the first time.

In summary, a multiplex assay using antibodies and bait proteins was developed to probe the status of β-catenin phosphorylation and function. The assay allows the investigation of the dynamic effects chemical compounds might have on the Wnt-signaling pathway and may therefore become an invaluable tool for drug discovery and systems biology processes. Compared with other biochemical methods, the proposed assay allows generating snapshots of β-catenin in biological systems in a fast and comprehensive manner. In the future, similar multiplex interaction assays, combined with sandwich immunoassays may have the potential to go beyond protein quantification by simultaneously providing relevant information about multiple diverse functions of proteins in cell signaling networks.

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[S] This article contains supplemental Figs. S1 to S3.

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