The following resources related to this article are available online at http://stke.sciencemag.org.
This information is current as of 25 January 2012.

| | |
|---|---|
| **Article Tools** | Visit the online version of this article to access the personalization and article tools:<br>**http://stke.sciencemag.org/cgi/content/full/sigtrans;5/206/pl1** |
| **Related Content** | The editors suggest related resources on *Science*'s sites:<br>**http://stke.sciencemag.org/cgi/content/abstract/sigtrans;5/206/eg2** |
| **References** | This article cites 28 articles, 16 of which can be accessed for free:<br>**http://stke.sciencemag.org/cgi/content/full/sigtrans;5/206/pl1#otherarticles** |
| **Glossary** | Look up definitions for abbreviations and terms found in this article:<br>**http://stke.sciencemag.org/glossary/** |
| **Permissions** | Obtain information about reproducing this article:<br>**http://www.sciencemag.org/about/permissions.dtl** |

PROTOCOL

# A Dual Array-Based Approach to Assess the Abundance and Posttranslational Modification State of Signaling Proteins

Katrin Luckert,[1]* Taranjit S. Gujral,[2]* Marina Chan,[2] Thomas O. Joos,[1] Peter K. Sorger,[2] Gavin MacBeath,[2]† Oliver Pötz[1]†

[1]NMI Natural and Medical Sciences Institute at the University of Tuebingen, Markwiesenstrasse 55, 72770 Reutlingen, Germany.
[2]Department of Systems Biology, Harvard Medical School, Boston, MA 02115, USA.

*These authors contributed equally to this work. †Corresponding authors. E-mail: poetz@nmi.de (O.P.); gavin_macbeath@harvard.edu (G.M.)

**A system-wide analysis of cell signaling requires detecting and quantifying many different proteins and their posttranslational modification states in the same cellular sample. Here, we present Protocols for two miniaturized, array-based methods, one of which provides detailed information on a central signaling protein and the other of which provides a broad characterization of the surrounding signaling network. We describe a bead-based array and its use in characterizing the different forms and functions of β-catenin, as well as lysate microarrays (reverse-phase protein arrays) and their use in detecting and quantifying proteins involved in the canonical and noncanonical Wnt signaling pathways. As an application of this dual approach, we characterized the state of β-catenin signaling in cell lysates and linked these molecule-specific data with pathway-wide changes in signaling. The Protocols described here provide detailed instructions for cell culture methods, bead arrays, and lysate microarrays and outline how to use these complementary approaches to obtain insight into a complex network at a systems level.**

## Introduction

Protein arrays have become powerful tools to investigate the status of signaling pathways in cells or tissues. The ability to perform multiplexed assays on hundreds to thousands of samples enables time-resolved studies of cells stimulated or perturbed in different ways. The data from these studies can then be used to infer the structure of the underlying network. Protein array technology is well suited for these types of investigations because it provides a way to measure many different proteins in parallel while consuming very little material (*1*, *2*). Over the past decade, two array platforms—bead-based arrays and lysate microarrays—have become well established in cell signaling research (Fig. 1). Both methods have been used to analyze signaling networks in a time-resolved fashion (*3–6*), and both methods offer multiplexing capabilities. In the case of bead arrays, a mixture of microspheres is used to detect and quantify different analytes in a sample. The beads are typically coated with capture antibodies specific to different analytes, and captured analytes are detected and quantified by using a mixture of fluorescently labeled detection antibodies (Fig. 1A). The identity of each bead is revealed by using an internal fluorescent color code. In the case of lysate microarrays, different samples are spotted onto a series of nitrocellulose-coated slides, and each slide is probed with a different antibody (Fig. 1B). In this case, the identity of each slide specifies the analyte and the location of each spot in the array specifies the sample. In both assays, posttranslational modifications can be detected by using posttranslational modification–specific antibodies.

One application of the bead-based assay is the acquisition of detailed information on a single protein. Because critical, highly connected nodes in signaling networks are often pleiotropic, it is important not just to quantify the abundance of the protein, but to obtain quantitative information on its different forms and on its interaction with other proteins. The specific state of a central signaling protein is often influenced by the surrounding network and, in turn, dictates downstream signaling. Thus, to understand the role of such a protein requires detailed information on not only the protein, but on its surrounding network as well. Here, we describe how to obtain such information in a time-resolved fashion, using, as an example, the response of hepatocarcinoma (HepG2) cells to stimulation with either a canonical Wnt ligand, Wnt3a, or a noncanonical ligand, Wnt5a.

In the case of Wnt signaling, the intracellular protein β-catenin is multifunctional, playing critical roles in both signaling and cell-cell adhesion complexes. β-catenin is also a proto-oncogene, and activating mutations in the gene that encodes β-catenin contribute to the genesis of common cancers, such as colorectal cancer and hepatocellular carcinoma (*7–9*). The different functions of β-catenin as a transcriptional coactivator and as a cell adhesion molecule are regulated by changes in protein abundance and phosphorylation state, both of which affect the ability of β-catenin to complex with other transcription factors or to interact with adhesion proteins, such as the
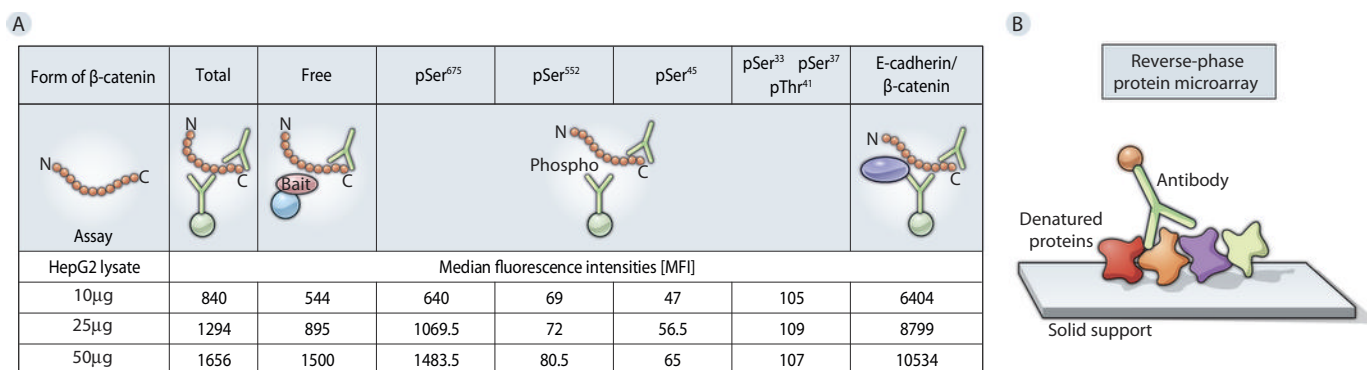
| Form of β-catenin | Total | Free | pSer$^{675}$ | pSer$^{552}$ | pSer$^{45}$ | pSer$^{33}$ pSer$^{37}$ pThr$^{41}$ | E-cadherin/ β-catenin |
|---|---|---|---|---|---|---|---|
| Assay | | | | | | | |
| HepG2 lysate | | | Median fluorescence intensities [MFI] | | | | |
| 10µg | 840 | 544 | 640 | 69 | 47 | 105 | 6404 |
| 25µg | 1294 | 895 | 1069.5 | 72 | 56.5 | 109 | 8799 |
| 50µg | 1656 | 1500 | 1483.5 | 80.5 | 65 | 107 | 10534 |

**Fig. 1. Monitoring β-catenin by bead array assay or lysate array. (A)** The β-catenin bead array panel uses antibodies and known interaction partners of β-catenin to study the phosphorylation status and its complexation status within the same multiplex assay system. Different amounts of protein extracts from untreated HepG2 cells were analyzed. The results are given in median fluorescence intensities (MFI). **(B)** A schematic of lysate microarrays (reverse-phase protein microarrays). In lysate microarrays, samples are immobilized on a nitrocellulose-coated slide and each slide is probed with a different antibody. The microarray format enables several thousand samples to be printed on a single slide, and multiplexing is achieved by printing many different copies of the same array.

cadherins (*10–12*). Increases in the abundance of cytoplasmic β-catenin and accumulation of the uncomplexed, transcriptionally active form of β-catenin are hallmarks of active β-catenin–dependent "canonical" Wnt signaling (*13*). Noncanonical signaling regulates cell polarity and cell movements and involves pathways, such as the planar cell polarity pathway, the Wnt to Jun N-terminal kinase pathway, or the Wnt to $Ca^{2+}$ signaling pathway (*14*).

The analytical methods described here are designed to provide a holistic view of the complex interactions mediated by β-catenin and how these interactions influence its function (*15, 16*). Data obtained with these methods can then be used to train computational models of Wnt signaling, which provide insight into the structure of the network and how best to intervene pharmacologically (*17–21*). More generally, the dual approach described here could be used to gain insight into other complex pathways with central signaling proteins, such the DNA damage response network and p53, or the epidermal growth factor (EGF) signaling network and the EGF receptor.

## Materials

### Reagents for Preparation of Buffers

Complete Protease Inhibitor Cocktail tablets (Roche)

Dimethylsulfoxide (DMSO) (Sigma-Aldrich)

Dithiothreitol (DTT) (Sigma-Aldrich)

Ethylenediaminetetraacetate (EDTA) (Sigma-Aldrich)

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (Thermo Fisher Scientific)

Glycerol (Sigma-Aldrich)

β-Glycerol phosphate (β-GP),

Phenylmethanesulfonylfluoride (PMSF)

Phosphatase Inhibitor Cocktail II (Sigma-Aldrich)

*N*-Hydroxysulfosuccinimide sodium salt (sulfo-NHS) (Thermo Fisher Scientific)

2-(*N*-Morpholino)ethanesulfonic acid (MES) (Sigma-Aldrich)

Sodium azide (Sigma-Aldrich)

Sodium chloride (Sigma-Aldrich)

Sodium dodecyl sulfate (SDS) (Sigma-Aldrich)

Sodium fluoride (NaF) (Sigma-Aldrich)

Sodium orthovanadate (Sigma-Aldrich)

Sodium phosphate ($Na_2HPO_4$) (Sigma-Aldrich)

Tris(hydroxymethyl)aminomethane (TRIS) (Sigma-Aldrich)

Triton X-100 (Sigma-Aldrich)

Tween 20 (Merck KGaA)

### Suspension Bead Array

Donkey serum and goat serum (Sigma-Aldrich)

Blocking Reagent for ELISA (enzyme-linked immunosorbent assay) (Roche Applied Science)

MagPlex Microspheres (beads) (Luminex Corp.)

Sterile phosphate-buffered saline (PBS) (PAA Laboratories GmbH)

**Antibodies for Bead Arrays**

*Capture antibodies*

Anti–β-catenin (β-catenin–specific) antibody (R&D Systems, #AF1329)

Anti–E-cadherin antibody (R&D Systems, #AF648)

Antibody against β-catenin phosphorylated at $Ser^{33}/Ser^{37}/Thr^{41}$ (Cell Signaling Technology, #9561)

> *Note: This antibody recognizes β-catenin phosphorylated at any combination of these sites.*

Antibody against β-catenin phosphorylated at $Ser^{45}$ (Cell Signaling Technology, #9564)

Antibody against β-catenin phosphorylated at $Ser^{552}$ (Cell Signaling Technology, #9566)

Antibody against β-catenin phosphorylated at $Ser^{675}$ (Cell Signaling Technology, #9567)

*Detection antibody*

Anti–β-catenin antibody (BD Biosciences, #610154)

*Fluorophor-labeled secondary antibody*

Antibody conjugated to phycoerythrin (PE) that recognizes mouse immunoglobulin G (IgG) heavy and light chains (PE-conjugated donkey antimouse IgG) (Jackson Immunoresearch, #115-116-146)

*Antibodies for noncovalent immobilization*

Polyclonal antibody that recognizes rabbit IgG heavy and light chains (donkey anti-rabbit IgG) (Jackson Immunoresearch, #711-005-152)

Polyclonal antibody that recognizes glutathione *S*-transferase (GST) (GE Healthcare, #27-4577-01)

**Bait Proteins for Bead Arrays**

Glutathione *S*-transferase E-cadherin cytosolic tail (GST-ECT)

Glutathione *S*-transferase T cell factor 4 (GST-TCF4)

> *Note: GST-ECT and GST-TCF4 were expressed in* Escherichia coli *and affinity-purified as previously described* (21*)*.

**Antibodies and Reagents for Lysate Microarrays**

*Antibodies against signaling proteins or controls*

Anti–β-actin antibody (Sigma, #A1978)

Antibody against Akt (protein kinase B) phosphorylated at $Ser^{473}$ (Cell Signaling Technology, #4058)

Anti-axin1 antibody (Cell Signaling Technology, #2087)

Anti-axin2 antibody (Cell Signaling Technology, #2151)

Anti–β-catenin antibody (Cell Signaling Technology, #9582)

Antibody against β-catenin phosphorylated at $Ser^{675}$ (Cell Signaling Technology, #9567)

Anti–casein kinase 1 (CK1) antibody (Cell Signaling Technology, #2655)

Anti–casein kinase 2α (CK2α) antibody (Cell Signaling Technology, #2656)

Antibody against cAMP response element-binding (CREB) phosphorylated at $Ser^{133}$ (Cell Signaling Technology, #9198)

Anti–Dishevelled 2 (Dvl2) antibody (Cell Signaling Technology, #3224)

Anti–Dishevelled 3 (Dvl3) antibody (Cell Signaling Technology, #3218)

Antibody against extracellular signal–regulated kinases (ERK1/2) phopshorylated at (Thr$^{202}$/Tyr$^{204}$) (Cell Signaling Technology, #4377)

Anti–low-density lipoprotein receptor–related protein 6 (LRP6) antibody (Cell Signaling Technology, #2560)

Antibody against LRP6 phosphorylated at Ser$^{1490}$ (Cell Signaling Technology, #2568)

Antibody against mitogen-activated protein kinase kinase 1/2 (MEK1/2) phosphorylated at Ser$^{217/221}$ (Cell Signaling Technology, #9121)

Antibody against protein kinase C (PKC) phosphorylated at Thr$^{410}$ (Cell Signaling Technology, #2060)

Antibody against Raf phosphorylated at Ser$^{289/296/301}$ (Cell Signaling Technology, #9431)

Antibody against signal transducers and activators of transcription (STAT) 1 phosphorylated at Tyr$^{701}$ (Cell Signaling Technology, #9167)

Antibody against STAT3 phosphorylated at Tyr$^{705}$ (Cell Signaling Technology, #9145)

Anti–TCF1 antibody (Cell Signaling Technology, #2203)

Anti-TCF3 antibody (Cell Signaling Technology, #2883)

### Detection antibodies and reagents

Anti-IRDye 800CW goat anti-mouse antibody (LI-COR Biosciences, #926-32210)

Anti-IRDye 680LT goat antirabbit antibody (LI-COR Biosciences, #926-68021)

Odyssey blocking buffer (OBB) (LI-COR Biosciences, #927-40000)

## Cell Culture and Sample Preparation

BCA protein assay kit (Pierce)

Complete Protease Inhibitor (Roche Applied Science)

Dulbecco's modified Eagle's medium (DMEM) (Cellgro, Mediatech)

Fetal bovine serum (FBS) (Omega, Cat No FB02)

Human HepG2 cells (ATCC HB-8065)

10,000 U/ml penicillin-streptomycin (100×) (Gibco)

Phosphatase Inhibitor Cocktail I and II (Sigma-Aldrich)

Trypsin EDTA 1× (Cellgro, Mediatech)

Recombinant human Wnt3a (R&D systems)

Recombinant mouse Wnt5a (R&D systems)

## Equipment

## Suspension Bead Array Procedure

1.5-ml reaction tubes, polypropylene, clear (Star lab)

Luminex 100 IS system (Luminex Corp.)

   *Note: The following alternatives to the Luminex 100 IS may be used: 200 IS system, or FLEXMAP 3D, MAGPIX (Luminex Corp.).*

Magnetic separator (Invitrogen)

Magnetic plate separator (Luminex Corp.)

Microplate assay sealing film (Thermo Fisher Scientific)

Sonification bath Sonorex (Bandelin Electronics)

xPONENT® Software (Luminex Corp.)

## Cell Culture and Sample Preparation

Standard equipment for cell culture: laminar flow cabinet, cell culture incubator ($CO_2$, 37°C), water bath, pipettes

Cell counter (Beckmann)

10-cm cell culture dishes

Cell strainer (BD Biosciences)

Centrifuge (Beckmann)

18 G × 1 1/2 in needles (BD Biosciences)

10-ml syringe (BD Biosciences)

24-well cell culture plates

## Production of Lysate Microarrays

Aushon 2470 microarrayer (Aushon Biosystems)

GenePix 4000 microarray scanner (Molecular Devices)

Near-infrared scanner (LI-COR Biosciences)

Nitrocellulose-coated slides (Grace Bio-labs)

Odyssey application software (LI-COR Biosciences)

ProPlate slide modules (Grace Bio-labs)

384-well V-bottom plates (Abgene)

96-well 0.2-μm filter plate (Pall Corp.)

96-well microplate, half-area, flat bottom, nonbinding surface (Corning)

96-well plate shaker (Thermo Mixer Comfort; Eppendorf)

## Recipes

### Recipe 1: Cell Culture Medium

Supplement DMEM with 10% FBS and 100 U/ml penicillin-streptomycin.

> *Note: The medium can be stored at 4°C and should be warmed to 37°C before adding to the cells.*

### Recipe 2: Serum-Free Cell Culture Medium

Supplement DMEM with 100 U/ml penicillin-streptomycin.

> *Note: The medium can be stored at 4°C and should be warmed to 37°C before adding to the cells.*

### Recipe 3: Wnt Ligand Solutions

Dissolve recombinant human Wnt3a or recombinant mouse Wnt5a in PBS to a final concentration of 200 μg/ml.

> *Note: This solution should be prepared immediately before use and kept on ice; 1 μl of ligand solution is needed per well of a 24-well plate (1/1000 dilution).*

### Recipe 4: NaCl Stock Solution

Prepare 50 ml of 5 M NaCl in ddH$_2$O and store at room temperature.

### Recipe 5: TRIS Stock Solution

Prepare 50 ml of 1 M TRIS, pH 7.4 in ddH$_2$O and store at room temperature.

### Recipe 6: 10% Triton Stock Solution

Prepare 50 ml by diluting Triton X-100 in ddH$_2$O to a final concentration of 10% (w/v) by using gentle rotation and store at room temperature.

### Recipe 7: 20% Tween Stock Solution

Prepare 50 ml by diluting Tween 20 in ddH$_2$O to a final concentration of 20% (w/v) by using gentle rotation and store at room temperature.

### Recipe 8: Bead Array Dilution Buffer

Prepare 50 ml of 150 mM NaCl, 50 mM Tris pH 7.4, and 1% Triton X-100 by mixing 1.5 ml of NaCl Stock Solution (Recipe 4), 2.5 ml of TRIS Stock Solution (Recipe 5), and 5 ml of 10% Triton Stock Solution (Recipe 6) with 41 ml ddH$_2$O.

*Note: This buffer can be stored for 4 weeks at 4°C or at −20°C for longer storage.*

### Recipe 9: Bead Array Cell Lysis Buffer

Supplement 10 ml Bead Array Dilution Buffer (Recipe 8) with 1× Complete Protease Inhibitor (1 tablet per 10 ml) and 1× Phosphatase Inhibitor Cocktail I and II immediately before use and store the buffer on ice.

*Note: Volume is sufficient for the preparation of 100 cell lysates grown in 24-well cell culture plates.*

### Recipe 10: Lysate Microarray Cell Lysis Buffer

Prepare a 20-ml volume of 50 mM Tris-HCl, 2% SDS, 5% glycerol, 5 mM EDTA, and 1 mM NaF.

Add the following reagents immediately before using:

Complete Protease Inhibitor Cocktail (1 tablet per 10 ml), phosphatase inhibitor cocktail 2 (1×), 10 mM β-GP, 1 mM PMSF, 1 mM sodium orthovanadate, and 1 mM DTT.

*Note: The buffer without the inhibitors can be stored at room temperature for several months or at −20°C for longer storage.*

### Recipe 11: Activation Buffer

Prepare 50 ml of 100 mM Na$_2$HPO$_4$, pH 6.2, and store at 4°C.

### Recipe 12: NHS Solution

Prepare 100 µl of a 50 mg/ml solution of Sulfo-NHS in DMSO.

*Note: This solution should be prepared immediately before use and kept on ice.*

### Recipe 13: EDC Solution

Prepare a 100-µl volume of a 50 mg/ml solution of EDC in Activation Buffer (Recipe 11).

*Note: This solution should be prepared immediately before use and kept on ice.*

### Recipe 14: Coupling Buffer

Prepare 50 ml of 50 mM MES, pH 5.0, and store at 4°C.

### Recipe 15: Washing Buffer

Prepare 100 ml by adding Tween 20 to PBS, pH 7.4 to a final concentration of 0.05% (v/v) Tween 20 and store at 4°C.

*Note: Volume is sufficient for the processing of 1 complete bead array assay procedure (96 samples).*

### Recipe 16: Bead Array Assay Buffer I

Dissolve Blocking Reagent for ELISA with 100 ml ddH$_2$O.

*Note: This buffer can be stored for 1 week at 4°C or at −20°C for longer storage.*

## Recipe 17: Storage Buffer

Prepare 5 ml by adding sodium azide to Bead Array Assay Buffer I (Recipe 16) to a final concentration 0.05% (w/v) and store at 4°C.

*Note: Handle sodium azide with care; it is toxic.*

## Recipe 18: Bead Array Assay Buffer II

Add Tween 20 to Bead Array Assay Buffer I (Recipe 16) to a final concentration of 0.1% (v/v). Prepare 50 ml vol.

*Note: This buffer can be stored for 1 week at 4°C or at –20°C for longer storage.*

## Recipe 19: Bead Array Detection Antibody Solution

Dilute the mouse antibody that recognizes β-catenin in Bead Array Assay Buffer II (Recipe 18) to a final concentration of 1 µg/ml immediately before use. Prepare 5 ml vol.

*Note: Volume is sufficient for the processing of 1 complete bead array assay procedure (96 samples).*

## Recipe 20: Bead Array Secondary Antibody Solution

Dilute the PE-conjugated donkey antimouse IgG in Bead Array Assay Buffer II (Recipe 18) to a final concentration of 2.5 µg/ml. Add donkey and goat sera to a final concentration of 1% (v/v) and incubate this solution for 2 hours at 4°C in the dark before use. Prepare 5 ml vol.

*Note: The donkey and goat sera are required to ensure a low background signal. Volume is sufficient for the processing of 1 complete bead-array assay procedure (96 samples).*

## Recipe 21: PBS with Tween-20 (PBST)

Prepare a 1-liter volume of PBS plus 0.1% Tween-20 and store at 4°C.

## Recipe 22: Slide Wash Buffer

Prepare 1 liter of 100 mM Tris-Hcl, pH 9.0 by dissolving Tris base in ddH$_2$O and adjusting pH with HCl. Filter with a sterile 0.22-µm filter.

*Note: This buffer can be stored at room temperature for several months.*

## Instructions

### Cell Culture, Exposure to Ligands, and Sample Collection

We used human HepG2 cells to study the activation of the Wnt signaling pathway. Previous studies have shown that treatment with Wnt3a at final concentration of 100 to 200 ng/ml activates the β-catenin/TCF pathway (*22*, *23*). The methods described can easily be adapted to other cell lines that respond to other stimuli. The optimal concentration of ligand should be determined in preliminary experiments based on cellular and molecular response.

Each experimental condition should be tested in biological triplicate. To generate comparable sample sets for bead array and lysate microarray analysis, perform two identical cell culture experiments in parallel. Careful attention to confluence and plating conditions is required and these variables should be standardized to the greatest extent possible.

1. Aspirate the medium from 75% confluent cells grown in Cell Culture Medium (Recipe 1) in 10-cm culture dishes and rinse the cells with a 5-ml volume of sterile PBS.

2. Add 1 ml of trypsin to each dish to promote cell dissociation and incubate the cells at 37°C for 5 min in the incubator.

3. Add 9 ml Cell Culture Medium (Recipe 1) to inactivate the trypsin.

4. Aspirate the cell suspension and separate clustered cells by carefully drawing the cell suspension through 18 gauge × 1 1/2–inch (0.318 m) needle with a syringe.

5. Strain the cell suspension with a cell strainer to remove remaining cell clusters.

*Note: HepG2 cells form clusters; therefore, trypsinized cells must be separated carefully before seeding a defined number of cells.*

6. Count the cells by using a particle counter.

7. Resuspend the cells at $1 \times 10^5$ cells per ml in Cell Culture Medium (Recipe 1).

8. Plate $1 \times 10^5$ cells per well of 24-well cell culture plates by adding 1 ml of the cell suspension to each well.

9. Grow the cells for another 24 hours.

10. Before beginning the experiment, starve the cells in 1 ml per well Serum-Free Cell Culture Medium (Recipe 2) in the 37°C incubator overnight.

11. Add a 1-μl volume of the appropriate ligand solution, Wnt3a or Wnt5a Ligand Solution (Recipe 3), to the cells at the appropriate time points.

   *Note: Wnt Ligand Solutions (Recipe 3) must be prepared immediately before use and kept on ice.*

12. Aspirate the medium and rinse the cells twice with a 500-μl volume of PBS.

13. Lyse the cells by adding 100 μl of either Bead Array Cell Lysis Buffer (Recipe 9) or Lysate Microarray Cell Lysis Buffer (Recipe 10) and incubate for either 30 min at 4°C with shaking (bead array samples) or 15 min at 4°C (lysate microarray samples).

   *Note: Bead Array Cell Lysis Buffer (Recipe 9) and Lysate Microarray Cell Lysis Buffer (Recipe 10) should be prepared immediately before use and kept on ice.*

14. Samples for lysate microarrays: Flash freeze and store at –80°C

15. Samples for analysis by bead array: Transfer samples to 1.5-ml reaction tubes for centrifugation.

16. Samples for analysis by bead array: Remove the cell debris by centrifugation at 15,000*g*, at 4°C for 30 min.

17. Samples for analysis by bead array: Transfer the supernatant to a clean tube and store at –80°C.

18. Before use, measure the total protein concentration in each sample by using the BCA Kit according to the manufacturer's instructions.

## Covalent Immobilization of Proteins on Magnetic Beads

Proteins can be directly conjugated to carboxy-modified magnetic beads with EDC/NHS chemistry resulting in an amide bond. These beads can be used directly for bead array–based sandwich immunoassays or protein-protein interaction assays. If buffer agents like glycine, stabilizing agents like BSA, or other additives impede the covalent immobilization through an amide linkage, site-directed noncovalent strategies can be used. For instance, tag- or species-specific antibodies can be used for the selective capture of recombinant proteins or antibodies. We used antibodies specific for total β-catenin and E-cadherin as capture molecules in assays for the determination of β-catenin and E-cadherin–β-catenin complexes.

1. Sonicate the bead stock and transfer 200 μl of each bead stock solution ($2.5 \times 10^6$ beads) to 1.5-ml reaction tubes (polypropylene, clear).

2. Place the tubes in a magnetic separator to collect the beads.

3. Remove the supernatant with a pipette and wash beads twice with 200 μl of Activation Buffer (Recipe 11).

   *Note: Do not allow beads to dry during washing steps. Vortex beads thoroughly throughout all washing or mixing steps.*

4. Resuspend beads in 80 μl of Activation Buffer (Recipe 11).

5. Add 10 μl each of NHS Solution (Recipe 12) and EDC Solution (Recipe 13) to the beads and activate beads for 20 min at room temperature in the dark under rotation.

   *Note: The NHS and EDC Solutions (Recipes 12 and 13) should be prepared immediately before use.*

6. Place the tubes in a magnetic separator to collect the beads. Remove the activation solution and discard.

7. Wash the beads 3 times with 500 μl of Coupling Buffer (Recipe 14). Collect the beads with the magnetic separator between washes.

8. Dilute the protein to be immobilized with Coupling Buffer (Recipe 14) to a concentration of 80 μg/ml.

9. Add 250 μl of the diluted protein solution to the beads and incubate at room temperature in the dark for at least 2 hours with rotation.

10. Place the tubes in a magnetic separator to collect the beads. Remove the supernatant and discard.

11. Wash the beads 3 times with 500 μl of Washing Buffer (Recipe 15). Collect the beads in the magnetic separator between washes.

12. Resuspend the coupled beads in 100 µl Storage Buffer (Recipe 17). Beads can be stored at 4°C until use.

> Note: Store coupled beads in the dark at 4°C.

## Bead Counting

1. Dilute the covalently coupled beads 1:500 with Bead Array Assay Buffer I (Recipe 16).

2. Transfer 100 µl/well of diluted beads to a microtiter plate and incubate the diluted beads for 30 min in a 96-well plate shaker rotating at 750 rpm at room temperature.

3. Measure the numbers of beads with a Luminex reader with the following settings:

> Sample size 50 µl
>
> Time-out 80 s
>
> Total beads 10,000
>
> Magnetic

4. Calculate the bead concentration as follows:

> Beads per µl = (number of beads/30 µl) × 500

## Noncovalent Immobilization of Antibodies and Recombinant Proteins on Magnetic Beads

Some proteins cannot be covalently coupled to carboxy-modified beads because of the presence of unfavorable additives, such as carrier protein or glycine buffer, or sodium azide or low protein concentration. In this case, the antibody or the bait protein can be coupled to the bead through a noncovalent immobilization strategy by using tag- or species-specific antibody-conjugated beads. We used antibodies that recognize phosphorylated β-catenin or GST-tagged bait proteins (GST-ECT and GST-TCF4) to illustrate this method. The indirect immobilization process involves several washing steps to remove excess unbound bait molecules, which can interfere with other assay components or the sample.

1. In separate 1.5-ml tubes (polypropylene, clear), incubate each of the GST-tagged bait proteins (1 µg of each protein) in 50 µl of PBS in the presence of $10^5$ beads coupled to the antibody that recognizes GST for 6 hours at room temperature with rotation.

2. In separate 1.5-ml tubes, incubate each of the phosphorylation-specific β-catenin antibodies (~250 ng) in the presence of $10^5$ beads conjugated to an antibody that recognizes rabbit IgG in 50 µl PBS for 4 hours at room temperature with rotation.

3. Place the tubes in a magnetic separator to collect the beads. Remove the supernatant and discard.

4. Wash each tube of beads three times with 200 µl PBS by using a magnetic separator.

5. Resuspend each bead population in 50 µl Bead Array Assay Buffer I (Recipe 16).

> Note: Beads cannot be stored and should be used directly in the assay.

## Suspension Bead Array Assay Procedure

Before the assay procedure, the cellular samples need to be adjusted to a consistent protein concentration. Depending on the cell type and the abundance of the protein of interest (in this case, β-catenin), 10 to 25 µg of total protein is usually appropriate for analysis. We suggest determining optimal amounts in separate preliminary experiments. A dilution series of cell culture lysates can be used to identify a suitable range of lysate amounts for detection of the proteins of interest.

Although we describe how to perform the process using a magnetic plate separator and manual washing in a 96-well plate, it is also possible to use a robot—for example, a magnetic bead transfer system (King Fisher, Thermo Fisher Scientific), which facilitates semiautomated washing and incubation (24).

1. Combine the coated beads in Bead Array Assay Buffer I (Recipe 16) so that there are 2000 beads per bead population in 20 µl of fully mixed beads.

2. Mix the beads by vortexing the diluted beads.

3. Adjust the protein concentration in each cellular sample so that each sample has the same protein concentration and keep the samples on ice.

> Note: We prepare our samples for analysis of β-catenin at 10 to 25 µg of total protein per well. For any given experiment, they should all be the same concentration.

4. Combine 20 µl bead mix and 40 µl of diluted cell sample in one well of the 96-well microplate, half-area, flat bottom, and with nonbinding surface. (This microplate is subsequently referred to as the assay plate.)

5. Use 20 µl of Bead Array Dilution Buffer (Recipe 8) as a reagent control sample.

6. Seal the plate with a microplate sealing film.

7. Incubate the samples with the beads in a 96-well plate shaker at 4°C and 750 rpm overnight.

8. Place assay plate on the magnetic plate separator for 2 min and remove the supernatant.

9. Wash the beads twice by adding and removing of 100 µl Washing Buffer (Recipe 15) per well and use the magnetic plate separator to collect the beads between washes.

10. Add 50 µl Bead Array Detection Antibody Solution (Recipe 19) per well and incubate the plate at room temperature and 750 rpm in a 96-well plate shaker for 60 min.

   *Note: This solution should be prepared immediately before use.*

11. Place assay plate on the magnetic plate separator and remove the supernatant.

12. Wash the beads twice by adding and removing of 100 µl Washing Buffer (Recipe 15) per well and use the magnetic plate separator to collect the beads between washes.

13. Add 50 µl Bead Array Secondary Antibody Solution (Recipe 20) per well and incubate the plate at room temperature and 750 rpm in a 96-well plate shaker for 45 min.

   *Note: This solution must be prepared at least 2 hours ahead because it must be preincubated with goat and donkey sera for 2 hours before use to minimize background signals and cross-reactivity of the polyclonal antibodies.*

14. Place the assay plate on the magnetic plate separator and remove the supernatant.

15. Wash the beads twice by adding and removing 100 µl of Washing Buffer (Recipe 15) per well and use the magnetic plate separator to collect the beads between washes.

16. Resuspend the beads in 100 µl Bead Array Assay Buffer II (Recipe 18) per well.

17. Place the assay plate containing the resuspended beads in the Luminex reader and read with the following standard settings:

   Doublet discriminator 7,500 to 15,000

   Sample volume 100 µl

   Minimal bead count per region 100

## Preparation of Lysate Microarrays

The microarray assay is performed by using printed slides created from a "source plate," which contains the cellular samples in the orientation matching the pin configuration of the microarrayer. The layout of the source plate depends on pin configuration of the microarrayer. We recommend that the design should allow the biological and technical replicates of each sample to be printed in one row (typically three biological replicates for each in duplicate). Because most antibodies exhibit a sigmoidal, rather than linear, relationship between analyte concentration and fluorescence, we correct for this nonlinearity by including twofold serial dilutions of a control lysate for each microarray, which are then used as an in-well calibration standard.

For this microarray assay, it is important that the lysate samples are cleared to remove viscous insoluble supernatant, which we achieve by filtering the samples using a filter plate.

1. Place a 50-µl volume of lysate array samples from step 14 of "Cell Culture, Exposure to Ligands, and Sample Collection" into each well of a 96-well plate fitted with a 96-well 0.2 µm plate filter.

2. Clarify the lysate array samples by centrifuging them through the 0.2-µm plate filter at 3000*g* for 60 min at room temperature.

   *Note: Filtered lysates can be stored at −80°C.*

3. Measure the total protein concentration in each sample by using the BCA Kit according to the manufacturer's instructions.

4. Pipette 7 to 10 µl of each lysate sample corresponding to ~10 µg of total protein into each well of a 384-well V-bottom plate. (This is the source plate.)

5. Pipette 7 to 10 µl of 6 twofold dilutions of the control lysate (HepG2 cells stimulated for 5 min with 200 ng/ml of Wnt3a and Wnt5a) at the bottom of each microarray.

   *Note: These 6 samples serve as an in-well calibration standard.*

6. Seal the plate with microplate sealing film.

7. Centrifuge the plate at 200*g* for 2 min at room temperature.

   *Note: Once the source plate is made, it can be stored at –80°C.*

8. Print the samples by using one pin-touch per sample onto nitrocellulose-coated slides at room temperature and 80% relative humidity and use the Aushon 2470 microarrayer. If the total protein concentration of the samples is less than 1 mg/ml, they can be printed with multiple depositions.

   *Note: Depending on the pin configuration and number of slides being printed, this process can take 12 to 18 hours and may be performed overnight. Once complete, the source plates should be stored at –80°C. The printed slides can be stored in a sealed container at room temperature.*

## Probing of Lysate Microarrays

1. Remove the printed slides from storage and wash 3 times for 5 min each wash with 10 ml PBST (Recipe 21) shaking at room temperature.

2. Wash the slides in a 5-ml volume of Slide Wash Buffer (Recipe 22) for 24 to 48 hours on an orbital shaker at room temperature.

   *Note: Refresh the Slide Wash Buffer (Recipe 22) at least four times during this time period.*

3. Wash the slides 3 times for 5 min each wash with 10 ml PBST (Recipe 21) shaking at room temperature.

4. Spin dry by centrifuging at 33*g* for 3 min at room temperature in a table-top centrifuge, and use a 50-ml conical tube.

5. Scan the slides at 532 nm by using the GenePix 4000 microarray scanner to verify the removal of the SDS present in the lysates, which is autofluorescent.

   *Note: If there is still residual autofluorescence, repeat steps 1 to 4. This step is important to ensure adequately low background fluorescence.*

6. Block the washed slides in 3 ml of OBB at room temperature for 1 hour with agitation. Cover the slides to avoid evaporation.

   *Note: Alternatively, slides can be blocked in PBST (Recipe 21).*

7. Dilute the antibody that recognizes the target and the antibody that recognizes β-actin 1:500 to 1:1000 in OBB and incubate slides in 3 ml of the diluted primary antibody solution for 24 hours on an orbital shaker at 4°C.

8. Wash with 10 ml PBST, shaking 4 times for 7 min each wash at room temperature.

9. Dilute the infrared labeled secondary antibodies (IRDye 800CW goat antimouse antibody and IRDye 680LT goat antirabbit antibody) 1:1000 in OBB and incubate slides in 3 ml of secondary antibody solution for 1 hour, shaking at room temperature.

10. Wash with 10 ml of PBST on orbital shaker 4 times for 7 min each wash at room temperature.

11. Spin dry by centrifuging at 33*g* for 3 min at room temperature in a table-top centrifuge and use a 50-ml conical tube.

## Scanning of Lysate Microarrays

The microarray slides are scanned in the 680-nm and 800-nm channels with an Odyssey imager.

1. Use the ProPlate tray and place the slide upside down on the plastic ledges. This ensures that the slide does not touch the glass surface of the scanner.

2. Set "z-offset" in the LI-COR scanner to 2 mm.

3. Scan and determine maximum pixel intensity on the slide (or a representative area of the slide) with the Odyssey Application Software.

4. Adjust the scanner sensitivity such that the maximum pixel intensity is ~30,000 in both channels.

   *Note: In our experience, we achieve a maximum pixel intensity of ~30,000 in the β-actin channel at a scanner sensitivity of ~6 to 8.*

## Troubleshooting

### Low or No Signal in the Bead Array Assay

For the assay to work, the protein of interest must be present in sufficient quantity to be detected. Therefore, it is important to use cells in which the pathway of interest or protein of interest is known to be present to serve as a positive control. Lack of signal in the positive control is an indication of technical problems with the assay rather than an indication that the protein is not present in sufficient quantity to be detected in the sample.

To analyze β-catenin signaling, we measured free β-catenin, which is the transcriptionally active form, in HepG2 cells. HepG2 cells express a mutant form of β-catenin that lacks N-terminal regulatory sites and thus is constitutively active. For cells that exhibit normal Wnt pathway activity and require Wnt ligand for activation, free β-catenin in the absence of added Wnt ligand is undetectable. Therefore, we suggest preparing positive control samples from human embryonic kidney (HEK) 293 cells exposed to Wnt3a or a glycogen synthase kinase 3 (GSK3) inhibitor, as previously described (*21*).

Once it has been determined from the positive controls that the problem is technical, there are several potential causes of the lack of signal. Coupling efficiency of the noncovalent immobilization procedure can be an issue. Regarding the GST-tagged bait proteins used here for the analysis of β-catenin activity, this can be assessed by incubation with PE-labeled antibody that recognizes GST. Whereas the bead-coated phosphorylation-specific antibodies that recognize β-catenin can be detected with a PE-labeled antibody that recognizes rabbit IgG. Additionally, the covalent immobilization of antibodies that recognize E-cadherin or total β-catenin can be tested by using a PE-labeled antibody that recognizes goat IgG.

Moreover, degradation of the bait molecules coating the beads will result in an undetectable signal. To exclude loss of signal caused by degradation, bait molecules can be analyzed separately by using SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

### High Background Signal on Lysate Microarrays

A high background signal on a reverse-phase array usually arises from a high degree of cross-reactivity by the primary antibody. If this is the case, the data are unreliable and should be excluded from the analysis, and alternative primary antibodies must be identified.

### Low or No Signal on Lysate Microarrays

Low or no signal on reverse-phase arrays can arise if the concentration of the primary antibody is too low (or if the stock of antibody is too old). Increasing the primary antibody concentration and using newer detection reagents should improve the signal.

## Notes and Remarks

### Advantages of Bead-Based Arrays

Bead-based arrays can be used in a focused way to obtain information on a specific signaling protein of interest (in this case, β-catenin) or a set of signaling proteins (*24*). Molecule-specific characteristics of the protein—such as abundance, posttranslational modifications, complex formation, and activity—can be detected in parallel (*21*). Overall, bead-based assay systems enable high sample throughput with low sample consumption (10 to 20 μg of total protein per sample) and are ideal for studies in which cells are analyzed under many stimulation conditions and many time points. In terms of assay development issues, capture and detection reagents can be tested rapidly, without the need for large quantities of material. Thus, additional analytes can be added to the existing multiplexed assay in a relatively straightforward fashion.

### Limitations of Bead-Based Arrays

In general, bead-based assay systems are limited in the number of proteins that can be analyzed in parallel (typically <25). Because of cross-reactivity, the signal-to-noise ratio decreases with an increasing number of detection antibodies in the system. Thus, bead-based assays are most appropriate for performing focused studies that involve a relatively small number of analytes but a large number of samples.

Regarding the development of such assays, two antibodies for each analyte are required: One is the capture (or bait) molecule, and the other is the detection molecule. Therefore, the availability of antibodies is a strong limiting factor. Moreover, the antibodies used in such assays should recognize its target in a native form, a fact that additionally limits the number of suitable antibodies.

The bead array panel described here provides molecular snapshots of one protein. Conditions have been optimized especially for extracting native β-catenin, its modified forms, and the intact cadherin–β-catenin complex. In general, the appropriate detergent has

to be investigated; salt concentration and inhibitor and buffer additives of the lysis buffer have to be optimized. This could be an issue if single assays are combined to a multiplex format, because the optimal extraction conditions for proteins are always slightly different. Thus, the extraction protocol for bead-based arrays detecting native proteins is a compromise and sensitivity of the singleplex can decrease in a multiplex format.

## Advantages of the Lysate Microarrays

One of the primary advantages of the microarray format is that it is compatible with large-scale investigations. Thousands of biological samples can be arrayed on a single slide, to provide information on cells subjected to many different stimulation conditions, time points, and other perturbations. It is also compatible with extremely small sample sizes; a single microarray spot contains as much lysate as would be obtained from a single cell. As little as 5 μl of a 1 mg/ml solution of lysate is needed for printing, and this is sufficient to print hundreds of arrays. In addition, lysate arrays are not limited with respect to their level of multiplexing. Because each array is probed separately, additional proteins can be quantified simply by probing additional arrays with new antibodies. Finally, the denaturing conditions used in this assay permit the uniform detection of proteins, regardless of whether or not the epitope is masked by protein-protein interactions.

## Limitations of the Lysate Microarrays

The major drawback of this method is that it requires highly selective antibodies. Sandwich-style immunoassays, such as those used in the bead-based assay system, require each protein to be recognized by two different antibodies with nonoverlapping epitopes and are therefore less subject to off-target signal. Reverse-phase arrays, in contrast, use only one antibody per analyte and are, therefore, more prone to interference from nonspecific antibody binding. Moreover, antibodies that perform well in Western blots do not necessarily yield quantitative data when used for lysate microarrays. It is therefore necessary to evaluate each antibody by direct comparison of data obtained by arrays and Western blots.

## An Example of Array-Based Cell Network Analysis

In this study, we used two complementary types of miniaturized proteomic methods to study canonical and noncanonical Wnt signaling. The combination of these methods enabled high-throughput collection of detailed information on the central signaling protein, β-catenin, and on the surrounding network.

We used a bead array panel (21) to study changes in several forms of β-catenin in response to Wnt3a and Wnt5a in HepG2 cells. A truncated form of β-catenin lacking residues 25 to 140 is abundant in HepG2 cells, along with a smaller amount of wild-type β-catenin (8, 25). Thus, they represent a model system in which to study a hyperactivated Wnt pathway. In the absence of added ligand, HepG2 cells had large amounts

of free and total β-catenin, and both pools contained wild-type and truncated β-catenin (Fig. 1A). The E-cadherin–β-catenin complex was abundant in these cells, which confirms an intact E-cadherin–mediated cell-adhesion mechanism in HepG2 cells (26). In addition, we observed increasing amounts of total β-catenin, free β-catenin, and Ser[675] phosphorylated β-catenin, but not that of Ser[33]/Ser[37]/Thr[41] Ser[33], and Ser[552] phosphorylated β-catenin, upon Wnt3a treatment (Fig. 2). In response to Wnt5a, no changes have been observed with the bead array panel.

To link molecule-specific data with pathway-wide changes in protein abundance, we used lysate microarrays to assay 21 cell signaling proteins or phosphorylated proteins involved in Wnt signaling (Fig. 3B). Differences in the response of the cells with
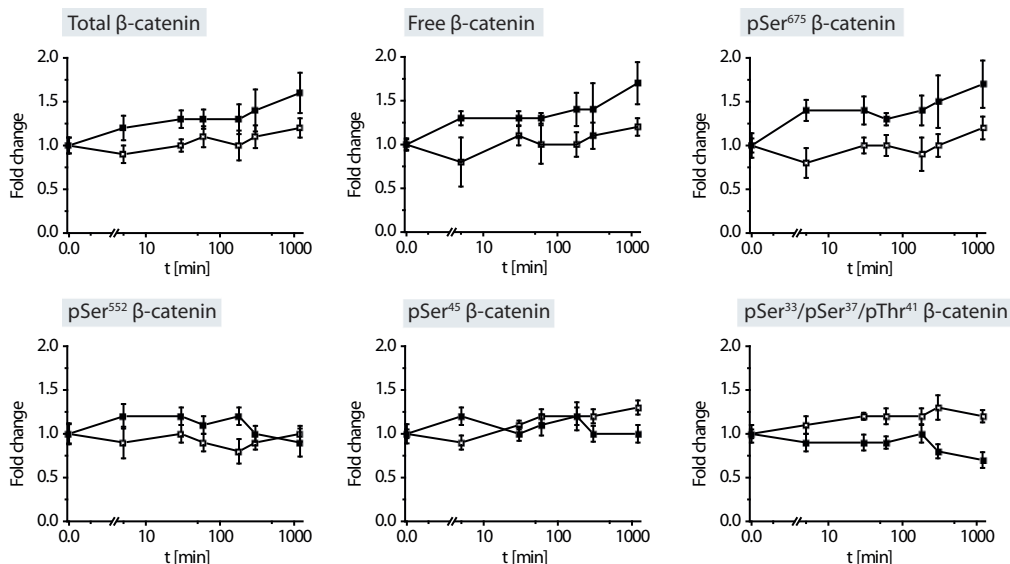
**Fig. 2. Snapshots of different forms of β-catenin monitored by bead array assay.** HepG2 cells were starved for 24 hours and then treated with 200 ng/ml recombinant Wnt3a (filled squares) or recombinant Wnt5a (open circles) for 5, 30, 60, 180, 300, and 1200 min. Each experiment was performed in biological triplicates. From each sample, 25 μg was analyzed by using the β-catenin bead array panel. Mean and standard deviation of fold-change were calculated from three independent experiments and are displayed as ratios (treated/untreated), including the corresponding error propagation.
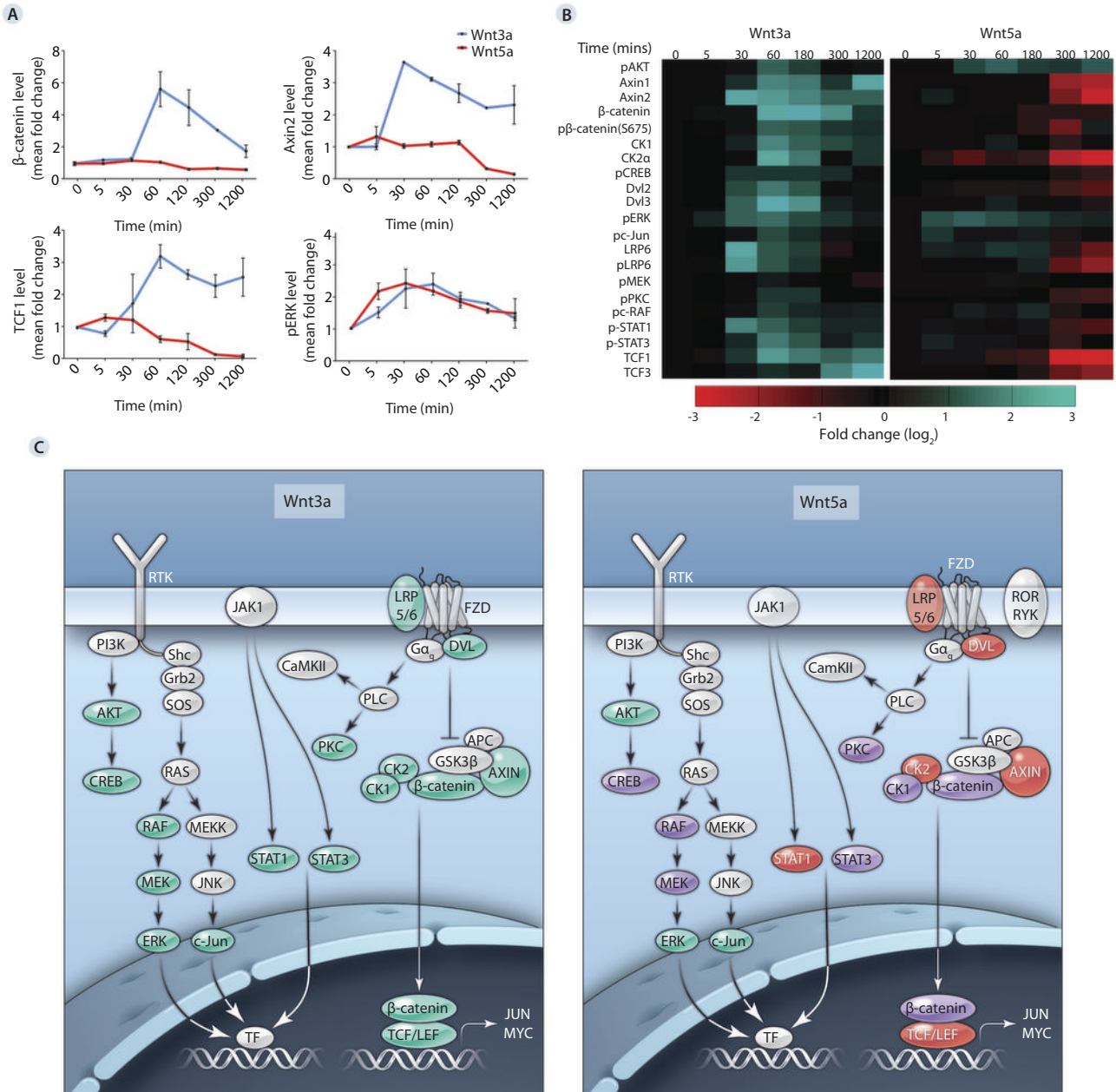
**Fig. 3. Monitoring the dynamic changes in proteins in response to Wnt ligands in HepG2 cells using lysate microarrays.** (**A**) Wnt3a and Wnt5a stimulate opposite changes in cellular content of the canonical Wnt signaling components, β-catenin, axin2, and TCF1. Both Wnt3a and Wnt5a stimulation caused similar changes in the phosphorylation of extracellular signal regulated kinase (pERK). (**B**) Heat maps showing changes in total levels of 10 proteins and phosphorylation status of 11 signaling proteins upon Wnt3a and Wnt5a stimulation. (**C**) A knowledge-based pathway diagram based on the heat plot (B) showing opposite effects of a canonical Wnt ligand (Wnt3a) and a noncanonical ligand (Wnt5a) in HepG2 cells. Green represents proteins (or phosphorylated proteins) that showed an increase, red represents proteins that showed a decrease, purple represents proteins that were measured but did not change, and gray represents proteins that were not measured.

regard to the amount of total β-catenin, TCF, and axin2 were apparent in cells exposed to Wnt3a or Wnt5a (Fig. 3A). We also detected Wnt3a-mediated activation of the lipoprotein related protein 6 (LRP6) Wnt co-receptor in HepG2 cells (detected as an increase in receptor phosphorylation) after 30 min (Fig. 3B). An increase in the amount of total β-catenin was detectable after a 1-hour exposure to Wnt3a (Fig. 3A). We also observed an increase in the amounts of Axin1 and Axin2, which are negative regulators of the canonical Wnt pathway (*27, 28*) in response to Wnt3a. Thus, the reverse-phase array data revealed an inducible canonical Wnt pathway in HepG2, even though these cells harbor hyperactivated β-catenin.

In contrast, the noncanonical Wnt ligand Wnt5a generally induced opposing effects on Wnt pathway proteins compared to those induced by Wnt3a (Fig. 3A). However, the overall amount of β-catenin was unchanged (Figs. 2 and 3A). Thus, we observed ligand-specific

responses of HepG2 cells in response to activation of either the canonical or noncanonical pathway (Fig. 3C). Consistent with previously published reports (*22*), we observed that activation of noncanonical signaling by Wnt5a impaired canonical Wnt activity at the level of the receptor and failed to increase the abundance of β-catenin.

## References and Notes

1. D. A. Hall, J. Ptacek, M. Snyder, Protein microarray technology. *Mech. Ageing Dev.* **128**, 161–167 (2007).
2. M. F. Templin, D. Stoll, J. M. Schwenk, O. Pötz, S. Kramer, T. O. Joos, Protein microarrays: Promising tools for proteomic research. *Proteomics* **3**, 2155–2166 (2003).
3. L. G. Alexopoulos, J. Saez-Rodriguez, B. D. Cosgrove, D. A. Lauffenburger, P. K. Sorger, Networks inferred from biochemical data reveal profound differences in toll-like receptor and inflammatory signaling between normal and transformed hepatocytes. *Mol. Cell. Proteomics* **9**, 1849–1865 (2010).
4. M. F. Ciaccio, J. P. Wagner, C. P. Chuu, D. A. Lauffenburger, R. B. Jones, Systems analysis of EGF receptor signaling dynamics with microwestern arrays. *Nat. Methods* **7**, 148–155 (2010).
5. J. Du, P. Bernasconi, K. R. Clauser, D. R. Mani, S. P. Finn, R. Beroukhim, M. Burns, B. Julian, X. P. Peng, H. Hieronymus, R. L. Maglathlin, T. A. Lewis, L. M. Liau, P. Nghiemphu, I. K. Mellinghoff, D. N. Louis, M. Loda, S. A. Carr, A. L. Kung, T. R. Golub, Bead-based profiling of tyrosine kinase phosphorylation identifies SRC as a potential target for glioblastoma therapy. *Nat. Biotechnol.* **27**, 77–83 (2009).
6. U. B. Nielsen, M. H. Cardone, A. J. Sinskey, G. MacBeath, P. K. Sorger, Profiling receptor tyrosine kinase activation by using Ab microarrays. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 9330–9335 (2003).
7. P. J. Morin, A. B. Sparks, V. Korinek, N. Barker, H. Clevers, B. Vogelstein, K. W. Kinzler, Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* **275**, 1787–1790 (1997).
8. A. de La Coste, B. Romagnolo, P. Billuart, C. A. Renard, M. A. Buendia, O. Soubrane, M. Fabre, J. Chelly, C. Beldjord, A. Kahn, C. Perret, Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 8847–8851 (1998).
9. P. Polakis, Wnt signaling and cancer. *Genes Dev.* **14**, 1837–1851 (2000).
10. K. M. Cadigan, M. Peifer, Wnt signaling from development to disease: Insights from model systems. *Cold Spring Harb. Perspect. Biol.* **1**, a002881 (2009).
11. R. L. Daugherty, C. J. Gottardi, Phospho-regulation of beta-catenin adhesion and signaling functions. *Physiology (Bethesda)* **22**, 303–309 (2007).
12. K. Willert, K. A. Jones, Wnt signaling: Is the party in the nucleus? *Genes Dev.* **20**, 1394–1404 (2006).
13. C. J. Gottardi, B. M. Gumbiner, Distinct molecular forms of beta-catenin are targeted to adhesive or transcriptional complexes. *J. Cell Biol.* **167**, 339–349 (2004).
14. S. L. McDonald, A. Silver, The opposing roles of Wnt-5a in cancer. *Br. J. Cancer* **101**, 209–214 (2009).
15. M. E. Cusick, H. Yu, A. Smolyar, K. Venkatesan, A. R. Carvunis, N. Simonis, J. F. Rual, H. Borick, P. Braun, M. Dreze, J. Vandenhaute, M. Galli, J. Yazaki, D. E. Hill, J. R. Ecker, F. P. Roth, M. Vidal, Literature-curated protein interaction datasets. *Nat. Methods* **6**, 39–46 (2009).
16. E. Pieroni, S. de la Fuente van Bentem, G. Mancosu, E. Capobianco, H. Hirt, A. de la Fuente, Protein networking: Insights into global functional organization of proteomes. *Proteomics* **8**, 799–816 (2008).
17. J. Saez-Rodriguez, L. G. Alexopoulos, M. Zhang, M. K. Morris, D. A. Lauffenburger, P. K. Sorger, Comparing signaling networks between normal and transformed hepatocytes using discrete logical models. *Cancer Res.* **71**, 5400–5411 (2011).
18. M. K. Morris, J. Saez-Rodriguez, D. C. Clarke, P. K. Sorger, D. A. Lauffenburger, Training signaling pathway maps to biochemical data with constrained fuzzy logic: Quantitative analysis of liver cell responses to inflammatory stimuli. *PLOS Comput. Biol.* **7**, e1001099 (2011).
19. B. L. Millard, M. Niepel, M. P. Menden, J. L. Muhlich, P. K. Sorger, Adaptive informatics for multifactorial and high-content biological data. *Nat. Methods* **8**, 487–493 (2011).
20. M. Sevecka, A. Wolf-Yadlin, G. MacBeath, Lysate microarrays enable high-throughput, quantitative investigations of cellular signaling. *Mol. Cell. Proteomics* **10**, M110.005363 (2011).
21. K. Luckert, F. Götschel, P. K. Sorger, A. Hecht, T. O. Joos, O. Pötz, Snapshots of protein dynamics and post-translational modifications in one experiment—beta-catenin and its functions. *Mol. Cell. Proteomics* **10**, M110.007377 (2011).
22. H. Yuzugullu, K. Benhaj, N. Ozturk, S. Senturk, E. Celik, A. Toylu, N. Tasdemir, M. Yilmaz, E. Erdal, K. C. Akcali, N. Atabey, M. Ozturk, Canonical Wnt signaling is antagonized by noncanonical Wnt5a in hepatocellular carcinoma cells. *Mol. Cancer* **8**, 90 (2009).
23. J. Liu, X. Ding, J. Tang, Y. Cao, P. Hu, F. Zhou, X. Shan, X. Cai, Q. Chen, N. Ling, B. Zhang, Y. Bi, K. Chen, H. Ren, A. Huang, T. C. He, N. Tang, Enhancement of canonical Wnt/β-catenin signaling activity by HCV core protein promotes cell growth of hepatocellular carcinoma cells. *PLoS ONE* **6**, e27496 (2011).
24. O. Poetz, T. Henzler, M. Hartmann, C. Kazmaier, M. F. Templin, T. Herget, T. O. Joos, Sequential multiplex analyte capturing for phosphoprotein profiling. *Mol. Cell. Proteomics* **9**, 2474–2481 (2010).
25. T. Cagatay, M. Ozturk, P53 mutation as a source of aberrant beta-catenin accumulation in cancer cells. *Oncogene* **21**, 7971–7980 (2002).
26. B. C. Fuchs, T. Fujii, J. D. Dorfman, J. M. Goodwin, A. X. Zhu, M. Lanuti, K. K. Tanabe, Epithelial-to-mesenchymal transition and integrin-linked kinase mediate sensitivity to epidermal growth factor receptor inhibition in human hepatoma cells. *Cancer Res.* **68**, 2391–2399 (2008).
27. E. H. Jho, T. Zhang, C. Domon, C. K. Joo, J. N. Freund, F. Costantini, Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Mol. Cell. Biol.* **22**, 1172–1183 (2002).
28. B. Lustig, B. Jerchow, M. Sachs, S. Weiler, T. Pietsch, U. Karsten, M. van de Wetering, H. Clevers, P. M. Schlag, W. Birchmeier, J. Behrens, Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. *Mol. Cell. Biol.* **22**, 1184–1193 (2002).