SUPPLEMENTARY INFORMATION

One-pot Synthesis of Heterodimeric Agonists that Activate the Canonical Wnt Signaling Pathway

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Experimental Section

Plasmids

The 7x TCF Wnt luciferase reporter[1] was constructed with a pVitro2-MCS vector with a hygromycin resistance gene for bacterial transformation (Invivogen). In the first cloning site (MCS-1), the ferritin promoter and CMV enhancer were removed and replaced with 7 repeats of the consensus TCF binding sequence followed by a minimal promoter (minP) and subsequently the firefly luciferase gene. Gene synthesis and cloning were performed by Gene Universal

The anti-Fzd Fab and anti-LRP6 Fab were engineered from Frizzled antibody and LRP6 antibody sequences obtained from patents by Gurney et al. and Jenkins et al. respectively[2, 3]. The expression plasmids were constructed by inserting the variable regions of the light chain and heavy chain sequences into the TGEX-LC and TGEX-FH vectors (Antibody Design Labs). The TGEX vectors code for the constant region of the human IgG heavy chain CH1 region with a C-term 6x His tag and the human kappa light chain CH1 region. One repeat of a GGGGS linker sequence followed by the SpyTag sequence were inserted between the CH1 region and the C-

terminal 6x His tag of the heavy chain of the anti-Fzd Fab. The SpyCatcher sequence was inserted at the C-terminus of LRP6 following a GGGGSKLGDIEFIKVNKGGGGS linker sequence. All plasmids were custom-synthesized and cloned into the TGEX vector by Gene Universal.

Transfection of 293F Cells for Obtaining Secreted Proteins

Forty five mL of HEK 293F (Thermo Scientific) mammalian cells suspended in Expi293 Expression Medium (Thermo Scientific) were grown up to a density of 2.9 million cells per mL in a 125-mL Erlenmeyer flask in a humidified incubator at 37 °C with 8% CO_2 concentration. Cultures for Fab expression were transfected with plasmids encoding the heavy chain and light chain in a 1:2 w/w ratio. Transfection was performed using the ExpiFectamine (Thermo Scientific) transfection reagent according to the manufacturer's recommendation. Recommended doses of transfection enhancers were added to the culture 20-24 hours after initial addition of transfection reagents. Post transfection, cells were incubated for 6 days and then pelleted. The Expi293F (Thermo Scientific) supernatant media was harvested to proceed with purification of the Fabs.

ELISA to Validate Binding of Ligands to Antigens

Commercial LRP6 ectodomain fused to a human IgG Fc region (LRP6-Fc) (R&D Systems) and Frizzled 2 (Fzd2) CRD fused to a human IgG Fc region (Fzd-Fc) (R&D Systems) were diluted in carbonate buffer (pH 9.6) to a concentration of 1 μg/mL. The antigens were used to coat a Maxisorp 96-well plate (Thermo Scientific) overnight at 4°C. Fabs were biotinylated with Sulfo-EZ-link NHS Biotin (Thermo Scientific) with a 50x molar excess of biotin at 4°C overnight. The next day, each well was blocked using 5% BSA solution in PBST (PBS containing 0.05% Tween-20) for 1 hour at room temperature. The blocked wells were then incubated with the

respective Fabs diluted to a concentration of 2 µg/mL in PBST with 1% BSA and then by the respective secondary anti-human antibody (Jackson Immunoresearch) or streptavidin (Thermo Scientific) HRP conjugates diluted in 1% BSA solution in PBST at a concentration recommended by the manufacturer. All wells were washed thoroughly with 200 µL of PBST in between incubation steps. Finally, the wells were incubated with 100 µL TMB (Thermo Scientific) solution for 15 minutes followed by the addition of 100 µL of 0.16 M sulfuric acid. Next, the absorbance at 450 nm was read using a BioTek Synergy plate reader.

Purifying Fzd-Fab-LRP6-Fab Heterodimers

The Expi media was dialyzed into 1x PBS twice for two hours to remove EDTA present in the media which would interfere with purification. The supernatant containing unreacted Fzd-Fab-SpyTag, LRP6-Fab-SpyCatcher and the Fzd-Fab-LRP6-Fab heterodimers was then purified using 1 mL Ni-NTA (Thermo Scientific) agarose resin with specific affinity for the His tag in 30 mL columns at 4 °C. Each Ni-NTA column was first equilibrated with 20 column volumes of equilibration buffer (50 mM Tris, 500 mM NaCl, 25 mM imidazole, 5% glycerol, pH 8.0). The supernatant was then passed through the column and the flow through discarded. The column was then washed using 50 mM imidazole buffer (pH 8.0) once to get rid of non-specific protein binding in the resin and the flow through was collected. The protein of interest was then eluted with 400 mM imidazole (pH 8.0) buffer in 2 mL fractions and all the fractions were collected. The eluted proteins were then concentrated down to less than 1 mL by centrifuging at 4500x g in Amicon 10kDa MWCO 15 mL spin filters (EMD Millipore). The 1 mL eluate was then purified in a HiLoad Superdex 200 column in the case of the heterodimer or a Superdex 75 column in the case of individual Fabs loaded on an AKTA Pure chromatography system (GE Healthcare) using size-exclusion chromatography at 4 °C. The fractions corresponding to the peaks in absorbance

at 280 nm were run on an SDS-PAGE gel and analyzed for purity using Coomassie Brilliant Blue Staining (Thermo Scientific). Fractions containing dimers were pooled and concentrated again in an Amicon 10kDa MWCO 15 mL spin filter. The purified proteins were then aliquoted and stored. For short-term use, purified heterodimers were stored in PBS containing >40% glycerol at -20°C. For long-term storage, purified fractions were frozen in -80°C in PBS containing >40% glycerol.

Luciferase Assay to Verify Activation of Canonical Wnt Signaling

Human embryonic kidney (HEK) 293T cells (CRL-11268) were purchased from the American Type Culture Collection (ATCC) and cultured in DMEM (high-glucose) supplemented with 10% Heat-Inactivated Fetal Bovine Serum (HI-FBS) (Gibco, Thermo Scientific) in a humidified incubator at 37 °C with 8% CO_2 concentration. HEK 293T cells were plated on a 6-well plate coated with poly-L-lysine and grown up to 80% confluency. The cells were then transfected with the 7x TCF reporter plasmid, trypsinized 24 hours after transfection (0.25% Trypsin-EDTA, Thermo Scientific), re-plated in a 96-well plate (Corning) coated with poly-L-lysine (Sigma-Aldrich). Transfected cells were then treated with different concentrations of heterodimers, Wnt-3a, or Fabs diluted in 1x DMEM media (Gibco) with 10% FBS (Gibco) as appropriate. Each treatment was done in triplicate. After 24 hours of treatment, cells were lysed in a passive lysis buffer (Promega) and centrifuged in the 96-well plate (Corning) at 2000x g for 10 minutes to remove debris from the cell lysate. The cleared cell lysates were then transferred to an opaque white 96-well plate (Corning). Luminescence signal was then measured from each well in a BioTek Synergy plate reader immediately following the addition of the Luciferase Assay Reagent (Promega).

Western Blot

HEK 293T cells were cultured in 6-well tissue culture plates (Corning) and grown up to 90% confluency. After treatment, the cells were washed in ice-cold DPBS (Corning) twice and 80 µL of lysis buffer, consisting of 0.5% (w/v) digitonin (pH 7.5) in DPBS, was added to each well. The lysis buffer was supplemented with N-ethylmaleimide (Sigma-Aldrich) at a concentration of 5 µM and phosphatase inhibitor (Sigma-Aldrich) and protease inhibitor cocktails (Thermo Scientific) at dilutions recommended by the manufacturer. The lysates were incubated on ice for 30-45 minutes for complete lysis and harvested using cell scrapers (Grainger). The lysates were then cleared by centrifuging at 13,000g for 15 minutes. The clear, debris-free supernatant was collected for further analysis for each lysate. These represented the whole-cell lysates. Because most cellular β-catenin is bound to the cell membrane and does not participate in Wnt signaling, we next obtained the cytoplasmic and nuclear extract by incubating the supernatant with Concanavalin A-Sepharose 4B beads (GE Healthcare) for 60 minutes at 4°C with continuous end-over-end mixing. The slurry was then centrifuged at 4000x g for 5 minutes. The membrane-associated proteins were bound to the Concanavalin A beads and the supernatant consisted of the cytoplasmic and nuclear fractions of the lysate. The beads were then washed 5 times with DPBS and denatured directly in 2x LDS Sampling Buffer (Thermo Scientific).

Lysates were then resolved using SDS-PAGE Gel Electrophoresis in 3-8% Tris-Acetate gels using an OWL P8DS (Thermo Scientific) gel-running apparatus. The proteins were then transferred using the Trans-Blot Turbo Transfer System (Bio-Rad) to a 0.2 µm pore size nitrocellulose membrane. For immunoblotting, the membrane was blocked in a blocking buffer (1x TBS with 0.1% Tween-20 and 5% non-fat dry milk or BSA; as per antibody manufacturer's recommendation) for 1 hour at room temperature. The membrane was then incubated overnight

at 4°C in primary antibody diluted in 5% BSA in TBST (1x TBS containing 0.1% Tween-20). The dilution used for the primary anti-β-catenin and anti-vinculin antibodies are 1:1000. Next, the membrane was washed three times with TBST for 5 minutes each and then incubated in secondary antibody diluted in the blocking buffer for 1 hour at room temperature with constant mixing. An HRP-conjugated anti-mouse antibody (Jackson Immunoresearch) was used to detect total β-catenin at a dilution of 1:10000. Vinculin was detected using an HRP-conjugated anti-rabbit antibody (Thermo Scientific) at a dilution of 1:3000. The membrane was then imaged in a ChemiDoc MP Imaging system (Bio-Rad) after 1 minute of incubation in the SuperSignal West Femto Maximum Sensitivity Substrate. The detected protein bands were quantified using the analysis tools provided in the Image Lab Software (Bio-Rad) and then validated using ImageJ.

Protein Quantitation by BCA Assay

The bicinchoninic acid (BCA) assay was performed using a kit purchased from Pierce using manufacturer's instructions. In brief, the supplied 2.0 mg/mL BSA standard was serially diluted two-fold in PBS. Next, 25 µL of each sample or standard were pipetted into the wells of a 96-well plate. The provided cupric sulfate solution was then diluted into a provided solution containing BCA at a 1:50 volume ratio. A volume of 200 µL of this reagent was added to each well and the plate was covered with tape and incubated at 37 ºC for 30 minutes. The absorbance at 562 nm was read and a standard curve generated from the BSA dilutions. A linear regression was performed to relate concentration to absorbance and the concentration of the samples was determined by interpolation.

Sample Preparation, Trypsin Digestion, Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS), and Peptide Identification

The proteins in each sample were reduced, alkylated and digested with trypsin according to the FASP protocol[4]. The peptides were analyzed by nano-LC-MS/MS, and peptide identification as previously described[5] with the following modifications. Reverse phase chromatography was performed using an in-house packed column (40 cm long X 75 μm ID X 360 OD, Dr. Maisch GmbH ReproSil-Pur 120 C18-AQ 1.9 μm beads) and a 120 min. gradient. The Raw files were searched using the Mascot algorithm (ver. 2.5.1) against a protein database constructed by combining the sequences of the heavy chain of the Fzd-Fab-SpyTag and the light chain of the LRP6-Fab-SpyCatcher and a contaminant database (cRAP, downloaded 11-21-16 from http://www.thegpm.org) via Proteome Discoverer 2.1. Additional modifications including FSK, FSKR, and FSKRDEDGK, which correspond to expected tryptic digest peptides that may be covalently linked to the aspartic acid residue in the SpyTag, were used and a "no enzyme" search was performed allowing cleavage at any residue. Only peptide spectral matches with expectation value of less than 0.01 ("High Confidence") were used. No cross-linked peptides were identified but spectral count analysis suggested that native peptides associated with the expected cross-link site were diminished. To verify this, the extracted ion current (XIC) for FSK from SpyCatcher and IVMVDAYKPTK from SpyTag was examined and found to be greatly reduced (Suppl. Fig. S3). Other peptides examined (e.g. VFPLAPSSK) generated spectral counts and XICs consistent in the heterodimer with what was observed for the monomeric proteins containing SpyCatcher and SpyTag suggesting that the primary structure was not altered except in the regions expected to be cross-linked by SpyTag-SpyCatcher Chemistry.

Dynamic Light Scattering

Glycerol-free aliquots of 100 µL of purified protein at a concentration of at least 1 mg/mL were placed into cuvettes and inserted into a DynaPro NanoStar device from Wyatt Technologies. The chamber was warmed to 25 ºC and ten acquisitions were recorded for each sample. Data were reported using an isotropic sphere model.

SEC Shifts

A sample of 5 µg of the purified heterodimer was run on a Superdex 200 gel filtration column from GE immediately after cleaning with 0.5 M NaOH and equilibration in PBS at 4 °C. Absorbance at 210 nm was recorded. Next, additional runs was performed with LRP6-Fc (R&D Systems) and Fzd2-Fc (R&D Systems) alone. Finally, 5 µg of the heterodimer were mixed with at least a 2 fold excess of LRP6-Fc or Fzd2-Fc, incubated for 5 minutes at room temperature, and the mixture was run on the column at 4 °C.

SDS-PAGE

After purification, samples of proteins were taken and mixed with 4x NuPAGE lithium dodecyl sulfate sample buffer from Invitrogen in a 3:1 volume ratio. Samples were then heated to 95 ºC for 10 minutes and allowed to return to room temperature. No reducing agent was added. Samples of 20 µL per well were then added to a NuPAGE 4-12% Bis-Tris gel and run at 100 V for 75 minutes at room temperature. The gel was then stained with SimplyBlue Coomassie stain from ThermoFisher, de-stained overnight, and imaged on a Bio-Rad ChemiDoc MP imaging system.

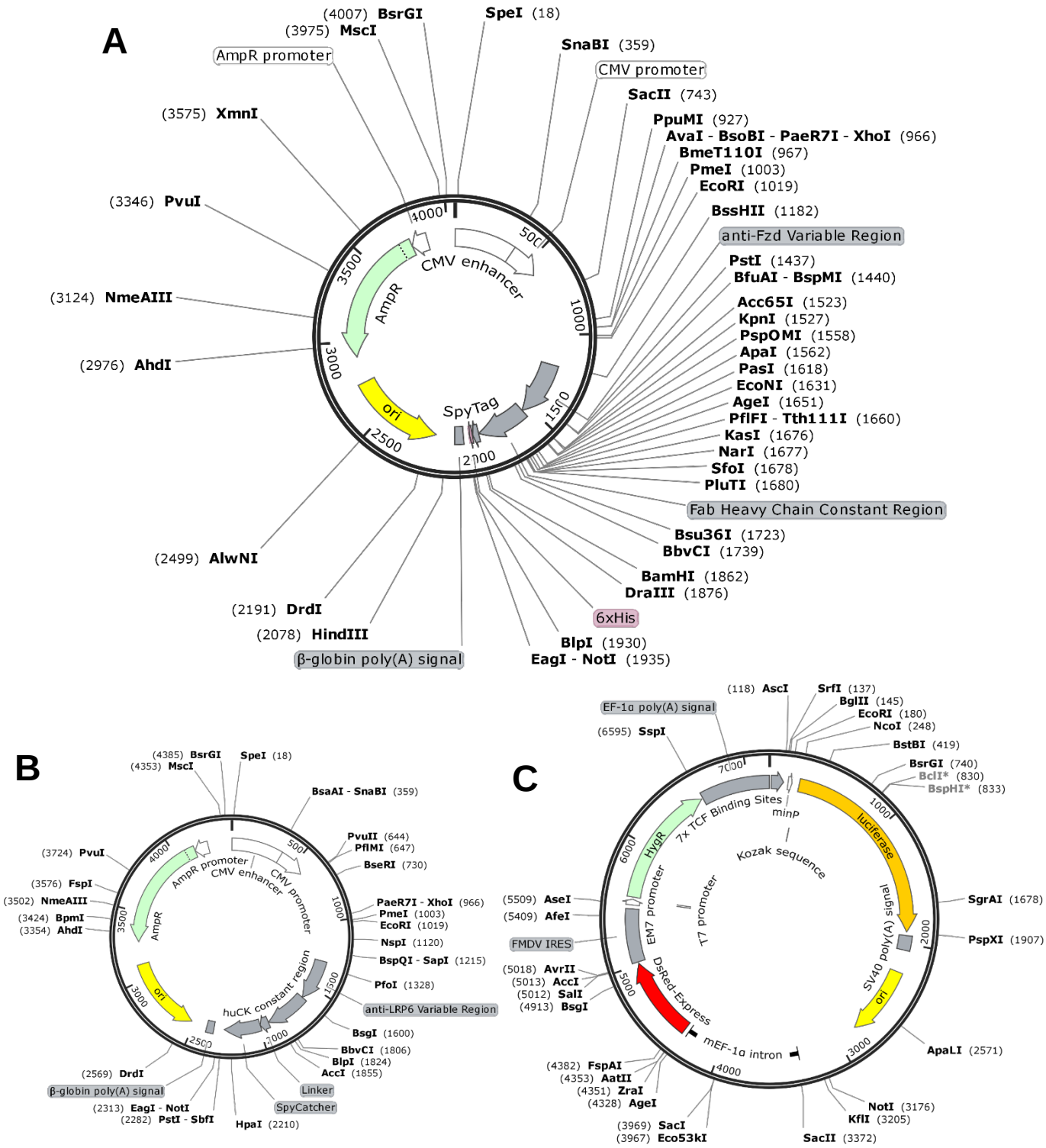

Figure S1. Vector maps for the plasmids used. A) Fzd-Fab-SpyTag in TGEX-FH; B) LRP6-Fab-SpyCatcher in TGEX-LC. C) 7x TCF Luciferase in pVitro2-hygro-DsRed.

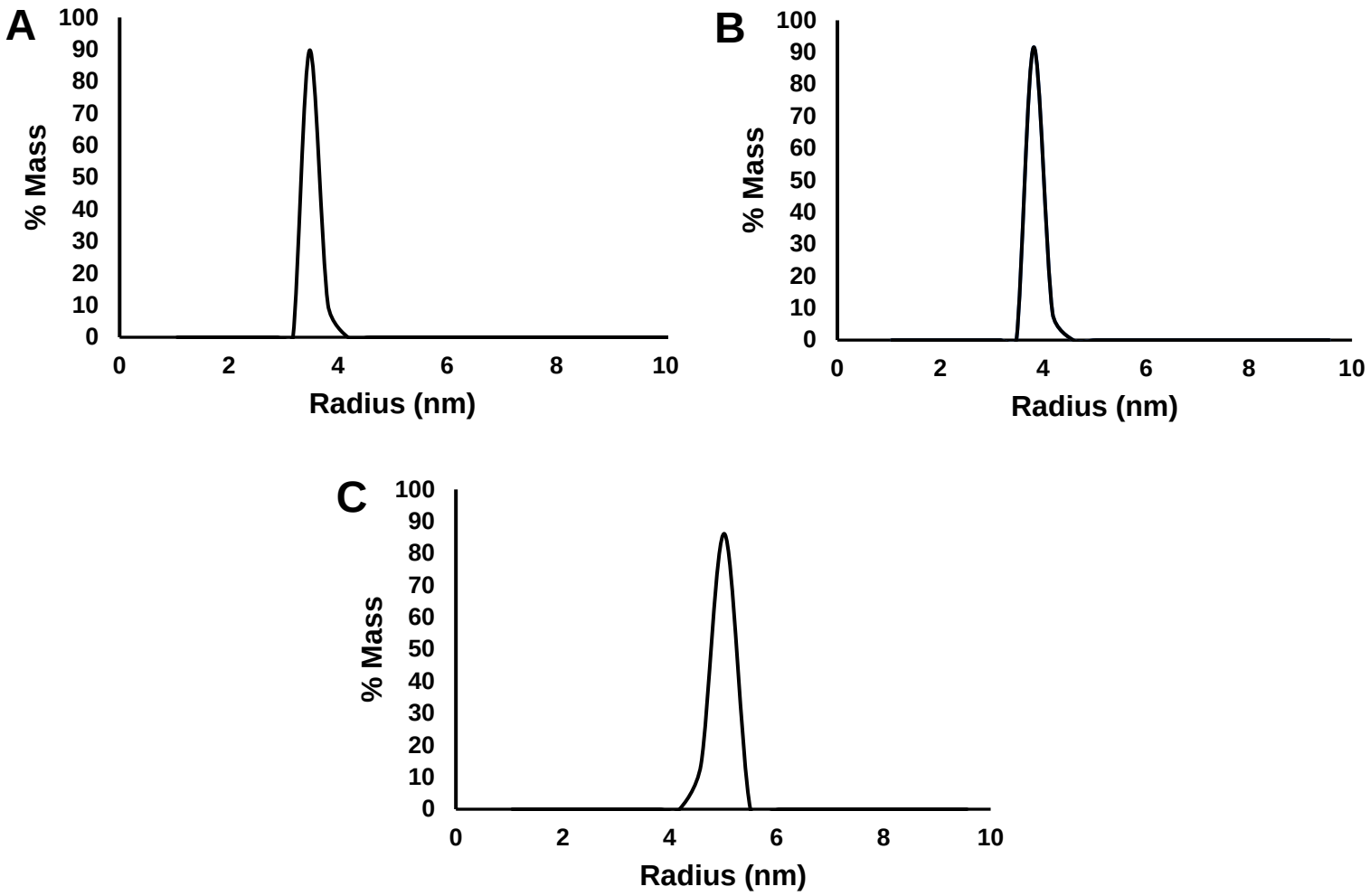

Figure S2. Characterization of Hydrodynamic Radius of Heterodimer and Monomeric Fabs. Dynamic Light Scattering analysis for purified: A) Fzd-Fab-SpyTag; B) LRP6-Fab-SpyCatcher; and C) Heterodimer. The data was generated from ten independent acquisitions using an isotropic sphere model.

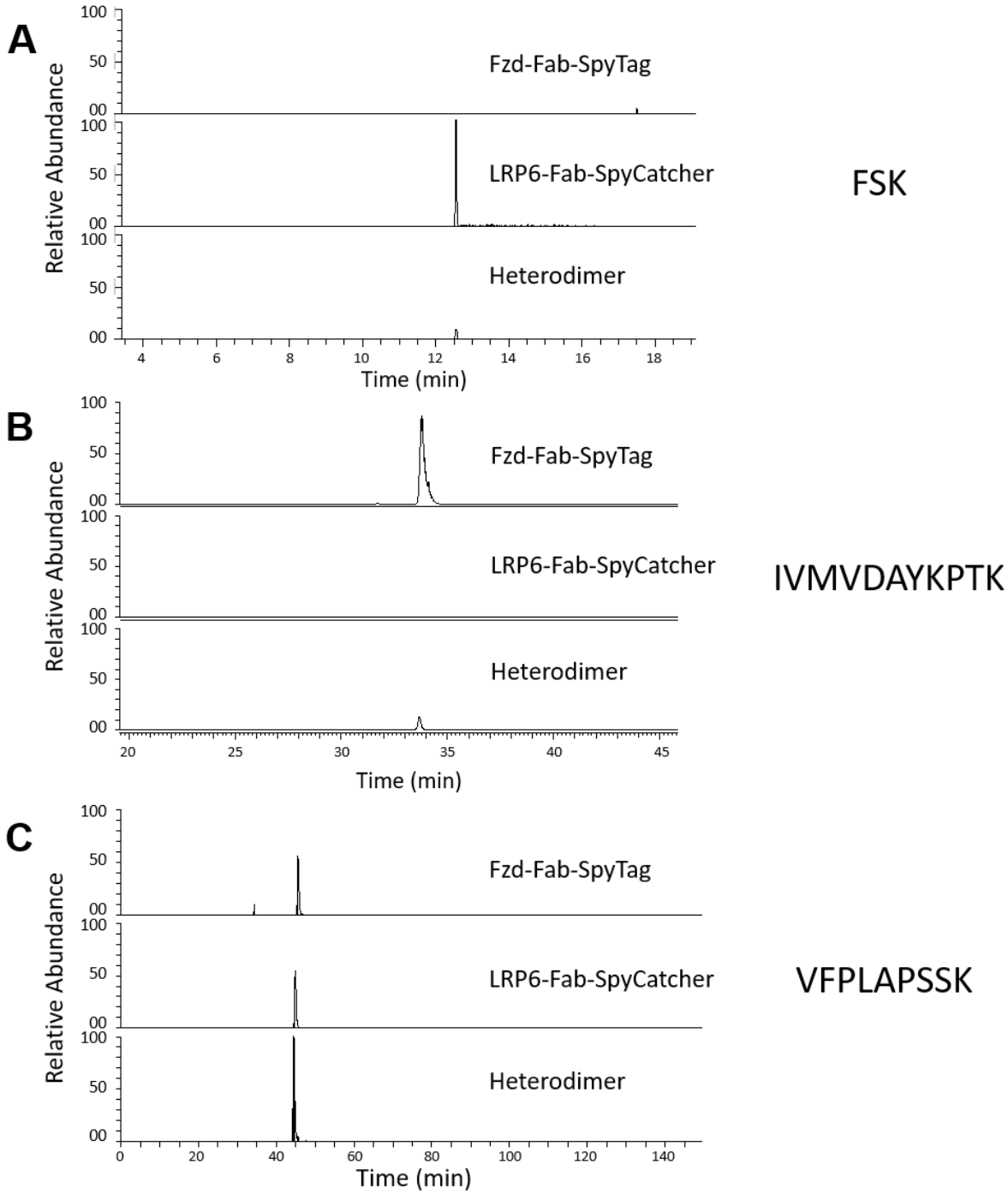

Figure S3. Characterization of Heterodimer Formation Reaction by LC-MS/MS.
Extracted Ion Currents (XIC) for peptides from the tryptic digest of Fzd-Fab-SpyTag, LRP6-Fab-SpyCatcher, and Heterodimer for : A) FSK ([M+H]+), corresponding to a sequence in LRP6-Fab-SpyCatcher that is not expected to be detected in the Heterodimer after isopeptide bond formation; B) Peptide fragment IVMVDAYKPTK ([M+2H]+2), part of the SpyTag sequence that is not expected to be seen in the Heterodimer; and C) Peptide fragment VFPLAPSSK ([M+2H]+2), corresponding to a sequence that is present in both Fzd-Fab-SpyTag, and LRP6-Fab-SpyCatcher and heterodimer formation is not expected to alter its presence. Each set of chromatographs are on the same scale (1.0E6, 2.0E8, and 6.5E7, for A, B, and C, respectively)

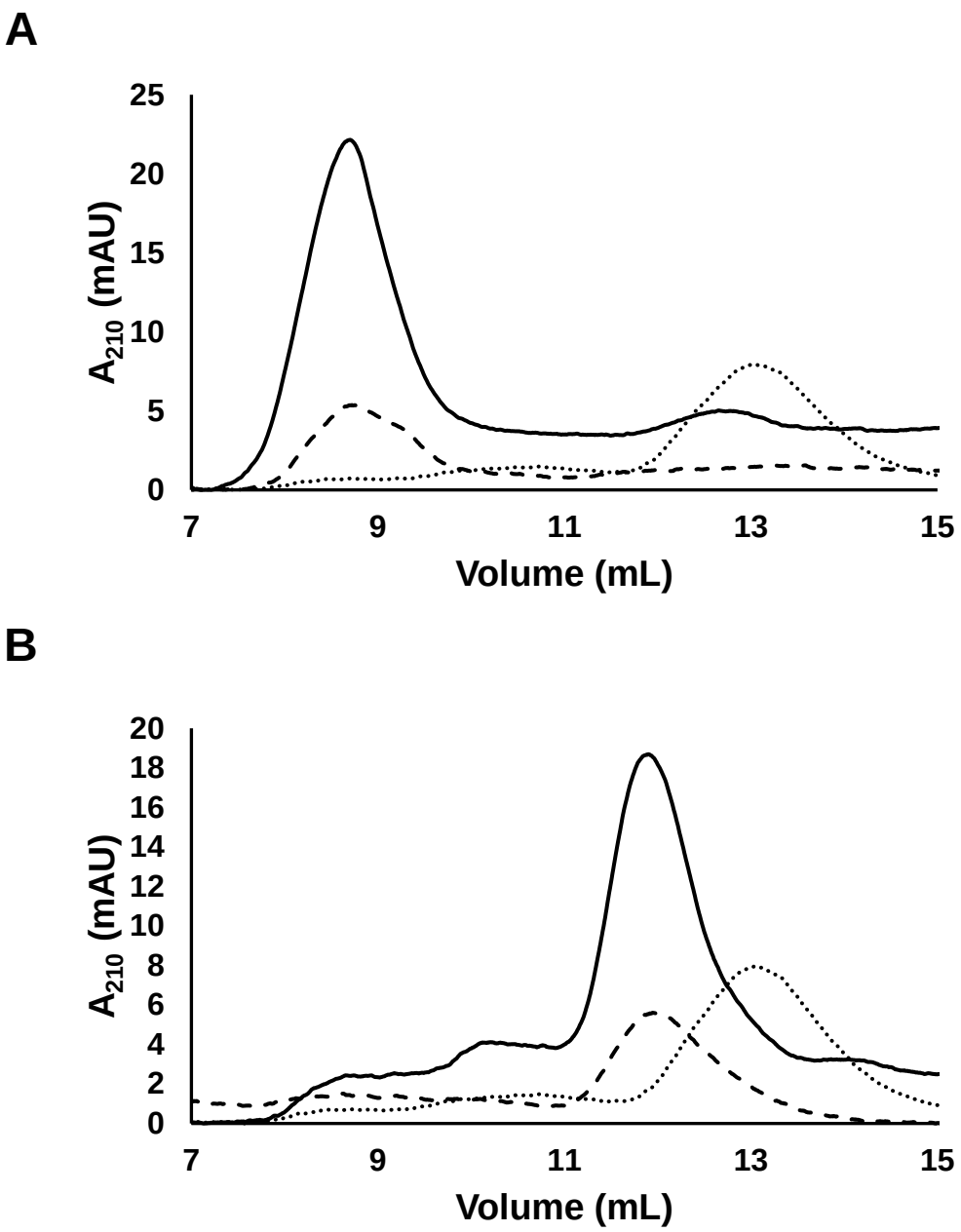

Figure S4. Characterization of Active Fraction of Heterodimer. A) Characterization by size exclusion chromatography of heterodimer alone (dotted line), LRP6-Fc (dashed line), and a mixture of heterodimer and LRP6-Fc (solid line). Analysis of peak areas suggests that 90% of the heterodimer shifts upon incubation with LRP6-Fc. B) Characterization by size exclusion chromatography of heterodimer alone (dotted line), Fzd-Fc (dashed line), and mixture of heterodimer and Fzd-Fc (solid line).

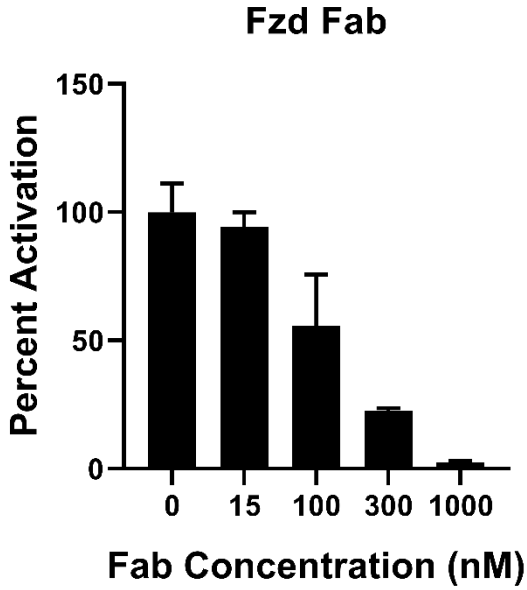

Figure S5. Characterization of Heterodimer Agonist Activity in the Presence of Competing Monomeric Fab. Bar graph compares Wnt-responsive luminescence signals from HEK 293T cells treated with 15 nM of heterodimer and Anti-Fzd Fab at concentrations ranging from 0 to 1000 nM.

References

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