Functional inhibition of β-catenin-mediated Wnt signaling by intracellular VHH antibodies

Laura E Newnham1,*, Michael J Wright1, Gill Holdsworth1, Kostas Kostarelos2, Martyn K Robinson1, Terence H Rabbitts3,#, and Alastair D Lawson1

1UCB; Slough, UK; 2Faculty of Medical and Human Sciences; University of Manchester; Manchester, UK; 3Weatherall Institute of Molecular Medicine; University of Oxford; John Radcliffe Hospital; Oxford, UK

#Previous address: Leeds Institute of Molecular Medicine; University of Leeds; Leeds, UK

Keywords: Wnt, β-catenin, intrabody, VHH, antibody, phage display

Abbreviations: APC, adenomatous polyposis coli; Bcl-9, B-cell CLL/lymphoma 9 protein; CDR, complementarity-determining region; CFU, colony forming unit; CK1, casein kinase 1, ELISA, enzyme-linked immunosorbant assay; FAP, familial adenomatous polyposis; GSK3β, glycogen synthase kinase 3 β, HCAb, heavy chain only antibody; HEK, human embryonic kidney; LRP, Low density lipoprotein receptor related protein; MOI, multiplicity of infection; PBMC, peripheral blood mononuclear cell; scFc, single chain IgG Fc domain; SEM, standard error of the mean; TOPflash, luciferase reporter gene downstream of TCF/Lef binding sites and a minimal promoter; VHH, variable domain of a HCAb

The Wnt signaling pathway is of central importance in embryogenesis, development and adult tissue homeostasis, and dysregulation of this pathway is associated with cancer and other diseases. Despite the developmental and potential therapeutic significance of this pathway, many aspects of Wnt signaling, including the control of the master transcriptional co-activator β-catenin, remain poorly understood. In order to explore this aspect, a diverse immune llama VHH phagemid library was constructed and panned against β-catenin. VHH antibody fragments from the library were expressed intracellularly, and a number of antibodies were shown to possess function-modifying intracellular activity in a luciferase-based Wnt signaling HEK293 reporter bioassay. Further characterization of one such VHH (named LL3) confirmed that it bound endogenous β-catenin, and that it inhibited the Wnt signaling pathway downstream of the destruction complex, while production of a control Ala-substituted complementarity-determining region (CDR)3 mutant demonstrated that the inhibition of β-catenin activity by the parent intracellular antibody was dependent on the specific CDR sequence of the antibody.

Introduction

Most mammalian genomes express 19 known Wnt genes, which encode a family of lipid-modified secreted proteins of approximately 40 kDa in size. Wnt signaling has a central role in adult tissue homeostasis, including control of stem cell proliferation in the gut and the hair follicle cycle, osteoblastogenesis and haematopoietic cell differentiation (for a review see ref 1). In resting cells, β-catenin turnover is regulated by the destruction complex, which contains the tumor suppressor adenomatous polyposis coli (APC), among other proteins. Activation of the canonical Wnt/β-catenin pathway by exogenous Wnt results in the stabilization and activation of β-catenin, which translocates from the cytoplasm into the nucleus where it interacts with numerous partners including the TCF/LEF family. Formation of the bipartite β-catenin/TCF transcription factor2 activates the transcription of Wnt responsive genes3 shown in Figure 1. Studies of β-catenin have revealed it to be a multi-functional protein which also has roles in cell-cell adhesion,4 as a component of the adherens junction, linking E-cadherin to the cell cytoskeleton.5 Structurally, β-catenin contains a series of 12 armadillo repeats, each of which is approximately 40 amino acids, surrounded by unstructured N- and C-terminal domains.6 Protein-protein interaction mapping experiments have demonstrated that all 3 β-catenin domains are involved in both signaling and cytoskeletal interaction.7,8 Over 38 β-catenin interaction partners have been...
Regulation of intracellular Wnt signaling is achieved through the modification of the interaction of β-catenin with its protein partners, generating multiple intracellular pools, phosphorylation states and conformational forms of β-catenin within the cell, as reviewed in Valenta et al.⁹

Mutations affecting the Wnt signaling pathway play a role in many diseases including, but not limited to, bone density disorders,¹⁰-¹² Alzheimer’s disease¹³ and numerous cancers. In fact mutations on this pathway are believed to be present in approximately 20% of all human cancers,¹⁴ with the majority of colorectal cancers bearing a mutation in the Wnt signaling pathway. One of the most frequent mutations is found in the APC gene (reviewed by Bienz and Clevers),¹⁵ causing the inherited condition familial adenomatous polyposis (FAP), which results from the loss of one allele of APC, leading to the formation of a high number of intestinal polyps and an increased predisposition to colorectal cancer. Additionally, mutations of the β-catenin gene have been identified in ovarian cancers,¹⁶ hepatocellular carcinomas,¹⁷ endometrial carcinomas¹⁸ and prostate cancer.¹⁹

Mutations that over-activate the transcription of Wnt/β-catenin target genes are completely dependent on transcriptionally active nuclear β-catenin, hence disrupting the transcriptional activity of β-catenin is of great therapeutic interest. A number of previous studies have identified small molecule²⁰-²⁷ and conventional antibody²⁸ inhibitors of the Wnt signaling pathway, shown in Figure 1.

Intracellular antibodies are antibody or antibody fragments located within a cell, sometimes in a particular cellular compartment, where they interact with their target antigen. Intracellular antibodies have been used in the study of several disease pathways including Ras-expressing tumors,²⁹ Alzheimer’s disease³⁰ and Huntington’s disease.³¹ Intracellular antibodies act in a complementary way to other techniques used to study intracellular protein function; for instance they do not prevent the gene protein product from being formed, like RNA interference, or rely on the expression of a mutated form of protein, but instead disrupt or alter the function of the endogenous protein in its native conformation inside the cell. Intracellular antibodies are of particular use in the study of multi-functional proteins, disrupting just one
function such as DNA binding rather than ablating total protein function. The antigen binding domains of heavy chain only antibodies (HCAbs), referred to as VHHs, are particularly suited for use as intracellular antibodies. VHHs contain ‘hallmark’ substitutions in framework 2, which are proposed to change this predominantly hydrophobic area in a VH where it packs against the VL, into a hydrophilic region in a VHH. This helps to explain the monomeric nature and high solubility of many individual VHH domains, which are desirable characteristics for intracellular antibodies. Here, we have applied VHH intracellular antibodies to the study of β-catenin biology. We generated and characterized VHH intracellular antibodies capable of specific inhibition of β-catenin’s co-activator of transcriptional activity. Such VHHs represent useful tools for the future study of the Wnt signaling pathway, probing novel sites for potential therapeutic intervention.

| Panning Round          | Input titre (cfu) | Output titre (cfu) | β-catenin binders (%) |
|------------------------|-------------------|--------------------|-----------------------|
| Low Wash Round 1       | $6.4 \times 10^{11}$ | $6.3 \times 10^{7}$ | 42.5                  |
| Low Wash Round 2       | $2.5 \times 10^{11}$ | $1.6 \times 10^{8}$ | 85                    |
| Low Wash Round 2 (without β-catenin) | $2.5 \times 10^{11}$ | $1.5 \times 10^{5}$ | —                     |
| High Wash Round 1      | $6.4 \times 10^{11}$ | $3.3 \times 10^{7}$ | 85                    |
| High Wash Round 2      | $1.7 \times 10^{11}$ | $5.5 \times 10^{8}$ | 100                   |
| High Wash Round 2 (without β-catenin) | $1.7 \times 10^{11}$ | $8.0 \times 10^{4}$ | —                     |
| In-solution Round 1    | $6.4 \times 10^{11}$ | $4.2 \times 10^{7}$ | 44                    |

### Results

#### Identification of anti-β-catenin VHHs

Generation of VHHs for use as intracellular antibodies targeted to β-catenin was achieved via immunisation of 2 llamas with purified full-length recombinant human β-catenin. A phage display library of VHH domains was then produced from llama peripheral blood mononuclear cells (PBMCs). The library contained approximately $2 \times 10^{8}$ members as determined by dilution titer, and sequence analysis of 158 individual members demonstrated that 78% contained in-frame VHH domains. Each of the VHH subfamilies previously identified in llamas was present, along with 8% of conventional VH sequences. The percentage of conventional VH sequences was in line with previous reports of some conventional VH sequences being found as HCAbs.

![Figure 2](image-url)
We selected β-catenin-specific VHHs from the immune phage library by panning against biotinylated β-catenin both in-solution and adsorbed directly to immunotubes. When panning β-catenin adsorbed to immunotubes, 2 stringencies of washing were used, termed Low and High wash. After only 2 rounds of phage display, incubation of the library with immunotubes coated with β-catenin showed approximately 1000-fold enrichment in phage binding compared with uncoated tubes, indicating that panning had successfully enriched β-catenin-specific VHHs. A single round of panning was carried out in-solution, in an effort to prevent over-selection and loss of VHH sequence diversity. Ninety-six clones were picked from the output of each of the individual rounds of panning that were undertaken. Monoclonal phage rescues were performed and screened.

Figure 3. Screen for function of VHH intracellular antibodies transiently transfected into HEK293 bioassay cells. Wnt signaling was induced by co-transfection of the Wnt1 gene. (A) Firefly luciferase activity, results are plotted as fold stimulation over empty vector transfected unactivated cells, all conditions performed in triplicate. (B) Renilla luciferase activity, results are plotted as raw signals. Data shows mean and SEM from a representative experiment. (C) Western blots of bioassay cells, cells from replicate bioassay plate wells were resuspended in LDS sample buffer (+DTT), and Western blots were performed with anti-myc antibodies. V refers to empty vector.
by enzyme-linked immunosorbant assay (ELISA) to reveal β-catenin-specific VHHs. Depending on the panning technique and round of panning, the percentage of β-catenin-specific VHHs recorded per screening plate varied from 43 to 100% (Table 1). Only a single binder was observed on an irrelevant protein (streptavidin, data not shown). In total, over 50 different variable regions sequences that bound to β-catenin by ELISA were identified, including representatives from each VHH subfamily described previously. The VHHs were further divided based on complementarity-determining region (CDR3) diversity into 12 different antibody ‘CDR3-families’ and 19 individual sequences that were not similar enough to be defined as family members. In this instance CDR3 families were defined as those containing 90% sequence identity in CDR3. In order to visualize the diversity of the VHH panel produced, we performed a principal components analysis (PCA) on a concatenated sequence of all 3 CDRs of each VHH. For the PCA 3 sequence variables were generated for each CDR residue representing size, electronic nature and hydrophobicity of the amino acid found there. The PCA then maps these sequence variables in a linear manner so that the complexity of the data is reduced but the variance between the data is maximized and described by principal components (PC) 1 and 2. The outcome of this analysis is shown in Figure 2 as a 2-dimensional (2-D) data plot. The distance between 2 points in Figure 2 is directly proportional to CDR sequence identity. Antibodies of identical CDR sequences resulted in the co-localization of data points on the 2-D plot. The wide spread of data points in Figure 2 shows the broad diversity of VHH antibody sequences identified.

**Functional inhibition of β-catenin activity**

All of the β-catenin binding VHHs were sub-cloned into a mammalian intracellular expression vector containing a myc-HIS tag, and screened for functional modification of β-catenin using a HEK293 bioassay, which permits measurement of Wnt-induced expression of the firefly luciferase reporter. To control for off-target effects of intracellular antibody expression, a vector constitutively expressing Renilla luciferase was co-transfected such that antibodies of interest would be expected to inhibit firefly luciferase expression, but not Renilla luciferase. Western blotting on parallel samples was used to confirm VHH expression levels (Fig. 3). Co-transfection of a Wnt1 expression plasmid and the anti-β-catenin VHHs demonstrated that these intracellular antibodies were generally well tolerated in the HEK293 reporter bioassay and gave firefly and Renilla luciferase activity signals that were comparable to the empty vector control (Fig. 3), indicating that they were neither toxic nor had a functional effect on β-catenin. In contrast, 3 intracellular antibodies (9, 16 and 17) demonstrated potential cellular toxicity since they produced marked decreases in firefly and Renilla luciferase activities.

In total, 4 active intracellular VHH antibodies (LL3, 12, 14, 15) specifically inhibited the Wnt-induced firefly luciferase signal, but not the constitutive Renilla luciferase signal compared to the vector only control. Expression of these antibodies was confirmed by Western blotting (Fig. 3). Intracellular antibody LL3 was selected for further characterization based on analysis of multiple factors including expression levels of VHH fragments, sequence diversity and amenability to CDR3 mutagenesis for retention of molecular integrity and ablation of specific binding. Only VHH LL3 fulfilled each of these criteria.

To confirm that the inhibition of Wnt signaling mediated by LL3 was a direct result of binding to β-catenin, Ala mutations...
were introduced to CDR3 to create the non-binding control antibody, LL3.CDRAAA (Fig. 4A). The binding properties of LL3.CDRAAA and the parent antibody, LL3, were compared as VHH fragments with single chain Fc (scFc) tags. LL3-scFc binding to β-catenin was confirmed by ELISA, while LL3.CDRAAA-scFc demonstrated no detectable specific binding to β-catenin when tested by ELISA (Fig. 4B). The affinity of the parent VHH, LL3-scFc, for β-catenin was determined by SPR to be 4nM.

The LL3.CDRAAA mutant was expressed at a similar level to the parent LL3 antibody in HEK293 reporter bioassay cells (Fig. 4C). To control for general variations in luciferase production caused by factors such as transfection variability and reduced cell viability, the Wnt-driven firefly luciferase signal was divided by the constitutive Renilla luciferase signal. Comparison of LL3 to LL3.CDRAAA demonstrated that inhibition of Wnt signaling was dependent on binding of LL3 to β-catenin (Fig. 4D), and no inhibition of β-catenin dependent firefly luciferase production or the Renilla luciferase signal occurred when LL3.CDRAAA was transfected into HEK293 reporter bioassay cells.

Characterization of anti-β-catenin intracellular antibody LL3

Intracellular antibody LL3 was further analyzed using LL3.CDRAAA as a control. In addition to stimulation by Wnt transfection, the HEK293 reporter bioassay was stimulated using Wnt3a conditioned media, or the GSK3β inhibitor LiCl2, which acts downstream of the cell-surface Wnt-Frizzled interaction. LL3 continued to show significant inhibition of Wnt signaling when the HEK293 reporter bioassay cells were stimulated with each of these methods (Fig. 5). The ability of LL3 to bind endogenous cellular β-catenin was confirmed in immunoprecipitation experiments where LL3 and LL3.CDRAAA with scFc tags were used to immunoprecipitate β-catenin from lysed HEK293 cells (Fig. 6). β-Catenin was detected from samples immunoprecipitated using LL3, but not LL3.CDRAAA.

To compare the relative expression levels of β-catenin and intracellular antibody LL3, we performed quantitative Western blots on lysed HEK293 reporter bioassay cells. From the blots, it was estimated that HEK293 cells contained approximately $3.0 \times 10^8$ molecules of intracellular antibody LL3, and approximately $9.5 \times 10^7$ molecules of β-catenin per cell. The figure for β-catenin was comparable with other reports of $5.4 \times 10^7$ molecules of β-catenin per HEK293 cell. Based on these figures, intracellular antibody LL3 was estimated to be in a large excess over β-catenin, in the region of 20,000 molecules of LL3 per β-catenin molecule.

β-Catenin is a multi-functional protein that is found in 3 distinct cellular compartments: the plasma membrane, the cytoplasm and nucleus. The effects of LL3 binding on the cellular localization of β-catenin were investigated via confocal microscopy using the myc-tag present on the intracellular antibody. β-Catenin was predominantly localized at the plasma membrane, where it is known to form part of the adherence junction. Expression of intracellular antibody LL3 appeared to be ubiquitous across the cell. The binding of LL3 continued to show significant inhibition of Wnt signaling when the HEK293 reporter bioassay cells were stimulated with each of these methods (Fig. 5). The ability of LL3 to bind endogenous cellular β-catenin was confirmed in immunoprecipitation experiments where LL3 and LL3.CDRAAA with scFc tags were used to immunoprecipitate β-catenin from lysed HEK293 cells (Fig. 6). β-Catenin was detected from samples immunoprecipitated using LL3, but not LL3.CDRAAA.

To compare the relative expression levels of β-catenin and intracellular antibody LL3, we performed quantitative Western blots on lysed HEK293 reporter bioassay cells. From the blots, it was estimated that HEK293 cells contained approximately $3.0 \times 10^8$ molecules of intracellular antibody LL3, and approximately $9.5 \times 10^7$ molecules of β-catenin per cell. The figure for β-catenin was comparable with other reports of $5.4 \times 10^7$ molecules of β-catenin per HEK293 cell. Based on these figures, intracellular antibody LL3 was estimated to be in a large excess over β-catenin, in the region of 20,000 molecules of LL3 per β-catenin molecule.

β-Catenin is a multi-functional protein that is found in 3 distinct cellular compartments: the plasma membrane, the cytoplasm and nucleus. The effects of LL3 binding on the cellular localization of β-catenin were investigated via confocal microscopy using the myc-tag present on the intracellular antibody. β-Catenin was predominantly localized at the plasma membrane, where it is known to form part of the adherence junction. Expression of intracellular antibody LL3 appeared to be ubiquitous across the cell and did not affect the localization of β-catenin at the plasma membrane, as seen by the yellow rings of β-catenin and LL3 co-localization at the plasma membrane (Fig. 7). These data suggest that inhibition of β-catenin’s function as a transcriptional co-activator by intracellular antibody LL3 did not affect the role of β-catenin at the plasma membrane. No discernible difference was detected in nuclear β-catenin levels in cells with or without the presence of LL3.
without intracellular antibody LL3 expression, which probably reflects the unstimulated nature of these cells, since Wnt signaling had not been activated during these experiments. The images captured by confocal microscopy demonstrate the difference in VHH protein expression levels between individual cells. It is clear that not all the cells in a sample became successfully transfected with VHH encoding DNA, and this may be why complete inhibition of β-catenin signaling was not achieved by intracellular antibody LL3.

β-Catenin contains many structural motifs and protein interaction domains. In order to identify which of these were involved in the binding of LL3 to β-catenin, a number of His-tagged-

---

**Figure 7.** Confocal microscopy of anti-β-catenin and intracellular antibody stained HEK293 bioassay cells. Fixed HEK293 bioassay cells were stained for intracellular antibody and β-catenin expression; the nuclear stain DAPI was also included. Anti-β-catenin and anti-intracellular antibody images were merged, and yellow donates co-localization. Pictures were taken using Leica TCS SP5 microscope, with 40× magnification.
truncated forms of β-catenin (Table 2) were produced. On a reducing Western blot, LL3 bound to truncations 2 and 4 as well as to full-length β-catenin (Fig. 8). The binding pattern indicated that the binding domain of LL3 resided between amino acid residues 164–390 corresponding to the first 6 armadillo repeats of β-catenin and was not a conformation-dependent epitope.

**Discussions**

To modify intracellular function, an intracellular antibody must be stably expressed inside a cell and must retain the ability to engage the target antigen with sufficient affinity to exert a functional effect. We identified the β-catenin-specific VHH, LL3, which was characterized in depth and demonstrated these attributes. LL3 exhibited specific function modifying activity when tested in the HEK293 Wnt-induced luciferase reporter bioassay, and while the stability of LL3 in the cell was not directly measured, the specific activity, expression and diffuse cellular staining indicated that the VHH fragment was stable and did not form visible aggregates inside the cell. These observations validated the selection of llama VHH domains for use as intracellular antibodies in this study and were in accordance with previous reports of VHHs as intracellular antibodies.\(^{39-44}\) Mutation of 3 residues in CDR3 of LL3 was sufficient to disrupt antigen binding; this was in line with another intracellular domain antibody study where mutation of 4 residues in CDR1 was sufficient to disrupt intracellular function.\(^{29}\)

A number of studies have investigated the effect of intracellular antibody pI on intracellular aggregation,\(^{45}\) and concluded that an overall negative charge at pH 7.4 was beneficial to intracellular antibody solubility. Consistent with this work the intracellular antibody LL3 studied here was predicted to have a pI value of 6.7.

Intracellular antibody LL3 showed functional inhibition of β-catenin co-transcriptional activity in the HEK293 bioassay stimulated with Wnt1, Wnt3a, and LiCl\(_2\). Wnt3a and Wnt1 are both thought to signal in a similar canonical fashion, but may use different sets of Frizzled co-receptors.\(^{46}\) LiCl\(_2\) is a potent inhibitor of GSK3\(_{\beta}\), which prevents β-catenin phosphorylation, hence acting as a stimulator of canonical Wnt signaling. The fact that LL3 could inhibit LiCl\(_2\) induction of firefly luciferase signal indicates that the interaction of intracellular antibody LL3 and β-catenin modulates signaling downstream of the GSK3\(_{\beta}\) phosphorylation of β-catenin, and thus downstream of the destruction complex (Fig. 1).

The epitope on β-catenin recognized by LL3 was shown to lay between residues 164 to 390. This is a broad region, and the actual LL3 binding epitope is likely to comprise considerably fewer residues. However, it is of interest that the 164–390 region coincides with the binding epitopes of Axin,\(^{47}\) Pontin\(^{52}\), Bcl\(_9\),\(^{48}\) E-cadherin,\(^{50}\) and TCF,\(^{51,52}\) and, consequently, LL3 may work by interfering with one or more of these β-catenin interactions. LL3 inhibited β-catenin transcriptional co-activation activity; hence, it is most likely that LL3 disrupts the interaction of β-catenin with a transcriptional enhancer such as TCF, Bcl\(_9\) or Pontin\(^{52}\). The potential sites of other molecules intervention in the Wnt signaling pathway are shown schematically in Figure 1. LL3 may inhibit a similar axis to small molecule compounds PKF 115–854 and CGP049090,\(^{53}\) in disrupting the β-catenin/TCF complex, or as carnosic acid inhibiting the interaction of β-catenin and Bcl\(_9\),\(^{21}\) or alternatively present a novel inhibitory axis between β-catenin and Pontin\(^{52}\).

### Table 2. Produced β-catenin fragments

| Fragment description                  | Amino acids | Primer sequences used in construction                                                                 | MW (kDa) |
|--------------------------------------|-------------|-------------------------------------------------------------------------------------------------------|----------|
| F1. N-terminus and first α helix     | 1–163       | GTACAGGCCATAGGATCCATATCAGGATTCGAGCGG                                                                 | 22.0     |
| F2. N-terminus and first 6 armadillo repeats | 1–390       | GTCACAGGCCATAGGATCCATATCAGGATTCGAGCGG                                                                 | 46.7     |
| F3. C-terminus and last 6 armadillo repeats | 391–781     | GTCACAGGCCATAGGATCCATATCAGGATTCGAGCGG                                                                 | 46.8     |
| F4. All 12 armadillo repeats and C-terminus | 138–781     | GTCACAGGCCATAGGATCCATATCAGGATTCGAGCGG                                                                 | 74       |
| F5. Full length                      | 1–781       | GTCACAGGCCATAGGATCCATATCAGGATTCGAGCGG                                                                 | 89.5     |

---

**Figure 8.** Western blots of small scale β-catenin fragment protein inductions. (A) Anti-His blot. (B) LL3 blot. F refers to fragment; the molecular weights for which are given in Table 2.
Although disruption of the β-catenin-Axin interaction cannot be ruled out, disruption of this axis would be expected to increase β-catenin transcriptional co-activation activity, rather than inhibit it when tested by the HEK293 reporter bioassay. Additionally disruption of the β-catenin-E-cadherin interaction would be expected to have modulated β-catenin accumulation at the plasma membrane, which was not witnessed by confocal microscopy of LL3 transfected cells.

Further study of the Wnt signaling pathway intracellular antibodies, such as LL3, may aid the discovery of drugs to target this pathway. The use of antibodies to guide small molecule drug discovery was recently reviewed. Analysis of the LL3-β-catenin complex crystal structure could identify biologically relevant conformations of β-catenin and opportunities for small molecule blockade of Wnt signaling.

In summary, we have described a novel VHH fragment that functions inside the cell to disrupt the transcriptional activating activity of β-catenin. Such well-tolerated tools will enable further study of the Wnt signaling pathway, with potential to validate sites for therapeutic intervention in multiple diseases.

**Materials and Methods**

**Generation of full length human β-catenin**

The full-length human β-catenin cDNA clone was obtained from Origene. β-Catenin was expressed and purified essentially as described.

**Generation of immune VHH phage library**

Two male llamas were immunised over 3 months with 5 doses of 0.25mg recombinant β-catenin in Freund’s adjuvant. PBMCs were collected from these animals and RNA purified using RNeasy kit (QiaGen). VH genes were amplified using primers ‘lлимmlibfor’ (SAGGTGCAGCTGGTRGAGTCTGGGGGAG) and ‘CH2lg primer’ by PCR following RT of purified RNA using oligo(dT) primers. VHH genes were specifically distinguished from the resulting PCR product based on size, re-amplified using primer ‘FR1for’ with ‘FR4rev’ and cloned into pASTT. The ligated product was electroporated into XL-1 Blue E. coli (Stratagene), and the library was estimated to contain 2 × 10^8 members. Phage particles were prepared essentially as described.

**Phage panning**

In the first panning experiment, the immune library was incubated in β-catenin-conjugated immunotubes blocked in 3% bovine serum albumin (BSA). Two separate schemes of washing were then performed: a Low wash protocol (5 washes in round 1 and 10 washes in round 2) and a High wash protocol (20 washes in round 1 and 40 washes in round 2), prior to elution of phage with 0.1 M HCl. In the second round of panning, input phage from both wash strategies were panned against both β-catenin conjugated and unconjugated immunotubes. One round of panning was also completed on antigen in solution. Recombinant biotinylated β-catenin was incubated (rolling) with 6 × 10^11 library phage particles, previously blocked in phosphate-buffered saline (PBS) containing 2.5% milk. β-Catenin and associated phage were then captured out of solution using M280 streptavidin-conjugated Dynabeads® (Invitrogen). The beads were washed by magnetic capture 4 times, and bound phage were eluted as above. Small scale phage rescues were performed for individual E. coli colonies picked from output phage infected colonies from each round of all panning conditions. Each sample was tested for binding to β-catenin or streptavidin by ELISA (further detailed below). A positive ‘hit’ was determined to be any sample with an antigen binding signal greater than 3 times the negative signal.

**Bioassay screening**

HEK293 cells stably transected with Tcf-firefly Luciferase reporter construct (as described by Holdsworth et al.) were grown in DMEM (Gibco® Life Technologies) supplemented with 10% fetal bovine serum, 2 mM glutamine and non-essential amino acids (Gibco® Life Technologies). Cells were seeded at 5 × 10^6/well in white poly-D-lysine coated 96-well plates and were transfected with expression plasmids (including the Renilla Luciferase pGL4.74 plasmid from Promega) using Lipofectamine 2000 (Gibco® Life Technologies) according to the manufacturer’s instructions. Plates were incubated for 40 hours at 37°C in 5% CO2. When required Wnt3a conditioned media or LiCl2 were added, 18 hours post transfection. Following the incubation, firefly and Renilla luciferase activity were measured using Dual-Glo® (Promega) as manufacturer’s instructions. Luminescence was recorded using a Biotek Synergy plate reader.

**ELISAs**

ELISA plates were coated overnight at 4°C. Washes were performed between each step of the assay, and consisted of 4 washes in PBS (containing 0.1% Tween20). All plates were blocked in PBS containing 3% BSA, and all samples were blocked in PBS containing 2.5% milk, for at least an hour prior to addition of screening samples to ELISA plates. Following the final wash, TMB (Calbiochem) was added, and the OD of plates was read at 630 and 490nm, with ΔOD recorded using a Biotek Synergy plate reader.

**β-Catenin binding ELISA**

ELISA plates were coated with streptavidin (2 μg/mL). Biotinylated β-catenin (1 μg/mL) was added in PBS, for 30 minutes. Blocked samples were added (phage samples or transient VHH-scFc samples). After an hour, bound samples were detected using a suitable antibody at 1 in 5,000 dilution in PBS (containing 3% BSA). Detection antibodies were goat anti-M13-HRP (GE Healthcare) or goat anti-mouse Fc HRP (Jackson ImmunoResearch).

**Streptavidin binding ELISA**

ELISA plates were coated with streptavidin (2 μg/mL). Blocked samples were added. After an hour bound samples were detected using a suitable antibody at 1 in 5,000 dilution in PBS (containing 3% BSA).
Confocal microscopy

HEK293 cells transfected with Myc-His tagged VHH constructs were attached to poly-D-lysine coated 8 well culture microscope slides in complete media. The cells were fixed in 4% paraformaldehyde and blocked/permeabilised in TBS block (containing 0.3% Triton 100, 1% BSA and 5% goat serum) for 1 hour. Anti-myc (9E10) Alexa Fluor® 488 (Gentaur), and anti-β-catenin (6B3) (Cell Signalling Technologies) were then added and incubated overnight at 4°C in a moist chamber. The anti-β-catenin detection antibody, anti-rabbit Alexa Fluor® 647 (Jackson ImmunoResearch), was added at a 1 in 1000 dilution, and incubated for 1 hour in the dark. Finally DAPI ProLong® Gold Antifade (Invitrogen) was added with a coverslip. Images were captured using a Leica TCS SP5 microscope.

VHH-scFc protein production

HEK293 cells were transfected with VHH-scFc expression vectors using 293fectin™ (Invitrogen), in accordance with the manufacturer’s instructions. The single chain mouse IgG Fc constructs comprised VHH, hinge, CH2 and CH3 domains, linked to another hinge, CH2, CH3 sequence through a 59 amino acid linker (GGSSTAGSGSGGAGGGSTTAGGSASGSGTGSGTGGASGASG). This construct had previously been shown to aid extracellular expression, purification and orientated immobilisation, and as a tag for VHHs. Transfected HEK293 cells were incubated at 37°C, with 5% CO2 on a shaking platform for 5–6 d. The cells were then harvested from the cultures by centrifugation at 423 g, and the VHH-scFc containing supernatant was stored at 4°C.

Immunoprecipitation

HEK293 bioassay cells, 2 × 10⁶, were washed in ice-cold PBS, and resuspended in 200 µL of ice-cold lysis buffer (including protease inhibitor cocktail). The samples were then rolled at 4°C for 30 minutes, followed by centrifugation for 15 minutes at 10,000 × g at 4°C. The HEK293 cell lysate supernatant was then removed into a fresh chilled microfuge tube. VHH-scFcs were conjugated to sheep anti-mouse Fc Dynabeads® (Invitrogen) as manufacturer’s instructions. The beads were then each added to 500 µL of HEK293 cell lysate and rolled at 4°C overnight. Each set of beads was magnetically captured out of solution and individually washed 3 times in fresh cell lysis buffer. Protein was then eluted from the beads by boiling in LDS sample buffer including dithiothreitol (DTT) (Invitrogen) for 5 minutes. Sam- ples were then analyzed by anti-myc and anti-rabbit Alexa Fluor® 647 (Gentaur) and anti-β-catenin (6B3) (Cell Signalling Technologies) and rabbit anti-penta HIS (Bethyl laboratories) followed by streptavidin HRP (Jackson ImmunoResearch) or anti-rabbit Fc HRP (Jackson ImmunoResearch) respectively for the detection of β-catenin. All membranes were developed with Pico Super Signal ECL (Pierce) for 5 minutes, and images captured using ImageQuant LAS 4000 (GE Healthcare) and densitometry of resulting bands analyzed by ImageQuant TL analysis software.

β-catenin fragment generation

BL21 Star™ DE3 cells (Invitrogen) expressing each of the β-catenin constructs (shown in Table 2) were grown in antibiotic selective 2xTY media supplemented with IPTG (final concentration of 0.3 mM), shaking overnight. Cells from the overnight culture were harvested by centrifugation at 4,000 × g for 5 minutes. The pelleted cells were lysed with 1× Bugbuster® (Novagen) containing Lysonase (Novagen) in 20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, 2 mM DTT) by rolling at room temperature for 20 minutes.

Disclosure of Potential Conflicts of Interest

Some of the authors hold shares and share options in UCB Pharma.

Acknowledgment

We would like to thank James Snowdon for his help on variable region diversity assessment and visualization.

Funding

This work was partially funded by the European Union FP7 ANTICARB program (HEALTH-2007-201587).

References

1. Clevers H. Wnt/β-catenin signaling in development and disease. Cell 2006; 127:468-90; PMID:17081971; http://dx.doi.org/10.1016/j.cell.2006.10.018
2. Daniels DL, Weis WI. Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. Nat Struct Mol Biol 2005; 12:364-71.
3. Shitashige M, Hirohashi S, Yamada T. Wnt signaling inside the nucleus. Cancer Sci 2008; 99:631-7; PMID:18177486; http://dx.doi.org/10.1111/j.1349-7006.2007.00716.x
4. Ozawa M, Barthault H, Kemler R. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. EMBO J 1989; 8:1711-7; PMID:2788974
5. Ben-Ze’ev A, Geiger B. Differential molecular interactions of beta-catenin and plakoglobin in adhesion, signaling and cancer. Curr Opin Cell Biol 1998; 10:629-59; PMID:9818174
6. Xing Y, Takeyama K, Liu J, Berndt JD, Zheng JJ, Moon RT, Xu W. Crystal structure of a full-length beta-catenin. Structure 2008; 16:478-87; PMID:18334222; http://dx.doi.org/10.1016/j.str.2007.12.021
7. Abele H, Schwartz H, Hoschuetzky H, Kemler R. Single amino acid substitutions in proteins of the armadillo gene family abolish their binding to alpha-catenin. J Biol Chem 1996; 271:1520-6; PMID:8576147
8. Hecht A, Litterst CM, Huber O, Kemler R. Functional characterization of multiple transactivating elements in beta-catenin, some of which interact with the TATA-binding protein in vitro. J Biol Chem 1999; 274:18017-25; PMID:10364252
9. Valenta T, Hausmann G, Baiker K. The many faces and functions of beta-catenin. EMBO J 2012; 31:2714-36; PMID:22617422; http://dx.doi.org/10.1038/emboj.2012.150

10. Bhaumik W, Mulling M, Patel N, Van Hal E, Olson P, Diogemgi M, Lacca C, Wuyn W, Van Den Eede J, Willem P, et al. Increased bone density in osteosclerosis is due to the deficiency of a novel secreted protein (SOST). Hum Mol Genet 2001; 10:537-43; PMID:11481857; http://dx.doi.org/10.1093/hmg/10.5.537

11. Gong Y, Lee RB, Fukui N, Rawadi G, Roman-Roman S, Reginato AM, Wang H, Cundy T, Gliozzi F, Lei D, et al. LDL receptor-related protein 5 (LRP5) affects bone accrual and body development. Cell 2001; 107:513-23; PMID:11719191; http://dx.doi.org/10.1016/S0092-8674(01)00571-2

12. Zheng HF, Tobias JH, Duncan E, Evans DM, Eriksson A, Galli M, et al. Wnt signaling pathway for mammalian cells. PLoS One 2012; 7:e51882; PMID:22363795; http://dx.doi.org/10.1371/journal.pone.0051882

13. Doyle PJ, Sade H, Hermans G, Gleddie SG, Hussack G, Arbahi-Gahroudi M, Seguin C, Savard ME, Mackenzie CR, Hall JC. Intracellular expression of a single domain antibody reduces cytotoxicity of 15-acycloxyxacin in yeast. J Biol Chem 2009; 284:55929-33; PMID:19783651; http://dx.doi.org/10.1074/jbc.M109.045047

14. Guergueva D, Li S, Walsh N, Mukerji A, Tanha J, Pandey S. Identification of single-domain, box-specific intrabodies that counter resist HBV to major variants raised against oxidative-stress-induced apoptosis. FASEB J 2006; 20:2626-8; PMID:17060401; http://dx.doi.org/10.1096/fj.06-36060e

15. Jeong SA, Jarman C, Teh MM, Holmborg N, Blake C, Verheyen ME. Immunomodulation of enzyme function in plants by single-domain antibody fragments. Nat Biotechnol 2003; 21:77-80; PMID:12485224

16. Angerer KL, Van HH, Verbrugghe P, Louwer-Seels G, Vanlandschoot P, llama-derived single-domain intrabodies inhibit secretion of hepatitis B viruses in mice. Hepatology 2009; 49:39-49; PMID:19085971; http://dx.doi.org/10.1002/hep.22607

17. Summanen M, Granqvist C, Tuominen RK, Vieripanta M, Verrips CT, Boonstra J, Blanchetor C, Ekoenski E. Kinetics of PKC epsilon activating and inhibiting llama single chain antibodies and their effect on PKC epsilon translocation in HeLa cells. PLoS One 2012; 7:e35930; PMID:22536418; http://dx.doi.org/10.1371/journal.pone.0035630

18. Verrecyse T, Pardon E, Vanstreels E, Steyaert J, Daelemans D. An intrabody based on a llama single domain antibody targeting the N-terminal alpha-helical intracytoplasmic domain of beta-catenin. Protein Eng Des Sel 2010; 23:489-98; PMID:20378699; http://dx.doi.org/10.1016/j.prepi.2009.12.002

19. Xing Y, Clements WK, Kimelman D, Xu W. Crystal structure of a beta-catenin/axin complex suggests a mechanism for the beta-catenin destruction complex. Genes Dev 2003; 17:2753-64; PMID:14600052; http://dx.doi.org/10.1101/gad.1142603

20. Bauer A, Huber O, Kembrer P, Pinzini M, an interaction partner of beta-catenin, binds to the TATA box binding protein. Proc Natl Acad Sci U S A 1998; 95:14787-92; PMID:9843967; http://dx.doi.org/10.1073/pnas.95.25.14787

21. Summanen M, Granqvist C, Kimelman D, Xu W. Crystal structure of a beta-catenin/axin complex suggests a mechanism for the beta-catenin destruction complex. Genes Dev 2003; 17:2753-64; PMID:14600052; http://dx.doi.org/10.1101/gad.1142603

22. Huber AH, Wisi W. The structure of the beta-catenin protein and the complex and the molecular basis of diverse ligand recognition by beta-catenin. Cell 2001; 105:391-402; PMID:11348595; http://dx.doi.org/10.1016/S0092-8674(01)00330-0

23. Gipsin T, Dahlberg C, Cho US, Hindi TR, Kimelman D, Xu W. Tcf4 can specifically recognize beta-catenin using alternative conformational structures. Nat Struct Biol 2001; 8:1048-52; PMID:11713475

24. Graham TA, Weaver C, Mao F, Kimelman D, Xu W. Crystal structure of a beta-catenin/Tcf complex. Cell
2000; 103:885-96; PMID:11136974; http://dx.doi.org/10.1016/S0092-8674(00)00192-6

53. Lawson AD. Antibody-enabled small-molecule drug discovery. Nat Rev Drug Disc 2012; 11:519-25; PMID:22743979; http://dx.doi.org/10.1038/nrd3756

54. Muyldermans S, Atarhouch T, Saldanha J, Barbosa JA, Hamers R. Sequence and structure of VH domain from naturally occurring camel heavy chain immunoglobulins lacking light chains. Protein Eng 1994; 7:1129-35; PMID:7831284; http://dx.doi.org/10.1093/protein/7.9.1129

55. Loset GA, Lobersli I, Kavlie A, Stacy JE, Borgen T, Kaustually L, Hvattum E, Simonsen B, Hovda MB, Brekke OH. Construction, evaluation and refinement of a large human antibody phage library based on the IgD and IgM variable gene repertoire. J Immunol Methods 2005; 299:47-62; PMID:15914190

56. Holdsworth G, Slocombe P, Doyle C, Sweeney B, Veverka V, Le Riche K, Franklin RJ, Compson J, Brookings D, Turner J, et al. Characterization of the interaction of sclerostin with the low density lipoprotein receptor-related protein (LRP) family of Wnt co-receptors. J Biol Chem 2012; 287:26464-77; PMID:22696217; http://dx.doi.org/10.1074/jbc.M112.350108