



# PAPTi: A Peptide Aptamer Interference Toolkit for Perturbation of Protein-Protein Interaction Networks

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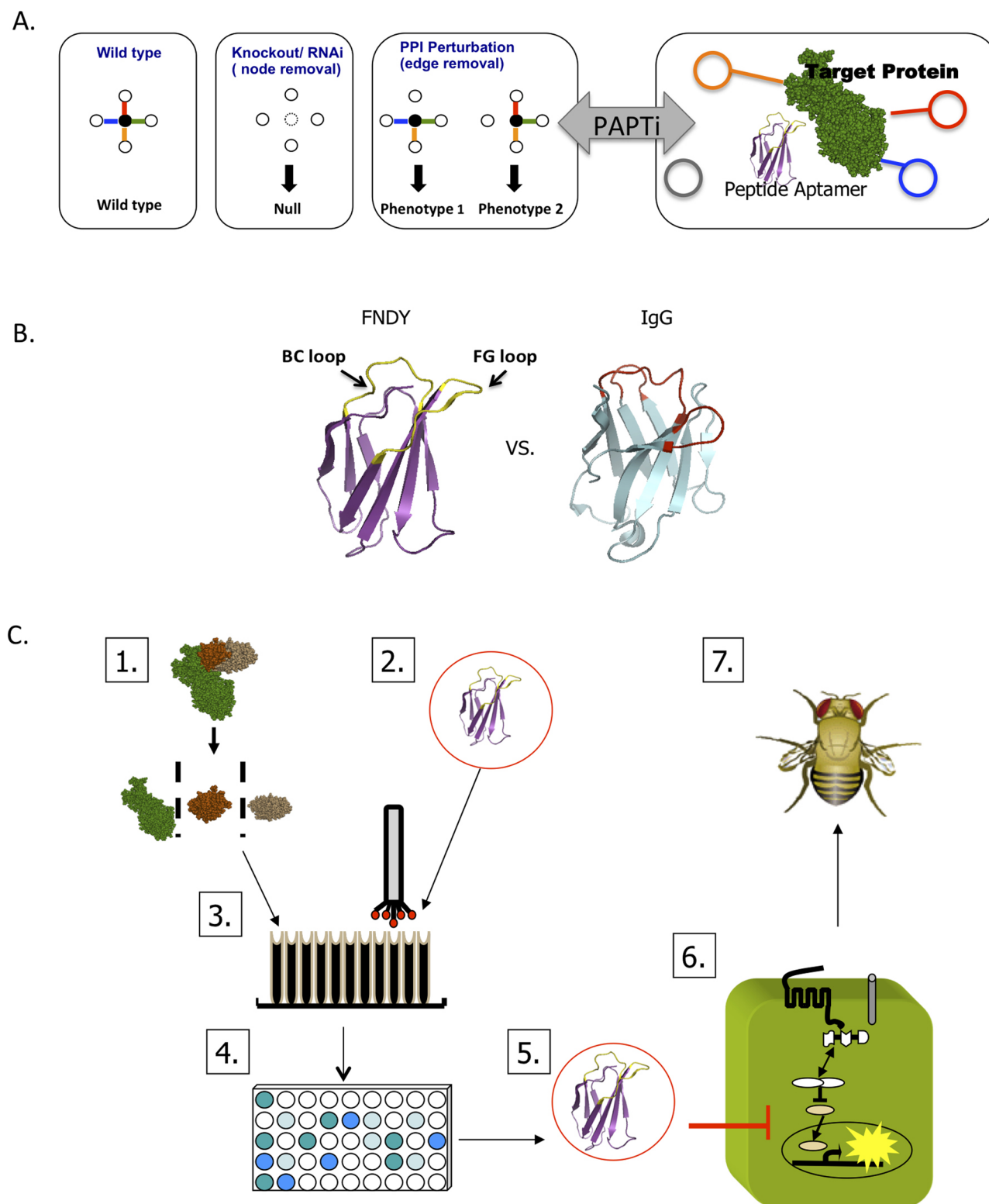
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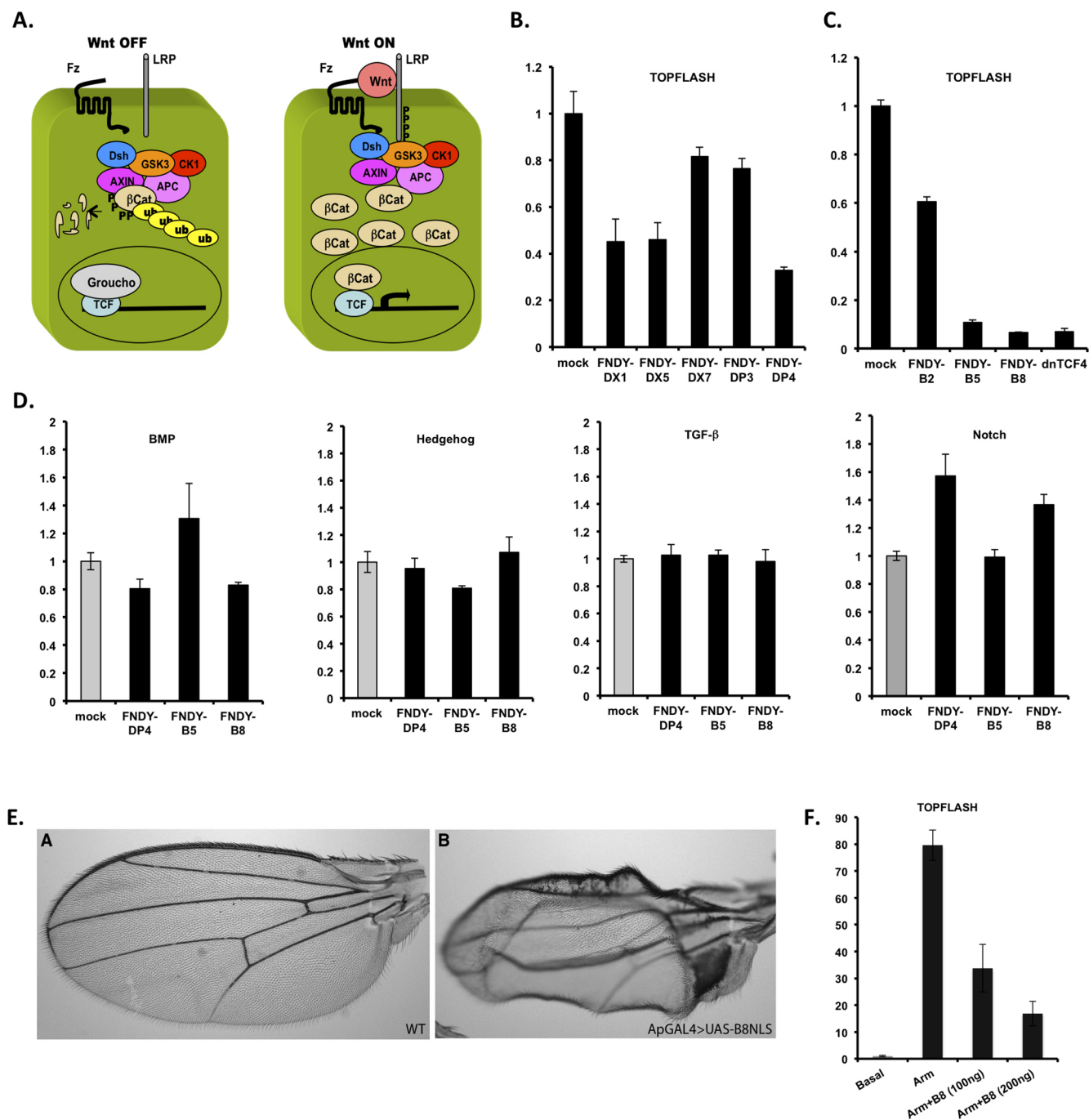
Signaling proteins often form dynamic protein-protein interaction (PPI) complexes to achieve multi-functionality. Methods to abrogate a subset of PPI interfaces without depleting the full-length protein will be valuable for structure-function relationship annotations. Here, we describe the use of Peptide Aptamer Interference (PAPTi) approach for structure-function network studies. We identified peptide aptamers against Dishevelled (Dsh) and  $\beta$ -catenin ( $\beta$ -cat) to target the Wnt signaling pathway and demonstrate that these FN3-based MONOBODYs (FNDYs) can be used to perturb protein activities both *in vitro* and *in vivo*. Further, to investigate the crosstalk between the Wnt and Notch pathways, we isolated FNDYs against the Notch Ankyrin (ANK) region and demonstrate that perturbing the ANK domain of Notch increases the inhibitory activity of Notch towards Wnt signaling. Altogether, these studies demonstrate the power of the PAPTi approach to dissect specific PPI interactions within signaling networks.

Statistical analyses have revealed that Protein-Protein Interaction (PPI) networks follow the “scale-free” highly connected rule with each protein (node) interacting on average with 3.6 partners and with some hub proteins having more than 6<sup>1</sup>. With this level of interconnectivity, cellular proteins can form robust PPI networks to maintain cellular functions. Strategies that abrogate a subset of interactions (edges) from a node without depleting the whole protein from the PPI network are thus valuable for the analysis of signaling protein multi-functionality and for the structure-function relationship annotations of specific PPI<sup>2</sup> (Fig. 1A). The most commonly used approaches for such “edgetic” analyses are mutagenesis screens<sup>2,3</sup> and chemical genetics<sup>4</sup>. Mutagenesis screens, whereby proteins generated from random mutagenesis are tested functionally (see for example<sup>3</sup>), are time consuming and often problematic, as the perturbation can result in prematurely truncated or misfolded proteins that behave as loss-of-function null alleles. Chemical genetics on the other hand uses bioactive small molecules as perturbation tools to mimic the effect of loss-of-interaction alleles<sup>4</sup>. Although a powerful approach, chemical genetics is limited, as most bioactive compounds available are enzyme inhibitors (mostly kinases) rather than protein-protein interface disruptors<sup>5</sup>, reflecting the fact that PPI interfaces are generally wide and shallow and therefore lack the deep hydrophobic pockets required for small molecules to be engulfed with high affinities and specificities<sup>6</sup>.

Peptide aptamers<sup>7</sup>, a class of affinity binders that consist of short stretches of variable peptide regions constrained within a protein scaffold<sup>7–9</sup>, provide a powerful alternative approach to target PPI interfaces. While they have been shown to be effective at inhibiting cell signaling both *in vitro* and *in vivo*<sup>10,11,12</sup>, the generation of peptide aptamers for functional studies in cell signaling still has not been extensively explored. Thus, we employed a Peptide Aptamer Interference (PAPTi) approach to select for aptamers that target PPI interfaces. Our PAPTi strategy (Fig. 1B, 1C) entails the engineering of a stable small protein scaffold, the human FN3 domain (Monobody)<sup>9,13</sup>, that can display variable protein binding sequences with high stability and affinity analogous to antibody-antigen interactions<sup>7,12</sup>. By constructing a randomized library with diversified amino acid sequences, target specific monobody peptide aptamers can be selected for functional PPI interference. Here, we describe the use of such a diversified PAPTi library to identify and validate inhibitory peptide aptamers toward the Wnt signaling network. The Wnt pathway is an evolutionarily conserved signaling pathway essential to animal development, stem cell self-renewal, and adult tissue homeostasis<sup>14</sup>. Conversely, dysregulation of Wnt signaling is commonly associated with human diseases such as cancer<sup>14</sup>. Consequently, peptide aptamers that perturb Wnt



**Figure 1 | Schematic representations of PAPTi platform.** (A) PPI network functional analysis. (B) FN3 monobody scaffold. Structure of the FN3 Monobody scaffold<sup>9,13</sup> (FN3, left) used in this study. The scaffold has an IgG fold similar to the antibody IgG (right). The randomized loops in this study (BC loop and FG loop) within the FN3 scaffold are colored in yellow. As a comparison, the three antigen recognition variable regions within the antibody IgG scaffold are colored in red. (C) PAPTi flowchart: (1) Target protein expression: Target proteins are divided into folded domains and each individual domain is expressed, purified and biotinylated. (2) Phage library construction: Diversified FN3 domain monobody peptide aptamers (FN3) are cloned in fusion with M13 phage pIII coat protein, resulting in a phage display library with  $2 \times 10^{10}$  complexity. (3) Selection of target binding phages. (4) ELISA validation: Individual phage clones are amplified and validated for target binding in 96-well ELISA plates. Positive clones are selected for further characterization. (5) Positive individual FN3 clones can be subcloned into various destination vectors for subsequent applications. (6) Cell-based reporter assays: To select functional peptide aptamers, candidate FN3s are transfected and expressed in reporter cells, and tested using luciferase-based reporter assays. (7) *In vivo* functional validation: FN3s are selected to generate transgenic animals.

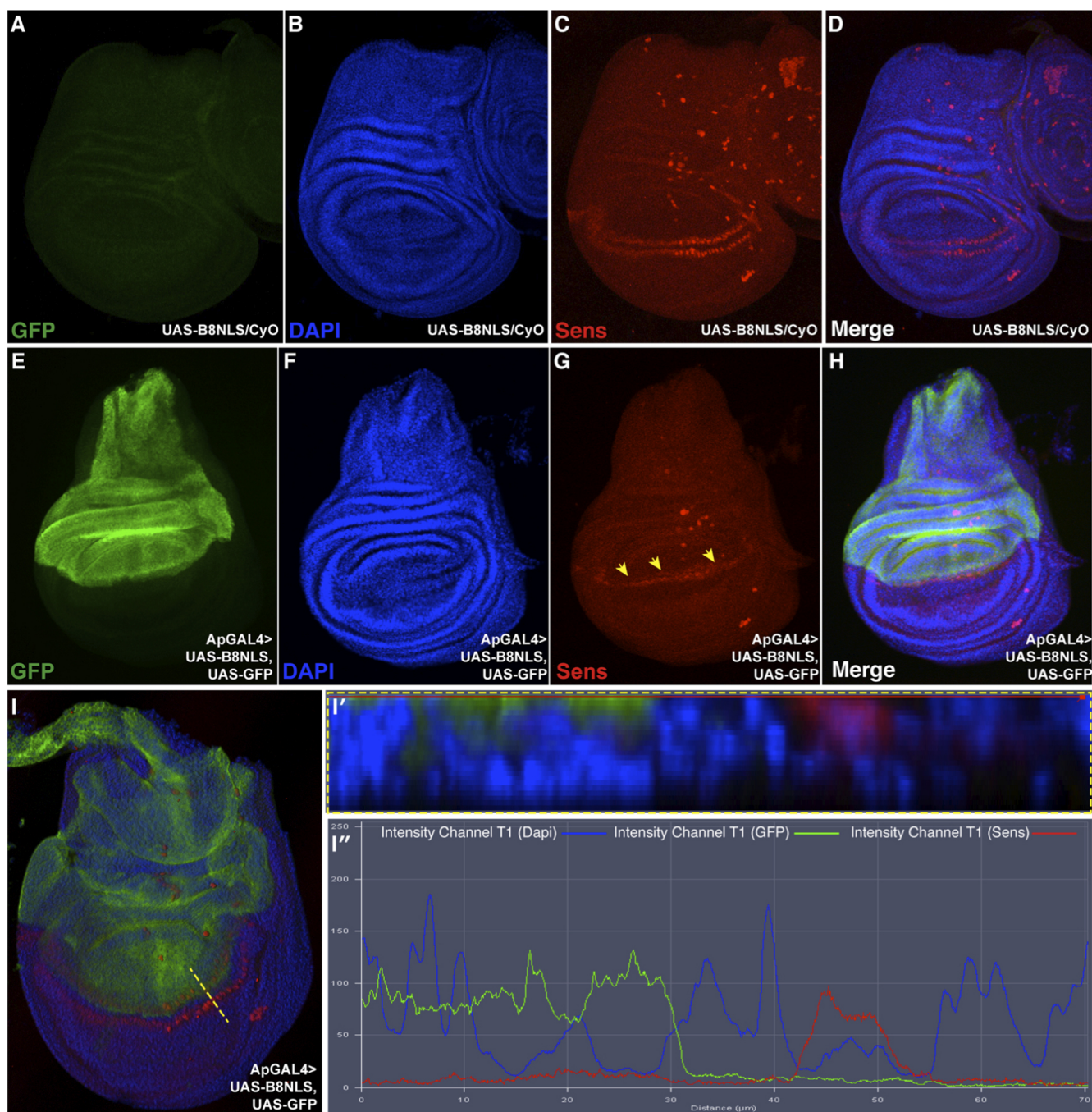


**Figure 2 | PAPTi of the canonical Wnt signaling pathway.** (A) The Wnt signaling pathway. The canonical Wnt signaling consists of Wnt (ligand), Frizzled (Fz) and LRP (receptor), Dsh (effector), GSK3/Axin/APC (destruction complex) and  $\beta$ -cat (transcriptional activator). (B–E) Cell-based activity assays of FNDYs. (B) FNDY-DX and FNDY-DP against Dsh inhibit TOPFLASH luciferase activity in HEK293T cells. (C) TOPFLASH validation of FNDYs against  $\beta$ -cat in HEK293 cells, and dominant negative (dn) TCF4 positive control. (D) FNDY-B8 inhibits TOPFLASH reporter activity as effectively as the dnTCF4 construct in the human colorectal cancer cell line DLD1. (D) FNDYs do not inhibit Hedgehog, TGF- $\beta$ , BMP, and Notch pathways, as indicated by the Gli-Luc, TGF $\beta$ -Luc, BMP-Luc, and CSL-Luc reporters, respectively. (Gray bar: stimulated reporter cells co-transfected with empty vector, Black bar: stimulated reporter cells co-transfected with FNDYs). (E) *In vivo* misexpression of  $\beta$ -cat FNDY peptide aptamers in the *Drosophila* wing results in severe growth defects, and blistering phenotypes. (A: Wild Type control adult wing; B: FNDY-B8-NLS expressing adult wing). Similar observations were made in transgenic flies expressing FNDY-B8-NLS under MS1096-GAL4 wing pouch driver (data not shown). (F) FNDY-B8 also inhibits fly Armadillo (Arm). TOPFLASH reporter activity was inhibited in HEK293T cells co-transfected with pCMV-Arm (15 ng/well) and FNDY-B8 (100 ng/well & 200 ng/well). (Y-Axis: Relative luciferase activity. Basal: Cells not activated by Arm).

signaling will be valuable tools in biology and translational medicine. In this report, we describe the identification of peptide aptamers that inhibit the canonical Wnt signaling effector proteins Dishevelled (Dsh) and  $\beta$ -catenin ( $\beta$ -cat). Furthermore, we demonstrate that

the PAPTi approach led us to discover that targeting Notch ANK repeats can be utilized as a mean to inhibit Wnt signaling. All together, this study provides a proof-of-concept for using PAPTi for functional PPI studies.





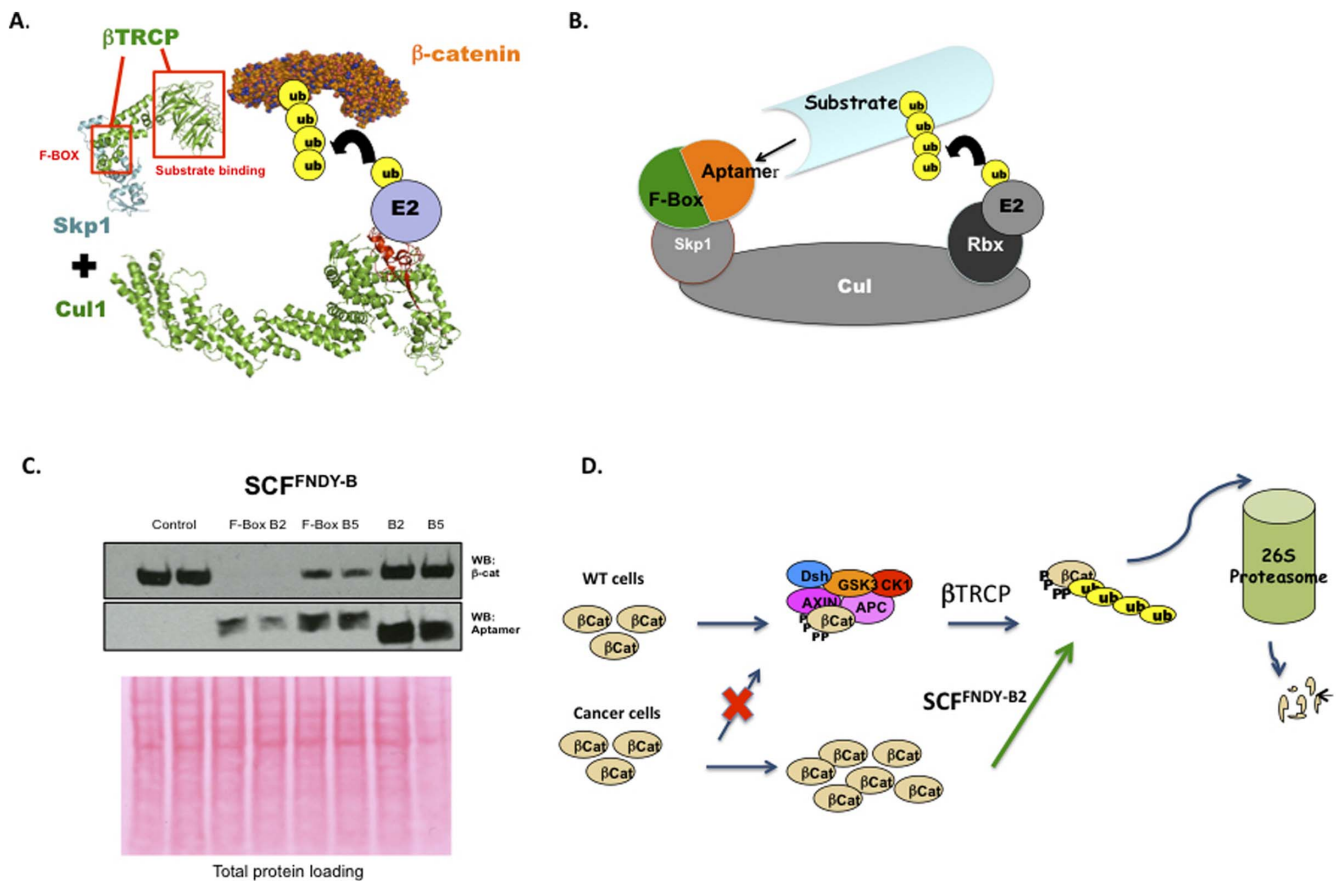
**Figure 3 | Efficacy of FNDY *in vivo*.** (A–D) Control imaginal discs displaying the normal expression of the Senseless (Sens) protein (red, panel C), in two rows of cells at the dorsal/ventral (D/V) boundary of 3<sup>rd</sup> instar larva. (E–H) Misexpression of *UAS:FNDY-B8-NLS* under the control of *apterous-GAL4* (ApGAL4), which is expressed only in the dorsal compartment, as shown by the co-expression of GFP (UAS-GFP) (E) results in a marked reduction of Sens expression specifically in the dorsal compartment (yellow arrowheads in G; also compare expression of Sens in dorsal versus ventral compartment in H). (I–I'') Quantitation of Sens expression in dorsal versus ventral compartment along a confocal Z-stack across the D/V boundary (denoted by the yellow dashed line in the X–Y image in panel I, and the Z-stack shown in I'). Note the significant loss of Sens staining (red) in the cells expressing GFP (green) in panel I', and as quantified using fluorescence intensity profile in panel I'', which shows a reciprocal relationship between the expression of GFP (green line) and expression of Sens (red line). These results indicate that the ectopic expression of *UAS:FNDY-B8-NLS* *in vivo* is sufficient to abrogate endogenous Wingless/Wnt signaling, most likely by acting on nuclear Arm/ $\beta$ -cat.

## Results

**PAPTi screen against Wnt signaling pathway.** Since Dsh and  $\beta$ -cat are the two major effectors of the canonical Wnt signaling pathway (Fig. 2A), we sought to set up a phage display PAPTi selection system (Fig. 1C, see Methods) to screen for peptide aptamers against these proteins. We expressed and purified the full-length human  $\beta$ -cat and

the three well defined domains of *Drosophila* Dsh (DIX, PDX and DEP domains, respectively) as targets for a peptide aptamer screen. From a phage display screen of a library with  $2 \times 10^{10}$  complexity, we obtained a collection of peptide aptamers [referred to as FNDYs (for FN3-based MONOBODY)]: FNDY-DX targeting the Dsh DIX domain, FNDY-DP against Dsh DEP, and FNDY-B against  $\beta$ -cat.





**Figure 4 | FNDY-F Box chimera as a tool for synthetically targeting protein degradation.** (A) Schematic representation of canonical  $\beta$ -cat degradation via the SCF <sup>$\beta$ -TrCP</sup> complex. Phosphorylated  $\beta$ -cat is recruited to the SCF <sup>$\beta$ -TrCP</sup> E3 ligase via the F-Box protein  $\beta$ -TrCP<sup>14</sup>. Illustrations of protein structures based on PDB 2GL7 ( $\beta$ -cat)<sup>28</sup>, PDB 1LDJ (*Cul1*)<sup>29</sup>, and PDB 1P22 ( $\beta$ -TrCP-Skp1)<sup>30</sup>. (B) FNDY-F Box SCF E3 ligase complex (SCF<sup>FNDY</sup>) restores  $\beta$ -cat degradation. (Upper) Replacing the WD-40 repeats domain of  $\beta$ -TrCP with variable peptide aptamer moieties to degrade desired target proteins via protein ubiquitination pathway. The method is reminiscent to the use of nanobodies to degrade GFP<sup>18</sup>. (Lower) HEK293T cells overexpressing non-phosphorylatable  $\beta$ -cat (S33Y, S37A, S41A, T45A) were transfected with either the FNDY peptide aptamer or SCF<sup>FNDY</sup>. 24 h post transfection, significant depletion of  $\beta$ -cat was achieved.

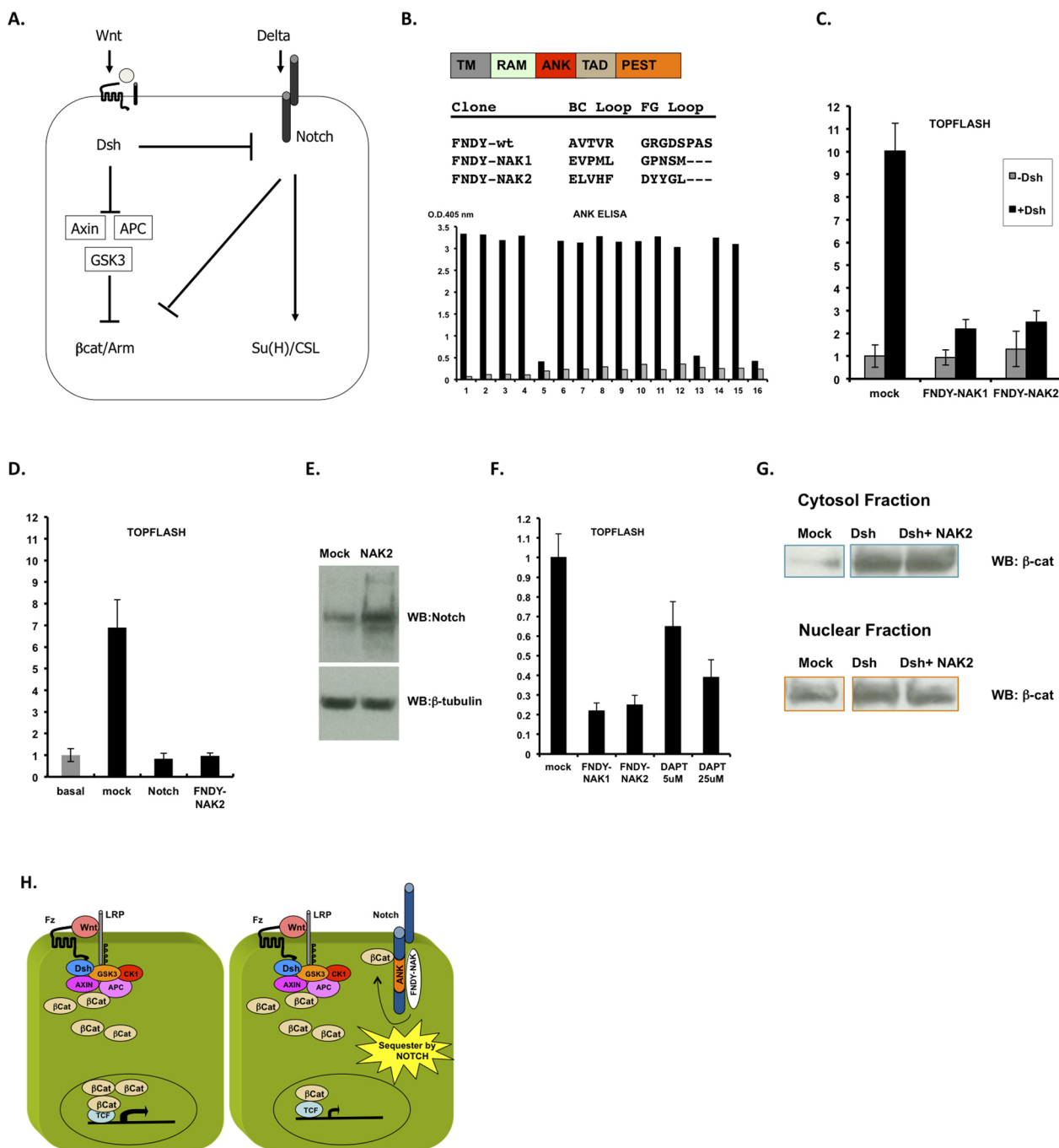
Interestingly, our screen against Dsh PDZ failed to yield high affinity binders, possibly due to the strong binding preference of PDZ domains for free carboxyl terminus peptides rather than internal peptide motifs.

Next, we used the Wnt pathway reporter TOPFLASH (dTF12), a construct with 12 multimerized LEF/TCF binding sites upstream of a heat shock minimum promoter driving the expression of luciferase, as a functional readout to select for FNDYs that can perturb Wnt signaling<sup>4</sup>. From the Dsh and  $\beta$ -cat screens, we identified bioactive clones that showed various degrees of inhibitory effects against either Dsh or  $\beta$ -cat (Fig. 2B, 2C; Table S1). Among the FNDYs tested in HEK293T reporter cells, FNDY-B8 exhibited the strongest activity and was as effective as dominant negative Tcf4 (Fig. 2C). Similar result was observed in the human colorectal cancer cells DLD1, a cancer cell line with truncated APC (Fig. S1). The effect of these peptide aptamers are Dsh or  $\beta$ -cat associated as the inhibitory effect of either FNDY-DP4, FNDY-B5 or FNDY-B8 was reversible upon transfection of increasing amount of Dsh or  $\beta$ -cat, respectively (Fig. S2). Further, the peptide aptamers are specific towards the Wnt pathway as they had no significant effects on Bone Morphogenetic Protein (BMP), Hedgehog (Hh), TGF- $\beta$  and Notch pathway activities (Fig. 2D), whereas Notch reporter activity was increased (~50%) by FNDY-DP4 (Fig. 2D), reminiscent to previous findings that Dsh act as a negative regulator of Notch<sup>15</sup> (see below for further discussion).

**FNDY-B8 perturbs *Drosophila* wing disc development.** We next asked whether FNDYs are suitable for functional PPI perturbation

*in vivo*. Because FNDY-B8-NLS exhibits strong inhibitory activity against Wnt signaling, we generated transgenic flies carrying inducible FNDY-B8-NLS (to enhance higher nuclear targeting *in vivo*, a 3X SV40 NLS sequence was added), using the Gal4/UAS system<sup>16</sup>. UAS:FNDY-B8-NLS flies crossed with the *apterous-Gal4* driver showed a disorganized wing phenotype: crumpled wing phenotype which likely results from differential growth in the dorsal versus ventral compartment, smaller wing size and large bubbles or blisters that appear on the wing blade (Fig. 2E), suggesting perturbed Wg signaling during wing development. A similar phenotype was observed with UAS:FNDY-B5-NLS, albeit less profound. In addition, consistent with human  $\beta$ -cat and *Drosophila* Armadillo (Arm) being highly homologous, FNDY-B8 also inhibited Arm in the TOPFLASH assay (Fig. 2E). To further demonstrate that the disorganized wing phenotype is Arm associated, we stained the wing disc and found that the endogenous Wg target, *senseless* (*sens*) was repressed upon FNDY-B8-NLS expression in the wing disc (Fig. 3). Furthermore, expression of B8-NLS did not influence cut staining on either side of the dorsoventral (D/V) boundary (Fig. S3), even though there is reduced *senseless* expression in the dorsal compartment, suggesting a specific effect of B8-FNDY in influencing expression of Wg targets rather than Notch targets. Collectively, these findings indicate that FNDY-B8-NLS is effective at blocking Wnt signaling *in vivo*.

**SCF<sup>FNDY</sup> as modules for synthetically targeting protein degradation.** To further expand the applications of peptide



**Figure 5 | Targeting Notch ANK repeats can inhibit Wnt signaling.** (A) Crosstalk between Wnt and Notch pathways. The canonical Notch signaling consists of Delta (ligand), Notch (effector) and Su(H)/CSL (transcription factor). Dsh has been shown to negatively regulate Notch<sup>15</sup>, and Notch can negatively regulate Wnt signaling<sup>20,23</sup>. (B) Selection of Notch ANK repeats aptamers (FNDY-NAK1 and FNDY-NAK2). (Upper) Notch intracellular domains. (Lower) ELISA validation of individual FNDY phage clones. Enriched Individual FNDY clones were tested on ELISA plates coated with Notch ANK repeats or control (streptavidin) proteins. Binding activities were quantitated colorimetrically by absorbance at 450 nm. (Gray bars: Streptavidin control protein; Dark bars: Notch ANK). In this assay, false positive clones (clone 5 & clone 13; clone 16 is wild-type, non-displaying M13 phage) were discarded. All the positive hits were sequenced and two distinct FNDY sequences (FNDY-NAK1 and FNDY-NAK2) were identified. (C) Both FNDY-NAK1 and FNDY-NAK2 antagonize Dsh activated TOPFLASH. HEK293T cells were transfected with Dsh to activate TOPFLASH luciferase activity. This luciferase activity is suppressed by co-expressing either FNDY-NAK1 or FNDY-NAK2. (D) Transfecting ΔEC Notch1 in HEK293T cells inhibits Dsh activated TOPFLASH reporter expression. FNDY-NAK2 shows a similar inhibitory effect in this assay. (E) Western blot (WB) of cytoplasmic Notch. Overexpressing FNDY-NAK2 stabilizes the cytoplasmic protein level of Notch1. The cytoplasmic fraction of HEK293 cell lysate was collected and blotted with Notch1 antibody (β-tubulin as a loading control). (F) γ-secretase inhibitor inhibits TOPFLASH activity. HEK293T cells were transfected with Dsh and treated with the γ-secretase inhibitor DAPT (5 μM and 25 μM). The inhibition of TOPFLASH caused by FNDY-NAK1 and FNDY-NAK2 are shown as a comparison. (Mock: cells transfected with Dsh; FNDY-NAK1/2: cells transfected with Dsh + FNDY-NAK1/NAK2; DAPT: cells transfected with Dsh and treated with DAPT). (G) Dsh overexpression lead to nuclear β-cat accumulation, which was decreased upon FNDY-NAK2 overexpression. (Note: In order to see significant β-cat accumulation in the nuclear fraction by western blot, higher amount of Dsh plasmid was transfected in the cells than that was used in TOPFLASH assays. FNDY-NAK2 was able to slightly reduce the ratio of nuclear β-cat).



aptamers in manipulating cellular signaling, we next used FNDYs as toolkit modules for protein modification such as ubiquitinylation. Since E3 ligases facilitate protein ubiquitinylation by placing the substrate targets and the activated ubiquitin into proximity (Fig. 4A), peptide aptamers can be used as a substrate recruiting module to create an artificial E3 ligase<sup>17</sup> (Fig. 4B). A similar approach, whereas a GFP nanobody in fusion with *Drosophila* Slimb F-Box (the deGradeFP system)<sup>18</sup>, has been shown to effectively deplete GFP-tagged fusion proteins *in vitro* and *in vivo*. Thus, we designed FNDYs in fusion with the human  $\beta$ -TrCP F-Box (residues 139-255) of the SCF $^{\beta$ -TrCP complex (termed SCF<sup>FNDY</sup> thereafter) to promote target protein degradation. We generated a number of SCF<sup>FNDY-B</sup> constructs and tested their activities toward  $\beta$ -cat degradation. Under physiological conditions when Wnt stimulation is absent,  $\beta$ -cat is undergoing constant degradation. However, in many cancer cells,  $\beta$ -cat protein accumulation is observed due to impaired degradation complex formation. To demonstrate that SCF<sup>FNDY-B</sup> can restore  $\beta$ -cat degradation, we transfected the cells with the non-phosphorylatable form of  $\beta$ -cat (S33Y, S37A, S41A, T45A  $\beta$ -cat), in which the phosphorylation by GSK3 $\beta$  is abolished and thereby impaired for  $\beta$ -TrCP WD40 domain binding. In these cells, both SCF<sup>FNDY-B2</sup> and SCF<sup>FNDY-B5</sup> were effective at promoting  $\beta$ -cat degradation, albeit SCF<sup>FNDY-B2</sup> was more effective (Fig. 4C) likely due to the favored orientation of the substrate being recruited to the SCF<sup>FNDY-B2</sup> E3 ligase complex. This result indicates that synthetic SCF<sup>FNDY-B2</sup> can be used to restore  $\beta$ -cat degradation for oncogenic Wnt signaling (Fig. 4C, 4D).

**FNDYs that targeting Notch ANK repeats domain can inhibit Wnt signaling.** To further explore the use of FNDYs to interrogate the function of protein domains, we tested the interaction between the Wnt and Notch pathways. Previous studies have shown that Notch synergizes or antagonizes Wnt signaling, arguing that Notch modulates Wnt signaling in a context dependent manner. Importantly, Notch can form direct PPI with Wnt pathway components to regulate Wnt signaling (Fig. 5A)<sup>15,19</sup>. In particular, the ANK repeats containing domain of Notch1 (Fig. 5B) has been shown to play a pivotal role in Notch activity, but has not yet been described as a regulatory region for Wnt pathway crosstalk. Thus, we generated FNDYs that target the ANK domain and asked whether they can affect any aspect of Notch and Wnt crosstalk (Fig. 5B). We identified two independent clones, FNDY-NAK1 and FNDY-NAK2 (Fig. 5B), which significantly inhibited TOPFLASH activity in HEK293T cells activated by either Dsh or  $\beta$ -cat (Fig. 5C, 5D). To validate the binding specificity of the peptide aptamers, we performed GST-pull down assays by incubating GST tag fusion proteins with lysates from HEK293 cells expressing individual FNDY constructs. FNDY-B constructs were found to be GST- $\beta$ -cat specific whereas FNDY-NAK1 and FNDY-NAK2 only recognize GST-Notch RAMANK (Notch1 1761-2127 which comprises the RAM and ANK domains) (Fig S4), indicating the Notch associated suppression of Wnt signaling by FNDY-NAK1 and FNDY-NAK2. In addition, because FNDY-NAK1 and FNDY-NAK2 are two independent clones with distinct variable sequences selected against the Notch ANK repeat region, it is unlikely that they both inhibit Wnt signaling due to nonspecific, off-target cross-reactivity with Wnt pathway components. Thus, this result suggests that Notch acts as a direct regulator of the Wnt/ $\beta$ -cat signaling via its ANK repeats domain.

A recent study has suggested that Notch acts as a direct negative regulator of  $\beta$ -cat by promoting its lysosomal degradation<sup>20</sup>. To test whether Notch negatively regulates Wnt signaling, we overexpressed  $\Delta$ EC Notch1<sup>21</sup>, an activated form of Notch1, in the Notch-responsive HEK293T cells (Fig S5, S6). In these cells, Notch1 negatively regulates Wnt signaling<sup>20</sup> (Fig. 5D) and FNDY-NAK2 could increase cytoplasmic Notch1 protein level (Fig. 5E). Since it has been

proposed that cytoplasmic, but not nuclear, Notch antagonizes Wnt signaling<sup>19,20</sup>, and that the ANK repeats interact with the RING type E3 ubiquitin ligase Deltex<sup>22</sup>, it is possible that FNDY-NAK1 and FNDY-NAK2 stabilize cytoplasmic Notch by interfering with the interaction between Notch and Deltex. Consistent with this hypothesis, Wnt pathway activity was decreased in HEK293T cells treated with DAPT, a  $\gamma$ -secretase inhibitor that prevents nuclear Notch accumulation<sup>20</sup> (Fig. 5F). Alternatively, as membrane associated Notch can also sequester  $\beta$ -cat accumulation<sup>23</sup>, FNDY-NAK2-mediated stabilization of Notch could more efficiently prevent  $\beta$ -cat to signal in the nucleus. In this regard, we observed that FNDY-NAK2 did not promote  $\beta$ -cat protein degradation (Fig. 5G). However, in the presence of high-level Dsh overexpression, FNDY-NAK2 could still slightly reduce the nuclear ratio of accumulated  $\beta$ -cat (Fig. 5G), suggesting that  $\beta$ -cat is sequestered but not degraded by the stabilized Notch. Altogether, these results illustrate the use of FNDYs to annotate the function of protein domains involved in signaling.

## Discussion

Altogether, our studies demonstrate the power of FNDYs for functional PPI perturbation and in particular to dissect specific PPI interactions within signaling networks. Specific PPI-mediated pathway crosstalk is essential to cellular signaling. Consequently, there exist particular interests in annotating the structure-function relationships of multi-domain signaling proteins in the PPI network. In particular, we demonstrated that the PAPTi approach led us to discover that targeting Notch ANK repeats may be further utilized as a mean to inhibit Wnt signaling in some types of cells. This provides a proof-of-concept for using PAPTi for “edgetic” functional PPI studies.

Compared to transgenic RNAi<sup>24</sup>, which allows perturbation of gene activity in a spatial and temporal manner, PAPTi allow specific protein domains to be targeted<sup>25</sup>. Further, in contrast to small molecules, the expression of peptide aptamers can be spatially and temporally controlled by conditional gene expression methods<sup>25</sup>. Not limited to PPI network biology, structural elucidation of Peptide Aptamer-Target interactions can also serve as a template for structure guided drug discovery<sup>25</sup>. For future extension of PAPTi coverage, it would be desirable to generate a large collection of bioactive, renewable FNDY peptide aptamers. Recently, an antibody consortium has reported its success in generating a large collection of renewable SH2 domain antibodies<sup>26</sup>. Similarly, owing to current available resources such as complete ORFeome collections of model organisms, high throughput screening platforms and sensitive proteomics methods, it should be feasible to scale up FNDY production to reach proteome-wide coverage for PPI structure-function analysis. Well-characterized FNDY clones from such a collection would enable edgetic analysis or PPI studies. As a resource platform, one of the parameters to ensure quality control of such reagents is their binding specificity, which could be addressed by incorporating a proteome-wide high density protein array<sup>27</sup> into the PAPTi workflow (Fig. 1C).

## Methods

**Phage display screen.** To construct the library, degenerate oligonucleotide pools encoding 5 randomized amino acid residues were ligated into the parental FN3 scaffold in the phagemid vector to replace the correspondent wild-type BC and FG loop sequences<sup>9,13</sup> (See Supplementary Information for details). The resulting FNDY library thus contained 10 randomized residues (5 random residues in each loop) that could be selected for target binding. The resulting plasmids were electroporated into XL1 Blue *E. coli* competent cells to create a phage display library with  $2 \times 10^{10}$  complexity. Target proteins were biotinylated (using biotinylation kit from Sigma-Aldrich) and immobilized on streptavidin coated ELISA plates (Nunc), and the phage library was then applied into the wells for capturing the target binding phages. After incubation at 4°C for 8 h, unbound phages were removed by extensive washing with TBST. The remaining target bound phages were absorbed by XL1 Blue *E. coli* and amplified. After three rounds of panning and enrichment, individual





phage clones were obtained and the phagemid DNA was sequenced to determine the selected sequences.

**FNDY expression constructs.** To express FNDY peptide aptamers in mammalian cells, selected FNDY ORF cDNAs were inserted into pEF-myc vectors (Invitrogen) by PCR cloning. For *Drosophila* expression, FNDY ORF cDNAs were cloned into the pUAST expression vector, which contains a 5 × Gal4 binding site in the promoter region. To generate transgenic flies, the pUAST-FNDY constructs were injected into fly embryos and the positive transformants were selected following standard procedure.

**Luciferase Reporter Assays.** For the Wnt TOPFLASH reporter assay, HEK293 cells cultured in 96-well plates were transfected with 100 ng/well TOPFLASH plasmid and 2.5 ng/well pCMV-RL plasmid together with or without 100 ng/well pEF-FNDY peptide aptamer plasmids and 10–25 ng/well pCMV-Dsh or pCMV-β-catenin plasmids. For the Notch reporter assay, cells were transfected with 100 ng/well of pcDNA3 ΔEC-NOTCH1 together with 100 ng pGL3 4xCSL-luc plasmid and 2.5 ng pCMV-RL plasmid. Similarly, for the Hedgehog reporter assay, 100 ng/well Gli-Luc reporter construct together with 2.5 ng/well pCMV-RL was used. For the BMP reporter assay, 100 ng/well XVENT-Luc reporter construct together with 2.5 ng/well pCMV-RL was used. For TGF-β reporter assay, 3xTP-Luc reporter construct together with 2.5 ng/well pCMV-RL was used. Luciferase activity was measured using the Dual-Glo luciferase assay kit and the firefly luciferase activity was normalized with Renilla luciferase activity.

**Transgenic Flies and Genetic Crosses.** To target the nuclear Arm more efficiently, a 3 × SV40 NLS sequence (DPKKKRKV) was inserted C-terminal to the FNDY-B8 ORF. The resulting pUAST-FNDY-B8-NLS construct was used to generate transgenic flies. *UAS:FNDY-B8-NLS* flies were crossed to the *apterous-Gal4* and *MS1096-Gal4* drivers (Gal4 drivers are described at <http://flybase.org/>). Flies were grown at 25°C and shifted to 29°C at the pupal stage. Adult flies were collected and their wing phenotypes scored under the microscope. Standard techniques were used for imaginal disc dissection, and antibody staining for Senseless (guinea pig anti-Sens used at 1:1000; gift from Dr. Hugo Bellen), GFP, and DAPI. The wild type discs used are from sibling control of the following genotype: *UAS:FNDY-B8/CyO*.

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## Authors contributions

J.Y., N.P. and R.D. conceived the idea and designed the experiments together. J.Y. performed the library screening and cell-based characterization. R.B. established and tested the transgenic *Drosophila* lines. T.G. performed the *Drosophila* wing disc staining. R.D. made the human β-catenin expression construct for protein purification. J.Y., R.B., R.D. and N.P. wrote the manuscript.

## Additional information

**Supplementary information** accompanies this paper at <http://www.nature.com/scientificreports>

**Competing financial interests:** The authors declare no competing financial interests.

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