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RESEARCH ARTICLE

Wnt proteins synergize to activate β-catenin signaling

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ABSTRACT

Wnt ligands are involved in diverse signaling pathways that are active during development, maintenance of tissue homeostasis and in various disease states. While signaling regulated by individual Wnts has been extensively studied, Wnts are rarely expressed alone, and the consequences of Wnt gene co-expression are not well understood. Here, we studied the effect of co-expression of Wnts on the β-catenin signaling pathway. While some Wnts are deemed 'non-canonical' due to their limited ability to activate β-catenin when expressed alone, unexpectedly, we find that multiple Wnt combinations can synergistically activate β-catenin signaling in multiple cell types. WNT1- and WNT7B-mediated synergistic Wnt signaling requires FZD5, FZD8 and LRP6, as well as the WNT7B co-receptors GPR124 (also known as ADGRA2) and RECK. Unexpectedly, this synergistic signaling occurs downstream of β-catenin stabilization, and is correlated with increased lysine acetylation of β-catenin. Wnt synergy provides a general mechanism to confer increased combinatorial control over this important regulatory pathway.

KEY WORDS: GPCR, Wnt signaling, Cancer, Development, Synergy

INTRODUCTION

Wnts are secreted morphogens that control myriad biological processes during development and adult tissue homeostasis in animals (Chien et al., 2009; Nusse and Varmus, 2012; Yu and Virshup, 2014). Aberrant Wnt signaling is associated with many pathological conditions (Clevers and Nusse, 2012). Nineteen distinct genes in the human genome encode Wnt ligands, which bind to a variety of receptors including ten frizzled proteins (FZDs) and the co-receptors low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6), as well as an increasing number of alternative receptors and co-receptors including the single transmembrane receptors RYK, ROR1/2, PTK7 and GPR124 (also known as ADGRA2) to trigger various downstream signaling pathways (MacDonald et al., 2009; Niehrs, 2012; Posokhova et al., 2015; Zhou and Nathans, 2014). Many Wnt ligands can stimulate β-catenin-induced gene transcription. In some settings, distinct Wnt genes utilize unique promoters and enhancers to drive expression with distinct developmental timing and tissue specificity. However, in both normal and disease states, multiple Wnt genes are often expressed in combination (Akiri et al., 2009; Bafico et al., 2004; Benhaj et al., 2006; Suzuki et al., 2004). For example, stromal cells that support the intestinal stem cell niche express at least six different Wnts at the same time (Kabiri et al., 2014). While in isolated instances, specific Wnt pairs have been shown to combine to enhance β-catenin signaling during embryonic development, whether this is a general phenomenon remains unclear (Cha et al., 2008; Cohen et al., 2012; Miller et al., 2012). Notably, increased expression of multiple Wnt ligands has been described in a number of cancers (Akiri et al., 2009; Bafico et al., 2004; Benhaj et al., 2006; Suzuki et al., 2004). The source of these cancer-associated Wnts is often from the cancer cells themselves, although stromal cells (including fibroblasts and hematopoietic cells) also produce multiple Wnts (Luga et al., 2012; Macheda and Stacker, 2008). Hence, Wnt ligands from multiple sources can converge on target cells in both physiological and pathological settings. How these Wnt ligands interact with each other to regulate β-catenin signaling is not well understood.

In the absence of Wnt ligand–receptor interaction, β-catenin released from membrane complexes is sequentially phosphorylated and targeted for ubiquitylation and proteasomal degradation by a cytosolic 'destruction complex' (reviewed in Anastas and Moon, 2013; MacDonald et al., 2009; Yu and Virshup, 2014). Wnt ligands bind to both FZD proteins and LRP5/6 receptors, with different Wnts interacting with two distinct interaction domains on the LRP5/6 receptors (Bourhis et al., 2010; Ettenberg et al., 2010; Gong et al., 2010). This initiates formation of multimeric signalosomes that suppress β-catenin phosphorylation by GSK3 (Gammons et al., 2016; reviewed in Kim et al., 2013; MacDonald et al., 2009). The stabilized β-catenin can then translocate to the nucleus to act as a transcriptional coactivator in conjunction with T cell factor (TCF) and lymphoid enhancer-binding factor (LEF) family transcription factors (reviewed in Cadigan and Waterman, 2012). β-catenin acts as a scaffold to recruit additional proteins to Wnt target gene promoters (reviewed in Mosimann et al., 2009; Valenta et al., 2012). The armadillo repeats of β-catenin recruit activators such as BCL9 and PYGO proteins, while the C-terminus interacts with many proteins involved in chromatin remodeling and transcription initiation including histone acetyltransferases (HATs) such as P300 (also known as EP300), CBP (also known as CREBBP) and KAT5 (also known as TIP60), histone methyltransferases (MLL1 and MLL2, also known as KMT2A and KMT2B), SWI/SNF factors (BRG1 and ISWI, also known as SMARCA4 and SMARCA5, respectively) and members of the PAF complex (reviewed in Mosimann et al., 2009; Valenta et al., 2012).

Using a recently constructed uniform Wnt expression library (Najdi et al., 2012), we screened 18 human Wnts in pairwise combinations for enhanced ability to activate a TCF/LEF reporter. Unexpectedly, multiple Wnt pairs were identified that, when co-expressed, synergize to potentiate β-catenin signaling. The mechanism of synergistic signaling from WNT1 and WNT7B was

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examined in depth. Synergy required both FZD5 and FZD8, as well as the recently identified WNT7A and WNT7B co-receptors GPR124 and RECK (Posokhova et al., 2015; Zhou and Nathans, 2014). The WNT7B–GPR124 interaction markedly stimulated K49 acetylation of β-catenin. Wnt synergy has important biological consequences, as co-expression of WNT1 and WNT7B regulated the expression of multiple genes and markedly enhanced the tumorigenicity of YCC11 gastric cancer cells. Co-expression of Wnts that synergize through novel pathways may have important combinatorial consequences in β-catenin-driven gene expression in diverse developmental, homeostatic and pathological processes.

RESULTS

Wnts cooperate to activate β-catenin signaling

We investigated the consequences of Wnt gene co-expression on β-catenin signaling, starting with HEK293 cells with a stably integrated SuperTopFlash reporter (STF, a β-catenin-activated TCF/LEF transcriptional reporter, denoted HEK293-STF; Veeman et al., 2003). Several pairs of Wnts showed synergistic interactions when nanogram quantities of Wnt expression plasmids were transfected (Table S1). For example, *WNT7B* expression alone did not activate the STF reporter, but it significantly potentiated the activity of both *WNT1* and *WNT3A* (Fig. 1A,B). *WNT7A* similarly synergized with *WNT1* (Fig. S1A). Another Wnt ligand, *WNT10B*, was found to potentiate the activity of *WNT3A* (Fig. 1B). Synergy was not simply a consequence of transfection, as it was also observed with purified proteins. Purified recombinant WNT10B, while inactive alone, potentiated signaling stimulated by recombinant WNT3A protein (Fig. 1C).

We found that the choice of Wnt pairs was important. For example, *WNT10B* did not potentiate the activity of *WNT1* (Fig. 1A). This correlates well with the finding that WNT1 and WNT10B interact with the same domain on LRP6, while WNT1 and WNT7B interact with different LRP6 domains (Gong et al., 2010). This effect is general, as Wnt synergy was also seen in YCC11 (gastric cancer) and HeLa (cervical cancer) cells (Fig. 1D,E; Fig. S1B,C). Demonstrating specificity for the Wnt pathway, neither *WNT1* nor *WNT7B* alone nor in combination activated either an nuclear factor (NF)-κB or an AP-1 reporter construct (Fig. S1D,E). Interestingly, *WNT10B*, which potentiated the activity of *WNT3A* on the STF reporter in HEK293 cells, did not have this effect in YCC11 cells (Fig. 1E). We speculate that this is due to differences in the Wnt receptors and co-receptors expressed in different cell types. Synergy was not due to increase in Wnt ligand expression upon co-expression of two Wnts (Fig. S6).

To quantitatively assess synergy, we performed Wnt titrations and calculated combination indices (CI) (Chou and Talalay, 1983, 1984) for the WNT combinations tested (*WNT1* and *WNT7B*; *WNT3A* and *WNT10B*) (described in detail in the Materials and Methods). A combination is defined as additive when the CI=1, synergistic when the CI<1 and antagonistic if the CI>1. Both the combination of *WNT1* and *7B* and the combination of *WNT3A* and *10B* had a CI of <<1, indicating they are highly synergistic (Fig. 1F).

In testing various pairwise combinations, again using nanogram quantities of Wnt expression plasmids (Najdi et al., 2012), we made several other intriguing observations. We found that two 'non-canonical' Wnts that did not activate Wnt/β-catenin signaling on their own in HEK293 cells could do so when co-expressed. *WNT2*, *WNT4*, *WNT9A* and *WNT9B* did not activate the STF reporter by themselves in HEK293 cells, but *WNT9B*, and to lesser extent, *WNT9A*, synergistically activated signaling when expressed in combination with *WNT2* or *WNT4* (Fig. 1F,G). In addition, *WNT6* and *WNT8A*, which interact with the same LRP6 domain as *WNT10B* (Gong et al., 2010), were found to inhibit the activity of *WNT10B* in HEK293 cells (Fig. S1F,G). Therefore, multiple sets of Wnts can interact in different ways to regulate β-catenin signaling activity in cells.

WNT1 and WNT7B cooperatively upregulate Wnt/β-catenin-responsive genes

We examined the consequences and requirement of the WNT1 and WNT7B interaction in more detail. We tested whether WNT1 and WNT7B co-operate in regulating the expression of endogenous Wnt/β-catenin target genes. The expression of *AXIN2*, *LEF1*, *NKD1* and *TCF7* was assessed in YCC11 cells by quantitative real-time RT-PCR (qRT-PCR) (Fig. 2A–D). *WNT1* and *WNT7B* alone each increased expression of these endogenous genes. In all cases, combined transfection of 100 ng of each Wnt plasmid produced at least twice the expression of the endogenous target genes as 200 ng of a single Wnt. Combination indices were calculated for these pairs as described above and a CI of <<1 was obtained for all the target genes (*AXIN2*, *LEF1*, *NKD1* and *TCF7*). Thus, Wnt synergy occurs on both a model reporter and on bona fide endogenous Wnt target genes.

To identify additional Wnt target genes that are synergistically regulated by combined WNT1 and WNT7B signaling, we performed a global transcriptome analysis by RNA-seq on YCC11 cells transfected with plasmids expressing *WNT1* (200 ng), *WNT7B* (200 ng) or both *WNT1* and *WNT7B* (100 ng each). This identified multiple additional genes synergistically up- and down-regulated by the WNT1 and WNT7B combination (Tables S2, S3; Fig. S1H,I,J). Taken together with the finding that multiple Wnt pairs can synergize, this result suggests that Wnt combinations have the potential to regulate the expression of multiple downstream pathways.

Synergistic signaling requires both FZD5 and FZD8

FZDs are the primary Wnt receptors on the cell surface and trigger various downstream signaling cascades based on the co-receptors involved (Niehrs, 2012). To test their involvement in *WNT1* and *WNT7B* synergistic signaling, we individually knocked down each FZD receptor that was expressed in both HEK293 and YCC11 cells (*FZD2*, *FZD3*, *FZD4*, *FZD5*, *FZD6*, *FZD7* and *FZD8*) using two independent siRNAs. Knockdown of either *FZD5* or *FZD8* reduced both the *WNT1* signal as well as *WNT1* and *WNT7B* synergy in HEK293-STF cells (Fig. 3A). There was no effect of the knockdown of other FZDs. Synergistic activation of endogenous *AXIN2* expression in YCC11 cells was likewise dependent on *FZD5* and *FZD8* expression (Fig. S1K). Consistent with a central role for FZD5 and FZD8 in combined Wnt signaling, their overexpression increased both the basal *WNT7B* signal and the synergistic signal (Fig. 3B). Thus, both *FZD5* and *FZD8* are required and rate limiting for *WNT1* and *WNT7B* signaling.

The WNT7B co-receptor GPR124 is required for synergy

GPR124 was recently identified as a co-receptor for WNT7A and WNT7B involved in Wnt/β-catenin signaling during central nervous system (CNS) angiogenesis (Posokhova et al., 2015; Zhou and Nathans, 2014). We therefore examined the involvement of GPR124 in *WNT7B* signaling and *WNT1* and *WNT7B* synergy. Consistent with published reports, siRNA-mediated knockdown of *GPR124* in HEK293-STF and YCC11 cells abrogated the *WNT7B*-induced STF reporter activity (Fig. 4A,B). Similar results were seen with multiple independent siRNAs (Fig. S2A–C). *GPR124* knockdown also abrogated the induction of *AXIN2* mRNA by *WNT7B* in YCC11 cells (Fig. 4C). Importantly, knockdown of

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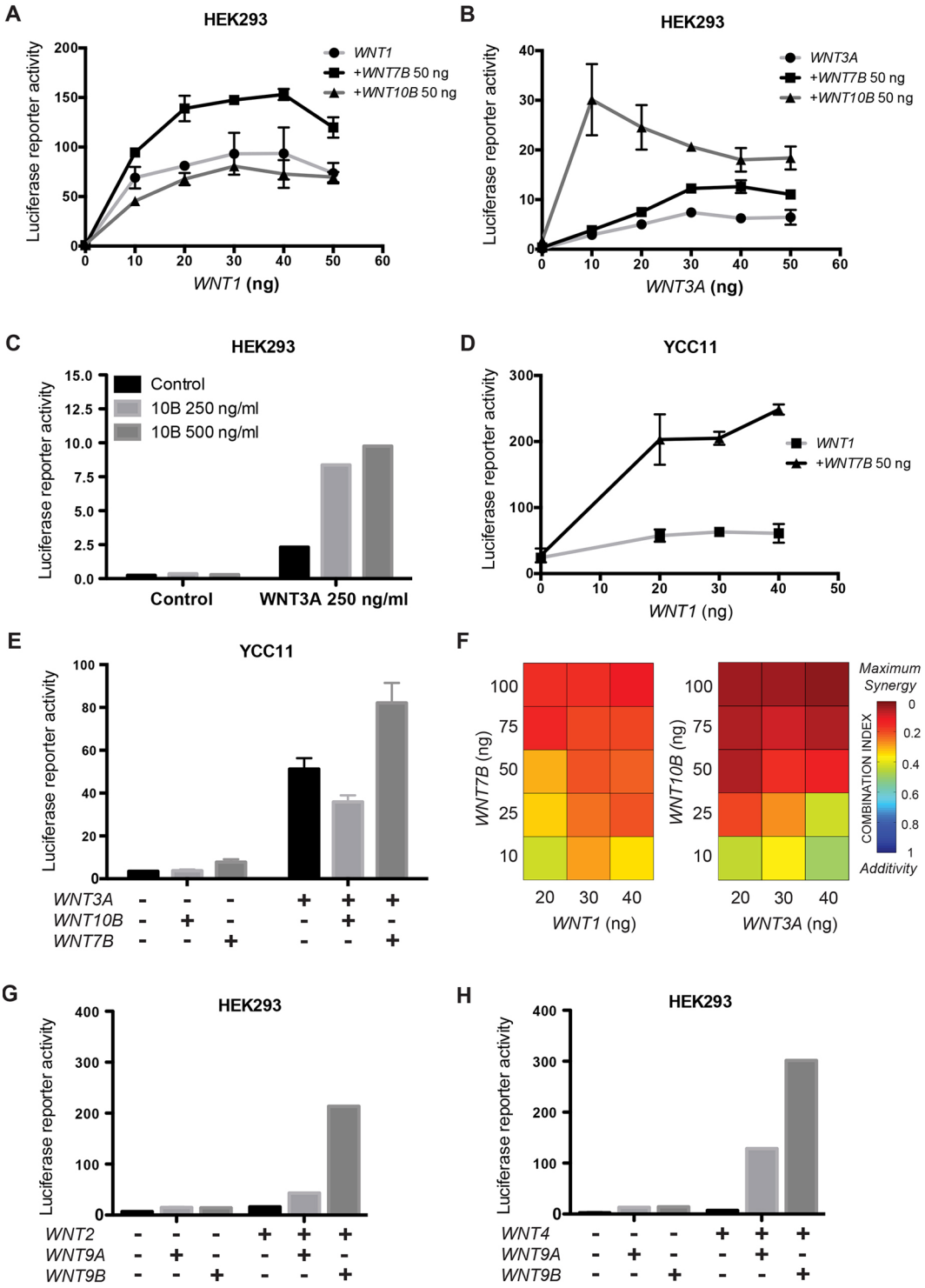

Fig. 1. Wnts specifically potentiate the activity of other Wnts. (A) *WNT7B*, but not *WNT10B*, synergizes with *WNT1*, demonstrating specificity in synergy. HEK293-STF cells were transfected with increasing amounts of *WNT1* expression plasmid in the absence or presence of 50 ng of *WNT7B* or *WNT10B* expression plasmid. Luciferase activity was measured 24 h post transfection and normalized as described in the Materials and Methods for transfection efficiency. (B) As in A, but with *WNT3A* instead of *WNT1*. *WNT10B* synergizes with *WNT3A* more robustly than does *WNT7B*. Graphs show mean±s.d. (n=3). (C) Purified Wnt protein stimulates synergistic signaling. HEK293-STF cells were treated with recombinant human WNT3A protein (250 ng/ml) in the absence or presence of recombinant human WNT10B (250 or 500 ng/ml). Luciferase activity was measured 24 h after the addition of Wnt proteins. Each point was performed in duplicate, and the study was repeated three times with similar results. Graph represents mean (n=2). (D) *WNT7B* synergizes with *WNT1* in YCC-11-STF cells. Cells were transfected with *WNT1* expression plasmid in the absence or presence of 50 ng of *WNT7B* and luciferase activity was measured as for A and B. Graph shows mean±s.d. (n=3). (E) *WNT7B* but not *WNT10B* synergizes with *WNT3A* in YCC-11-STF cells and luciferase activity was measured as for D. 50 ng of each expression plasmid was used. Graph shows mean±s.d. (n=3). (F) Combination Index (CI) calculated for the indicated Wnt combinations shows that they have a high degree of synergy. (G,H) 'Non-canonical' Wnts activate β-catenin signaling in combination. *WNT2*, *WNT4*, *WNT9A* and *WNT9B* expression constructs alone (50 ng each) do not activate the STF reporter, but specific combinations give up to a 30-fold increase in signaling. Each point was performed in duplicate, and the study was repeated three times with similar results. Graphs represent mean (n=2).

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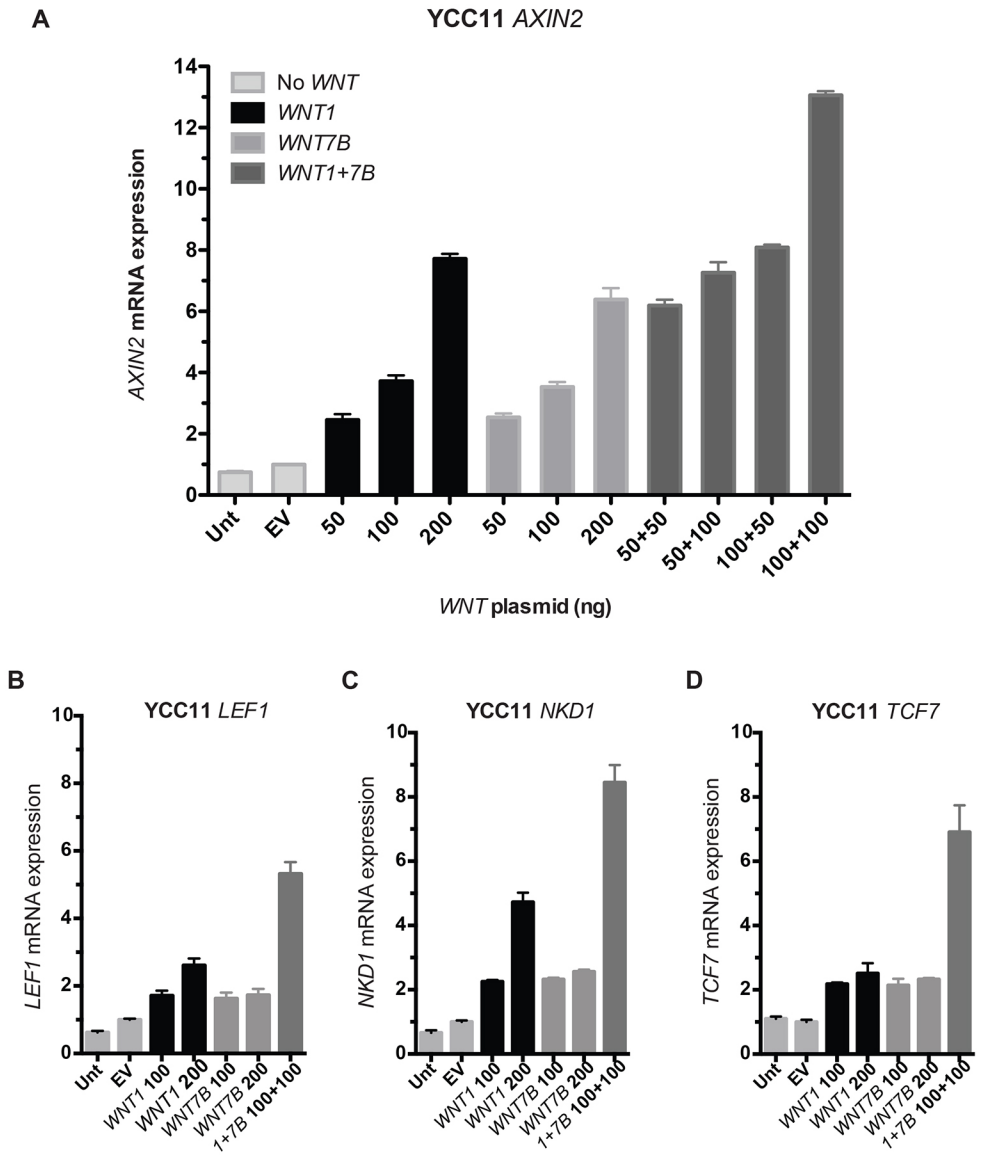

Fig. 2. WNT1 and WNT7B synergistically upregulate canonical Wnt target genes in YCC11 cells. (A) *AXIN2* mRNA abundance in YCC11 cells was assessed by qRT-PCR 24 h after transfection with the indicated combinations of *WNT1* and *WNT7B* expression plasmids. 50 ng of *WNT1* in combination with *WNT7B* is more potent than 100 ng *WNT1* or *WNT7B* alone. The CI was <0.65 for 50 ng, and <0.3 for 100 ng of Wnts, demonstrating strong synergy. Results are mean±s.d. (n=3). P<0.0001 for *WNT1* or *WNT7B* (200 ng) vs *WNT1* +*WNT7B* (100 ng each) (ANOVA with Tukey's multiple comparison test). (B–D) The same analysis as in A was performed for the β-catenin target genes *LEF1*, *NKD1* and *TCF7*. The CI ranged from 0.002–0.21, indicating strong synergy. P<0.0001 for *WNT1* or *WNT7B* (200 ng) vs WNT1+ WNT7B (100 ng each) (ANOVA with Tukey's multiple comparison test). Unt, untransfected; EV, empty vector.

GPR124 had no effect on WNT1 signaling, but markedly reduced WNT1 and WNT7B synergy (Fig. 4A–C; Fig. S2A). Conversely, overexpression of GPR124 increased *WNT7B* signaling in both HEK293-STF and YCC11 cells (Fig. S2E,F) and increased WNT1 and WNT7B synergy (Fig. S2G). We conclude that WNT1 and WNT7B synergy requires engagement of GPR124 by WNT7B.

The glycophosphatidylinositol (GPI)-anchored membrane protein RECK has also been shown to be a part of the WNT7–GPR124 complex at the membrane required for β-catenin signaling (Vanhollebeke et al., 2015). Consistent with this, siRNA-mediated knockdown of RECK also decreased *WNT7B*-induced luciferase reporter activity and synergy in both HEK293-STF and YCC11 cells (Fig. 4D,E; Fig. S2D) and reduced *WNT7B*-induced *AXIN2* mRNA expression in YCC11 cells (Fig. 4F). Therefore, WNT7B signaling at the membrane requires GPR124 and RECK along with FZD5 and FZD8 to signal alone and to potentiate the WNT1-induced signaling.

WNT1 and WNT7B synergy is downstream of β-catenin stabilization

To investigate the mechanism of WNT1 and WNT7B cooperation, events downstream of the Wnt–receptor interaction were examined. Prior to the identification of GPR124 as a WNT7A and WNT7B co-receptor, Miller et al. reported that WNT7A and WNT7B cooperate in foregut development, and this cooperation was mediated through the PDGF pathway (Miller et al., 2012). However, while we found that GPR124 knockdown reduced synergy, in our system inhibition of PDGFR (and other growth factor receptor tyrosine kinases) had no effect on WNT1 and WNT7B synergy (Fig. S2H,I).

Binding of Wnt ligands to their FZD receptors and LRP6 co-receptor leads to LRP6 phosphorylation followed by inactivation of the β-catenin destruction complex (Clevers and Nusse, 2012; MacDonald et al., 2009; Yu and Virshup, 2014). To test the role of LRP6 in WNT1 and WNT7B synergy, we used DKK1, which binds to and blocks LRP6 function. Recombinant DKK1 decreased total Wnt/β-catenin signaling but did not reduce synergy of the residual signal (Fig. 5A). LRP6 S1490 phosphorylation was increased by *WNT1* but not *WNT7B*; however, the combination of Wnts did not lead to additional LRP6 phosphorylation (Fig. 5B; Fig. S3A, Fig. S8). Thus, synergy does not appear to occur at the level of LRP6 activation. The *WNT3A* and *WNT10B* synergy on the other hand was inhibited by DKK1 (Fig. S3B) indicating that the WNT3A and WNT10B synergy

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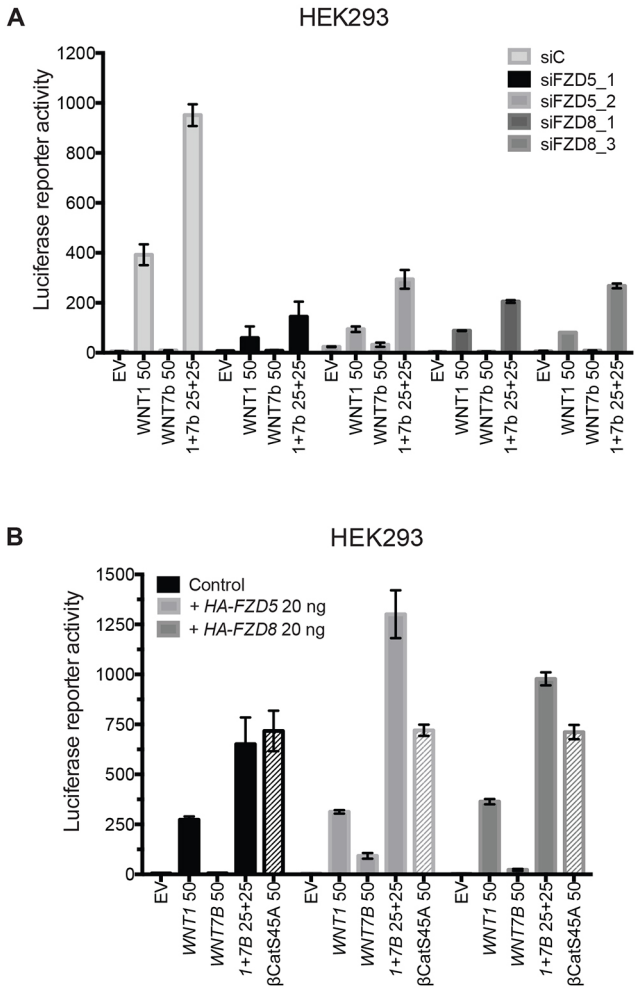

Fig. 3. FZD5 and FZD8 are both required for Wnt synergy. (A) Knockdown of either *FZD5* (siFZD5_1 and siFZD5_2) or *FZD8* (siFZD8_1 and FZD8_3), each with two independent siRNAs, markedly diminished β-catenin signaling as measured with the STF reporter. All experiments were performed in triplicate in 24-well plates and repeated three times with similar results. Graph shows mean±s.d. (n=3). The Wnt combinations led to a significantly greater signaling than WNT1 alone regardless of FZD knockdown (P<0.001). siC, control siRNA. (B) FZD overexpression increases synergy. A synergy experiment with *WNT1* and *WNT7B* was performed in the absence or presence of 20 ng *HA-FZD5* or *HA-FZD8* plasmid. As a control, signaling induced by β-catenin S45A was not affected by FZD expression. EV, empty vector.

may require LRP6 engagement via different propeller domains (Ettenberg et al., 2010; Gong et al., 2010). Supporting a central role for LRP6 in Wnt synergy, DKK1 also inhibited *WNT2* or *WNT4* synergistic signaling with *WNT9B* (Fig. S3C).

As expected, we found that β-catenin is required for signaling and synergy. siRNA-mediated knockdown of β-catenin abrogated the WNT1 signal (Fig. 5C). However, the residual β-catenin still functions better in the presence of combined *WNT1* and *WNT7B* expression. To test whether the synergistic signal is due to increased stabilized β-catenin, we probed for total β-catenin by western blotting. However, *WNT7B* co-expression did not cause any further increase in stabilized β-catenin beyond that caused by expression of *WNT1* alone (Fig. 5B).

After cytoplasmic stabilization, nuclear translocation of β-catenin is the next step in signaling. Surprisingly, there was no additional increase in nuclear β-catenin with combined WNT1 and WNT7B co-expression in HEK293 cells (Fig. 5D). We also examined the levels of non-phosphorylated 'active' β-catenin and found that there was no increase in 'active' β-catenin upon WNT1 and WNT7B co-expression (Fig. 5E). We therefore considered whether WNT7B directly enhanced the activity of nuclear β-catenin. *WNT7B* expression, which alone produces no STF activity in HEK293 cells, enhanced the activity of a stabilized β-catenin with an S45A mutation (Fig. 5F), albeit to a lesser extent (<1.5-fold) than the combination of WNT1 and WNT7B (>3-fold). Calculation of combination indices for *WNT7B* and β-catenin-S45A also yielded a CI of <<1, indicative of synergy. Interestingly, this effect is not unique to WNT7B as several other 'non-canonical' Wnts (*WNT2*, *WNT9B* and *WNT10B*) that did not signal alone could also modestly activate β-catenin-S45A signaling (Fig. 5G). These results suggest that the 'non-signaling' Wnts (WNT2, WNT7B, WNT9B and WNT10B) can signal directly to stabilized β-catenin, but that formation of a two-Wnt signaling complex at the membrane is a much more potent means of generating synergy in the nucleus.

WNT1 and WNT7B synergistic signaling causes increased acetylation of β-catenin

We next examined events downstream of β-catenin stabilization and nuclear entry. Recruitment of CBP/p300 to β-catenin is one of the key events that occurs after β-catenin translocates to the nucleus and has been proposed to be important for the transcriptional activity of the β-catenin–TCF complex (Levy et al., 2004). The acetylation status of β-catenin was therefore examined by immunoblotting with a β-catenin K49Ac-specific antibody. WNT1 and WNT7B individually each induced acetylation of β-catenin to a greater or lesser extent in YCC11 and HEK293 cells (Fig. 6A,B; Fig. S7). However, β-catenin K49Ac was markedly increased when *WNT1* and *WNT7B* were co-expressed. This suggests an increase in specific nuclear acetyltransferase activity or a decrease in deacetylase activity in response to WNT7B signaling.

We tested whether CBP was responsible for the synergistic signaling. CBP acetylates β-catenin on K49 (Wolf et al., 2002). Treatment with the β-catenin–CBP interaction inhibitor ICG-001 (Emami et al., 2004) indeed globally decreased β-catenin signaling. However, the IC_{50} of inhibition of signaling by ICG-001 was the same for WNT1 alone as for the WNT1 plus WNT7B synergistic signal (Fig. S3D). Since ICG-001 is a binding inhibitor, this suggests there is no increase in the CBP bound to the complex during synergistic signaling.

GPR124 is the co-receptor responsible for the WNT7B signaling in HEK293 and YCC11 cells. *GPR124* knockdown decreased both the WNT7B-induced β-catenin K49Ac, and the synergistic β-catenin acetylation induced by WNT1 and WNT7B (Fig. 6C; Fig. S3E). Thus, the WNT7B interaction with GPR124 activates β-catenin acetylation and synergistic β-catenin signaling. Ongoing studies aim to define the signaling pathway from GPR124 to nuclear protein acetylation.

The functional importance of β-catenin acetylation is not well established. To test whether modification of β-catenin on K49 is required for synergy, endogenous β-catenin was knocked down using siRNA and rescued with either siRNA-resistant wild-type β-catenin or the K49R mutant. We found that the K49R acetylation mutant of β-catenin could signal and synergize with *WNT7B* as well as with the wild type (Fig. S5A). Similarly, using a second approach, we found that *WNT7B* could synergize with the K49R mutant of stabilized β-catenin S45A to the same extent as it does with the wild-type β-catenin S45A (Fig. S5C,D). In both instances, the K49R mutation had no effect on β-catenin signaling. These findings suggest that Wnt synergy enhances nuclear protein

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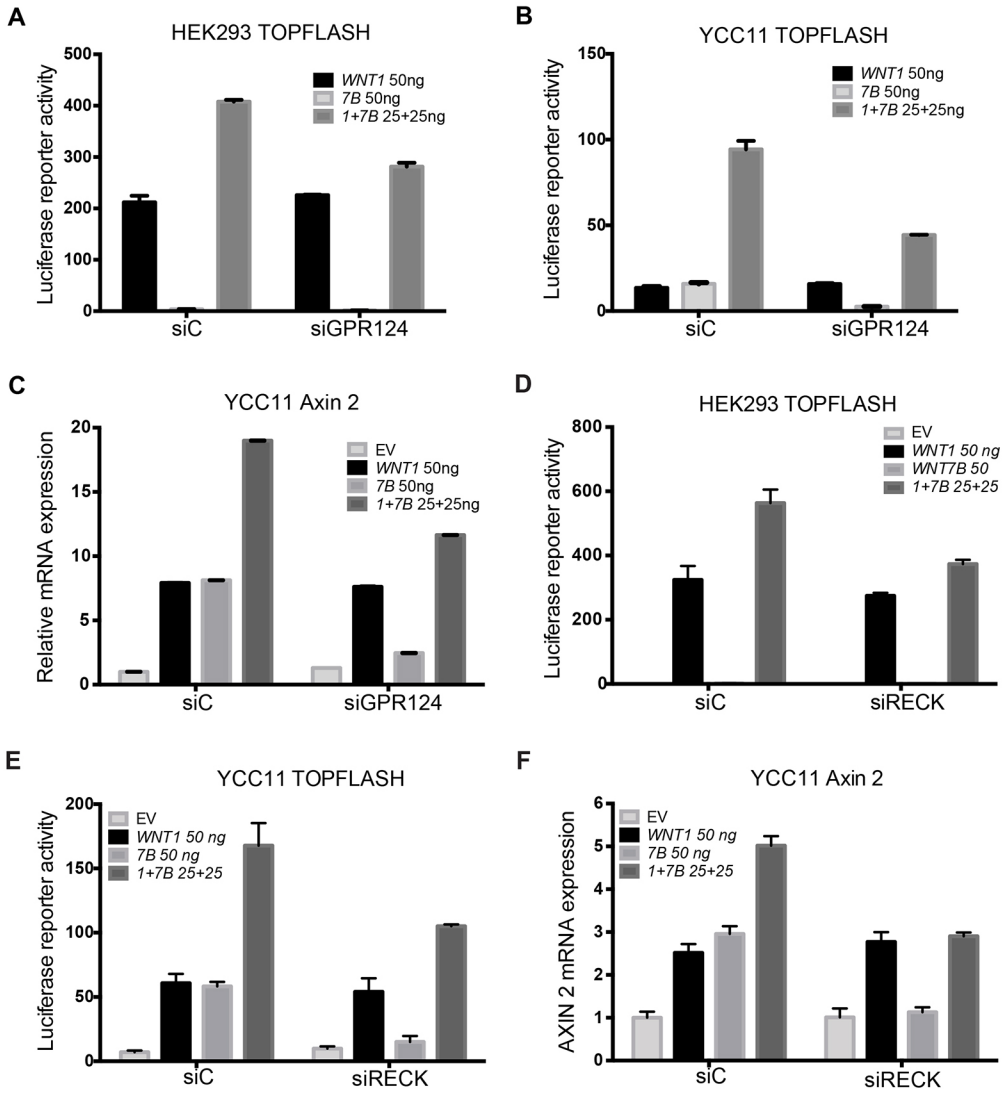

Fig. 4. GPR124 is the receptor for WNT7B responsible for synergistic signaling. Knockdown of *GPR124* in (A) HEK293-STF and (B) YCC11 cells with an siRNA pool (siGPR124; 100 nM) decreases the synergy from *WNT1+WNT7B* co-expression. siRNA was introduced 24 h prior to *WNT* transfection, and signaling was assessed 24 h later. Individual siRNAs had a similar effect (Fig. S2A). *WNT1+WNT7B* synergy was significantly reduced upon GPR124 knockdown (P<0.0001). (C) *GPR124* knockdown inhibits synergy and *WNT7B* signaling as determined by measuring the amount of endogenous *AXIN2*. The experiments were performed as in B except *AXIN2* was assessed by qRT-PCR and normalized to *HPRT1*. *WNT7B* signaling and *WNT1+WNT7B* synergy was significantly reduced upon GPR124 knockdown (P<0.0001). (D,E) Knockdown of RECK in (D) HEK293-STF and (E) YCC11 cells with an siRNA pool (siRECK; 100 nM) reduced *WNT1+WNT7B* synergy. WNTs were transfected 24 h post siRNA transfection and luciferase reporter activity was measured 24 h later. (F) Knockdown of RECK also inhibits *WNT7B* signaling and *WNT1+WNT7B* synergistic upregulation of the endogenous *AXIN2*. Graphs represent mean±s.d. (n=3). EV, empty vector; siC, control siRNA.

acetylation, and that acetylation of β-catenin K49 correlates with, but is not essential for, Wnt synergy.

Subsets of gastric cancers co-express multiple Wnt genes

Multiple Wnt genes appear in the genomes of all metazoans including sponges, suggesting synergy between Wnts may be a central feature of this pathway (Nichols et al., 2006). To test whether increased co-expression of Wnt genes might play a role in cancer, we assessed the expression of Wnt genes in a cohort of 201 primary gastric cancer samples previously stratified by gene expression signature into mesenchymal (Fig. 7, orange), proliferative (Fig. 7, cyan) and metabolic (Fig. 7, purple) groups (Lei et al., 2013). The data suggest that there is increased expression of distinct combinations of specific Wnt ligands in different subsets of gastric cancers (Fig. 7A). For example, *WNT2B* and *WNT9A* expression clusters in the mesenchymal group, *WNT5A* is expressed primarily in the proliferative group, and multiple Wnts including WNT1, WNT3 and WNT7 are expressed in the metabolic group. Wnt gene expression can be in either cancer cells themselves, and/or from cells in the stroma. Additionally, Wnt gene expression in cancers and stroma may be induced by the interactions in the local microenvironment. Consistent with a key role for stroma, expression profiling of 37 gastric cancer cell lines (Ooi et al., 2009) grown in plastic in the absence of stroma did not show similar Wnt-high subsets (Fig. S4). We next asked whether significant Wnt gene expression occurs in tumor stroma. We examined six gastric cancer patient-derived xenografts propagated in NSG mice, where the cancer cells are human but the stroma is murine. Using human- or mouse-specific PCR primers (Table S4), we found multiple but different mouse (i.e. stromal) Wnts were upregulated, with samples GC38, GC84 and GC47 having high human and mouse Wnt expression, and GC72, GC66 and GC45 having low expression (Fig. 7B,C). Taken together, the data suggest that Wnt synergy can occur in a subset of gastric cancers that have coordinate upregulation of multiple Wnts produced both in cancer and stromal cells.

WNT1 and WNT7B synergy increases clonogenicity of YCC11 cells

As multiple Wnts are co-expressed in a subset of gastric cancers, we tested the biological consequences of WNT1 and WNT7B synergy in the gastric cancer cell line YCC11. *WNT1* alone, *WNT7B* alone or *WNT1* plus *WNT7B* were stably expressed in YCC11 cells by lentiviral transduction and the ability of these cells to form colonies in soft agar was assessed (Fig. 8). Cells expressing *WNT1* alone were hindered in their ability to form colonies, while *WNT7B* expression alone had no effect. The combination of *WNT1* and

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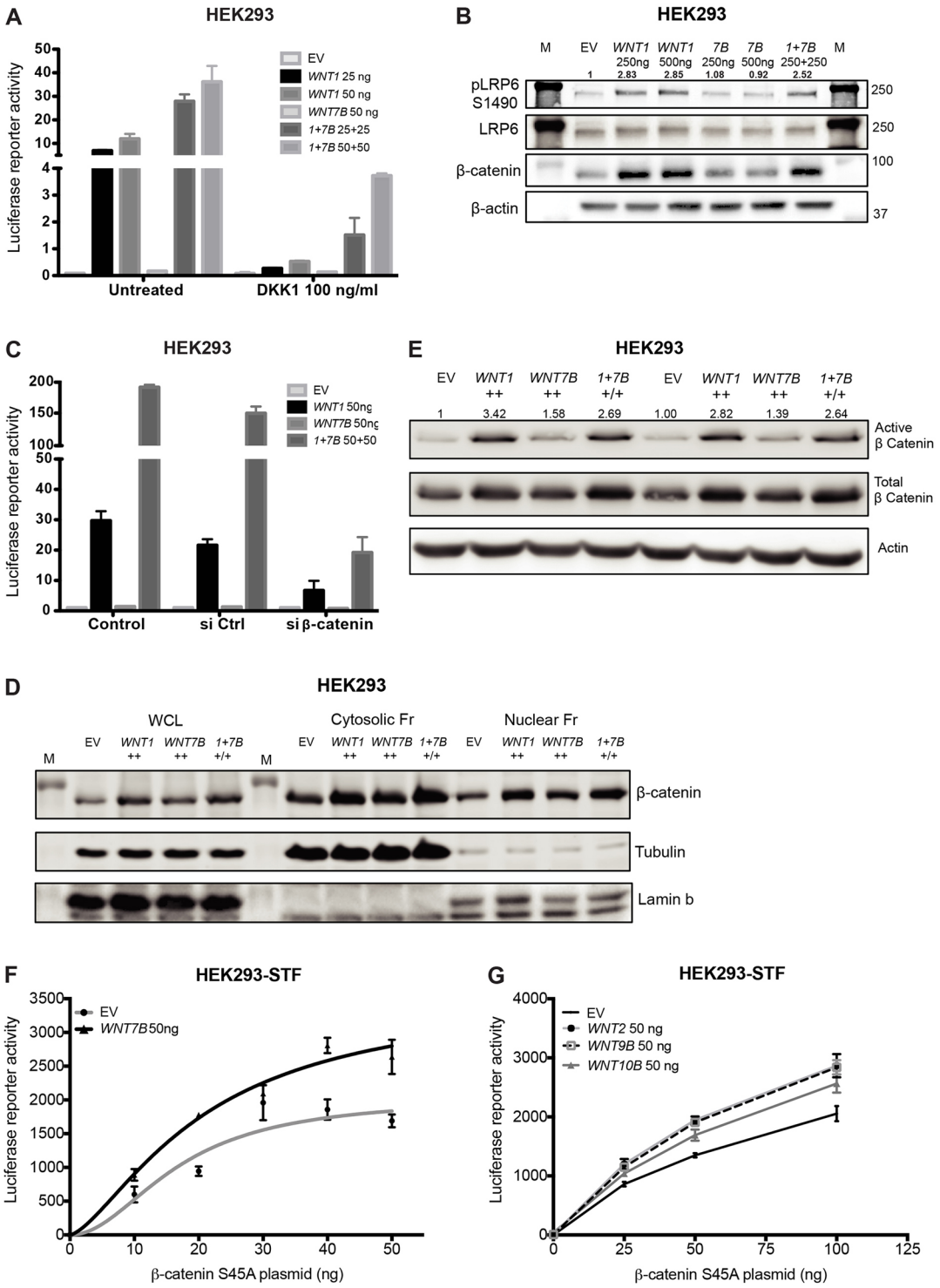

Fig. 5. See next page for legend.

WNT7B doubled the number of colonies and also increased their size. The data are consistent with Wnt co-expression promoting tumorigenesis by synergistic regulation of downstream target genes.

DISCUSSION

Multiple Wnts potently regulate diverse biological responses. Wnt genes are often coordinately expressed, but they are most often studied individually. Here, using a library of 19 Wnts, we assessed interactions between multiple Wnts and find synergy to be a general phenomenon. We find that specific combinations of Wnts signal synergistically through both the well-known FZD5 and FZD8 receptors and the recently described WNT7 receptor GPR124 and its co-receptor RECK. One surprising consequence of synergy between WNT1 and WNT7A occurs at the level of GPR124-dependent increased β-catenin acetylation. The data suggest a model whereby 'non-canonical' Wnts such as WNT7B interact with

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Fig. 5. Synergistic WNT1 and WNT7B signaling is downstream of LRP6 phosphorylation and β-catenin stabilization. (A) DKK1 inhibits WNT signaling but not *WNT1+WNT7B* synergy. HEK293-STF cells were transfected with *WNT1* and *WNT7B* expression plasmids as indicated. Recombinant purified DKK1 (100 ng/ml) was added 5 h post transfection. Signaling was measured 24 h post *WNT* transfection. (B) Synergy does not increase LRP6 phosphorylation. HEK293 cells in 60 mm dishes were transfected with the indicated WNT expression plasmids and lysed 24 h post transfection. Lysates were analyzed by SDS-PAGE and immunoblotted for LRP6 phosphorylated at S1490 (pLRP6 S1490), total LRP6 and β-catenin. The blot is representative of three independent experiments shown in Fig. S8. The numbers above the blot represent the ratio of pLRP6 to total LRP6 normalized to empty vector (EV) (=1). (C) siRNA-mediated knockdown of β-catenin reduces WNT signaling but not *WNT1+WNT7B* synergy. HEK293-STF cells were transfected with 50 nM siRNA against β-catenin (si β-catenin). Plasmids encoding *WNT1* and *WNT7B* (50 ng) were transfected as indicated 48 h post siRNA transfection. Signaling was measured 24 h post WNT transfection. (D) Synergy does not increase the nuclear abundance of β-catenin. Cytoplasmic and nuclear lysates were prepared from HEK293 cells transfected with the indicated WNT expression constructs (100 mm dishes; 1.5 µg each of *WNT1* or *WNT7B*). At 24 h post transfection, lysates were analyzed by immunoblotting for β-catenin. WCL, whole-cell lysate; Cytosolic Fr, cytosolic fraction; Nuclear Fr, nuclear fraction. Tubulin and lamin b were used as controls for cytoplasmic and nuclear protein fractions, respectively. The blot is representative of three independent experiments. ++, represents 2× of either *WNT1* or *WNT7B*; +/+, 1× of *WNT1* with 1× of *WNT7B*. (E) Synergy does not increase the amount of unphosphorylated 'active' β-catenin. HEK293 cells in 6-well plates were transfected with the indicated WNT expression plasmids and lysed 24 h post transfection. Lysates were analyzed by SDS-PAGE and immunoblotted for unphosphorylated and total β-catenin. The blot is representative of three independent experiments. The numbers above the blot represent the ratio of unphosphorylated to total β-catenin normalized to empty vector (=1). ++, represents 2× of either *WNT1* or *WNT7B*; +/+, 1× of *WNT1* with 1× of *WNT7B*. (F) *WNT7B* modestly potentiates β-catenin signaling downstream of β-catenin stabilization. STF reporter activity in HEK293 cells transfected with β-catenin S45A expression plasmid (10–50 ng) in the absence or presence of 50 ng of *WNT7B* expression plasmid in a 24-well plate. Firefly luciferase readings were taken 24 h post transfection. The potentiation of β-catenin S45A-induced signaling by *WNT7B* was significant at $P<0.0001$ for all concentrations of S45A except 10 ng ($P<0.05$) and 30 ng of S45A (not significant). (G) Other 'non-canonical' Wnts can further partially activate stabilized β-catenin. STF reporter activity in HEK293 cells transfected with the indicated amounts of β-catenin S45A expression plasmid in the absence or presence of 50 ng of *WNT2*, *WNT9B* or *WNT10B* expression plasmid in a 24-well plate. Firefly luciferase readings were taken 24 h post transfection. Quantitative results are mean±s.d. ($n=3$). EV, empty vector; siCtrl, control siRNA; M, marker lanes.

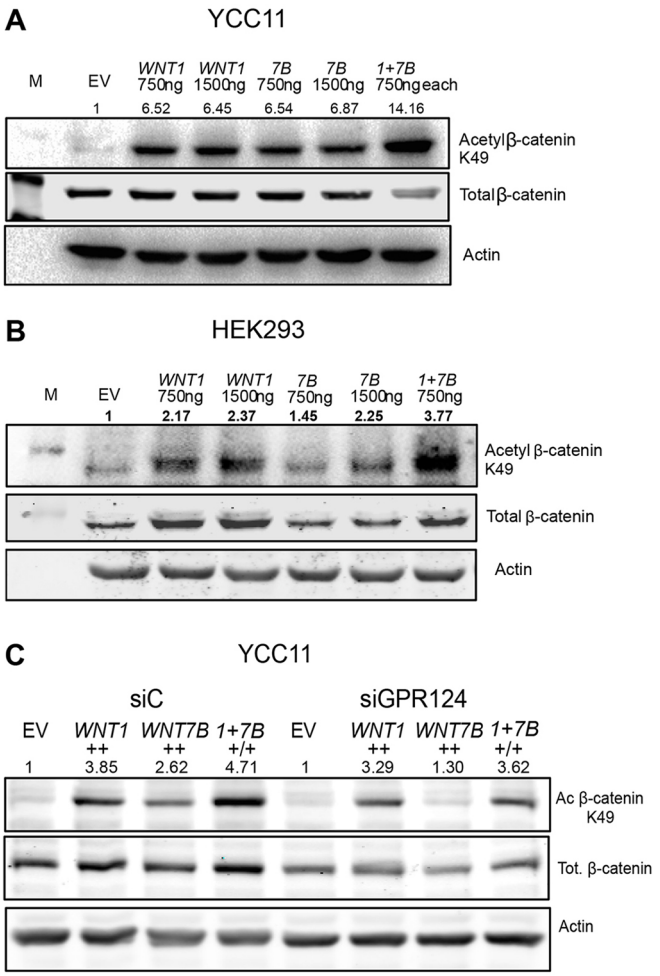

Fig. 6. β-catenin K49 acetylation is stimulated by WNT1 plus WNT7B signaling and requires GPR124. (A) *WNT1* and *WNT7B* were expressed as indicated in YCC11 cells and after 24 h lysates were analyzed for acetylated β-catenin K49 and total β-catenin. 100 mm dishes were used. Plasmid amounts used were proportional to the surface area so as to maintain a constant plasmid-to-surface-area ratio. The blot is representative of the three independent experiments that are shown in Fig. S7. (B) As in A, but in HEK293 cells. The numbers above the blot represent the ratio of acetylated β-catenin (K49) to total β-catenin normalized to empty vector (EV) (=1). (C) The increase in β-catenin acetylation is transduced through GPR124. YCC11 cells in 12-well plates were transfected with 100 nM of either control (siC) or a pool of siRNAs against *GPR124* (siGPR124). Plasmids encoding *WNT1* (100 ng), *WNT7B* (100 ng), and *WNT1* and *WNT7B* together (50 ng each) were transfected 24 h post siRNA transfection. Lysates were made 24 h post WNT transfection and probed for acetylated β-catenin K49 and total β-catenin. The blot is representative of three independent experiments. The numbers above the blot represent the ratio of acetylated β-catenin to total β-catenin normalized to empty vector (=1). ++, represents 2× of either *WNT1* or *WNT7B*; +/+, 1× of *WNT1* with 1× of *WNT7B*. Individual siRNAs had a similar effect (Fig. S3E).

specific FZDs and LRP5/6 in the presence of a third co-receptor, in this case GPR124, to signal through currently unknown pathways to enhance β-catenin activity, nuclear lysine acetylation and, hence, gene expression.

GPR124 is a putative G-protein-coupled receptor required for angiogenesis in the brain, and was recently shown to be a co-receptor for WNT7 family members (Posokhova et al., 2015; Zhou and Nathans, 2014). GPR124 is likely to be important in vasculature outside of the brain, as it was also identified as tumor endothelial marker 5 (TEM5), a transcript enriched in the vasculature of human colorectal cancer and murine tumors (Carson-Walter et al., 2001) and which is itself upregulated by TGF-β (Anderson et al., 2011) and Rac signaling (Vallon et al., 2010). Whether activated GPR124 couples to a G-protein, or whether it signals to the nucleus via other mechanisms is not currently known.

β-catenin acetylation by CBP/p300 has been frequently observed during canonical Wnt/β-catenin pathway activation (Hecht et al., 2000; Levy et al., 2004) and may play a role in signaling at β-catenin-responsive promoters. Our data suggest that WNT7B and GPR124 signaling either increases the activity of the β-catenin K49 acetyltransferase (CBP) (Wolf et al., 2002), or decreases the activity of the deacetylase. Of course, changes in the activity of these enzymes may also regulate the acetylation of other important lysine residues leading to changes in promoter activation. We have not examined the acetylation status of other lysine residues on β-catenin (e.g. K345) due to lack of good antibodies. Involvement of other HATs that may acetylate additional lysine residues on β-catenin or other proteins cannot be ruled out based on our current data. Additional work is needed to clarify the landscape of lysine acetylation changes in response to WNT7B and GPR124 signaling. We found the acetylation of β-catenin on lysine 49 to be a

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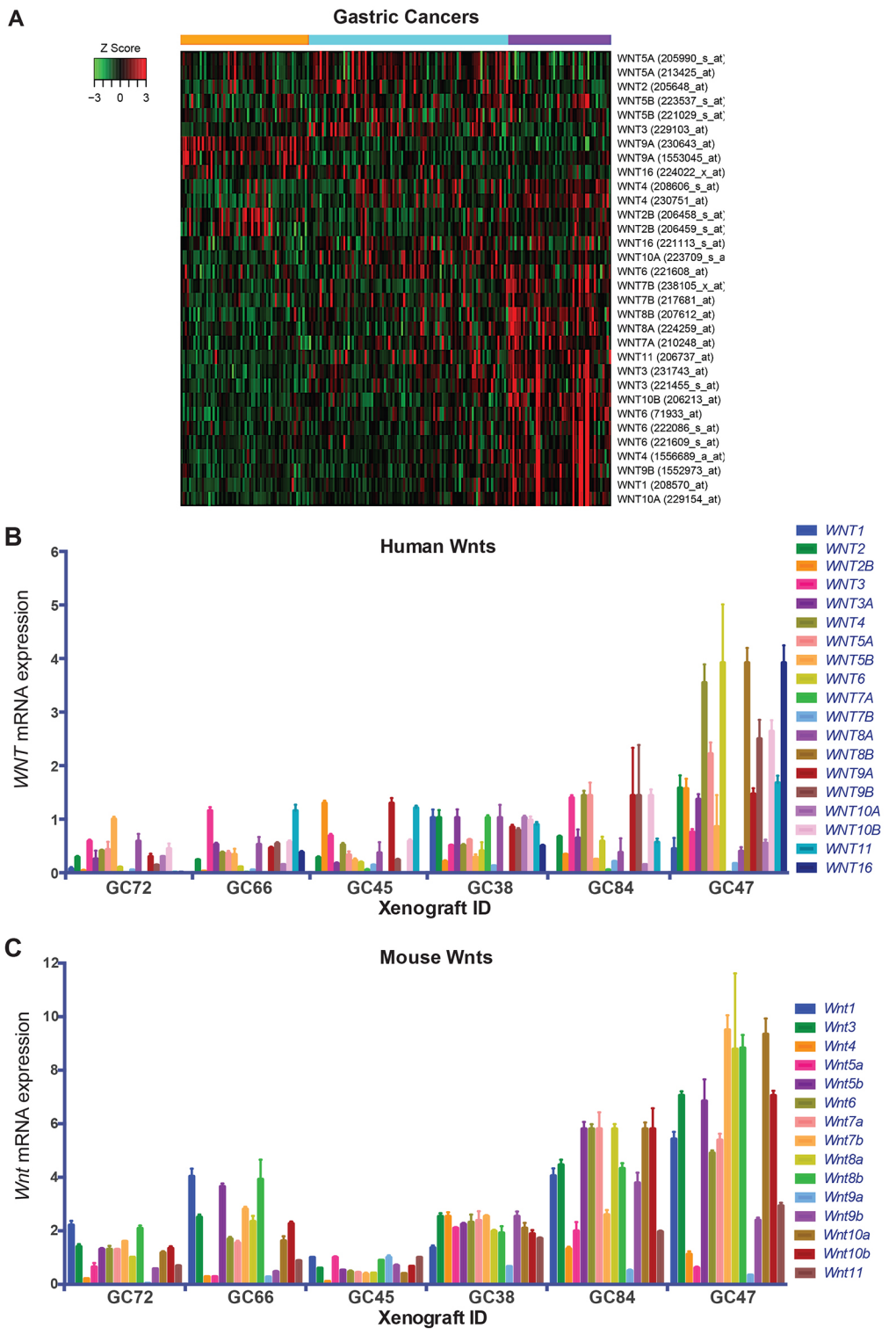

Fig. 7. Multiple Wnts are coordinately upregulated in cancer. (A) A subset of gastric cancers express multiple Wnts. Gene expression profiling data from 201 previously reported gastric cancers was clustered into three groups, invasive (orange), proliferative (blue) and metabolic (purple) as described previously (Lei et al., 2013), and Wnt gene expression examined. The metabolic subtype expressed multiple Wnts, while the proliferative subtype was *WNT5A* high. (B) Wnt-high gastric cancer xenografts express both human and mouse Wnts. Relative expression of human WNTs in six human gastric cancer patient-derived xenografts propagated in NSG mice was assessed by qRT-PCR using human-specific *WNT* primers. (C) Relative expression of mouse Wnts was assessed in the same samples using mouse-specific *Wnt* primers. Results are mean±s.d. (n=3).

consequence of increased acetyltransferase (or reduced deacetylase) activity and that it was not required for synergy as the K49R mutant of β-catenin could signal and synergize with WNT7B as well as did the wild type (Fig. S5).

Miller et al. (2012) reported that WNT2 and WNT7B cooperate in foregut development. They found that WNT2 and WNT7B cooperation was specific to the mesenchymal cell lineage and did not occur in epithelial cells. We confirmed that WNT2 and WNT7B did not synergize in HEK293 cells. Conversely, they did not see synergy between WNT1 and 7B in mesenchymal cells, while we found robust synergy between these Wnts in multiple cell types of epithelial origin. The differences may well be related to expression patterns of known and novel Wnt receptors in various cell types. Further synergistic partners may be discovered by conducting similar screens in different cell types or model systems.

One clue to mechanism is the correlation between synergy pairs and the ability of different Wnts to bind to different domains on LRP6 (Bourhis et al., 2010; Ettenberg et al., 2010; Gong et al.,

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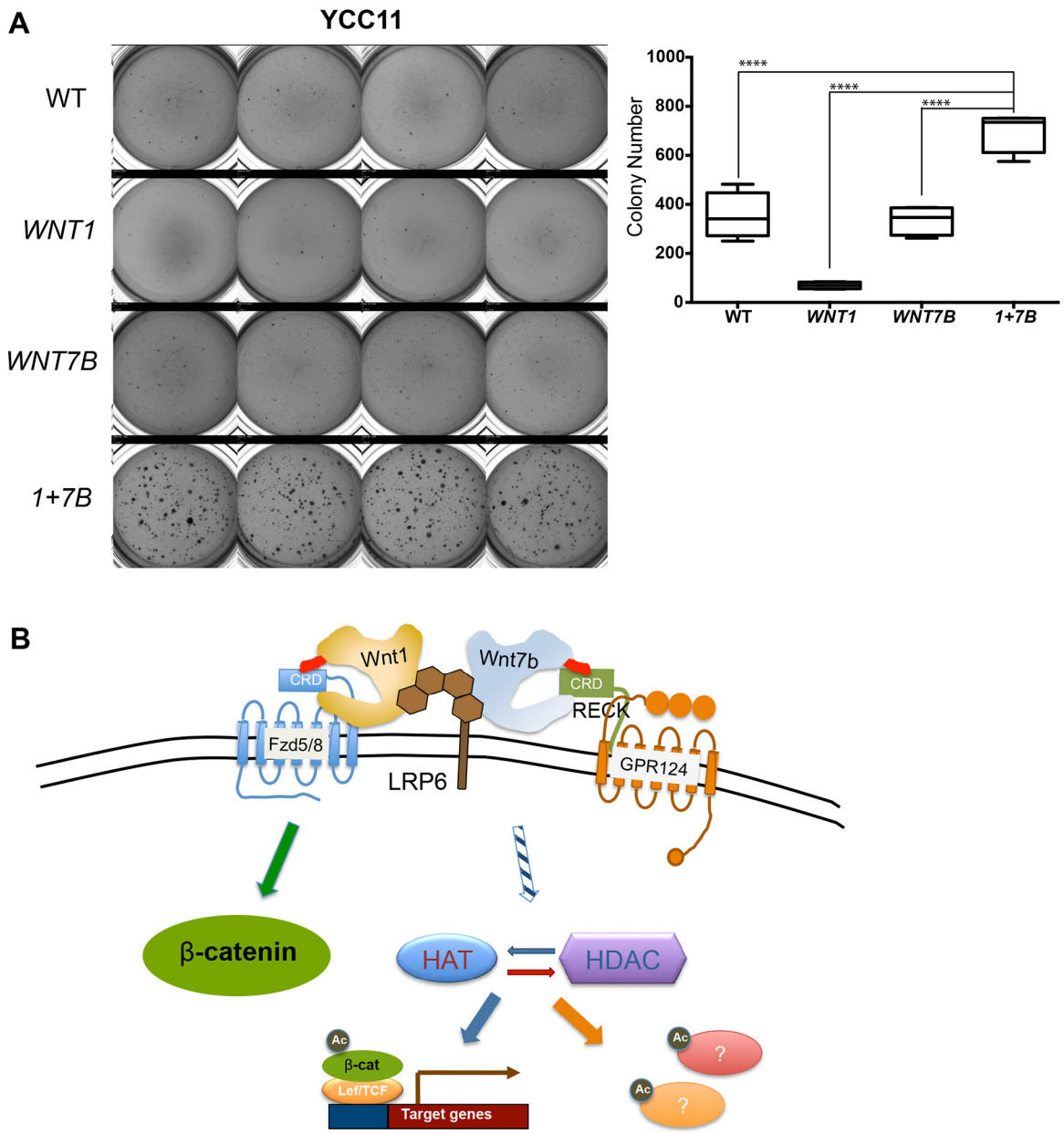

Fig. 8. *WNT1* and *WNT7B* co-expression enhances transformation of YCC11 cells, and a model of WNT1 and WNT7B synergy. (A) Soft agar assay with YCC11 cells stably expressing *WNT1*, *WNT7B* or both as indicated. The assay was performed twice with similar results. The quantification shows the results from one assay. The box represents the 25–75th percentiles, and the median is indicated. The whiskers show the minimum and maximum values. ****P<0.0001 (one-way ANOVA with multiple comparisons using Tukey's multiple comparison test). (B) A speculative model of WNT1 and WNT7B synergy. WNT1 binds to LRP6, FZD5 and FZD8 and signals to stabilize β-catenin. WNT7B binds to a different domain of LRP6 and RECK–GPR124 (and potentially to FZD5 and FZD8 as well, not shown in the model for the sake of simplicity). This nucleates the formation of multimeric membrane receptor complexes to activate two signaling pathways. WNT1 causes β-catenin stabilization, while the WNT7B–RECK–GPR124 complex also leads to alteration of the HAT/HDAC balance in the nucleus, causing increased lysine acetylation and transcriptional activation.

2010). We speculate that the synergy of the Wnt pairs may be due to the formation of a multimeric complex comprising multiple LRP6/WNT/FZD and alternative receptors such as GPR124/RECK at the membrane. A speculative model (based on Bourhis et al., 2010) is presented in Fig. 8D.

Wnt ligand synergy in activating the Wnt/β-catenin (and possibly other) pathways has substantial physiological relevance as many of these ligands are co-expressed at low individual levels in various organs (Farin et al., 2012) and during development (Witte et al., 2009). WNT7B has been shown to be involved in the development of lung (Rajagopal et al., 2008), kidney (Yu et al., 2009), nervous system (Stamatakou and Salinas, 2014) and pancreas (Afelik et al., 2015), and is implicated in the pathogenesis of breast (Yeo et al., 2014), pancreatic (Arensman et al., 2014), prostate (Zheng et al., 2013) and bladder cancer (Bui et al., 1998). The ability of WNT7B to potentiate the activity of canonical Wnts may play a significant part in its contribution towards these processes of development and disease progression. The synergy with other Wnts may be needed for proper pathway activation *in vivo*. This provides a further regulatory mechanism for fine-tuning Wnt signaling activity in time and space. Intersecting fields of Wnt expression can focus signaling into discrete areas. Wnt synergy may also explain how high

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β-catenin signaling activity can be maintained without concurrent high expression of any single ligand. Since WNT7B can further activate a stabilized mutant of β-catenin (S45A), this provides an additional route for Wnt ligands to function in cancers with stabilized β-catenin and perhaps even adenomatous polyposis coli (APC) mutations. The study of Wnt interactions will provide additional insights into the complex role that Wnts play in development and disease.

MATERIALS AND METHODS

Reagents

HEK 293-STF cells (with the stably integrated Super8×TOPFlash reporter) were a kind gift from Kang Zhang (Institute for Genomic Medicine, University of California, San Diego, La Jolla, CA) (Xu et al., 2004). HeLa cells were obtained from American Type Culture Collection (ATCC). YCC11 cells were a kind gift from Dr Patrick Tan (Programme in Cancer and Stem Cell Biology, Duke-NUS Medical School, Singapore). Authenticity of the cell lines was not validated. The cells were routinely tested and regularly confirmed to be mycoplasma free. All cells were grown in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque) containing 4.5 g/l glucose, penicillin-streptomycin, 10% fetal bovine serum (FBS) and 1 mM sodium pyruvate in a humidified incubator with 5% CO_2. Recombinant DKK1, human WNT3A and human WNT10B were purchased from R&D Systems (Minneapolis, MN). Super8×TOPFlash reporter (Addgene Plasmid #12456; deposited by Randall Moon; Veeman et al., 2003). Expression plasmids for FZD5 and FZD8 were a kind gift from Jeffrey Rubin (Laboratory of Cellular and Molecular Biology, NCI, NIH, Bethesda, MD). The expression plasmid for GPR124 was a kind gift from Bradley St Croix [Tumor Angiogenesis Section, Mouse Cancer Genetics Program (MCGP), NCI at Frederick, NIH, Frederick, MD] (Posokhova et al., 2015). Wnt expression constructs were prepared as part of the Open Source Wnt project (Addgene Kit #1000000022) (Najdi et al., 2012). The plasmid for β-catenin expression, pCS2+Myc:β-catenin, was mutated to be resistant to β-catenin siRNA #11 (see below) and K49R using site-directed mutagenesis. The plasmid for stabilized β-catenin, pCS2+Myc:β-catenin S45A, was mutated to K49R by using site-directed mutagenesis. All patient samples were collected with informed patient consent from National University Hospital Singapore according to the National Healthcare Group Domain Specific Review Board (DSRB) guidelines (DSRB-B/07/367). All animal experiments were conducted with the approval of Institutional Animal Care and Use Committee (IACUC) in the National University of Singapore.

Gene expression analysis

We utilized previously reported gene expression data for 201 primary gastric tumors (Gene Expression Omnibus GSE15459 and GSE34942) (Lei et al., 2013). Based on the gene expression profiles, these 201 gastric tumors were classified into three molecular subtypes: mesenchymal (orange), proliferative (cyan), and metabolic (purple) (Fig. 7). We extracted WNT genes (probesets) from the gene expression profiles and generated a heatmap on the gene expression values (robust multi-array average) with clustering on WNT genes. We obtained previously reported gene expression data for 37 gastric cancer cell lines (Gene Expression Omnibus GSE22183) (Ooi et al., 2009). All the gastric cancer cell lines except MKN7 were classified into one of the three molecular subtypes using the gastric cancer classifier GC-Class developed in Lei et al. (2013), while MKN7 was unclassifiable. Similarly, we generated the heatmap using the WNT gene expression values with clustering on WNT genes (Fig. S4).

SuperTOPFlash assays

SuperTOPFlash (β-catenin-activated TCF/LEF transcriptional reporter; STF) assays were performed in 24-well plates, transfecting 400 ng of total plasmid/well, composed of indicated amounts of WNT, 100 ng of mCherry expression plasmid and 100 ng of SuperTOPFlash (where needed) by using Lipofectamine 2000 (11668019, ThermoFisher Scientific). Lysates were prepared in PBS with 0.6% NP-40 with complete protease inhibitor cocktail without EDTA (Sigma-Aldrich, St. Louis, MO) and firefly luciferase activity was measured with a luciferase assay kit according to the manufacturer's recommendations (Promega, Madison, WI) by using a Tecan Infinite M200 plate reader (Tecan Trading AG, Switzerland). All assays unless otherwise indicated were performed in triplicate and graphed as mean±s.d. Each experiment was repeated at least three times with similar results.

For siRNA-mediated knockdown experiments, cells were plated in 12- or 24-well plates and transfected with the indicated siRNAs (at indicated concentration) using Lipofectamine 2000 (ThermoFisher Scientific, Waltham, MA). Wnt expression plasmids were transfected 48 h later and cells were harvested after a further 24 h incubation and analyzed for luciferase activity or by qRT-PCR. The siRNAs used are as follows: control/non-targeting (#D-001810-01-05), β-catenin (#J-093415-11, 5′-G-CGTTTGGCTGAACCATCA-3′), *FZD8* (#L-003962, #1: 5′-AGACAG-GCCAGATCGCTAA-3′, #3: 5′-TCACCGTGCCGCTGTGTAA), *GPR124* (#L-005540, #5: 5′-GAGCGAAACTACCGTCTAA-3′, #8: 5′-CGACTA-AACATATCTGGAA-3′) and *RECK* (#L-011474) were from GE-Dharmacon (Lafayette, CO). The targeting sequences for the other FZD siRNAs are as follows: *FZD2* (5′-CGGTCTACATGATCAAATA), *FZD5* (#1: 5′-TCCTCTGCATGGATTACAA-3′, #2: 5′-AGACGGACAAGCTGGAGA-A-3′).

Calculation of combination index

The extent of synergy between two factors (WNT1 and WNT7B, or WNT3A and WNT10B) was quantified using by determining the combination index (Chou and Talalay, 1983, 1984). The combination index, CI, was originally conceptualized in combination therapy to study the extent of interaction between two drugs, where CI=1 indicates purely additive effects. The CI is derived from the median-effect equation which is a unified theory of multiple mass-action equations (Chou, 2006). A combination is classified as synergistic when CI<1, or as antagonistic when CI>1.

To compute the CI for pairs of canonical and non-canonical Wnts, such as *WNT1* and *WNT7B*, or *WNT3A* and *WNT10B*, we first obtained dose–response curves for each Wnt individually. We then stimulated the cells with pairs of Wnt ligands, using a matrix of dose combinations, and we measured the effects with the same SuperTOPFlash assay. As the CI for a pair of drugs is not necessarily constant across different doses, we used the matrix of dose combinations to compute the combination index (CI) for each treatment (Fig. 1F). An alternative method would have been to use serial dilutions of combination at a constant ratio, such as $D_{can}:D_{non\text{-}can}=(IC_{50})_{can}:(IC_{50})_{non\text{-}can}$ (where 'can' is the 'canonical Wnt'). Constant ratio experiments permit simulation of estimated CIs for a generalized range of dose combinations. However, they require that different batches of cells be used for determining the IC_{50} and for quantifying the combination effects. This introduces batch effects, which are problematic to analyze. More importantly, the non-canonical Wnts have a very weak SuperTOPFlash response by themselves, causing their IC_{50} to be far beyond the physiological range, and producing a poor signal-to-noise ratio in the non-canonical single-Wnt curve. In contrast, our matrix design (non-constant ratio combinations) allowed us to use dose combinations that are of practical relevance. We then used the Chou–Talalay method to quantify the CI for those experimentally verified combination data points.

Responses to combination treatments (referred to as f_A) were normalized such that 1.0 would represent the maximum response to a saturating dose. The theoretical maximum response to each individual Wnt was estimated by assuming that the maximum measured effect was 80% of the saturated maximum effect. To verify the impact of the 80% assumption, we repeated the CI analysis over a range of assumed percentages (10, 20, 30…100%) and found that the calculated CI values and CI trends were insensitive to the assumption.

The computation of CI requires computing a linear fit between log(dose) versus $\log[f_A/(1-f_A)]$ for each of the individual stimuli. Lines with higher slope (higher efficacy of each dose) yield lower synergism when computing the CI. Since the exact linear relationship is uncertain (e.g. due to experimental error) and the CI values might be sensitive to the slope of the fitted line, we fitted an ensemble of lines to the points (for each Wnt) and selected the one with maximum slope (which would give the lowest

synergy), to be the most conservative linear fit for the relationship between log(dose) and log[$f_A/(1-f_A)$].

For each combination of doses (D_{can} and $D_{non\text{-}can}$), we used the linear fit (computed above) to interpolate how much of the canonical Wnt alone ($^{equiv}D_{can}$) would be necessary to achieve the effect of the combination dose. The process was repeated to find the equivalent single dose of the non-canonical Wnt ($^{equiv}D_{non\text{-}can}$). Finally, we used the combination doses and equivalent single doses to compute the CI according to the Chou–Talalay method:

$$CI = \frac{D_{can}}{(^{equiv}D_{can})} + \frac{D_{non-can}}{(^{equiv}D_{non-can})}.$$

The CI computation was performed using MATLAB scripts and verified using Compusyn (Chou and Martin, 2005).

qRT-PCR

RNA was isolated using an RNeasy kit (cat. #74106, Qiagen, Hilden, Germany), reverse transcribed with the i-script RT kit (cat. #170-8891, Bio-Rad Hercules, CA), and quantified on a Bio-Rad CFX96 real-time cycling machine using the SsoFast EvaGreen PCR assay (cat #172-5200, Bio-Rad). Sequences for the human- and mouse-specific Wnt and housekeeping primers and other primers used are given in Table S4.

Western blotting

Cells (HEK293 or YCC11) were transfected with the indicated Wnt expression plasmids and lysates were made 24 h post transfection. Nuclear and cytoplasmic extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kit (PI-78835, ThermoFisher Scientific, Waltham, MA) following the manufacturer's recommendations. Whole-cell lysates were made in 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The proteins were separated on 8 or 10% SDS polyacrylamide gels and transferred onto PVDF membrane (Hybond, GE Healthcare, Pittsburgh, PA). Membranes were blocked in 5% BSA in 1× TBS with 0.1% Tween-20 for 1 h and incubated overnight at 4°C with primary antibodies. Antibody against β-catenin (cat. #610154) was from BD Biosciences (Franklin Lakes, NJ) and was used at 1:1000 dilution. Antibodies against acetylated β-catenin (K49, cat. #9030), non-phosphorylated (active) β-catenin (cat. #8814), LRP6 (cat. #2560), phospho-LRP6 (Ser1490, cat. #2568) were from Cell Signaling Technology (Danvers, MA) and were used at 1:1000 dilution. Antibody against Eg5 (4H3-IF12) was from Cell Signaling (cat. #4203) and was used at 1:2000 dilution. Antibodies against actin (cat. #3280) and tubulin (cat. #52623) were from Abcam (Cambridge, UK) and were at 1:1000 dilution, and antibody against lamin b (cat. # SC-6213), used at 1:1000 dilution, was from Santa Cruz Biotechnology (Dallas, TX). Antibody against V5 was from Invitrogen (cat. #R96025) and was used at 1:5000 dilution. Antibody against WNT1 was from Genetex (cat #GTX111182) and was used at 1:1000 dilution and anti-WNT3A antibody was a generous gift from Shinji Takada (Okazaki Institute for Integrative Bioscience and National Institute for Basic Biology, Okazaki, Japan) (culture supernatant used at 1:100 dilution).

After incubation with primary antibodies, blots were washed and incubated with horseradish peroxidase-conjugated (Bio-Rad; dilution 1:5000) or IR dye-conjugated secondary antibodies (ThermoFisher Scientific; dilution 1:15,000). The blots were detected using SuperSignal West Dura substrate for chemiluminescence (cat. #34075, ThermoFisher Scientific, Waltham, MA). The membranes were imaged for chemiluminescence (ImageQuant LAS 4000, GE Healthcare, Pittsburgh, PA) or fluorescence (Odyssey, LI-COR, Lincoln, NE).

Soft agar colony formation assay

YCC11 cells were infected with lentiviral constructs expressing human *WNT1*, *WNT7B* or both and stable cell lines generated using antibiotic selection (puromycin and/or blasticidin). YCC11 cells stably expressing human *WNT1*, *WNT7B* or both were seeded in soft agar (bottom 0.6%, top 0.36%) in 24-well plates at a density of 5000 cells per well. The medium was replenished twice a week and colonies were quantified after 4 weeks using Crystal Violet staining and manual counting.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5 for Mac (GraphPad Software, La Jolla, CA) using one-way or two-way ANOVA, correcting for multiple comparisons using Tukey's test. Significance for all tests was set at $P<0.05$ unless otherwise stated.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.A. performed the majority of the studies and wrote the manuscript. Z.L. and S.G.R. supplied and analyzed gastric cancer gene expression data. S.K. performed initial assays identifying Wnt synergy in unexpected Wnt pairs. N.S.J. and L.T.-K. advised on synergy studies and analyzed synergy data. N.H. assisted in analysis of RNAseq data. D.M.V. supervised the studies, and co-authored the manuscript.

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Supplementary information

Supplementary information available online at
http://jcs.biologists.org/lookup/doi/10.1242/jcs.198093.supplemental

References

Afelik, S., Pool, B., Schmerr, M., Penton, C. and Jensen, J. (2015). Wnt7b is required for epithelial progenitor growth and operates during epithelial-to-mesenchymal signaling in pancreatic development. *Dev. Biol.* **399**, 204-217.

Akiri, G., Cherian, M. M., Vijayakumar, S., Liu, G., Bafico, A. and Aaronson, S. A. (2009). Wnt pathway aberrations including autocrine Wnt activation occur at high frequency in human non-small-cell lung carcinoma. *Oncogene* **28**, 2163-2172.

Anastas, J. N. and Moon, R. T. (2013). WNT signalling pathways as therapeutic targets in cancer. *Nat. Rev. Cancer* **13**, 11-26.

Anderson, K. D., Pan, L., Yang, X.-M., Hughes, V. C., Walls, J. R., Dominguez, M. G., Simmons, M. V., Burfeind, P., Xue, Y., Wei, Y. et al. (2011). Angiogenic sprouting into neural tissue requires Gpr124, an orphan G protein-coupled receptor. *Proc. Natl Acad. Sci. USA* **108**, 2807-2812.

Arensman, M. D., Kovochich, A. N., Kulikauskas, R. M., Lay, A. R., Yang, P.-T., Li, X., Donahue, T., Major, M. B., Moon, R. T., Chien, A. J. et al. (2014). WNT7B mediates autocrine Wnt/β-catenin signaling and anchorage-independent growth in pancreatic adenocarcinoma. *Oncogene* **33**, 899-908.

Bafico, A., Liu, G., Goldin, L., Harris, V. and Aaronson, S. A. (2004). An autocrine mechanism for constitutive Wnt pathway activation in human cancer cells. *Cancer Cell* **6**, 497-506.

Benhaj, K., Akcali, K. C. and Ozturk, M. (2006). Redundant expression of canonical Wnt ligands in human breast cancer cell lines. *Oncol. Rep.* **15**, 701-707.

Bourhis, E., Tam, C., Franke, Y., Bazan, J. F., Ernst, J., Hwang, J., Costa, M., Cochran, A. G. and Hannoush, R. N. (2010). Reconstitution of a frizzled8.Wnt3a. LRP6 signaling complex reveals multiple Wnt and Dkk1 binding sites on LRP6. *J. Biol. Chem.* **285**, 9172-9179.

Bui, T. D., O'Brien, T., Crew, J., Cranston, D. and Harris, A. L. (1998). High expression of Wnt7b in human superficial bladder cancer vs invasive bladder cancer. *Br. J. Cancer* **77**, 319-324.

Cadigan, K. M. and Waterman, M. L. (2012). TCF/LEFs and Wnt signaling in the nucleus. *Cold Spring Harb. Perspect. Biol.* **4**, a007906-a007906.

Carson-Walter, E. B., Watkins, D. N., Nanda, A., Vogelstein, B., Kinzler, K. W. and St Croix, B. (2001). Cell surface tumor endothelial markers are conserved in mice and humans. *Cancer Res.* **61**, 6649-6655.

Cha, S.-W., Tadjuidje, E., Tao, Q., Wylie, C. and Heasman, J. (2008). Wnt5a and Wnt11 interact in a maternal Dkk1-regulated fashion to activate both canonical

and non-canonical signaling in Xenopus axis formation. *Development* **135**, 3719-3729.

Chien, A. J., Conrad, W. H. and Moon, R. T. (2009). A Wnt survival guide: from flies to human disease. *J. Invest. Dermatol.* **129**, 1614-1627.

Chou, T.-C. and Martin, N. (2005). *CompuSyn for Drug Combinations and for General Dose-Effect Analysis, Software and User's Guide: A Computer Program for Quantitation of Synergism and Antagonism in Drug Combinations, and the Determination of IC50 and ED50 and LD50 Values*. Paramus, NJ, USA: ComboSyn Inc.

Chou, T.-C. and Talalay, P. (1983). Analysis of combined drug effects: a new look at a very old problem. *Trends Pharmacol. Sci.* **4**, 450-454.

Chou, T.-C. and Talalay, P. (1984). Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* **22**, 27-55.

Chou, T.-C. (2006). Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol. Rev.* **58**, 621-681.

Clevers, H. and Nusse, R. (2012). Wnt/β-catenin signaling and disease. *Cell* **149**, 1192-1205.

Cohen, E. D., Miller, M. F., Wang, Z., Moon, R. T. and Morrisey, E. E. (2012). Wnt5a and Wnt11 are essential for second heart field progenitor development. *Development* **139**, 1931-1940.

Emami, K. H., Nguyen, C., Ma, H., Kim, D. H., Jeong, K. W., Eguchi, M., Moon, R. T., Teo, J.-L., Oh, S. W., Kim, H. Y. et al. (2004). A small molecule inhibitor of β-catenin/cyclic AMP response element-binding protein transcription. *Proc. Natl Acad. Sci. USA* **101**, 12682-12687.

Ettenberg, S. A., Charlat, O. and Daley, M. P. (2010). Inhibition of tumorigenesis driven by different Wnt proteins requires blockade of distinct ligand-binding regions by LRP6 antibodies *Proc. Natl. Acad. Sci. USA* **107**, 15473-15478.

Farin, H. F., Van Es, J. H. and Clevers, H. (2012). Redundant sources of Wnt regulate intestinal stem cells and promote formation of Paneth cells. *Gastroenterology* **143**, 1518-1529.e7.

Gammons, M. V., Renko, M., Johnson, C. M., Rutherford, T. J. and Bienz, M. (2016). Wnt signalosome assembly by DEP domain swapping of dishevelled. *Mol. Cell* **64**, 92-104.

Gong, Y., Bourhis, E., Chiu, C., Stawicki, S., DeAlmeida, V. I., Liu, B. Y., Phamluong, K., Cao, T. C., Carano, R. A. D., Ernst, J. A. et al. (2010). Wnt isoform-specific interactions with coreceptor specify inhibition or potentiation of signaling by LRP6 antibodies. *PLoS ONE* **5**, e12682.

Hecht, A., Vleminckx, K., Stemmler, M. P., van Roy, F. and Kemler, R. (2000). The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates. *EMBO J.* **19**, 1839-1850.

Kabiri, Z., Greicius, G., Madan, B., Biechele, S., Zhong, Z., Zaribafzadeh, H., Edison, H., Aliyev, J., Wu, Y., Bunte, R. et al. (2014). Stroma provides an intestinal stem cell niche in the absence of epithelial Wnts. *Development* **141**, 2206-2215.

Kim, S.-E., Huang, H., Zhao, M., Zhang, X., Zhang, A., Semonov, M. V., MacDonald, B. T., Zhang, X., Abreu, J. G., Peng, L. et al. (2013). Wnt stabilization of β-catenin reveals principles for morphogen receptor-scaffold assemblies. *Science* **340**, 867-870.

Lei, Z., Tan, I. B., Das, K., Deng, N., Zouridis, H., Pattison, S., Chua, C., Feng, Z., Guan, Y. K., Ooi, C. H. et al. (2013). Identification of molecular subtypes of gastric cancer with different responses to PI3-kinase inhibitors and 5-fluorouracil. *Gastroenterology* **145**, 554-565.

Levy, L., Wei, Y., Labalette, C. and Wu, Y. (2004). Acetylation of β-catenin by p300 regulates β-catenin-Tcf4 interaction. *Mol. Cell. Biol.* **24**, 3404-3414.

Luga, V., Zhang, L., Viloria-Petit, A. M., Ogunjimi, A. A., Inanlou, M. R., Chiu, E., Buchanan, M., Hosein, A. N., Basik, M. and Wrana, J. L. (2012). Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration. *Cell* **151**, 1542-1556.

MacDonald, B. T., Tamai, K. and He, X. (2009). Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev. Cell* **17**, 9-26.

Macheda, M. L. and Stacker, S. A. (2008). Importance of Wnt signaling in the tumor stroma microenvironment. *Curr. Cancer Drug Targets* **8**, 454-465.

Miller, M. F., Cohen, E. D., Baggs, J. E., Lu, M. M., Hogenesch, J. B. and Morrisey, E. E. (2012). Wnt ligands signal in a cooperative manner to promote foregut organogenesis. *Proc. Natl Acad. Sci. USA* **109**, 15348-15353.

Mosimann, C., Hausmann, G. and Basler, K. (2009). β-Catenin hits chromatin: regulation of Wnt target gene activation. *Nat. Rev. Mol. Cell Biol.* **10**, 276-286.

Najdi, R., Proffitt, K., Sprowl, S., Kaur, S., Yu, J., Covey, T. M., Virshup, D. M. and Waterman, M. L. (2012). A uniform human Wnt expression library reveals a shared secretory pathway and unique signaling activities. *Differentiation* **84**, 203-213.

Nichols, S. A., Dirks, W., Pearse, J. S. and King, N. (2006). Early evolution of animal cell signaling and adhesion genes. *Proc. Natl Acad. Sci. USA* **103**, 12451-12456.

Niehrs, C. (2012). The complex world of WNT receptor signalling. *Nat. Rev. Mol. Cell Biol.* **13**, 767-779.

Nusse, R. and Varmus, H. (2012). Three decades of Wnts: a personal perspective on how a scientific field developed. *EMBO J.* **31**, 2670-2684.

Ooi, C. H., Ivanova, T., Wu, J., Lee, M., Tan, I. B., Tao, J., Ward, L., Koo, J. H., Gopalakrishnan, V., Zhu, Y. et al. (2009). Oncogenic pathway combinations predict clinical prognosis in gastric cancer. *PLoS Genet.* **5**, e1000676.

Posokhova, E., Shukla, A., Seaman, S., Volate, S., Hilton, M. B., Wu, B., Morris, H., Swing, D. A., Zhou, M., Zudaire, E. et al. (2015). GPR124 functions as a WNT7-specific coactivator of canonical β-catenin signaling. *Cell Rep.* **10**, 123-130.

Rajagopal, J., Carroll, T. J., Guseh, J. S., Bores, S. A., Blank, L. J., Anderson, W. J., Yu, J., Zhou, Q., McMahon, A. P. and Melton, D. A. (2008). Wnt7b stimulates embryonic lung growth by coordinately increasing the replication of epithelium and mesenchyme. *Development* **135**, 1625-1634.

Stamatakou, E. and Salinas, P. C. (2014). Postsynaptic assembly: a role for Wnt signaling. *Dev. Neurobiol.* **74**, 818-827.

Suzuki, H., Watkins, D. N., Jair, K.-W., Schuebel, K. E., Markowitz, S. D., Dong Chen, W., Pretlow, T. P., Yang, B., Akiyama, Y., van Engeland, M. et al. (2004). Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nat. Genet.* **36**, 417-422.

Valenta, T., Hausmann, G. and Basler, K. (2012). The many faces and functions of β-catenin. *EMBO J.* **31**, 2714-2736.

Vallon, M., Rohde, F., Janssen, K.-P. and Essler, M. (2010). Tumor endothelial marker 5 expression in endothelial cells during capillary morphogenesis is induced by the small GTPase Rac and mediates contact inhibition of cell proliferation. *Exp. Cell Res.* **316**, 412-421.

Vanhollebeke, B., Stone, O. A., Bostaille, N., Cho, C., Zhou, Y., Maquet, E., Gauquier, A., Cabochette, P., Fukuhara, S., Mochizuki, N. et al. (2015). Tip cell-specific requirement for an atypical Gpr124- and Reck-dependent Wnt/β-catenin pathway during brain angiogenesis. *Elife* **4**, 2807.

Veeman, M. T., Slusarski, D. C., Kaykas, A., Louie, S. H. and Moon, R. T. (2003). Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. *Curr. Biol.* **13**, 680-685.

Witte, F., Dokas, J., Neuendorf, F., Mundlos, S. and Stricker, S. (2009). Comprehensive expression analysis of all Wnt genes and their major secreted antagonists during mouse limb development and cartilage differentiation. *Gene Expr. Patterns* **9**, 215-223.

Wolf, D., Rodova, M., Miska, E.A., Calvet, J. P. and Kouzarides, T. (2002). Acetylation of beta-Catenin by CREB-binding Protein (CBP). *J. Biol.Chem.* **277**, 25562-25567.

Xu, Q., Wang, Y., Dabdoub, A., Smallwood, P. M., Williams, J., Woods, C., Kelley, M. W., Jiang, L., Tasman, W., Zhang, K. et al. (2004). Vascular development in the retina and inner ear: control by Norrin and Frizzled-4, a high-affinity ligand-receptor pair. *Cell* **116**, 883-895.

Yeo, E.-J., Cassetta, L., Qian, B.-Z., Lewkowich, I., Li, J. F., Stefater, J. A., Smith, A. N., Wiechmann, L. S., Wang, Y., Pollard, J. W. et al. (2014). Myeloid WNT7b mediates the angiogenic switch and metastasis in breast cancer. *Cancer Res.* **74**, 2962-2973.

Yu, J. and Virshup, D. M. (2014). Updating the Wnt pathways. *Biosci. Rep.* **34**, 593-607.

Yu, J., Carroll, T. J., Rajagopal, J., Kobayashi, A., Ren, Q. and McMahon, A. P. (2009). A Wnt7b-dependent pathway regulates the orientation of epithelial cell division and establishes the cortico-medullary axis of the mammalian kidney. *Development* **136**, 161-171.

Zheng, D., Decker, K. F., Zhou, T., Chen, J., Qi, Z., Jacobs, K., Weilbaecher, K. N., Corey, E., Long, F. and Jia, L. (2013). Role of WNT7B-induced noncanonical pathway in advanced prostate cancer. *Mol. Cancer Res.* **11**, 482-493.

Zhou, Y. and Nathans, J. (2014). Gpr124 controls CNS angiogenesis and blood-brain barrier integrity by promoting ligand-specific canonical wnt signaling. *Dev. Cell* **31**, 248-256.