Frizzled-8 integrates Wnt-11 and transforming growth factor-β signaling in prostate cancer

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Wnt-11 promotes cancer cell migration and invasion independently of β-catenin but the receptors involved remain unknown. Here, we provide evidence that FZD8 is a major Wnt-11 receptor in prostate cancer that integrates Wnt-11 and TGF-β signals to promote EMT. FZD8 mRNA is upregulated in multiple prostate cancer datasets and in metastatic cancer cell lines in vitro and in vivo. Analysis of patient samples reveals increased levels of FZD8 in cancer, correlating with Wnt-11. FZD8 co-localizes and co-immunoprecipitates with Wnt-11 and potentiates Wnt-11 activation of ATF2-dependent transcription. FZD8 silencing reduces prostate cancer cell migration, invasion, three-dimensional (3D) organotypic cell growth, expression of EMT-related genes, and TGF-β/Smad-dependent signaling. Mechanistically, FZD8 forms a TGF-β-regulated complex with TGF-β receptors that is mediated by the extracellular domains of FZD8 and TGFBR1. Targeting FZD8 may therefore inhibit aberrant activation of both Wnt and TGF-β signals in prostate cancer.
Prostate cancer is the most commonly diagnosed cancer and the second leading cause of death in men in Western countries. Owing to the essential role of the androgen receptor (AR) in the normal growth and development of the prostate gland, and also in prostate carcinogenesis, men with prostate tumors initially respond well to androgen deprivation therapy. However, most patients eventually experience disease progression to a more aggressive state, defined as castration-resistant prostate cancer (CRPC). Although a new generation of drugs that target AR signaling is extending the lives of patients with CRPC, the development of treatment resistance remains an issue. Consequently, the identification of targets not involving AR could lead to the development of more effective treatments.

Wnt proteins are a family of cysteine-rich secreted lipoglycoproteins that play fundamental roles in development and disease. Dysregulation of Wnt signaling at the level of ligands, receptors, or effectors is observed in several types of cancer, including colon, lung, breast, and prostate. Wnt proteins bind to transmembrane Frizzled (FZD) receptors and a variety of co-receptors (LRP4-6, ROR1/2, and RYK) to activate β-catenin-dependent and β-catenin-independent signals. Our understanding of the mechanisms by which Wnt proteins stimulate different signaling responses is incomplete, but they are likely to involve the activation of distinct Wnt receptors in specific cell contexts.

A hallmark of β-catenin-dependent Wnt signaling is the stabilization and nuclear translocation of β-catenin, which binds to Tcf/LEF family of transcription factors and exerts effects on the expression of genes that affect cell proliferation and cell fate specification. β-catenin-independent Wnt signals are more diverse, but can be sub-divided into the Planar Cell Polarity (PCP) and the Wnt/Ca²⁺ signaling pathways. PCP signaling involves the small GTPases Rho, which activates Rho-associated kinase, and Rac, which is linked to activation of Jun-N-terminal kinase (JNK) and AP-1 transcription factors and regulates cell migration. Wnt/Ca²⁺ signals stimulate Ca²⁺ release from the ER and activate G-proteins, protein kinase C (PKC), and calcium/calmodulin-dependent kinase II, which regulate prostate cell growth, survival, invasion, and angiogenesis.

Wnt-11 is predominantly a β-catenin-independent Wnt14 that activates PKC and JNK to increase ATF2-dependent gene expression and can also inhibit β-catenin-dependent Wnt signaling. Wnt-11 associates with Fzd-7 in Xenopus embryo and Fzd-5 in zebrafish, Fzd-4 in mouse cardiomyocytes, and Fzd-4 and Fzd-8 in the developing mouse kidney. The response to Wnt-11 is highly context-dependent and therefore likely to depend on the presence of Wnt co-receptors, among which Wnt-11 has been reported to associate with Ror2 in zebrafish and Ryk in Xenopus.

While Wnt-11 is best known for its role during embryonic development, it has also been linked to different types of cancer. In prostate cancer, WNT11 mRNA levels are elevated in a subset of high-grade prostate tumors, CRPC xenografts, and tumor metastases. Inhibition of AR signaling increases WNT11 gene expression, and Wnt-11, in turn, inhibits AR-dependent transcriptional activity and AR-dependent proliferation. Wnt-11 also promotes prostate tumor cell survival, migration, invasion, and neuroendocrine-like differentiation (NED). However, the receptors that transduce Wnt-11 signals in prostate cancer are not known. Here, we addressed this question, focusing on Wnt-11 receptors required for prostate cancer cell migration and invasion. We find that FZD8 is a major Wnt-11 receptor in prostate cancer and show that it is upregulated in metastatic disease, where it plays a crucial role in mediating crosstalk between Wnt and TGF-β signaling pathways during the epithelial-to-mesenchymal transition (EMT), which is important for prostate cancer cell migration and invasion.

### Table 1 Wnt receptor expression in prostate cancer cell lines

| Receptor | UP in NED | UP in AI PCa | UP in mPCA | Restricted expression |
|----------|-----------|--------------|------------|-----------------------|
| FZD2     | x         | x            | x          |                       |
| FZD3     |           |              |            |                       |
| FZD4     |           | x            |            |                       |
| FZD5     |           | x            |            |                       |
| FZD6     |           |              |            |                       |
| FZD7     |           |              |            | x                     |
| FZD8     | x         |              |            |                       |
| FZD9     |           |              |            |                       |
| FZD10    |           |              |            |                       |
| VANGL1   |           |              |            |                       |
| VANGL2   | x         |              |            |                       |
| ROR1     |           |              |            |                       |
| ROR2     |           |              | x          |                       |
| RYK      |           | x            |            |                       |
| PTK7     |           | x            |            |                       |
| LGR4     |           | x            |            | x                     |
| LGR5     |           |              |            | x                     |
| LRP4     |           | x            |            |                       |
| LRP5     |           |              |            |                       |
| LRP6     |           |              |            |                       |
| GPC4     |           |              |            |                       |
| MuSK     |           |              |            |                       |

Note: Summary of Wnt receptor mRNA expression in prostate cancer (PCa) cell lines with x denoting expression in lines with features of neuroendocrine-like differentiation (NED), androgen-independence (AI), capacity for metastasis (Met), and restricted expression to one line (RE) (for details see Supplementary Fig. 1); FZD2, FZD4 and FZD8 (highlighted in bold) were highly expressed in cells with more aggressive features of PCa; FZD1 was not detected.
FZD8 is required for Wnt-11/ATF-2 signaling. After identifying Wnt receptors with increased expression in prostate cancer, immunofluorescence assays were used to determine which FZD receptors, when transfected, co-localized with Wnt-11. Wnt-11 co-localized strongly with FZD8 and moderately with FZD4 and FZD10, but not with other FZD family members (Fig. 1a and Supplementary Fig. 3). To determine which FZD receptors might be capable of transducing Wnt-11 signals, we examined their effects on ATF2-dependent signaling. Wnt-11 activates ATF2 in a variety of contexts, including cardiac tissue morphogenesis, Xenopus embryonic development, and avian facial morphogenesis. Wnt-11/ATF-2 signaling in prostate cancer cells was measured using an ATF2-dependent luciferase reporter (Fig. 1b). ATF2-dependent transcriptional activity in PC-3M cells was significantly enhanced upon transfection of seven members of the FZD family (Fig. 1c). Among these, FZD8 and FZD10 showed the strongest induction, which was further increased by Wnt-11 (Fig. 1c). Next, gene reporter assays were carried out in PC-3M cells transfected with siRNAs targeting the more highly expressed FZD family members. FZD8 silencing reduced Wnt-11 activation of ATF2-dependent transcription by 40% and FZD2 silencing reduced it by 20%, whereas silencing FZD3, FZD4, and FZD5 had no effect (Fig. 1d). FZD8 silencing also resulted in a small reduction in FZD5 expression (Supplementary Fig. 4a). However, FZD5 silencing did not affect Wnt-11 activation of ATF2-dependent transcription (Fig. 1d) and an unrelated Dicer-substrate FZD8 siRNA also inhibited Wnt-11 activation of ATF2-dependent transcription (Supplementary Fig. 4b), consistent with endogenous FZD8, rather than FZD5, mediating the Wnt-11 response. Importantly, the effect of FZD8 siRNA on ATF2 transcriptional activity was rescued by transfection of mouse FZD8 (Supplementary Fig. 4c). Together, these findings indicate that Wnt-11 activation of ATF2-dependent transcription requires endogenous FZD8.

ATF2 is a component of the AP-1 complex and Wnt-11 can also regulate AP-1 signaling. Consistent with this, FZD8 silencing reduced AP-1-dependent gene reporter activity (Fig. 1e). FZD8 can also transduce Wnt/b-catenin signals. However, FZD8 silencing did not inhibit b-catenin/Tcf-dependent transcriptional activity in PC-3M cells (Fig. 1f). Moreover, FZD8 silencing reduced expression of ATF2, but not the b-catenin/Tcf target gene AXIN2, which actually increased (Fig. 1g). Together, these data support a role for FZD8 in b-catenin-independent Wnt signaling in prostate cancer.

The association of Wnt proteins with FZD receptors is an essential step in activation of both canonical and non-canonical Wnt signals. To determine if Wnt-11 and FZD8 formed a stable complex, immunoprecipitation (IP) analysis was carried out using PC-3M cells transfected with tagged forms of Wnt-11 and FZD8. The results indicated that Wnt-11 and FZD8 form a stable complex (Fig. 1h).

FZD8 is required for cell invasion and EMT. Wnt-11 is required for prostate cancer cell migration and invasion. To determine the role of FZD8 in this context, cell migration and invasion assays were carried out using PC-3M and another metastatic cell line, DU145. FZD8 silencing reduced cell migration (Fig. 2a and Supplementary Fig. 5a) and invasion (Fig. 2b and Supplementary Fig. 5b) in both cell lines. Of note, FZD8 silencing also had a small effect on cell number, reducing it by up to 20% (Supplementary Fig. 5c, d), but this effect was taken into account when measuring effects on migration and invasion. Stable FZD8 knockdown using two different lentiviral shFZD8 constructs also reduced prostate cancer cell invasion (Supplementary Fig. 5e). The extent of FZD8 silencing was confirmed by q-RT-PCR (Supplementary Fig. 5f) and effects on cell number (Supplementary Fig. 5g) were taken into account. Together, these results indicate that FZD8 contributes to the migratory and invasive activities of metastatic prostate cancer cells. A recent study reported small molecule inhibitors of Wnt signaling that target the WNT-binding site on FZD8. Two of these inhibitors reduced PC-3M cell migration (Fig. 2c) without affecting cell proliferation (Supplementary Fig. 5h).

The EMT is a reversible process in which epithelial cells acquire mesenchymal properties by altering their morphology, cellular architecture, adhesion, and migratory capacities. Since Wnt signals play roles both in EMT and tumor progression, we hypothesized that Wnt11-FZD8 signals drive EMT. To test this, the effect of FZD8 silencing on EMT-associated gene expression was examined (Fig. 2d and Supplementary Fig. 6a). FZD8 silencing in PC-3M cells reduced expression of the mesenchymal genes CDH2 and VIM, and the mesenchymal transcription factors SNAI1, TWIST1, and ZEB1, but not SNAI2. In addition, FZD8 silencing increased expression of the epithelial cell marker CLDN1 (Fig. 2d). FZD8 silencing had similar effects on EMT-related genes in DU145 cells (Supplementary Fig. 6a). However, it did not affect CLDN1 or TWIST1 and reduced SNAI2 (Supplementary Fig. 6a), possibly reflecting the more epithelial nature of DU145 cells or the different extent of FZD8 silencing achieved (Fig. 2f and Supplementary Fig. 6c). Western blot analysis indicated that FZD8 silencing also affected EMT at the
**Fig. 1** FZD8 is required for Wnt-11/ATF-2 signaling in prostate cancer. 

a Confocal microscopy analysis of PC-3M cells transfected with 1D4-tagged FZD6, FZD8, or FZD10 (red) and Wnt-11 (green) for 24 h (for other FZD family members, see Supplementary Fig. 2); low magnification images of single color and dual channels and high magnification images of single cells are shown; anti-1D4 and goat anti-Wnt-11 (R&D) were used to detect FZDs and Wnt-11, respectively; blue staining shows cell nuclei (DAPI), images are representative of three independent experiments, scale bars 25 µm. Quantification of colocalization was determined by ImageJ (see Methods) using 10 cells per experiment. Numbers in the images correspond to average Pearson correlation coefficient ± standard deviation.

b Relative ATF2 luciferase/renilla activity in C4-2B and PC-3M cells transfected with empty vector pcDNA (V) or Wnt-11; results are normalized to empty vector.

c Relative ATF2 luciferase/renilla activity in C4-2B cells transfected with empty vector pRK5 (V) or Wnt-11 and FZD 1-10; results are normalized to empty vector.

d Relative ATF2 luciferase/renilla activity in PC-3M cells transfected with control siRNA (siCtrl) or the indicated siRNAs and then with Wnt-11; results are normalized to siCtrl.

e Relative AP1 luciferase/renilla activity in PC-3M cells transfected with siCtrl or siFZD8 and then with Wnt-11; results are normalized to siCtrl.

f Relative β-catenin/TCF activity (TOPFlash/FOPFlash) in PC-3M cells transfected with siCtrl or siFZD8 and then with Wnt-11; results are normalized to siCtrl.

g Q-PCR analysis showing mRNA expression of the indicated genes, relative to 36B4, in PC-3M cells transfected with control (siCtrl) and FZD8 siRNAs.

h Western blots of anti-1D4 and anti-PA immunoprecipitates (IP) and extracts (Input) from PC-3M cells transfected with 1D4-tagged FZD8 and PA-tagged Wnt-11 plasmids. Extracts were probed with PA, 1D4, or FZD8 (LS bio) antibodies; blots are representative of three independent experiments. Error bars in b–g show SD from four independent experiments (*p < 0.05, **p < 0.001 by Student’s t-test or ANOVA with Tukey post hoc test where required).
**Fig. 2** FZD8 is required for prostate cancer cell migration, invasion, and expression of epithelial–mesenchymal transition (EMT) genes. 

**a** Migration assays for PC-3M cells transfected with control (siCtrl) and FZD8 siRNAs; values are relative to siCtrl and normalized to viable cell number of transfected cells plated in parallel (Supplementary Fig. 5c); error bars show SD for six independent experiments (*p < 0.05 by Student’s t-test). Representative images are on the right, scale bar 100 µm. 

**b** Invasion assays for PC-3M cells transfected with control (siCtrl) and FZD8 siRNAs, values are relative to siCtrl and normalized to viable cell number (Supplementary Fig. 5d); error bars show SD for four independent experiments (*p < 0.05 by Student’s t-test). Representative images are on the right, scale bar 100 µm. 

**c** Migration assays for PC-3M cells treated with DMSO or inhibitors 1 (I1) and 2 (I2) at 10 µM for 24 h; values are relative to DMSO and normalized to viable cell number (Supplementary Fig. 5h), error bars show SD for four independent experiments (*p < 0.05 by ANOVA with Tukey post hoc test). Representative images are below the graph, scale bar 100 µm. 

**d** Q-PCR analysis showing expression levels of the indicated EMT genes, normalized to 36B4, in PC-3M cells transfected with control (siCtrl) or FZD8 siRNAs; error bars show SD for four independent experiments (*p < 0.05, **p < 0.001 by Student’s t-test). 

**e** Extracts from PC-3M cells transfected with control or FZD8 siRNAs were blotted for the indicated proteins; graph shows average relative protein levels, as determined by densitometry analysis of blots from three independent experiments (*p < 0.05 by Student’s t-test), normalized to HSP60 and relative to control siRNA (siCtrl). 

**f** Relative expression levels of FDZ8 and WNT11 measured by Q-PCR in PC-3M cells transfected with control (siCtrl) and FZD8 siRNAs; error bars represent SD of four independent experiments (*p < 0.05, **p < 0.001 by Student’s t-test).
Fig. 3 FZD8 is required for prostate cancer cell invasion in organotypic 3D cultures. a Representative images from live-cell imaging of 3D cultures of control (siCtrl) and FZD8-silenced PC-3M cells at days 1–9; scale bar 100 μm. b FZD8 expression levels in siRNA-transfected cells at days 0, 4, and 8 of 3D culture. Error bars indicate SD from three independent experiments (*p < 0.05, **p < 0.001 by ANOVA with Tukey post hoc test, ns indicate non-significant). c Representative segmentation of live-cell spinning disk confocal images of FZD8-silenced PC-3M cell organoids cultured in 3D for 9 days. Organoids were segmented and analyzed by AMIDA; apoptotic cells are in red (ethidium homodimer-1) and live cells in green (calcein). d Heatmaps and graphical p-value matrix of morphometric parameters measured by AMIDA and found to be altered by FZD8 silencing (red: increased, and blue: decreased). p-values displayed in the figure are Bonferroni-corrected from t-tests, comparing siFZD8 and siCtrl. e Box and whisker plots of selected parameters from heatmaps; p = 0 indicates p < 0.001. For explanation of the morphometric parameters, see Supplementary Table 4. f Confocal microscopy analysis of organoids derived from siCtrl and FZD8-silenced PC-3M cells at day 9 of growth in 3D culture; immunostaining for vimentin (Vim) is shown in green and for laminin-α1 (Lam) in red, blue staining shows cell nuclei (Draq5), scale bar 100 μm.
protein level, reducing the levels of N-cadherin and vimentin and increasing that of claudin 1 (Fig. 2e). Furthermore, bioinformatics analysis indicated that both FZD8 and WNT11 expression correlated with SNAI1, SNAI3, TWIST1, and TWIST2, and negatively correlated with CDH1 and CTNNB1 (Supplementary Fig. 6b). WNT11 has itself been described as an EMT gene in kidney epithelial cells42, and we observed that FZD8 silencing also reduced WNT11 expression (Fig. 2f and Supplementary Fig. 6c), and found a positive correlation between FZD8 and WNT11 expression in the MSKCC dataset (Supplementary Fig. 6d). These results suggest a positive-feedback loop in which FZD8 signals lead to increased WNT11 expression.

**FZD8 is required for cell invasion in 3D organotypic cultures.**

To investigate the role of FZD8 in prostate cancer cell invasion further, we used an organotypic cell culture model in which acinar structures (organoids) are formed that display physiologically relevant cell–cell and cell–matrix interactions, epithelial polarization and differentiation, recapitulating human cancer histology15. PC-3 and PC-3M metastatic prostate cancer cells initially differentiate into hollow organoids (days 4–5) and later spontaneously de-differentiate into invasive stellate structures (days 8–12)15. Cells were transfected with FZD8 siRNA and cultured as organoids, monitoring morphology, polarization, and growth for up to 9 days using the IncuCyte® system. Cells transfected with control siRNA initially matured into well-differentiated organoids and then formed invasive and multi-cellular structures at days 7–8 (Fig. 3a and Supplementary Fig. 7a). In contrast, FZD8-silenced cells matured into well-differentiated organoids but did not form invasive structures (Fig. 3a and Supplementary Fig. 7a). Of note, these effects were completely evident at the endpoint of the assay, despite recovery of basal FZD8 mRNA expression by day 8 (Fig. 3b and Supplementary Fig. 7b).

To quantify the phenotypic changes, at least 1000 organoids were examined using high-content, automated morphometric...
image data analysis (AMIDA) software, which allows segmentation and quantitative measurement of images with different shapes, sizes, and textures. Organoids were live-stained with calcein and ethidium homodimer at the endpoint of the 3D culture, visualized by confocal microscopy and images segmented (Fig. 3c and Supplementary Fig. 7c), and analyzed using AMIDA. FZD8 silencing significantly reduced the severity (AppIndex) and length (MaxApp) of invasive multicellular structures, accompanied by reductions in the numbers of small filopodia-like cellular extensions (Roughness) and organoid size (Area), as well as by a rounder shape (Roundness) (Fig. 3d, e and Supplementary Fig. 7d, e). Organoid morphology was also examined by immunostaining. Laminin-α1 was not detectable in control organoids but was clearly observed in FZD8-silenced organoids, indicating the presence of a basal lamina characteristic of well-differentiated structures (Fig. 3f and Supplementary Fig. 7f). On the other hand, vimentin staining highlighted invasive cells migrating from control cell organoids but not from FZD8-silenced organoids, reflecting a reduction in invasive properties of the latter (Fig. 3f and Supplementary Fig. 7f). Interestingly, some FZD8-silenced organoids showed a total absence of vimentin staining (Fig. 3f). These 3D cell culture data further support a role for FZD8 in promoting tumor cell invasion in prostate cancer.

![Fig. 5](image-url) FZD8 and Wnt-11 expression correlates with prostate cancer progression. **a** Immunohistochemical staining of FZD8 and Wnt-11 in adjacent sections of prostate cancer (Gleason 4+3) and an area of benign prostate from the same patient; scale bar 25 μm. **b** Immunohistochemical staining of FZD8 and Wnt-11 in a section of prostate cancer (Gleason 4+3) from another patient; scale bar 25 μm. High magnification images for FZD8, Wnt-11, epithelial pan-cytokeratin (CK), and H&E are also shown. Arrows show disseminated tumor cells positive for FZD8, Wnt-11, and epithelial cytokeratins. **c** Stratification of FZD8 and Wnt-11 expression in cancer (T) and benign (N) epithelia and stroma (**p < 0.001 by Pearson Chi-square test with correction). **d** Immunohistochemical staining for FZD8, Wnt-11, and vimentin in sections of a PC-3M cell lymph node metastasis from a mouse orthotopic xenograft; scale bars 50 μm. Squares highlight regions shown at higher magnification below.
FZD8 silencing reduces tumor growth in vivo. To validate the functional role of FZD8 in prostate cancer cells in vivo, we used the chorioallantoic membrane (CAM) model. The CAM is a highly vascular membrane in chicken eggs that enables efficient tumor cell grafting and growth, mimicking a physiological cancer environment. Control and FZD8-silenced PC-3 cells were grafted onto the exposed CAM at developmental day 7 (EDD7). At EDD10 the tumors formed by FZD8-silenced PC-3 cells were significantly smaller than those formed by control cells (Fig. 4a–c). Evaluation of FZD8 levels at the moment of cell grafting and when tumors were excised indicated that silencing was maintained during the course of the assay (Fig. 4d). Analysis of the expression of vimentin, which contributes to prostate cancer cell invasion (Fig. 4e). Of note, immunostaining for vimentin distinguishes prostate cancer cells from CAM cells, which are negative for this marker (Fig. 4e). FZD8 and Wnt-11 correlate with prostate cancer progression. In order to evaluate FZD8 and WNT11 gene expression during prostate cancer progression, bioinformatic analyses were performed using the MSKCC dataset. These revealed a clear upregulation of FZD8 in prostate cancer, compared to normal prostate, and in prostate cancer metastases, compared to primary tumors. Both FZD8 and WNT11 were upregulated in high Gleason score tumors and in tumors that had spread to lymph nodes (Supplementary Fig. 8a). Expression of WNT11 correlated significantly (p = 0.014) and FZD8 showed a trend (p = 0.057) with increased biochemical recurrence (Supplementary Fig. 8b). Further analysis indicated that elevated FZD8 and WNT11 expression was more prevalent in patients with recurrent disease, as compared to disease-free patients (p = 0.049, Fisher’s exact test, two-sided) (Supplementary Fig. 8c), supporting the relevance of WNT-11/FZD8 silencing to prostate cancer progression.

To confirm the in silico data, immunohistochemistry for FZD8 and Wnt-11 was carried out in tissue arrays (TMAs) comprising sections of benign and malignant prostate from prostate cancer patients (Supplementary Table 5). This revealed significantly higher levels of FZD8 and Wnt-11 in tumor cells, compared to in benign epithelium (Fig. 5a–c, Supplementary Table 7) and correlations in the levels of FZD8 and Wnt-11 in benign and tumor epithelia and stroma (Table 3). FZD8 and Wnt-11 were also both significantly lower in tumor stroma than in the benign stroma (Fig. 5c, Supplementary Table 7), suggesting that elevated expression in cancer epithelial cells is accompanied by reduced expression in tumor-associated stroma. Even though FZD8 and Wnt-11 levels were higher in prostate cancer than in benign prosthetic epithelium, there was no significant correlation with Gleason score in this patient cohort (Supplementary Table 8). FZD8 and Wnt-11 were also detected in lymph node metastases after orthotopic implantation of mice with PC-3M cells (Fig. 5d), consistent with a role for Wnt-11/FZD8 signaling in metastasis. Vimentin staining was used to distinguish prostate cancer cells from host cells.

**Table 3** Correlation analysis of FZD8 and Wnt-11 expression

| FZD8 | Wnt-11 | Chi-square; Fisher exact |
|------|--------|--------------------------|
| Cancer | Low    | High  | <0.0001; 0.000071 |
| Low  | 17     | 5     |  |
| High | 18     | 46    |  |
| Benign | Low | High | No value; 0.039 |
| Low  | 61     | 8     |  |
| High | 14     | 7     |  |
| Cancer stroma | Low | High | 0.0063; 0.011 |
| Low  | 46     | 17    |  |
| High | 10     | 14    |  |
| Benign stroma | Low | High | No value; 0.027 |
| Low  | 5      | 9     |  |
| High | 8      | 68    |  |

Note: p < 0.001 by Pearson Chi-square test with correction and Fisher’s exact test, two-sided

FZD8 regulates TGF-β/Smad signaling. Crosstalk between TGF-β and Wnt signaling during development has been studied extensively. The best-defined venue for crosstalk is the nucleus, where Smad proteins have been reported to associate with β-catenin/Tcf/LEF complexes to regulate gene expression. Smads also associate with AP-1 transcription factors, which mediate β-catenin-independent Wnt signaling. TGF-β is a recognized master regulator of EMT, regulating expression of SNAIL/TWIST/ZEβ, which control cadherin switching, matrix metalloproteinases (MMPs), plasminogen activator inhibitor-1 (PAI-1), and vimentin. Since FZD8 is required for Wnt-11/ATF-2 and TGF-β signaling, we hypothesized that it plays a role in TGF-β signaling. Consistent with this, FZD8 silencing reduced TGF-β activation of a Smad-dependent gene reporter (Fig. 6a and Supplementary Fig. 9a). Inhibition of TGF-β/Smad-dependent transcription was also observed using an unrelated Dicer-substrate FZD8 siRNA (Supplementary Fig. 9b). Although FZD8 silencing reduced TGF-β activation of the Smad-dependent gene reporter, it did not block it completely, suggesting FZD8 is not the only effector of TGF-β in this context. FZD8 silencing also reduced expression of MMP9 and PAI1 (Fig. 6b and Supplementary Fig. 9c) and TGF-β-dependent increases in SMAD2 phosphorylation and SMAD2/3 levels (Fig. 6c and Supplementary Fig. 9d).

Since TGF-β signaling increases cell invasion, we determined the effect of FZD8 silencing on TGF-β-induced cell invasion. FZD8 silencing significantly reduced invasion both in PC-3M and DU145 cells (Fig. 6d and Supplementary Fig. 9e) without affecting cell number (Supplementary Fig. 9f, g). To determine if the effects of FZD8 silencing on EMT gene expression were mediated via inhibition of TGF-β signaling, we examined the expression of EMT genes upon TGF-β treatment. In DU145 cells, TGF-β increased VIM, SNAI1/2, and ZEB1 and reduced CDH1 (Supplementary Fig. 10a). TGF-β also increased FZD8 and WNT11 expression (Supplementary Fig. 10c). FZD8 silencing inhibited TGF-β-induced expression of VIM, SNAI1/2, ZEB1, MMP9, and PAI-1 and increased expression of CLDN1 (Fig. 6e). Together, these results indicate that FZD8 plays a role in TGF-β-mediated cell invasion and EMT gene expression.

**Association of FZD8 with TGF-β receptors.** Since FZD8 is involved both in Wnt-11/ATF-2 and TGF-β/Smad signaling and ATF2 has been reported to associate with Smad3, we examined the possibility that Wnt-11/FZD8 signaling activates TGF-β/Smad signaling via ATF2. To assess the contribution of ATF2, we used Δ-ATF2, a dominant-negative form of ATF2 that binds and inhibits ATF2 and its AP-1 family partners. Δ-ATF2 significantly reduced TGF-β activation of Smad-dependent transcription (Supplementary Fig. 11a), indicating that ATF2 and/or other AP-1 family members are required for TGF-β/Smad signaling. Inhibition was also observed using a reporter containing Smad and AP-1-binding sites (3TP-lux, Supplementary Fig. 11b).
Moreover, Δ-ATF2 reduced TGF-β-induced expression of VIM, SNAI2, and ZEB1 and increased expression of CDH1 in DU145 cells (Supplementary Fig. 11c), suggesting ATF2 is required for expression of a subset of TGF-β-regulated EMT genes. Thus, FZD8 may promote TGF-β/Smad-dependent signaling via activation of ATF2. Given the reported association of ATF2 with Smad3, we wished to determine if this was affected by FZD8 silencing. However, we were unable to detect a stable complex between ATF2 and Smad3 by IP (Supplementary Fig. 11d).

We next hypothesized that crosstalk between FZD8 and TGF-β signaling might take place at the membrane. TGF-β signaling is initiated by ligand binding to two transmembrane receptor kinases (TGFβRI and RII), with ligand binding to TGFβRII a
Fig. 7 FZD8 associates with TGF-β receptors. a Western blots of anti-1D4 immunoprecipitates (IP) and extracts (inputs) from PC-3M cells transfected for 24 h with 1D4-tagged FZD8, Flag-tagged TGFβRI, and HA-tagged TGFβRII plasmids were probed for TGFβRII (HA), TGFβRI (Flag), and FZD8 (1D4); blots are representative of three independent experiments. b Confocal immunofluorescence analysis of PC-3M cells transfected with 1D4-tagged FZD8 (red) and Flag-tagged TGFβRI or HA-tagged TGFβRII (green) for 24 h. Images are representative of three independent experiments; 1D4 epitope or goat anti-FZD8 antibody was used to detect FZD8 and Flag and HA epitope tag antibodies were used to detect TGFβRI and TGFβRII, respectively; blue staining shows cell nuclei (DAPI); scale bar 25 μm. c Western blots of Protein A/G-agarose pull-downs (IP IgG) and extracts (inputs) from PC-3M cells transfected for 24 h with FZD8-CRD-IgG, LRP6-IgG, Flag-tagged TGFβRI, and HA-tagged TGFβRII plasmids were probed for TGFβRI (Flag), and FZD8 (IgG); blots are representative of three independent experiments. d Western blots of Protein A/G-agarose pull-downs (IP IgG) and extracts (inputs) from PC-3M cells transfected for 24 h with FZD8-CRD-IgG, Flag-tagged TGFβRII, and Flag-tagged TGFβRII extracellular domain (ΔTGFβRII) plasmids were probed for TGFβRII (Flag) and FZD8 (IgG); blots are representative of three independent experiments. e Western blots of anti-1D4 immunoprecipitates (IP) and extracts (inputs) from PC-3M cells transfected for 24 h with plasmids encoding 1D4-tagged FZD8, Flag-tagged TGFβRII, and HA-tagged TGFβRII, treated with 0.3 ng ml⁻¹ TGF-β for 30 min were probed for TGFβRII (HA), TGFβRI (Flag), and FZD8 (1D4); extracts were also probed for pSMAD3 and GAPDH as a loading control. Blots are representative of three independent experiments; graph shows average TGFβRII (HA) and TGFβRI (Flag) levels in 1D4 IPs, as determined by densitometry, in control (−) and TGF-β-treated (+) cells transfected with all three receptors, normalized to 1D4 from three independent experiments (*p < 0.05 by Student’s t-test). f Cartoon depicting crosstalk of Wnt-11 and TGF-β signaling at the level of the receptors and transcription factors. Dashed arrow indicates potential regulation of SMAD2/3 by ATF-2, based on other published studies (see text).
prerequisite for binding to TGFβRII54. To test if FZD8 associated with TGFβ receptors, PC-3M cells were transfected with plasmids expressing epitope-tagged FZD8, TGFβRII, and RII and subjected to IP analysis. A stable interaction was observed between FZD8 and both TGFβRI and TGFβRII (Fig. 7a). In addition, FZD8 partially co-localized with both TGFβRI and TGFβRII in cells (Fig. 7b). FZD8 has an extracellular N-terminal cysteine-rich domain (CRD) that binds to Wnt proteins. To determine if the FZD8 CRD was involved in the association of FZD8 with TGFβ receptors, we used a plasmid encoding FZD8 CRD fused to IgG. This fusion protein associated with TGFβRI but not with TGFβRII (Fig. 7c), suggesting FZD8 associates with TGFβRI and its interaction with TGFβRII (Fig. 7a) is mediated by endogenous TGFβRI. The extracellular domain of LRP5 fused to IgG did not bind either TGFβRI or TGFβRII (Fig. 7c). A tagged form of the TGFβRI extracellular domain readily associated with FZD8 CRD-IgG (Fig. 7d), consistent with the interaction between FZD8 and TGFβRII involving the extracellular domains of both proteins. Together, these results indicate that the association between FZD8 and the TGFβ receptor complex involves the FZD8 CRD and the extracellular domain of TGFβRII. Next, experiments were performed in cells treated with or without exogenous TGF-β. Western blotting for phosphorylated SMAD3 indicated that transfection of TGFBR1/2 was sufficient to activate TGF-β signaling and that this was not affected by exogenous TGF-β (Fig. 7e). Treatment with TGF-β reduced the interaction between FZD8 and TGFβRII, whereas there was no significant effect on its association with TGFβRI (Fig. 7e). These results are consistent with FZD8 interacting with TGFβRII via TGFβRI and with TGF-β treatment reducing binding of TGFβRII to the FZD8–TGFβRII complex. Taken together, these observations support a model in which FZD8, by interacting both with Wnt-11 and TGFβRII, is able to play a pivotal role in integrating Wnt and TGF-β signals to drive EMT and invasion in prostate cancer (Fig. 7f).

Discussion

The development of CRPC is a critical problem in patients with prostate cancer and there remains an urgent need to identify targets that do not function by activating AR to develop more effective therapies. Given that Wnt-11 is upregulated in CRPC and upon AR inhibition28,29, components of the Wnt-11 signaling pathway could provide such targets. Here, we have identified FZD8 as a major Wnt-11 receptor in prostate cancer that may be a useful therapeutic target.

Our findings show that FZD8, like Wnt-11, is highly expressed in more aggressive prostate cancer cell lines. Expression of FZD8 mRNA is also elevated in tumor samples of prostate cancer datasets. We further show that both Wnt-11 and FZD8 protein levels are higher in prostate tumor cells than in prostate epithelial cells in benign tissue, consistent with a role for FZD8 in transducing autocrine Wnt-11 signals. We also observed a trend for increased expression of FZD8 and Gleason score. That this was not significant might be attributed to the low number of high Gleason score samples in the TMA. Analysis of larger patient cohorts will be required to determine the prognostic value of FZD8 in prostate cancer. In addition to the correlations between WNT11 and FZD8 and genes involved in EMT (Supplementary Fig. 6b), FZD8 expression strongly correlated with the presence of the TMPRSS2-ERG gene fusion and ERG mRNA expression (Supplementary Fig. 2d), an observation confirmed in a second dataset (Grasso; Oncomine™ database, Supplementary Fig. 2e). Further studies will be required to determine the relevance of this potentially interesting observation.

Studies in several cell types have found that Wnt-11 activates protein kinases, such as PKC and JNK35, which can lead to activation of ATF/CREB family transcription factors36 and inhibition of β-catenin/Tcf/LEF32,39. Wnt-11 activates ATF2 in a variety of cell contexts37,38. In prostate cancer cells, Wnt-11 and several FZDs activated ATF2-dependent transcription. Among them, FZD8 and FZD10 showed the strongest effects and also potentiated the effect of Wnt-11 and co-localized with Wnt-11. FZD10 mRNA was only detected in VCaP cells, which express the highest level of Wnt-11, suggesting that FZD10 may be a functional Wnt-11 receptor in a subset of prostate tumors. However, FZD10 was not upregulated in prostate tumor datasets. In contrast, FZD8 was upregulated in prostate tumor datasets, correlated with WNT11 expression in prostate cancer cell lines and FZD8 protein levels correlated with Wnt-11 in prostate TMA s. In addition, FZD8 and Wnt-11 formed a stable complex in PC-3M cells. Together, these observations are consistent with FZD8 as a receptor of Wnt-11 in metastatic prostate cancer. Wnt-11 is also upregulated upon hormone-depletion of LNCaP cells, where it is required for NED28,29. However, FZD8 expression was reduced in hormone-depleted LNCaP cells (Supplementary Fig. 1), suggesting other FZD family members transduce Wnt-11 signals in this context.

FZD8 silencing reduced Wnt-11 activation of ATF2-dependent and AP-1-dependent transcription, but did not affect β-catenin/Tcf/LEF-dependent gene reporter activity, consistent with Wnt-11/FZD8 transducing a β-catenin-independent signal. FZD2 silencing also reduced Wnt-11 activation of ATF2-dependent transcription, albeit to a lesser extent than silencing of FZD8. However, FZD2 mRNA levels were not upregulated in prostate tumor datasets and FZD2 did not co-localize with Wnt-11 in PC-3M cells. FZD4 mRNA levels were upregulated in prostate tumor datasets but FZD4 silencing did not affect Wnt-11 activation of ATF-2-dependent transcription and FZD4 did not colocalize with Wnt-11, so FZD4 is unlikely to transduce Wnt-11 signals in prostate cancer.

While this study focused on FZD8 class Wnt receptors, Wnt co-receptors are also anticipated to play a role in the response to Wnt-11. Several Wnt co-receptors were highly expressed in the majority of the prostate cancer lines examined, although none of them matched the WNT11 expression profile (Supplementary Fig. 1). Moreover, only PTK7 mRNA levels were upregulated in more than one prostate cancer dataset. Further studies will be needed to determine which Wnt co-receptors are important for Wnt-11 signaling in prostate cancer.

Previous studies have reported the involvement of Wnt-11 in prostate cancer migration and invasion29. Consistent with the role of FZD8 as a Wnt-11 receptor, silencing of FZD8 reduced prostate cancer cell migration and invasion. In keeping with the importance of Wnt signaling in tumor progression and EMT41, FZD8 silencing reduced mesenchymal gene and protein levels, which may account for its requirement for prostate cancer cell migration and invasion.

A number of small molecule inhibitors have been developed that target Wnt signaling in cancer. The best known of these are porcupine inhibitors, which block Wnt secretion, tankyrase inhibitors, which inhibit β-catenin-dependent Wnt signaling by stabilizing Axin, and drugs that target β-catenin interactions with transcription factors8. The elucidation of the structure of the XWnt8-Fzd3 CRD complex55 has accelerated the development of drugs targeting the Wnt pathway at the receptor level56,57. The increased expression of FZD8 in a significant proportion of prostate tumors and the demonstrated inhibitory effect of FZD8 silencing on prostate cancer cell migration and invasion suggests that inhibition of Wnt-FZD8 interactions may be a
useful approach for treatment of patients with metastatic prostate cancer. Consistent with this, we found that small molecule inhibitors that target the WNT-binding site on FZD8 reduced prostate cancer cell migration. Cross-talk between TGF-β and Wnt signals has been studied extensively. The FZD requirement for expression of EMT-related genes prompted us to explore its role in TGF-β signaling, finding that it is required for TGF-β effects on Smad phosphorylation, Smad-dependent gene reporter activity, expression of the TGF-β target genes PAI1 and MMP9, TGF-β-dependent cell invasion, and of a subset of TGF-β-regulated mesenchymal genes. While TGF-β increased MMP9 and PAI1 expression, it did not affect expression of EMT-related genes in PC-3M cells, apart from increased CDH2 (Supplementary Fig. 10b), in contrast to what was observed in DU145 cells. This is consistent with the more mesenchymal and invasive character of PC-3M cells, as compared to DU145 cells. Of particular interest, we observed cross-talk between TGF-β signaling and Wnt-11/ATF2 signaling. Dominant-negative ATF2 reduced TGF-β/Smad gene reporter activity and TGF-β-induced expression of mesenchymal genes, indicating a requirement for ATF2 in the TGF-β regulation of EMT genes. However, we were unable to confirm the previously reported interaction between ATF2 and Smad proteins. The requirement for FZD8 in TGF-β signaling in prostate cancer is reminiscent of studies in lung fibroblasts, where TGF-β-induction of WNT5A, WNT5B, and FZD8 is required for the expression of genes encoding extracellular matrix proteins and myofibroblast differentiation markers.

As far as we are aware, this is the first report of an association between a member of the Fzd family and the TGF-β receptor complex. The interaction is mediated by the FZD CRD and the extracellular domain of TGFβRI and is affected by the presence of exogenous TGF-β. The molecular events that take place subsequent to TGF-β binding are complex, in the absence of ligand, TGFβRI and TGFβRII may occur as monomers, homodimers, and heterodimers. TGFβRII homodimers and heterodimers are stabilized by contacts between the receptor cytoplasmic domains, whereas TGFβRI homodimers do not require the TGFβRI cytoplasmic domain. TGF-β binding to TGFβRII homodimers leads to recruitment of TGFβRII homodimers and formation of a heterohexameric complex of TGF-β, TGFβRI, and TGFβRII dimers. TGFβRII then phosphorylates and activates TGFβRI, which propagates the signal. Treatment of PC-3M cells with TGF-β for 30 min reduced the association between FZD8 and TGFβRII without significantly affecting FZD8 binding to TGFβRII (Fig. 7e), suggesting that FZD8 plays a role in signal transduction subsequent to TGFβRII phosphorylation and activation of TGFβRII. However, the exact events that take place in the FZD8–TGFβR complex will require detailed study of the endogenous receptors. While this manuscript was in preparation for submission, Li et al. reported that FZD8 promotes bone metastasis in prostate cancer. The authors’ observations that FZD8 is upregulated in prostate cancer and promotes prostate cancer cell migration and invasion are consistent with ours. However, they propose that FZD8 activates Wnt/β-catenin signaling by increasing WNT3A expression. In contrast, our results indicate that Wnt/β-catenin signaling activity is low in prostate cancer and is not affected by FZD8 silencing. How these contrasting mechanisms can be consolidated will require further studies.

In summary, our results suggest that by interacting with TGFβRII, FZD8 can coordinate Wnt and TGF-β signals to promote expression of EMT genes and increase prostate cancer cell migration and invasion. FZD8 may therefore be a useful therapeutic target in metastatic prostate cancer, since blocking its activity has the potential to inhibit aberrant activation of both Wnt and TGF-β signals.

Methods

**Cell culture and reagents.** PC-3, LNCaP, and VCaP cells were obtained from the American Type Culture Collection. PC-3M cells were provided by Scott Fraser and Mustafa Djamgoz (Imperial College London). LNCaP and C4-2B cells were from Charlotte Bevan (Imperial College London), and DU145 cells were from John Masters (University College London) and Magali Williamson (Kings College London). Cell lines were authenticated by DNA profiling (Eurofins Genomics, Germany) and cells for routine use (and all cells for routine use in the lab) for motility assays were cultured for up to 6 months after thawing. LNCaP, C4-2B, PC-3M, and DU145 cells were cultured at 37 °C, 5% CO2 in RPMI-1640 with GlutaMAX (Invitrogen) supplemented with 10% fetal bovine serum (FBS; First Link, UK) and antibiotics (100 units ml−1 penicillin, 100 µg ml−1 streptomycin, Invitrogen, UK). Cell line treatment of LNCaP cells was by culture in RPMI with 5% charcoal-stripped serum (First Link, UK) for 3 or 6 days. VCaP cells were cultured in DMEM/Ham's F12, 10% FBS, antibiotics, 0.5% sodium pyruvate, and 1.5 mM glutamine. For transfection, cells were cultured in OptiMEM (Invitrogen). For gene reporter assays, TGF-β (R&D Systems) at 0.1 or 1 ng ml−1 was added 4 h after transfection and incubated for 24 h. For q-RT-PCR, cells were treated with 1 ng ml−1 TGF-β for 24 h. For invasion assays, cells were pre-treated with 5 ng ml−1 TGF-β for 4 h and the same concentration was added when cells were plated on the transwell filters for 48 h. For evaluation of TGF-β-induced SMAD phosphorylation, 1 ng ml−1 TGF-β was added for 30 min to cells 48 h after transfection. Inhibitor 1 (3253-0367) and 2 (sodium L-8302), which were provided by ChemDiv Inc. (CA, USA) were used at 10 µM in 24 h migration assays.

**Plasmids and siRNAs.** Plasmids used were pcDNA Wnt11−1 and PA-tagged Wnt11−1, a gift from Junichi Takagi, pRL-tk (Promega), Super8XTOP/FOP-Flash13, AP-1-luciferase52, and CAGA12-luc55, which contains 12 CAGA boxes treated as PAI1 promoter. ATF2-luciferase56 was kindly provided by Christof Niehrs (Mainz, Germany) and pRK5 mFzd1-10-D1Δ4 was given from Chris Garcia and Jeremy Nathans (Addgene #42263-42272). CMV500-Δ-TF2 and its empty vector CMV500 was from Charles Vinson (Addgene #33362, #33348). pSTP-lux was a gift from Joan Massague and Jeff Warna (Addgene #11767), pRK5 TGFβRI-Flag57 and pRK5-Flag-ALK5-β (ecto) were from Rik Derynick (Addgene #14831, Addgene #31720), pCMV5B TGFβRII wild-type (N-terminal HA) was from Jeff Warna (Addgene #24801), pRK5 mFz8CRD Ig (Addgene #16689)58 and pc2S LRP6-Δ Ig (Addgene #27279) were from Xi He. SMARTpool siRNAs were purchased from Dharmacon, Thermofisher and dsRNA from Integrative DNA Technologies (IDT, Leuven Belgium). The siRNAs and dsRNA are listed in Supplementary Table 1.

**Cell transfection.** For gene reporter assays, 100,000 (C4-2B) or 80,000 (PC-3M and DU145) cells per well were plated in 12-well plates. After 24 h, cells were washed with OptiMEM and transfected with reporter constructs using Lipofectamine LTX with PLUS (Life Technologies), as instructed by the manufacturer. For transient experiments, 60,000 PC-3M or DU145 cells per well were plated in 12-well plates. After 24 h, cells were washed with OptiMEM (Life Technologies) to remove antibiotics and transfected with 25 or 50 nM of siRNA or dsRNA using RNAMix (Invitrogen), according to the manufacturer's directions. Silencing was evaluated after 48 h. In both cases, fresh media were added to the cells 4 h after transfection.

**Generation of stable knockdown cells.** For stable knockdown of FZD8, lentiviral pLKO and shFZD8 constructs (TRC Lentiviral Human siRNA, RHS4533, Dharmacon) were used. Lentiviruses were produced as previously described94. Lentiviral infection of PC-3M cells was performed twice by addition of cell media from transfected 293FT cells at 24 and 48 h. FZD8 mRNA levels were checked by q-PCR.

**RNA extraction and quantitative real-time PCR.** Total cellular RNA from prostate cancer cells was extracted using TRizol (Invitrogen Life Technologies, Burlington, ON), according to the manufacturer’s protocol. For the silencing experiments, RNA was extracted using illustra® RNAspin Mini Isolation Kit (GE Healthcare). For RNA extraction from tumors growing on the chick CAM, tissues were broken up with a tissue homogenizer (Qagen) for a few seconds and then lysed in 600µl of RNA lysis buffer and RNA extracted using illustra® RNAspin Mini Isolation Kit (GE Healthcare). In all cases, 2µg total RNA was used for reverse transcription using M-MLV Reverse Transcriptase and RNaseOUT Ribonuclease Inhibitor (Invitrogen), according to the manufacturer’s instructions. Quantitative-PCR was performed using PerfeCta SYBR® Green Supermix, Low Rox (Quanta, Barcelona, Spain) in a Viia7 Real-Time PCR System (Applied Biosystems, Madrid, Spain) with the following conditions: Taq polymerase activation 95 °C 3 min, denaturation 95 °C 15 s, annealing/extension 62 °C 1 min, melting curve 95 °C 15 s, 60 °C 1 min, 95 °C 15 s, 40 cycles. Relative levels of mRNA were determined according to the ΔΔCT method, relative to the housekeeping gene 36B4. Primers are listed in Supplementary Table 2.

**Gene reporter assays.** Transfections were normally carried out in triplicate wells 24 h after plating. Cells were transfected with 250 ng of AT2/AT1-P1/TOP/FOP luciferase reporters, 50 ng pRL-tk, and 200 ng empty vector or Wnt-11 plasmid. For CAGA
luciferase activity, cells were transfected with 450 ng of reporter and 50 ng pRL-tk. After 24 h, cells were harvested for luciferase analysis, and the luciferase/renilla ratio was calculated.

**Cell migration and invasion assays.** 250,000 PC-3M or DU145 cells were plated in 6-well plates and transfected with FDEZβ siRNA for 48 h. Afterwards, cells were trypsinized and resuspended in RPMI with 1% FBS, and 50,000 cells/well were plated in 24-well plates with media containing 20% FBS in the lower chamber. As a control for cell viability, cells were plated at the same density in 24-well plates. Migration and invasion were evaluated after 48 and 96 h, respectively. Non-migrated/invasive cells were removed using a cotton swab, and migrated/invasive cells were stained with crystal violet for 30 min. Images were recorded with a Leica confocal microscope.

**Immunoprecipitation.** Luciferase activity was measured using the Dual Glo Luciferase Assay System (Promega) or Luciferase Assay Kit (PJK, Germany) as instructed by the manufacturers. Gene reporter activities were calculated as luciferase/renilla ratios.

**Immunofluorescence.** PC-3M cells were plated on coverslips at 40,000 cells per well in a 24-well plate. 24 h after plating, cells were transfected with 170 ng Wnt-11 and 80 ng FZD plasmid or 120 ng TGFRβⅠ/II and 90 ng Fzd8-D14. The following day, cells were fixed in 4% paraformaldehyde in PBS (Santa Cruz) for 20 min at RT. Immunostaining was performed using an antibody cocktail containing the following: Anti-FZD (1:500, Cell Signaling), Anti-Wnt-11 (1:500, Cell Signaling), and Anti-HA (1:500, Cell Signaling). Coverslips were mounted using Vectashield mounting medium with DAPI (Vector Labs). Stained cells were visualized using a confocal microscope (Leica TCS SP2) with a 63× oil immersion objective. A detailed explanation of the parameters has been described previously. From two independent experiments, the staining efficiency was calculated as the ratio of the mean fluorescent intensity of the stained cells to the mean fluorescent intensity of the negative control.

**Immunohistochemistry.** Paraffin blocks containing lymph node metastases surgically removed from mice 31 days after orthotopic implantation of 2 × 106 PC-3M-luc cells were provided by Gencore (Piscataway, NJ, USA). Sections from these blocks were fixed in 4% paraformaldehyde in PBS and mounted on 3-μm-thick slides. Tissues were dehydrated through a series of ethanol and dried at 60 °C. Antigen retrieval was performed using an IncuCyte (Essen Bio science, Hertfordshire, UK). Images were captured every 2 h for 9 days. To monitor silencing efficiency during 3D culture, siRNA-transfected cells were replated in 12-well plates and RNA extracted at days 4 and 8 and analyzed for gene expression using q-RT-PCR.

**3D imaging acquisition and morphometric analyses.** At the experimental end-points, 3D multicellular structures were stained using 1 μM Calcein AM (Invitrogen) and ethidium homodimer-1 (Invitrogen) in medium at 37°C for 30 min.

**Tumor growth assay on the CAM.** Fertilized White Leghorn chicken eggs were cleaned with water and 70% ethanol. Eggs were placed in an egg incubator with the sharp end down (embryo development day 0, EDD0). Incubation was performed at 37°C under constant humidity (60%) and rotation. No method of randomization was used. Separation of the developing CAM was induced on EDD4 by cutting a 2 mm diameter hole at the sharp end. After covering holes with tape, eggs were returned to the incubator. At EDD7, holes were enlarged to a diameter of approximately 1 cm and a plastic ring was set above the blood vessels of the CAM. 500,000 PC3 cells/egg (previously transfected with siRNAs for 48 h) were suspended in PBS and Matrigel (1:1) and 20 μl cells per egg implanted in the middle of the ring. At EDD16, eggs were placed on ice for 1 h to anesthetize the embryos. Holes were enlarged to 2 mm and embryos were phagocytized.

**Clinical samples.** TMA cores were generated at the Imperial College Experimental Cancer Medicine Centre using samples provided by the Imperial College Healthcare NHS Trust Tissue Bank (ICHTB; project R15043), which is supported by the National Institute for Health Research (NIHR) Biomedical Research Centre, based at Imperial College Healthcare NHS Trust and Imperial College London. Tissues were obtained from surgical resections from 99 prostate cancer patients following patient consent and approval from the local research ethics committee (ref: ICHTB HTA; licence: 12275; REC Wales approval: 12/WA/0196). The clinical characteristics of the samples are summarized in Supplementary Table 5. Other investigators may have received samples from these same tissues. TMA cores contained two cores from regions containing cancer and two cores from regions without cancer from each patient. A histopathologist (I.C.) examined representative haematoxylin and eosin-stained sections to evaluate their pathology.

**Immunohistochemical staining.** Paraffin blocks containing lymph node metastases surgically removed from mice 31 days after orthotopic implantation of 2 × 106 PC-3M-luc cells were provided by Gencore (Piscataway, NJ, USA). Sections from these blocks were fixed in 4% paraformaldehyde in PBS and mounted on 3-μm-thick slides. Tissues were de-paraffinized and from TMA cores were de-paraffinized with Histo-Clear II (National Diagnosis) and then transferred through four changes of 100, 96, 70%, 50% ethanol and water. Antigen retrieval was performed in a pressure cooker filled with sodium citrate buffer at pH 6.0. Endogenous peroxidase activity was quenched for 10 min with 3% hydrogen peroxide. Blocking was performed for 15 min with Avidin Biotin blocking kit (Vector Labs). Samples were washed with PBS and blocked with 5% horse serum for 30 min at room temperature to reduce non-specific staining. After washing, primary antibodies to
FZD8, Wnt-11, Vimentin, and pan-Cytokeratin (CK) (Supplementary Table 3) were applied overnight at 4°C. Sections were incubated with biotinylated secondary antibody (Vector Labs) for 30 min followed by Vectastain Elite ABC reagent (Vector Labs) for 30 min. Liquid diaminobenzidine (DAB) (Dako) was used as a chromogenic agent for 1–2 min and sections were counterstained with Mayer’s haematoxylin. Images were taken on an AxioImager D1 light microscope (Zeiss).

Statistical analysis. Results are presented as the mean ± standard deviation (SD). All experiments were repeated at least three times. Statistical evaluations were performed with GraphPad Prism 5.0 (GraphPad, La Jolla, CA, USA) using two-sided Student’s t-test for single comparisons or one-way analysis of variance (ANOVA) with post hoc Tukey for multiple group comparisons. A two-tailed p-value ≤ 0.05 was considered to indicate statistical significance. For TMA analysis, patients were divided into low (0, 1) and high (2, 3) FZD8 and Wnt-11 expression and Gleason scores ≥ 4 + 3 and ≤ 3 ≤ 4 and analyzed by one-way v2 test, Chi-squared test with Yates correction or Fisher’s exact test, two-sided. Correlation analysis was calculated using Phi-correlations to test association between expression of FZD8 and Wnt-11. All TMA analyses were performed using SPSS v16 (IBM Corp., Somers, NY, USA).

Data availability. All data generated or analyzed during this study are included in this article or the Supplementary Information files, or available from the authors upon request.

Received: 24 July 2017 Accepted: 29 March 2018
Published online: 01 May 2018

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Acknowledgements
We thank Iskander Aurékoketsu and Giacomo Domenici for assistance generating stable knockdown cells, Inmaculada Lépiz-Sánchez for support with correlation quantification, and Maria Vivanco for critical reading of the manuscript. We also thank Jenny Steele and Naina Patel for help with the TMAs, Ignacio Zabalza (Galdakao Hospital) for advice interpreting the immunohistochemistry, Arkaitz Carracedo for anti-human vimentin, Junshi Takagi (Osaaka University) for PA-Wnt-11, Pedro Lazo (IBMMC) for HA-ATF2 and HA-c-JUN, Wei Cui for Flag-SMAD3, and Christof Niehrs (Mainz, Germany) for ATF2-luciferase. We gratefully acknowledge funding from the Basque Department of Education (BFI-2010-129 and PRE_2015_0076), the Ministry of Science and Innovation (MICINN SAF2014-51966-R, SAF2017-84092-R), EMBO (STF_7003), Harris Family Charitable Trust, Academy of Finland (Phenotypic Screening for Cancer Drug Discovery/ Consortium, PESCADoR 309372), Sigrid Jusélius Foundation, Finnish Cancer Organizations and Magnus Ehrnrooth Foundation, and infrastructure support from the Department of Industry, Tourism and Trade (Elkartek) and Department of Innovation Technology of the Government of the Autonomous Community of the Basque Country, the Center of Excellence Severo Ochoa (2017-2021), the Cancer Research UK Imperial Centre, the Imperial Experimental Cancer Medicine Centre and the National Institute for Health Research Imperial Biomedical Research Centre.

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V.M.G.: Concept and design, collection and assembly of data, data analysis and interpretation, and writing of manuscript; I.G.E.: administrative and technical support, collection and assembly of data; M.A.: collection and analysis of data of 3D culture assays; M.C.P.: technical performance, data analysis, and interpretation of CAM assays; L.S.: data analysis of CAM assays, financial support; M.N.: data interpretation of 3D culture assays and administrative support; J.C.: histopathology; J.W.: administrative and financial support; R.M.K.: conception and design, financial support, data analysis and interpretation, and writing of manuscript.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-04042-w.

Competing interests: The authors declare no competing interests.

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