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Frizzled-7 is required for Wnt signaling in gastric tumors with and without Apc mutations.

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Abstract

A subset of gastric cancer (GC) patients have mutations in genes that participate in or regulate Wnt signaling at the level of ligand (Wnt) receptor (Fzd) binding. Moreover, increased Fzd expression is associated with poor clinical outcome. Despite these findings, there are no in vivo studies investigating the potential of targeting Wnt receptors for treating GC, and the specific Wnt receptor transmitting oncogenic Wnt signaling in GC is unknown. Here we use inhibitors of Wnt/Fzd (OMP-18R5/Vantictumab) and conditional gene deletion to test the therapeutic potential of targeting Wnt signaling in preclinical models of intestinal-type gastric cancer and ex vivo organoid cultures. Pharmacological targeting of Fzd inhibited the growth of gastric adenomas in vivo. We identified Fzd7 to be the predominant Wnt receptor responsible for transmitting Wnt signaling in human gastric cancer cells and mouse models of GC, whereby Fzd7-deficient cells were retained in gastric adenomas but were unable to respond to Wnt signals and consequently failed to proliferate. Genetic deletion of Fzd7 or treatment with Vantictumab was sufficient to inhibit the growth of gastric adenomas with or without mutations to Apc. Vantictumab is currently in phase Ib clinical trials for advanced pancreatic, lung, and breast cancer. Our data extend the scope of patients that may benefit from this therapeutic approach as we demonstrate that this drug will be effective in treating gastric cancer patients regardless of Apc mutation status.

Statement of significance

The Wnt receptor Fzd7 plays an essential role in gastric tumorigenesis irrespective of Apc mutation status therefore targeting Wnt/Fzd7 may be of therapeutic benefit to gastric cancer patients.

Introduction

Gastric cancer (GC) is a common malignancy, ranking in the top 4 of global cancer incidence [1]. Often due to advanced stage diagnosis, gastric cancer patients have a very poor 5-year survival rate [1]. This highlights a desperate need for novel clinical treatments as there are very few approved targeted therapies for GC [2, 3]. Gastric cancer is divided histologically into two groups; intestinal-type and diffuse-type, with intestinal-type being more prevalent. Members of the cell-surface Frizzled (Fzd) receptor family are deregulated or overexpressed in several cancer types, including GC [4]. Wnts are lipid-modified glycoproteins that initiate signal transduction by binding to Fzd via a palmitate group, which is appended by the palmitoyltransferase Porcupine (PORCN) [5, 6]. Wnts also bind cell surface co-receptors, such as Lrp5/6, forming a ternary complex [7]. Formation of the Wnt-receptor complex leads to inhibition of a multiprotein 'destruction complex' comprised of Axin, glycogen synthase kinase-3 (GSK3), calcium kinase-1 (CK1) and adenomatous polyposis coli (APC), which targets β-catenin for proteosomal degradation. Newly synthesised cytoplasmic β-catenin can now escape degradation, accumulate and translocate to the nucleus, where it forms a transcriptionally active complex with T-cell factor (TCF)/lymphoid enhancing factor (LEF) family of transcription factors to induce Wnt target gene transcription [8]. However, deregulated Wnt signaling can initiate cell transformation and subsequent carcinogenesis [8].

Furthermore, several Wnt/Fzd antagonists [9] are epigenetically silenced through promoter hyper-methylation, including *DKK3* (67.6% of gastric tumors [10]), *sFRP1* (91%), *sFRP2* (96%), *sFRP5* (65%) [11], whilst others such as the E3 ligase *RNF43*, which regulates Fzd turnover on the cell surface [12], are mutated in 54% and 4.8% of microsatellite instable (MSI) and microsatellite stable (MSS) gastric tumors, respectively [13]. Exogenous re-introduction of sFRP or DKK can significantly reduce gastric tumor burden in *APC* or *β-catenin*-mutant gastric cancer cells by attenuating Wnt signaling [11, 14]. Critically, this provides proof-of-principle that modulation of ligand/receptor signaling components can further regulate Wnt signaling irrespective of downstream mutations that constitutively activate the pathway, which has been reported in colorectal cancer cells [15-18]. Together, these data strongly implicate a role for Wnt/Fzd in GC which could be exploited for targeted therapy.

We recently demonstrated that Frizzled-7 (Fzd7) regulates stem cell function in the gastric and intestinal epithelium [19, 20]. In addition, *FZD7* is abundantly expressed in human gastric cancer tissue [21-23], which is also associated with poor patient outcome [24]. Despite compelling

103 evidence implicating Fzd receptors in GC, there has been no formal investigation of the
104 therapeutic benefit of targeting Fzd receptors in GC *in vivo*. These types of *in vivo* studies are
105 crucial to fully understand the potential of novel therapeutic strategies due to the complex cellular
106 and molecular interactions of a tumor, which can directly inform clinical trials and cannot be
107 replicated *in vitro*. Our results demonstrate that Fzd receptors, specifically *Fzd7*, are rate-limiting
108 for the growth of gastric adenomas with or without *Apc* mutations *in vivo*. These findings have
109 significant clinical utility as targeted Fzd therapeutics (OMP-18R5/Vantictumab), currently being
110 tested in other solid cancer types (http://www.oncomed.com/Pipeline), can now be extended to
111 GC patients with and without *APC* mutations.
112

113 **Materials and Methods**

114 **Mice**

115 The *Tff1Cre*^{ERT2} [25], *Fzd7*$^{fl/fl}$ [20], *Apc*^{580} *(Apc*$^{fl/fl}$*)* [26], *c-Myc*$^{fl/fl}$ *[27], Rosa26LacZ* [28] and *gp130*$^{F/F}$
116 [29] are previously described. Mice were interbred to generate compound mice with appropriate
117 alleles on an inbred C57Bl/6 genetic background. Mice were co-housed using appropriate
118 littermates as controls. All animal experiments were approved by the Animal Ethics Committee,
119 Office for Research Ethics and Integrity, University of Melbourne.
120

121 **Treatments**

122 *In vivo* Cre induction was performed in 8-10 week old mice with a single daily intraperitoneal (ip)
123 injection of 2mg of tamoxifen/mouse/day over four consecutive days. *gp130*$^{F/F}$ mice aged 8-9
124 weeks were injected ip with 20mg/kg of OMP-18R5 (OncoMed) or vehicle control
125 (2.5%DMSO+IgG) twice weekly over the course of 30 days at which point animals were sacrificed
126 and tissues harvested.
127

128 **Tumor xenografts**

129 A total of 4x10^{6} cells in 100µl of PBS were injected subcutaneously into the hind flank of 6-8 week
130 old nude mice (nu(ncr)-foxn1 nu/nu). 7 mice were used for each cohort which were treated with
131 20mg/kg OMP-18R5 or vehicle control (2.5%DMSO+IgG) once tumors were palpable, five days
132 following injection of cells. Xenografts were measured with calipers twice a week to monitor tumor
133 growth.
134
135
136

137 **Tissue collection and histological analysis**
138 Mouse stomachs were isolated, flushed with PBS, fixed overnight at 4°C in 10% neutral buffered
139 formalin (NBF) and processed for immunohistochemistry and immunofluorescence as we
140 previously described [20, 30, 31], with antibodies used on Table S2.
141
142 **Isolation and culture of normal and tumor organoids**
143 Organoids were cultured from mouse stomachs as previously described [31]. Adenomas from
144 *gp130*$^{F/F}$ mice were isolated from the stomach, washed in PBS, roughly minced and incubated in
145 digestion buffer (Dispase I (125µg/ml), Collagenase IV (75U/ml) and DMEM+2.5% FCS) at 37°C
146 until epithelial fragments dissociate from tumor bulk. Dissociated cells were passed through a
147 70µM cell strainer, counted, centrifuged and resuspended in Matrigel. *In vitro* Cre recombinase
148 was activated by treating gastric organoid cultures with 100nM 4-hydroxytamoxifen (4-OHT) as
149 previously described [31]. R-Spondin and Wnt conditioned medium were withdrawn from
150 *Tff1Cre*$^{+}$*;Apc*$^{fl/fl}$ organoid cultures following 4-OHT treatment. Differential interference contrast
151 (DIC) images were captured as Z-sections and final image generated as previously described
152 [20, 32].
153
154 **RNA extraction and analysis**
155 Gastric glands were homogenized in TRizol and total RNA purified, DNAse treated, quantified
156 and subjected to quantitative reverse transcriptase PCR (qRT-PCR). qRT-PCR and calculating
157 gene expression levels relative to the house-keeping gene 18S ($2^{-\Delta\Delta CT}$) were performed as
158 previously described [16].
159
160 **MTT assay**
161 Following treatment, gastric organoids were mechanically dissociated, washed with ADF,
162 resuspended in fresh Matrigel and seeded in a flat bottom 96 well tissue culture plate for
163 enumeration using the MTT assay performed exactly as we previously described [19, 20].
164
165 **Cell culture and transfection**
166 Human gastric cancer cell lines (MKN28, MKN74, MKN7, MKN1, AGS and MKN45) were
167 maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS)
168 (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) and L-Glutamine (Invitrogen) and were
169 not taken past passage 15 for experimental use. All cells were tested for Mycoplasma,
170 authenticated and cultured at 37°C in 5% CO_2. Gastric cancer cells were transfected with Short-

171 hairpin RNA (shRNA) and expression constructs designed to knockdown and stably express
172 FZD7 respectively, as previously described [16, 33] or MSCV-MYC from Addgene (18119).
173
174 **Soft agar colony assay**
175 Cells were cultured in 60mm tissue culture dishes until 50% confluency and transfected with 5µg
176 of plasmid DNA using Lipofectamine LTX (Invitrogen) following manufacturer's instructions. After
177 48hrs incubation, cells were washed in PBS, detached using trypsin, resuspended
178 RPMI+10%FCS, counted and mixed with pre-warmed 1% agar/RPMI culture medium to a final
179 concentration of 500 cells/well of a 6-well plate. Once agar/cell suspensions solidified, cultures
180 were overlaid with RPMI+10% FCS culture medium and incubated at 37°C in 5% CO_2 for 14 days.
181 For Wnt inhibition experiments, cells were treated with OMP-18R5 (10µg/ml), IWP-2 (10µM) [34]
182 or vehicle control (2.5%DMSO+IgG) 3 days after plating. Treatments were removed and replaced
183 every 4 days over the 2 weeks. Cells were fixed in 4%PFA and stained with crystal violet and
184 colonies consisting of ≥50 cells scored and imaged.
185
186 **Genomic recombination PCR**
187 Conventional PCR to detect the *Fzd7* and *Apc* mutant alleles following recombination in genomic
188 DNA extracted from compound transgenic mice was performed as previously described [20, 35].
189 See also supplementary experimental procedures.
190
191 **Luciferase assay**
192 Cells were cultured in 24-well tissue culture plates until 50% confluency and transfected with a
193 total of 1µg plasmid DNA/well (500ng of SuperTOPflash or SuperFOPflash TCF reporter plasmids
194 expressing firefly luciferase [36], plus 500ng of either "control" or "treatment" DNA, plus 2ng of
195 renilla luciferase plasmid to normalize transfection efficiency). Cells were transfected using
196 Lipofectamine LTX with Plus reagent (Invitrogen) according to manufacturer's instructions. Cells
197 were harvested 48hr later and analysed using the dual luciferase reporter assay system
198 (Promega). Ratio of luciferase/renilla reporter activity was calculated and results expressed
199 relative to control cultures.
200
201 **Analysis of gastric adenocarcinoma genomic dataset**
202 Analysis of somatic mutations and copy number alterations (CNA) for a panel of 21 Wnt pathway
203 genes was performed on the TCGA stomach adenocarcinoma dataset [37] using the cBioPortal

204 platform [38]. Only samples with sequencing and CNA data were assessed across all molecular
205 subtypes, n = 287.
206
207 **Statistical analysis**
208 Data are expressed as mean ± SEM, where mean represents number of mice (≥ 3 per genotype)
209 or number of independent experiments (≥3). Statistical tests used are Mann-Whitney with Prism7
210 (GraphPad software) where *P* values of ≤ 0.05 were considered significant. Heatmap generated
211 in R version 3.0.2 using the *heatmap* function in the stats base package. Raw Ct values were
212 transformed to delta Ct values using β2M as housekeeping gene.
213
214 **Results**
215 **Gastric cancer cells require cell intrinsic Wnt signaling for growth**
216 Gastric cancer, like many malignancies, is genetically heterogeneous, which complicates
217 identifying non-redundant signaling pathways suitable for targeted therapy. To investigate the
218 expression of Fzd receptors, which transmit oncogenic Wnt signals, we performed qRT-PCR for
219 all 10 mammalian Fzd genes on a panel of human GC cell lines. Several Fzd receptors were
220 abundantly expressed, including *FZD7* (Figs. 1A, B and Supplementary Fig. S1A-D), suggesting
221 these might be attractive therapeutic targets. Although the pan-Fzd antibody, OMP-18R5
222 (Vanticumab) has shown efficacy in several solid cancer types [39], its therapeutic potential for
223 GC has not been explored. MKN28 (*APC* mutant), MKN74 (*APC* mutant) and MKN45 (*APC* wild-
224 type) GC cells treated with OMP-18R5 formed significantly fewer anchorage-independent
225 colonies compared to vehicle control treated cells (Figs. 1C, D and Supplementary Fig. S1E). Of
226 note, MKN28 and MKN45 cells grown as conventional 2D monolayers do not show growth
227 inhibition following OMP-18R5 treatment (Supplementary Fig.1F and G), which highlights the
228 importance of testing drug efficacy in conditions that better mimic tumor biology. This suggests
229 that cell intrinsic Wnt ligands are required for the 3D-growth of GC cells, which we confirmed by
230 treatment with IWP-2, which prevents Wnt secretion [40] (Figs. 1C and D). TOPflash assays and
231 qRT-PCR demonstrate that either IWP-2 or OMP-18R5 treatment inhibit Wnt signaling in GC cells
232 (Figs. 1E-H). These data demonstrate cell intrinsic secretion of Wnt ligands and Fzd receptor
233 availability are required for the sustained growth of GC cells. To determine whether Fzd regulates
234 the growth of established gastric tumors, MKN28 and MKN45 cells were subcutaneously injected
235 into the hind flanks of nude mice and allowed to develop into palpable gastric tumors. Compared
236 to vehicle control treated gastric tumor xenografts, OMP-18R5 treated mice had significantly
237 smaller gastric tumors (Supplementary Figs. 2A-D), which demonstrates Fzd inhibition is sufficient

238 to block the initiation (Figs 1C and D) and progression (Supplementary Figs. 2A-D) of human
239 gastric cancer cells.
240
241 **Inhibiting Fzd receptors limits gastric tumorigenesis in vivo**
242 We next utilised the well-characterised *gp130*$^{F/F}$ mouse-model of intestinal-type gastric
243 tumorigenesis [29, 41], which develop prominent antral lesions with adenomatous hyperplasia to
244 explore the relative expression of Fzd receptors. Compared to normal gastric epithelium,
245 upregulation of several Fzds was observed in *gp130*$^{F/F}$ gastric adenomas (Figs. 2A-C), supporting
246 expression levels observed in human GC cells (Figs. 1A, B, and Supplementary Figs. S1A-D).
247 Expression of Wnt ligands and target genes are also increased in *gp130*$^{F/F}$ gastric adenomas
248 compared to non-adenoma gastric epithelium (Figs. 2A-C, Table S1). To determine if Fzd
249 inhibition could also reduce the growth of antral gastric adenomas *in vivo*, we treated 8-week-old
250 *gp130*$^{F/F}$ mice, which at this age have small antral gastric adenomas (Supplementary Fig. S2E),
251 with OMP-18R5 twice a week for 30 days, following published protocols (Supplementary Fig. S3A)
252 [39]. Gastric adenomas were significantly smaller and fewer in OMP-18R5-treated *gp130*$^{F/F}$ mice
253 compared to vehicle control treated mice (Figs. 2D-F), which was associated with a significant
254 reduction in the expression of Wnt target genes and cell proliferation (PCNA IHC) (Figs. 2G-J).
255 As previously reported [39], no toxicity was observed in OMP-18R5-treated mice, which displayed
256 consistent bodyweight, no signs of morbidity and no reduction in proliferation of normal non-
257 adenoma gastric epithelial cells for the duration of treatment (Supplementary Figs. S3B-D). These
258 data strongly suggest Fzd receptors are rate-limiting for the growth of gastric adenomas *in vivo*,
259 and in human GC cells *in vitro.* Given that Wnts and Fzds can be expressed by non-epithelial
260 cells, we established gastric organoids from *gp130*$^{F/F}$ antral adenomas using defined culture
261 conditions to determine if the anti-growth effects observed in *gp130*$^{F/F}$ mice following OMP-18R5
262 treatment was systemic or cell intrinsic. *gp130*$^{F/F}$ gastric adenoma organoids treated with OMP-
263 18R5 or IWP-2 displayed reduced viability (MTT assay) and growth compared to vehicle control
264 treated organoids (Figs. 2K-M). This data confirms that Wnt ligands and Fzd receptors are
265 required cell intrinsically for the growth of gastric adenoma cells *ex vivo*.
266
267 **Targeted FZD7 knockdown reduces gastric cancer colony formation**
268 Inhibition of cell growth following OMP-18R5 treatment suggest that one of several Fzds targeted
269 by OMP-18R5 (FZD1, 2, 5, 7 and 8) is responsible for transmitting Wnt signals to GC cells. Gene
270 expression analysis narrows this down to *FZD2* and/or *FZD7*, as *FZD1*, *FZD5* and *FZD8* are
271 undetectable in these cell lines (Figs. 1A and B). We have previously shown that Fzd2 is unable

272 to compensate for the loss of *Fzd7* in the intestinal epithelium [20], which may indicate Fzd7 plays
273 a predominant role in Wnt signal transmission in gastric tissue. Indeed, *FZD7* is commonly
274 upregulated in a variety of different cancer types, including gastric cancer, which is associated
275 with poor clinical outcome [24, 42]. To determine the specific requirement of *FZD7* for the growth
276 of human GC cells we performed colony formation assays. Cells transfected with FZD7-targeted
277 shRNA (shFZD7) [16] had a marked decrease in colony growth, compared to scrambled shRNA
278 (shSCRAM) or empty vector (EV) controls (Figs. 3A and B), associated with decreased Wnt
279 signaling (Figs. 3C and D). These data suggest that Fzd7 is the predominant Wnt receptor
280 transmitting oncogenic Wnt signaling in GC cells. Importantly, growth inhibition following FZD7-
281 knockdown was rescued by co-transfection with a full-length FZD7 expression construct [33],
282 demonstrating the specificity of the shRNA and FZD7-regulated growth in human GC cells
283 (Supplementary Figs. S4A and B).

284

285 **Conditional deletion of Fzd7 from $gp130^{F/F}$ gastric tumors reduces cell proliferation**

286 To determine the functional requirement of Fzd7 for gastric adenoma growth *in vivo*, we
287 conditionally deleted *Fzd7* in the gastric adenomas of 8-week old $Tff1Cre^{ERT2/+};gp130^{F/F};Fzd7^{fl/fl}$
288 mice ($Cre^{+};gp130^{F/F};Fzd7^{fl/fl}$) (Supplementary Fig. S4C), which allows robust recombination in
289 these adenomas [25]. Tamoxifen injected $Cre^{+};gp130^{F/F};Fzd7^{fl/fl}$ mice developed significantly
290 smaller and fewer antral gastric adenomas than their Cre-negative ($Cre^{-};gp130^{F/F};Fzd7^{fl/fl}$)
291 tamoxifen-treated littermates (Figs. 3E-G and Supplementary Fig. S4D), supporting our previous
292 *in vitro* experiments demonstrating *FZD7* inhibition is sufficient to block gastric adenoma growth
293 (Figs. 3A-D).

294

295 **Fzd7 deficient cells are retained in gastric tumors and fail to proliferate**

296 The growth of $gp130^{F/F}$ gastric adenomas requires Stat3 [43]. Therefore we performed p-Stat3
297 IHC and *Socs3* qRT-PCR which identified no alterations in Stat3 activity, and did not cause the
298 reduced growth of gastric adenomas in $Cre^{+};gp130^{F/F};Fzd7^{fl/fl}$ mice (Figs. 4A and B). This identifies
299 that Fzd7-mediated Wnt signaling is rate-limiting for Stat3-driven gastric adenomas, which have
300 no Wnt-activating mutations. Deletion of *Fzd7* in normal, non-transformed gastric epithelium
301 causes repopulation with Fzd7-proficient cells [19]. To monitor if repopulation occurs in
302 $Cre^{+};gp130^{F/F};Fzd7^{fl/fl}$ adenomas, we performed PCR for the recombined *Fzd7* floxed allele
303 ($Fzd7^{\Delta}$), which we have previously shown is lost during repopulation in the normal gastric
304 epithelium following *Fzd7* deletion [19]. However, in gastric adenomas of $Cre^{+};gp130^{F/F};Fzd7^{fl/fl}$
305 mice 30 days post tamoxifen, we detect robust recombination of the $Fzd7^{\Delta}$ allele, demonstrating

306 that *Fzd7* deleted cells are retained in these adenomas (Fig. 4C). In support, the expression of
307 *Fzd7* and many Wnt pathway components and target genes remain low in these adenomas (Fig.
308 4D, Supplementary Fig. S4E and Table S1). This suggests that the mechanism underlying smaller
309 gastric adenomas following *Fzd7* deletion is due to retention of *Fzd7*-deficient cells in the
310 adenoma that are unable to respond to proliferative Wnt signals, and thus fail to proliferate (Fig.
311 4E). To investigate this further, we performed IHC on serial sections to detect recombined (β-gal$^+$,
312 *Fzd7* deleted) cells and proliferating cells (PCNA$^+$) in *Cre$^+$;gp130$^{F/F}$;Fzd7$^{fl/fl}$;LacZ* mice and
313 observed a marked co-localisation of non-proliferative (PCNA$^-$) cells with recombined cells (β-
314 gal$^+$) (Fig. 4F).
315
316 To monitor cellular changes following *Fzd7* deletion in *Cre$^+$;gp130$^{F/F}$;Fzd7$^{fl/fl}$* mice, IHC for
317 apoptosis (Caspase-3) and differentiation (Muc5a and Gastrin) was performed (Supplementary
318 Fig. S4F). Muc5a$^+$ and Gastrin$^+$ cells were increased following *Fzd7* deletion in
319 *Cre$^+$;gp130$^{F/F}$;Fzd7$^{fl/fl}$* mice compared to *Fzd7*-proficient gastric adenomas (*Cre$^-$;gp130$^{F/F}$;Fzd7$^{fl/fl}$*).
320 This also suggests that gastric adenomas do not repopulate following *Fzd7* deletion, as
321 repopulation in the normal gastric epithelium following *Fzd7* deletion is associated with reduced
322 cell differentiation [19]. No change in the frequency of Caspase-3$^+$ cells was observed
323 (Supplementary Fig. S4D), indicating that deletion of *Fzd7* from adenoma cells does not trigger
324 apoptosis.
325

326 **Cell intrinsic Wnt signaling via Fzd7 is required for Wnt-driven gastric adenomas**

327 The *gp130$^{F/F}$* mice and MKN45 GC cells are wild-type for *APC*, and have no known Wnt-activating
328 mutations, suggesting that targeting Fzd7 may be effective in gastric adenomas and GC cells
329 without mutations to the Wnt pathway. However, some of the GC cell lines that responded to Fzd
330 therapy (MKN28 and MKN74) have mutant *APC* (https://portals.broadinstitute.org/ccle),
331 suggesting that Fzd therapies can be effective in gastric adenomas with and without mutant *APC*.
332 In silico analysis of GC patient datasets identify mutations in several genes that regulate Wnt
333 signaling, demonstrating that this pathway is aberrantly activated in GC (Supplementary Fig.
334 S5A). To functionally investigate this, gastric organoids established from *Tff1Cre$^{ERT2/+}$;Apc$^{fl/fl}$*
335 (*Cre$^+$;Apc$^{fl/fl}$*) mice were treated with tamoxifen, to truncate *Apc,* and showed significant increase
336 in growth and proliferation (Fig. 5A), which was confirmed by Ki-67 staining and increased cell
337 viability (MTT assay) (Figs. 5A-C). A concordant increase in Wnt target gene expression was
338 observed in hyperproliferative *Apc* mutant organoids (Fig. 5D). Treatment of *Apc* mutant
339 organoids with IWP-2 or OMP-18R5 prevented upregulation of the Wnt pathway and blocked

340 organoid proliferation (Figs. 5A-D), demonstrating that cell intrinsic Wnt secretion and Fzd
341 receptors are required for gastric cells to activate Wnt signaling and regulate growth, even after
342 mutation of *Apc* (Figs. 5A-D).
343

344 *Fzd7* expression was increased in *Apc* mutant gastric organoids and subsequently downregulated
345 in IWP-2 or OMP-18R5 treated organoids (Fig. 5E), therefore we examined whether Fzd7 is
346 responsible for transmitting Wnt signaling in *Apc* mutant gastric adenoma cells *in vivo*. 30 days
347 following tamoxifen, $Cre^{+};Apc^{fl/fl}$ mice developed multiple, large intestinal-type gastric adenomas
348 with extensive hyperplasia in the antral stomach (Figs. 6A and B), which were not observed in
349 tamoxifen-treated $Cre^{-};Apc^{fl/fl}$ mice (Fig. 6A). Remarkably, co-recombination of *Apc* and *Fzd7*
350 alleles in $Cre^{+};Apc^{fl/fl};Fzd7^{fl/fl}$ mice inhibited the ability of *Apc* mutant cells to develop antral
351 adenomas (Figs. 6A and B). Gastric adenomas of $Cre^{+};Apc^{fl/fl};Fzd7^{fl/fl}$ mice had significantly less
352 $PCNA^{+}$ cells compared to $Cre^{+};Apc^{fl/fl}$ mice (Figs. 6A and C). In common with $gp130^{F/F}$ tumors,
353 deletion of *Fzd7* in *Apc* deficient gastric adenomas also results in retention of *Fzd7*-deficient cells
354 as monitored by expression of the $Fzd7^{\Delta}$ allele (Fig. 6D).
355

356 As expected, Wnt signaling is increased in gastric adenomas of $Cre^{+};Apc^{fl/fl}$ mice, however, Wnt
357 signaling is not elevated in the non-adenoma antral epithelium of $Cre^{+};Apc^{fl/fl};Fzd7^{fl/fl}$ mice (Fig.
358 6E). This is supported by IHC for the surrogate markers of active Wnt signaling, β-catenin and
359 Myc (Supplementary Fig. S6A). IHC revealed a decrease in $Muc5a^{+}$ and $Gastrin^{+}$ cells following
360 *Apc* mutation (Supplementary Fig. S6B), while tamoxifen-treated $Cre^{+};Apc^{fl/fl};Fzd7^{fl/fl}$ mice display
361 a modest restoration of mucus-secreting and gastrin-producing cells, similar to that observed in
362 $Cre^{+};gp130^{F/F};Fzd7^{fl/fl}$ mice (Supplementary Fig. S4D). Collectively, these data demonstrate that
363 *Apc*-mutant gastric phenotypes require functional *Fzd7.*
364

365 **Fzd7-dependant Myc expression is required for the growth of gastric adenomas.**

366 The transcription factor c-Myc is a well-characterised β-catenin/TCF target gene in the
367 gastrointestinal tract as c-*Myc* is required for all intestinal tumor phenotypes following *Apc*-
368 mediated activation of Wnt signaling [35]. Myc is upregulated in our gastric adenoma mouse
369 models and human GC cell lines, and inhibition of Fzd7 prevents this upregulation (Figs. 2H, 3C,
370 4D, 5D and 6E). Conditional deletion of *c-Myc* in $Tff1Cre^{ERT2/+};Apc^{fl/fl};c\text{-}Myc^{fl/fl}$ ($Cre^{+};Apc^{fl/fl};Myc^{fl/fl}$)
371 mice showed complete absence of antral adenoma formation and Wnt activation compared to
372 $Cre^{+};Apc^{fl/fl}$ mice (Supplementary Fig. S7), indicating Fzd7-dependant expression of Myc is
373 required for the growth of *Apc* mutant gastric adenomas.

374 To determine whether elevated levels of MYC can rescue GC cell growth suppression following
375 *FZD7* knockdown, GC cells were co-transfected with FZD7shRNA and MSCV-MYC expression
376 plasmids and grown as colonies in soft agar for 2 weeks. Compared to control (EV) transfected
377 cells, co-transfected cells (FZD7shRNA and MSCV-MYC) showed no difference in the number of
378 colonies formed (Supplementary Fig. S7G-I), which suggests that overexpression of MYC is able
379 to rescue the growth suppressive effects of FZD7 knockdown in GC cells.
380
381 **Discussion**
382 Expression of Fzd receptors is deregulated in several cancers, including gastric cancer [4, 21,
383 42]. Here we show for the first time that Fzd receptors are rate-limiting for the growth of gastric
384 adenomas *in vivo*. We further elucidate that Fzd7 is the predominant Wnt receptor transmitting
385 cell-intrinsic Wnt signals in human GC cells.
386
387 *In vitro* studies have shown that targeted inhibition of Fzd is sufficient to block growth of GC cells
388 [24, 44]. However, it is well documented that *in vitro* studies do not fully recapitulate the complex
389 cellular and molecular interactions present in tumors [45]. Here, we demonstrate that gastric
390 adenomas require Fzd7 for optimal growth using genetic and pharmacological strategies in two
391 independent mouse models. Our findings support our previous work [39] demonstrating that
392 targeting multiple Fzd receptors blocks the growth of several different cancers, which we now
393 extend to GC. Using *ex-vivo* adenoma-derived organoids we demonstrate these anti-growth
394 effects are cell intrinsic as OMP-18R5 blocks the growth of gastric adenoma-derived organoids in
395 the absence of immune or stromal cells.
396
397 As previously observed in the normal gastric epithelium [19], genetic inhibition of *Fzd7* in gastric
398 adenomas induces upregulation of other *Fzd* genes (Table S1), however, these are insufficient to
399 compensate and promote gastric adenoma growth. This suggests that specific targeting of Fzd7
400 is an attractive therapeutic strategy for the treatment of gastric cancer.
401
402 Deletion of *Fzd7* in the normal gastric epithelium triggers repopulation [19] which could be a
403 possible explanation for why *Fzd7*-deficient gastric adenomas are smaller. Epithelial repopulation
404 is an effective tissue mechanism that helps the gastric epithelium to survive the harsh conditions
405 of the stomach. Here we show that repopulation is not preserved in gastric adenomas, which
406 contain aberrant cell signaling and tissue architecture, and therefore *Fzd7*-deficient cells remain
407 in the adenoma but are unable to respond to Wnt signals and thus do not proliferate.

408 One feature of inflammation-associated tumors in the gastrointestinal tract is phosphorylated
409 Stat3 (p-Stat3), which regulates many cancer hallmarks [43]. Gastric adenomas in *gp130*^{FF} mice
410 do not harbor any Wnt-activating mutations [41], however, they display high levels of Wnt
411 signaling. Stat-3 has been shown to activate Wnt signaling, which would allow pathway activation
412 in the absence of Wnt mutations in *gp130*^{FF} adenomas [46, 47]. Indeed, Wnt and gp130/Stat3
413 signaling operate in parallel during gastric tumorigenesis as active p-Stat3 levels remain high in
414 *Fzd7* deleted adenomas, demonstrating that Wnt/Fzd7 signaling is rate-limiting for Stat3-driven
415 gastric adenomas. Similarly, mTORC1 signaling is also rate-limiting for *gp130*^{FF} adenoma growth
416 independent of Stat3 [41].
417
418 Recent large-scale sequencing of human gastric tumors has identified environmental and genetic
419 factors associated with increased pathology, which include aberrant Wnt signaling [48-50].
420 Importantly, these genomic studies are yet to be validated with functional interrogation *in vivo*,
421 which are essential to understand the therapeutic potential of targeting Wnt signaling in gastric
422 cancer [21]. We and others have demonstrated that *Fzd7* inhibition is sufficient to block Wnt
423 signaling in cells with mutant *APC* [17, 51]. Interestingly, ~37% of *APC* mutant gastric tumors are
424 mutant for *RNF43* (regulates Fzd on the cell surface [12]), demonstrating that Fzd is deregulated
425 in a subset of *APC* mutant gastric tumors (http://www.cbioportal.org/). Interestingly *RNF43* and
426 *APC* mutations are mutually exclusive in colon tumors suggesting that CRC and GC cells
427 preferentially select different Wnt mutations that confer optimal or 'just-right' levels of Wnt
428 signaling required for tumor growth [52, 53].
429
430 Furthermore, we have shown that Myc is required for the gastric adenoma phenotypes associated
431 with *Apc* mutation. These findings are reminiscent of the role played by Myc in the intestinal
432 epithelium following *Apc* mutation [35], and thus place the Wnt/Fzd7/Myc signaling axis as an
433 attractive therapeutic target for gastric cancer. Encouragingly, next generation bromodomain
434 (BET) inhibitors are effective in killing patient-derived GC cells [54]. Importantly, this provides
435 justification for testing a combination of BET and Wnt inhibitors in GC, which we have previously
436 shown is effective at blocking the growth of human colon cancer cells [55].
437
438 New generation PORCN inhibitors are in clinical trials for solid tumors, which our results show
439 may be effective in gastric cancer, however these target the secretion of all Wnt ligands.
440 Collectively, we demonstrate that targeted inhibition of Wnt receptors, specifically Fzd7, is rate-
441 limiting for the growth of gastric adenomas with and without *Apc* mutations. This provides a broad

scope for the application of this therapeutic strategy for the treatment of GC, with potentially less side effects than targeting all Wnt secretion with PORCN inhibitors, and will directly inform clinical trials to treat GC patients with OMP-18R5 (Vantictumab), which only targets 5 out of the 10 Fzd family.

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578

Figure Legends

Figure 1. Inhibition of Wnt or Fzd blocks gastric cancer cell growth.

A. qRT-PCR for *FZD* gene expression in MKN28 gastric cancer cells. Expression shown relative to housekeeper (β2M), n=4 biological replicates.
B. qRT-PCR for *FZD* gene expression in MKN74 gastric cancer cells. Expression shown relative to housekeeper (β2M), n=4 biological replicates.
C. Quantification of cell colonies (>50 cells) from MKN28 gastric cancer cells grown in agar for 2 weeks following treatment with vehicle control (2.5%DMSO+IgG), IWP-2 (10μM) or OMP-18R5 (10μg/ml). Treatments were replaced every 4 days for the duration of 2 weeks. Individual experiments were repeated three times. Colonies were counted with ImageJ (*= p<0.05, mean ±SEM, Mann-Whitney).
D. Quantification of cell colonies (>50 cells) from MKN74 gastric cancer cells grown in agar for 2 weeks following treatment with vehicle control (2.5%DMSO+IgG), IWP-2 (10μM) or OMP-18R5 (10μg/ml). Treatments were replaced every 4 days for the duration of 2 weeks. Individual experiments were repeated three times. Colonies were counted with ImageJ (*= p<0.05, mean ±SEM, Mann-Whitney).
E. TOPflash assay on MKN28 cells treated 24hrs with DMSO, IWP-2 (10μM) or OMP-18R5 (10μg/ml) (**= p<0.005, mean ±SEM, n=9 biological replicates, Mann-Whitney). Individual experiments were repeated three times.
F. TOPflash assay on MKN74 cells treated 24hrs with DMSO, IWP-2 (10μM) or OMP-18R5 (10μg/ml) (**= p<0.005, mean ±SEM, n=9 biological replicates, Mann-Whitney). Individual experiments were repeated three times.
G. qRT-PCR for *CD44* in MKN28 and MKN74 cells described in E and F (mean ±SEM, n=6 biological replicates, Mann-Whitney). Individual experiments were repeated twice.
H. qRT-PCR for *AXIN2* in MKN28 and MKN74 cells described in E and F (mean ±SEM, n=6 biological replicates, Mann-Whitney). Individual experiments were repeated twice.

Figure 2. Inhibition of Fzd receptors reduces cell intrinsic Wnt signaling and gastric adenoma burden.

A. qRT-PCR for Wnt ligands in *gp130*$^{F/F}$ adenomas compared to normal gastric epithelium (*= p<0.05, mean ±SEM, n=4 mice, Mann-Whitney).
B. qRT-PCR for Fzd receptors in *gp130*$^{F/F}$ adenomas compared to normal gastric epithelium (*= p<0.05, mean ±SEM, n=4 mice, Mann-Whitney).
C. qRT-PCR for Wnt target genes in *gp130*$^{F/F}$ adenomas compared to normal gastric epithelium (*= p<0.05, mean ±SEM, n=4 mice, Mann-Whitney).
D. Whole mount images of 8-9 week old *gp130*$^{F/F}$mice treated with control IgG or OMP-18R5 over the course of 30 days and harvested. Black and white arrows show gastric tumors.
E. Weights of gastric adenomas from mice described in D (***= p<0.001, mean ±SEM, n=9 mice, Mann-Whitney).
F. Quantification of gastric adenomas in mice described in D (***= p<0.001, mean ±SEM, n=9 mice, Mann-Whitney).
G. qRT-PCR for Fzd receptors in mice described in D (**= p<0.005, mean ±SEM, n=9 mice, Mann-Whitney).
H. qRT-PCR for Wnt target genes in mice described in D (**= p<0.005, mean ±SEM, n=9 mice, Mann-Whitney).

625 I. Immunohistochemistry for PCNA on adenomas sections from mice described in D. Scale
626 bars = 100μm.
627 J. Quantification of $PCNA^+$ cells from adenomas sections described in I (*= $p<0.05$, mean
628 ±SEM, n=4 mice, Mann-Whitney).
629 K. Representative DIC images of *gp130*$^{F/F}$ adenoma-derived organoids treated with vehicle
630 control (2.5%DMSO+IgG), IWP-2 (10μM) or OMP-18R5 (10μg/ml) and cultured for 5 days.
631 Green arrows indicate viable organoids. Red arrows indicate dying/atrophic organoids.
632 Scale bar = 200 μm
633 L. MTT viability assay performed on organoids described in K (*= $p<0.05$, mean ±SEM, n=3
634 biological replicates, Mann-Whitney).
635 M. Measurement (diameter) of organoids described in K. Measurements were quantified in
636 ImageJ (*= $p<0.05$, mean ±SEM, n=3 biological replicates, Mann-Whitney).
637

638 **Figure 3. Targeted inhibition of Fzd7 reduces gastric cancer clonogenicity and adenoma**
639 **burden.**
640 A. Representative DIC images of MKN28 and MKN74 cells transfected with empty vector
641 (EV), scrambled (shSCRAM) or FZD7-specific shRNA (FZD7shRNA) and grown in agar.
642 Scale bars = 200μm
643 B. Quantification of cell colonies from experiment described in A (*= $p<0.05$, mean ±SEM,
644 n=3 biological replicates, Mann-Whitney). Individual experiments were repeated twice.
645 C. qRT-PCR for Wnt taget genes on MKN28 and MKN74 cells transfected with empty vector
646 (EV), scrambled (shSCRAM) or FZD7-specific shRNA (Fzd7shRNA) (*= $p<0.05$, mean
647 ±SEM, n=3 biological replicates, Mann-Whitney).
648 D. TOPflash assay on MKN28 and MKN74 cells described in C (***= $p<0.001$, mean ±SEM,
649 n=9 biological replicates, Mann-Whitney). Individual experiments were repeated three
650 times.
651 E. Representative images of tamoxifen-treated *Tff1Cre*$^{ERT2/-}$ (*Cre*$^-$) or *Tff1Cre*$^{ERT2/+}$ (*Cre*$^+$)
652 stomachs following *Fzd7* deletion. Black arrows indicate gastric tumors.
653 F. Weights of gastric adenomas per mouse described in E (**= $p<0.005$, mean ±SEM, n=7
654 mice, Mann-Whitney).
655 G. Quantification of gastric adenomas per mouse described in E (**= $p<0.005$, mean ±SEM,
656 n=7 mice, Mann-Whitney).
657

658 **Figure 4. Deletion of *Fzd7* from gastric tumors decreases cell proliferation.**
659 A. Immunohistochemistry (IHC) for p-Stat3 on adenoma sections from *Fzd7*$^{fl/fl}$*;gp130*$^{F/F}$ mice
660 (*Cre*$^-$ or *Cre*$^+$) 30 days after tamoxifen treatment. Scale bars = 100μm.
661 B. qRT-PCR for *Socs3* on gastric adenomas from mice described in A (*= $p<0.05$, mean
662 ±SEM, n=4 mice, Mann-Whitney).
663 C. Conventional PCR to detect recombination of *Fzd7*$^{fl/fl}$ allele ($Fzd7^{\Delta}$) in gastric adenomas
664 from mice described in A.
665 D. qRT-PCR for Wnt target genes in gastric adenomas from mice described in A (**=
666 $p<0.005$, mean ±SEM, n=7 mice, Mann-Whitney).
667 E. Quantification of $PCNA^+$ cells from adenoma sections described in A (*= $p<0.05$, mean
668 ±SEM, n=3 mice, Mann-Whitney).
669 F. Representative IHC images for β-galactosidase (detecting allelic recombination) and
670 PCNA (proliferation) on serial sections from *Tff1Cre*$^-$*;Fzd7*$^{fl/fl}$*;gp130*$^{F/F}$*;LacZ* or
671 *Tff1Cre*$^+$*;Fzd7*$^{fl/fl}$*;gp130*$^{F/F}$*;LacZ* mice 30 days following tamoxifen. Note, yellow dashed

672 lines demarcate areas of allelic recombination, which correspond to reduced proliferation
673 and black dashed lines represent areas of non-recombined cells. Scale bars = 100µm.
674
675 **Figure 5. Wnt/Fzd inhibition reduces *Apc* mutant gastric organoid proliferation.**
676 A. Representative DIC and immunofluorescence images of *Tff1Cre;Apc*$^{fl/fl}$ organoids treated
677 for 24hrs with tamoxifen (tmx, 100nM), IWP-2 (10µM) or OMP-18R5 (10µg/ml). Green
678 arrows indicate hyperproliferative organoids. Red arrows indicate growth-constrained
679 organoids. Scale bars = 200µm.
680 B. MTT viability assay performed on organoid cultures described in A (***= $p<0.001$, mean
681 ±SEM, n=3 biological replicates, Mann-Whitney). Individual experiments were repeated
682 twice.
683 C. Measurement of organoid size (µm) from cultures described in A (***= $p<0.001$, mean
684 ±SEM, n=3 biological replicates, Mann-Whitney).
685 D. qRT-PCR for Wnt target genes on organoid cultures described in A (*= $p<0.05$, mean
686 ±SEM, n=3 biological replicates, Mann-Whitney).
687 E. qRT-PCR for Fzd receptors on organoid cultures described in A. Expression of Fzd shown
688 as Log_2 ratio.
689
690 **Figure 6. Deletion of *Fzd7* rescues gastric adenoma formation following *Apc* truncation.**
691 A. Representative whole mount and IHC (PCNA) on wild-type (*Tff1Cre*$^{-}$*;Apc*$^{fl/fl}$), *Apc* mutant
692 (*Tff1Cre*$^{+}$*;Apc*$^{fl/fl}$) and *Apc/Fzd7* mutant mice (*Tff1Cre*$^{+}$*;Apc*$^{fl/fl}$*;Fzd7*$^{fl/fl}$) 30 days following
693 tamoxifen. Black arrows indicate gastric adenomas in top panels. Scale bars = 100µm.
694 B. Weights of gastric adenomas from harvested mice described in A (**= $p<0.005$, mean
695 ±SEM, n=7 mice, Mann-Whitney).
696 C. Quantification of PCNA^{+} cells in adenoma sections from mice described in A (***= $p<0.001$,
697 mean ±SEM, n=3 mice, Mann-Whitney).
698 D. Conventional PCR for recombined *Fzd7* (Fzd7$^{\Delta}$) and *Apc* (Apc$^{\Delta}$) alleles in mice described
699 in A.
700 E. qRT-PCR for Wnt target genes on tamoxifen-treated mice described in A (**= $p<0.005$,
701 mean ±SEM, n=4 mice, Mann-Whitney).
702
703