sion was undetectable in VilER-Cre-Fzd7KO$^{-/-}$ mice, but some residual expression of *Fzd5* could be observed in VilER-Cre-Fzd5KO$^{-/-}$ animals (Fig.23).

Next, these models were crossed with VG animals to generate VG-VilER-Cre-Fzd5KO$^{-/-}$ and VG-VilER-Cre-Fzd7KO$^{-/-}$. These mice were allowed to develop their phenotype for 6 weeks before receiving 80 mg/Kg of tamoxifen intraperitoneally. A second dose (80mg/kg) was applied on week 9 to target cells that had escaped recombination (escaper cells). Samples were collected 120 days after birth. H&E staining revealed that both mouse models developed lesions in comparable size and numbers to VG controls (Fig.24). Examination of *Fzd5* and *Fzd7* levels of expression by single-channel ISH indicated that, whereas Fzd7 transcripts remained undetectable over the course of the experiments in VilER-Cre-Fzd7KO$^{-/-}$ animals, polyps from VG-VilER-Cre-Fzd5KO$^{-/-}$ mice were completely repopulated by *Fzd5+* escaper cells by the endpoint (Fig.25).

In order to assess whether *Fzd5* KO levels in VilER-Cre-Fzd5KO$^{-/-}$ mice could be maintained short term, a second experiment was conducted where these animals were injected with 1mg of tamoxifen daily for a total of 5 days, and samples were collected on the day of the last injection. Although the levels of expression of *Fzd5* in test animals were lower than in VG controls, *Fzd5* staining spanned the whole intestine, specifically in the ECF compartment. This suggests that cells that escape recombination have a strong competitive advantage with respect to their KO neighbours, which allows them to repopulate the tissue in a short time-frame (Fig.S1).

4.11 Characterization of Advanced VG Polyps

Despite the fact that the majority of VG lesions did not express detectable levels of *Anxa1* nor *Lgr5* via *In Situ Hybridisation*, some advanced VG polyps from aged animals (31-38 weeks old at collection) were positive for these SC markers (Fig.26D,E).

In these samples, *Lgr5* expression correlated with nuclear localization for β-catenin (Fig. 27A.B). For this reason, Lgr5 was used as a marker of presumed Wnt activation. In order to investigate whether these read-outs for high WNT activation co-occurred with acquisition of Wnt alterations, WES and bulk RNA-Sequencing data from advanced Lgr5+ VG polyps were analysed and compared to intermediate Lgr5- paired cases. Raw data was processed using the same pipelines described earlier in this section. Unlike their Lgr5- counterparts, 2 out of 3 Lgr5+ polyps had mutations in β-catenin (Fig.27E). The remaining sample carried a *Ptprk-Rspo3* fusion, which was detected by the *STAR-fusion* algorithm and confirmed with brown ISH for *Rspo3*

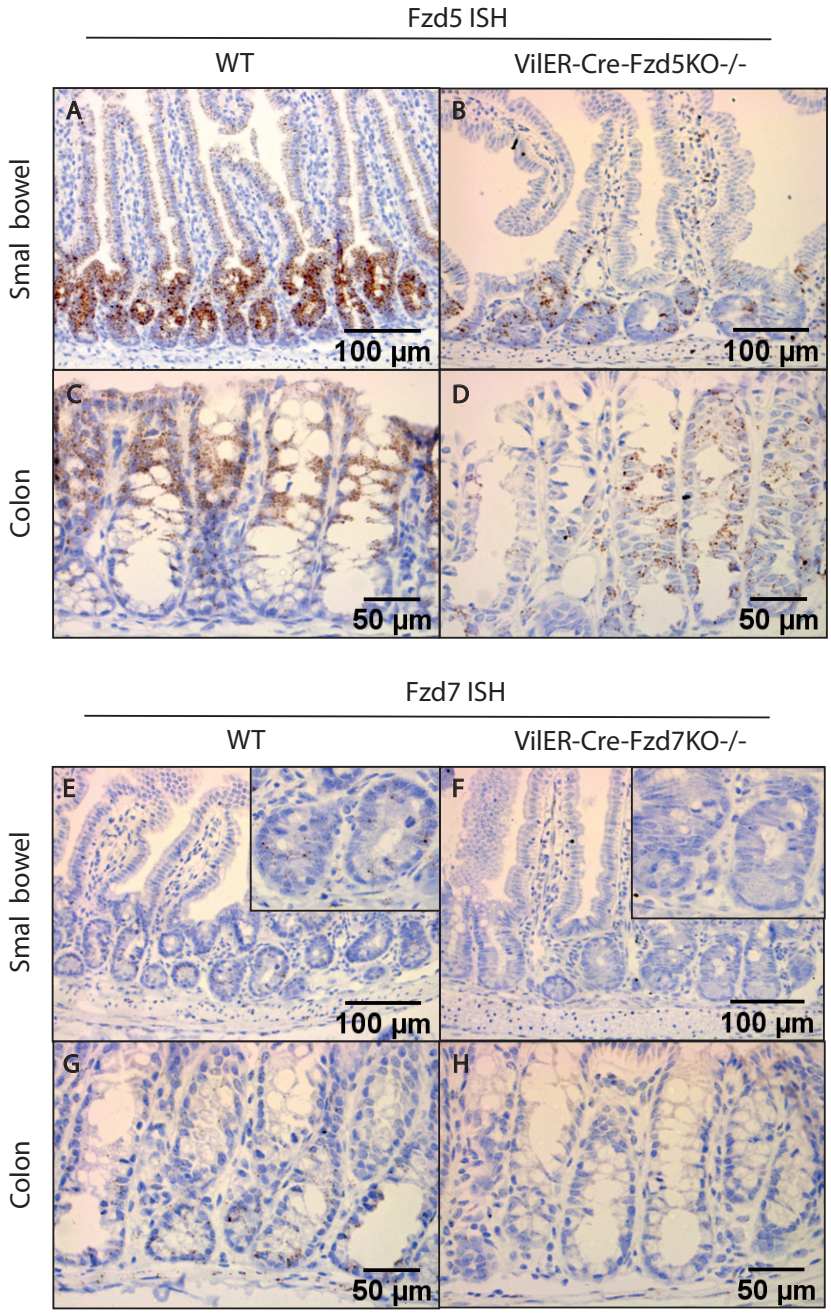

Figure 23: Expression of *Fzd5* and *Fzd7* in VilER-Cre-Fzd5KO$^{-/-}$ and VilER-Cre-Fzd7KO$^{-/-}$ animals. A-H. *In Situ Hybridisation* of *Fzd5* (A-D) and *Fzd7* (E-H) in the small bowel (A,B,E,F) and colon (C,D,G,H) of VilER-Cre-Fzd5KO$^{-/-}$(A-D) and VilER-Cre-Fzd7KO$^{-/-}$(E-H) animals. Details show conventional crypts.

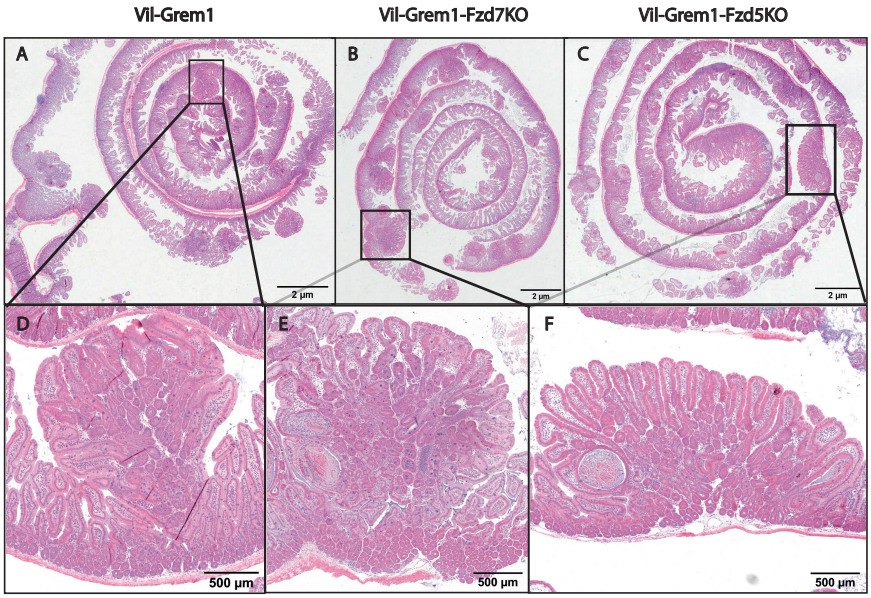

Figure 24: There are no difference in terms of polyp burden in VG-VilER-Cre-Fzd7KO$^{-/-}$ and VG-VilER-Cre-Fzd5KO$^{-/-}$ versus paired Vil1-*GREM1* controls. A,B,C. Gut roll of the small bowel from a Vil1-*GREM1* (A) VG-VilER-Cre-Fzd7KO$^{-/-}$ (B) and VG-VilER-Cre-Fzd5KO$^{-/-}$ (C) animal. D,E,F. Close-ups showing a polyp from a Vil-GREM1 (D) VG-VilER-Cre-Fzd7KO$^{-/-}$ (F) and VG-VilER-Cre-Fzd5KO$^{-/-}$ (G) animal

(Fig.27F). This evidence suggests that epithelial cell intrinsic WNT mutations are selected in advanced stages of VG pathology.

Furthermore, RNAseq data was processed as described in Chapter 3 in order to obtain CBC and RSC GSVA scores, and calculate the Stem Cell Index for these lesions. When each SC population was evaluated independently, there was a clear increase in both CBC and RSC scores in Wnt-disrupted polyps versus their non-mutated counterparts (Fig.27G,H). However, despite a tendency for lesions with Wnt alterations to have lower Stem Cell Index values, this difference was not significant (Fig. 27I). This suggests that both SC populations may have been acquired at similar proportions in advanced VG polyps, with no competitive advantage of one phenotype over the other.

Finally, RNAseq processed counts from these samples were used to interrogate whether the patterns of expression of Frizzled receptors were altered upon acquisition of Wnt alterations. Whereas *Fzd5* was upregulated in histologically intermediate samples, advanced polyps lost expression of this transcript (Fig.28A). ISH for *Fzd5* and *Lgr5* in paraffin-embedded sections of advanced polyps revealed that the patterns

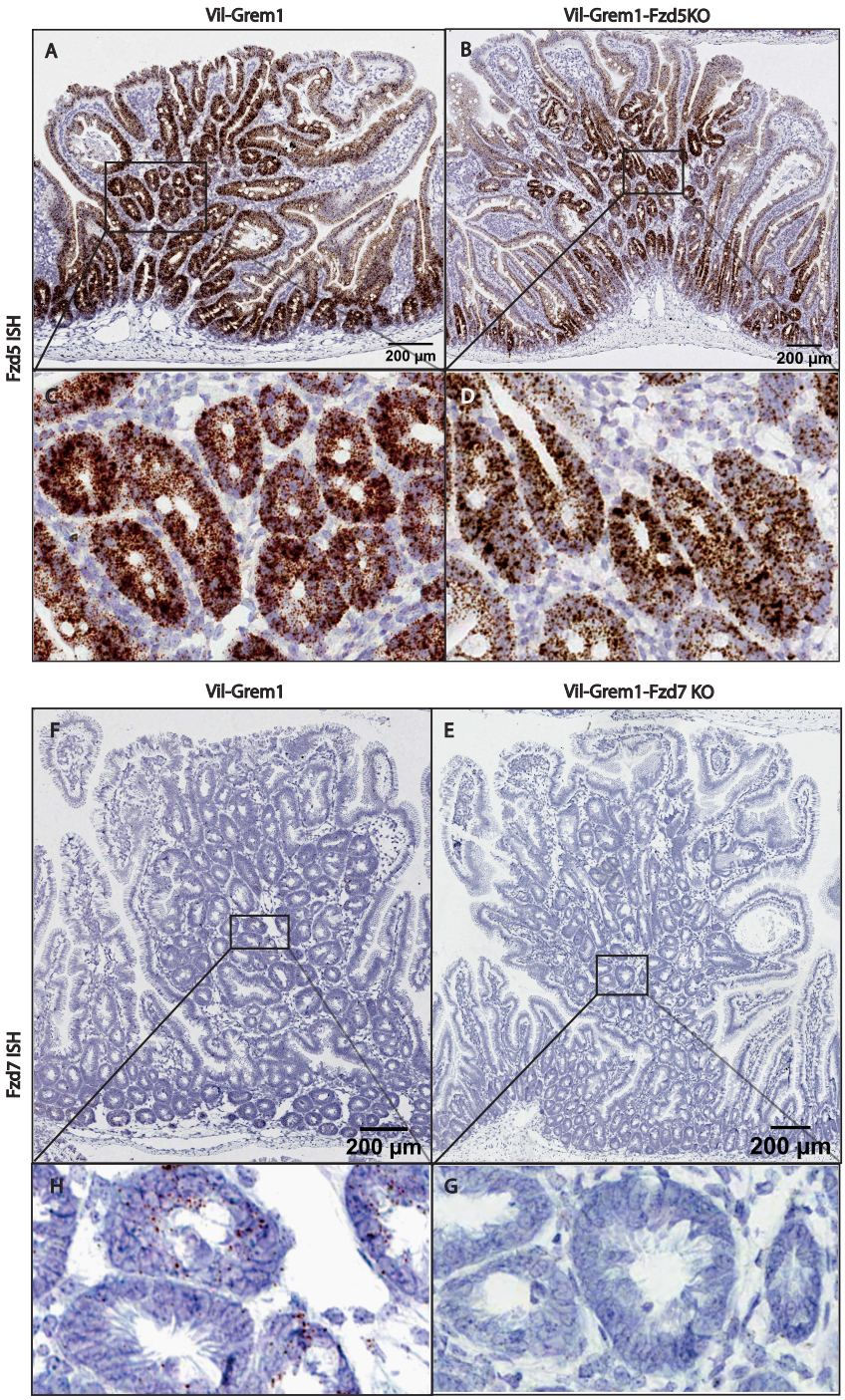

Figure 25: Levels of long-term *Fzd5* and *Fzd7* downregulation in VG-VilER-Cre-Fzd7KO$^{-/-}$ and VG-VilER-Cre-Fzd5KO$^{-/-}$ animals. A-D *In Situ Hybridization* of *Fzd5* in VG (A,C) and VG-VilER-Cre-Fzd5KO$^{-/-}$(B,D) samples. C,D. Close-ups of ectopic crypts. E-H *In Situ Hybridization* of *Fzd7* in Vil1-*GREM1* (E,G) and VG-VilER-Cre-Fzd7KO$^{-/-}$(F,H) samples. G,H. Close-ups of ectopic crypts.

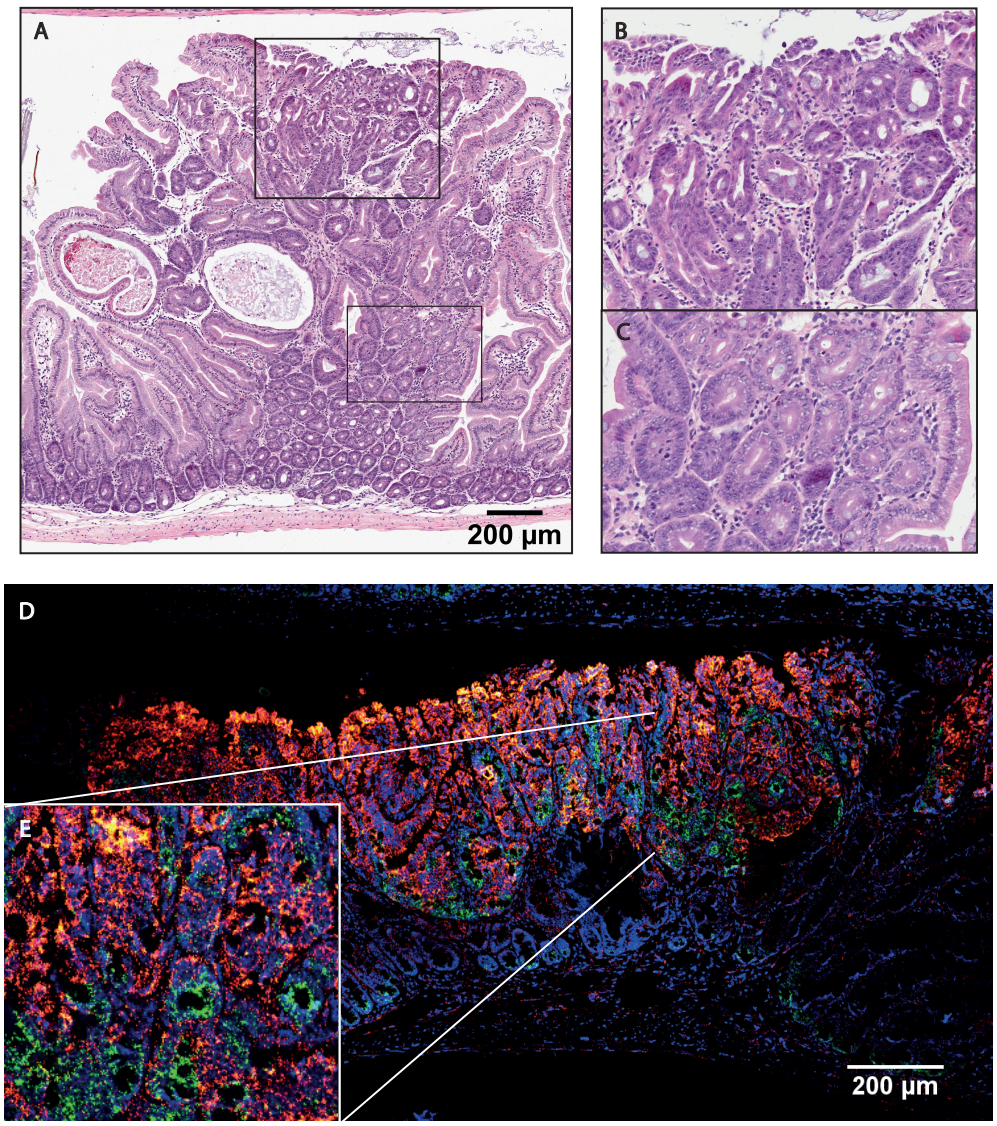

Figure 26: Advanced Vil1-*GREM1* polyps acquire expression of CBC and RSC markers. A-C H&E of an advanced VG polyp. D-E. *In Situ Hybridisation* for *Lgr5* (green) and *Anxa1* (red) in an advanced Vil1-*GREM1* polyp. B. Detail of ectopic crypts.

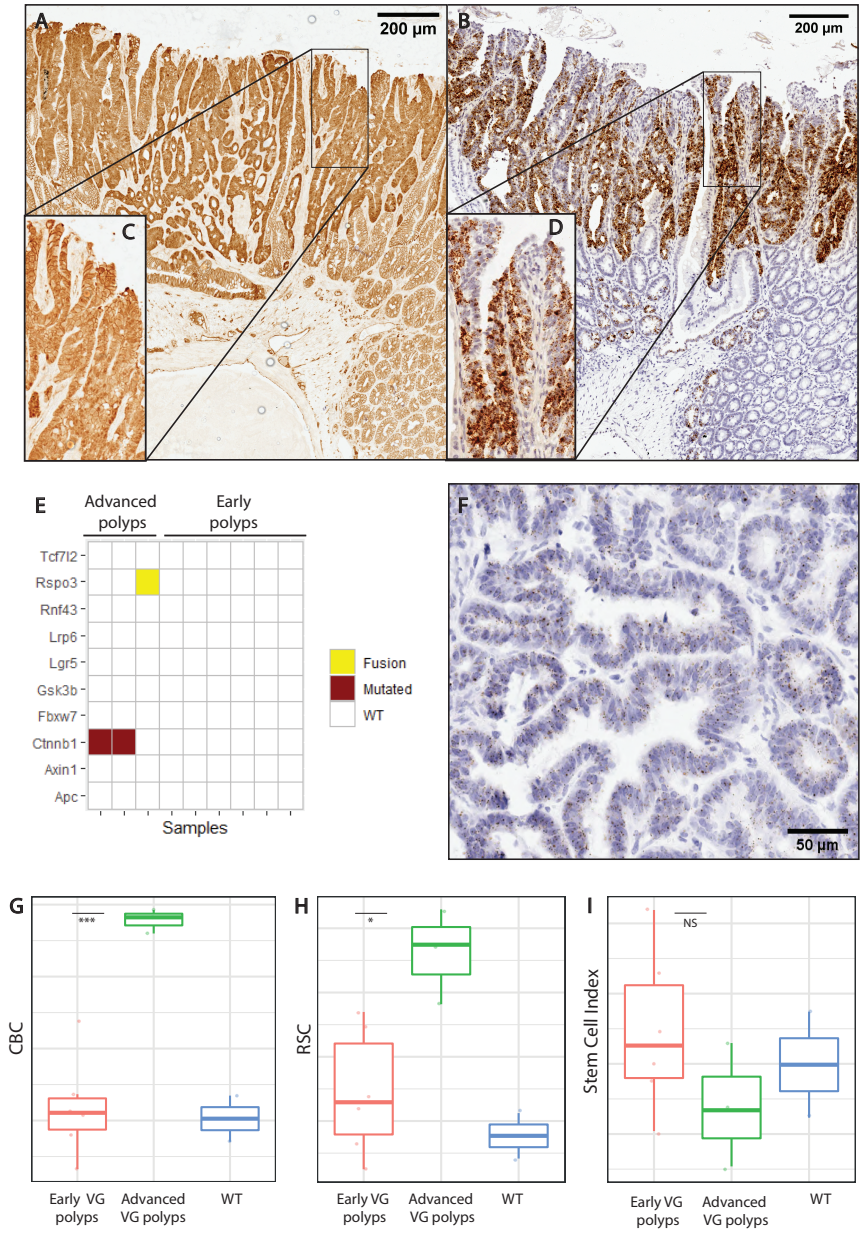

Figure 27: Advanced polyps acquire cell-intrinsic forms of Wnt activation. A. β-catenin immunohistochemistry. B *Lgr5* ISH. Details show areas of high Wnt activity (C,D). E. Diagram showing the mutation alterations found in intermediate and advanced polyps F. ISH for *Rspo3* in the ectopic crypts of a Vil1-*GREM1* polyp with a *Ptprk-Rspo3* fusion. G,H. GSVA scores of WT samples, intermediate and advanced Vil1-*GREM1* polyps for the CBC [7] (G) and RSC (H) signatures. I. Stem cell Index of WT samples, advanced and intermediate Vil1-*GREM1* polyps. The index was calculated by subtracting the CBC from the RSC score. Differences between advanced and intermediate Vil1-*GREM1* polyps were analysed using a t-test and Holm correction was applied. Asterisks indicate a p-value <0.05 (*), <0.01(**), <0.001 (***) or >0.05 (NS).

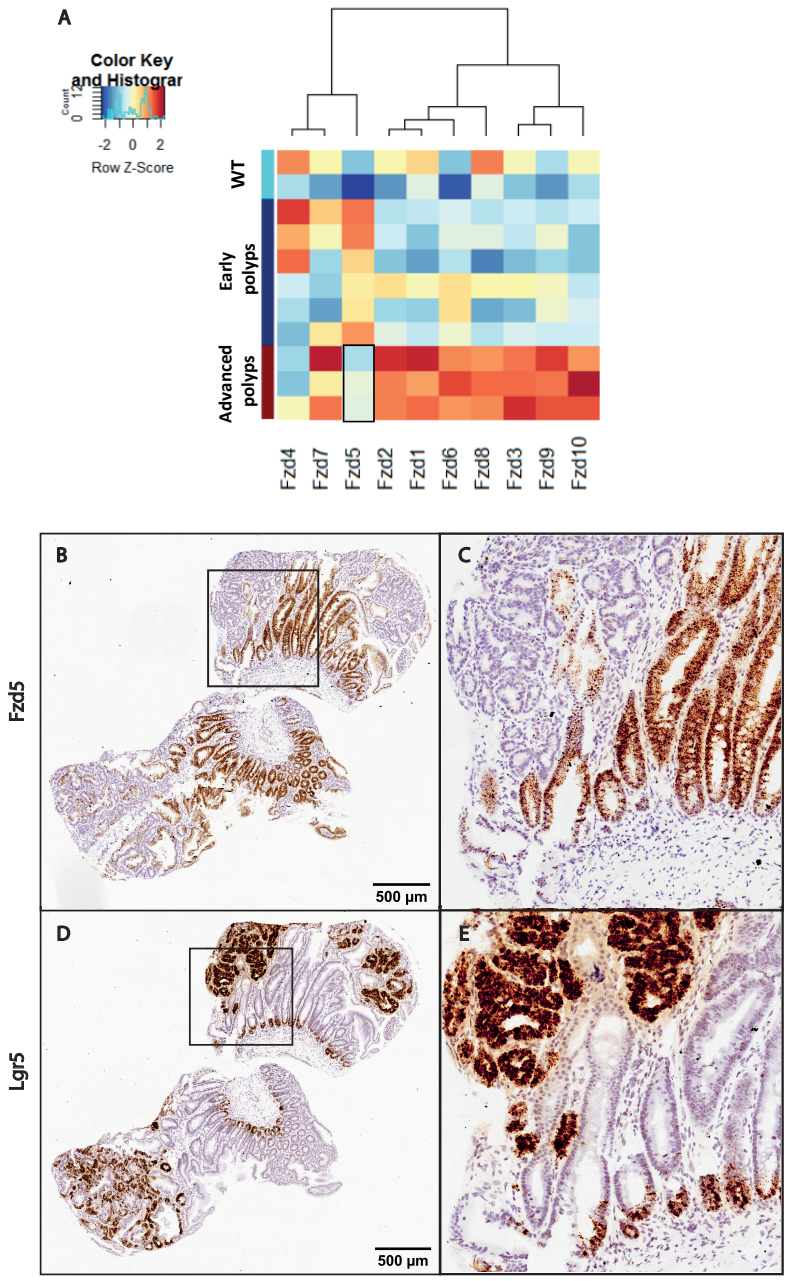

Figure 28: The expression patterns of *Lgr5* and *Fzd5* are inversely correlated in Vil-GREM1 polyps. A. Heatmap showing the log-transformed normalized counts for Frizzled receptors in WT villi, intermediate and advanced Vil1-*GREM1* polyps. B-E. *In Situ Hybridisation* of *Fzd5* and *Lgr5* in polyps of Vil-GREM1 animals. C,E. Close-up of the polyps.

of expression of these transcripts were mutually exclusive (Fig. 28B-D). Instead of Fzd5, logCPM counts of *Fzd1, Fzd3, Fzd6, Fzd7, Fzd8, Fzd9* and *Fzd10* were clearly

increased in advanced lesions (Fig.12A), indicating that any potential cell-extrinsic Wnt stimulation would be mediated by a different set of receptors in these samples.

4.12 Discussion

The HMPS is an hereditary form of CRC that is initiated by the aberrant epithelial expression of Gremlin-1 and the consequent disruption of the homeostatic gradients that control intestinal cell identity. These patients experience a form of mixed polyposis which is characterized by the appearance of ECF. Davis et al generated a mouse model which phenocopied this human condition by expressing the gene *GREM1* under the epithelial promoter *Villin*. However, the nature of the SC within the ECF of these animals and their WNT requirements remained unknown. Here, a combination of molecular biology and histopathologic techniques were used to investigate these open questions. Despite Gremlin-1 being a ISC niche factor and its important role in regeneration, neither *Lgr5* nor *Anxa1* were detected in early VG polyps. Furthermore, the CBC and RSC scores from these lesions were comparable to those from WT intestines. Collectively, this points towards an alternative progenitor/stem cell population taking the lead in early stages of VG pathogenesis. However, *Lgr5+* CBC and *Anxa1+* RSC were detected in advanced polyps, which reflects the changes in stem cell composition that lesions undergo as they progress and acquire driver gene mutations.

The mechanisms by which Gremlin-1 is mediating the polyposis observed in VG animals have not been fully elucidated, but evidence by us and others suggest that a variety of factors may be involved in this process. The most straight-forward effect of *GREM1* overexpression in the intestinal epithelium is a net decrease in differentiation cues, which has a direct effect in epithelia cell identity. However, several lines of evidence suggest that activation of fibroblastic populations, as an indirect consequence of the disrupted BMP cross-talk, may also be contributing to tissue transformation. In the stomach, deletion of the *Bmp receptor 1a* (*Bmpr1a*) in subepithelial fibroblasts results in stromal activation, which drives the formation of gastric tumours within 90 days [238]. In the intestine, deletion of Bmpr1a specifically in the epithelial compartment is insufficient to drive tumorigenesis and ectopic crypt formation[239]. However, specific deletion of this gene in Foxl1+ subepithelial myofibroblasts drives stromal activation, hyperplasia within 90 days, and the formation of colon polyps at 1 year of age[240], which suggests that dysregulation of the fibroblast compartment may be a key element in CRC models driven by BMP disruption.

One of the mechanisms by which stromal BMP inhibition may be contributing to tissue transformation is the altered production of morphogens. Indeed, VG villi experience an upregulation of stromal sources of WNT, specifically *Wnt2b* and *Wnt5a*. These ligands were co-expressed with the fibroblast markers *Foxl1* and *Gli* respectively, suggesting that GREM1 upregulation caused the activation of these stromal populations and the consequent ectopic expression of Wnt ligands. Importantly, Wnt5a has been shown to drive the formation of new crypts, a processed known as crypt fission, in settings of tissue damage[55], which suggests that this may be one of the key factors promoting the appearance of ECF in VG. Allaire et al. found increased levels of Wnt5a in the colon of animals harbouring *Bmpr1a* stromal mutations, but they did not report any increase in canonical Wnt ligands, unlike what was observed in VG animals. Given that the phenotype of VG is most pronounced in the small bowel[132], it is possible that differences between the small bowel and colon partially account for the observed differences.

Furthermore, changes in the folding of the tissue may also alter the homeostatic functional compartmentalization of the intestine. Work by Shyer et al showed how changes in the villus architecture alter epithelial-stromal interaction during intestinal development, which ultimately determines cell identity and the structure of the crypt-villus units[241]. As they beautifully demonstrated, the formation of the villi modifies the interactions between the epithelial and stromal compartments, which causes SC to be relegated to the base of the crypts. The key element that drives these changes is the bigger surface-area-to-volume ratio found at the tips of the villi, which causes an accumulation of Bmp ligands and drives epithelial differentiation. Importantly, changes in the intestinal architecture where observed in the intestines of VG animals, which presented with enlarged villi. Hence, the reduced number of epithelial cells that interact with the underlying stroma in VG villi may alter the epithelial-stromal interactions and favour the acquisition of stem cell properties.

Overall, this data seems to indicate that Bmp inhibition not only directly alters the epithelium and stroma of VG animals, but it also disturbs the interactions normally established between these two compartments. Whether there are key cues that drive ECF formation or they arise as a result of the abnormal folding of the tissue is a question that remains to be answered.

Importantly, treatment of VG animals with a Porcupine inhibitor was enough to prevent the development of polyps and ECF in early lesions, thus proving the importance of environmental sources of Wnt in Gremlin-1 driven pathology. However, the intestines of these animals retained the enlarged villi structures suggestive of a

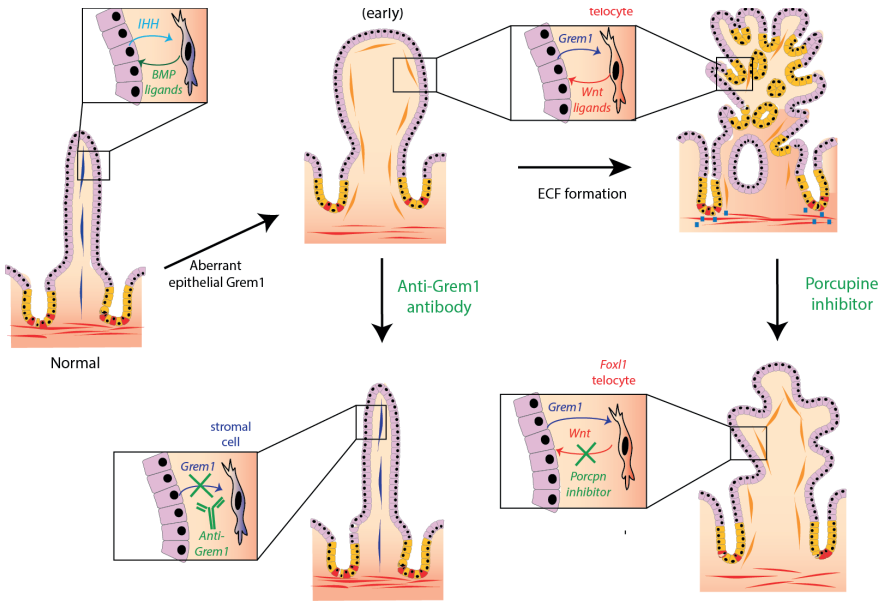

Figure 29: Proposed model of the Vil1-*GREM1* pathogenesis. In this hypothesis, Gremlin-1 driven transformation in Vil1-*GREM1* animals occurs through the stromal remodelling of the villi compartment and the consequence expression of Wnt ligands in ectopic regions. This causes the generation of stem cell niches outside of the base of the crypts, which leads to the formation of ectopic crypts. Treatment with a blocking GREM1 antibody completely reverts this transformation, whereas Porcupine inhibition prevents the development of ectopic crypts and polyps, but can not stop the stromal remodelling initiated by Gremlin-1 upregulation.

reactive stromal compartment. Transcriptomic analysis of VG lesions revealed an aberrant upregulation of *Fzd5* in early VG lesions. However, the low recombination efficiency of our VilER-Cre-Fzd5KO$^{-/-}$ model added to the rapid expansion of escaper crypts, rendered the experiments using this model uninformative. Consequently, the role of Frizzled5 in Gremlin-1 driven pathogenesis could not be confirmed. All in all, the fact that every ECF in VG-VilER-Cre-Fzd5KO$^{-/-}$ animals was completely repopulated by *Fzd5+* cells suggests a competitive advantage for the cells that escaped recombination. Further experiments will be needed to confirm this hypothesis.

WNT dysregulation is a common phenomena in CRC[138], and it can act as a tumour-initiating event [136, 137]. A detailed genomic and transcriptomic analysis of VG polyps revealed that, despite most of them relying on cell extrinsic Wnt ligands to drive ECF proliferation, some late lesions acquire cell-intrinsic forms of Wnt activation (eg. *Ctnnb1* mutations, *Ptprk-Rspo3* fusions), generating epithelial cell Wnt autonomy and reducing the reliance on a micro-environmental ligand sources. Hence, although the activation of a Wnt-producing, reactive stroma may render cell-

intrinsic Wnt-disrupting events unnecessary for initiating Bmp-driven transformation, this traits may be playing a part in the progression of VG polyps. Interestingly, acquisition of Wnt alterations came with a profound upregulation of the R-spondin receptor *Lgr5*, and a equally pronounced reduction on *Fzd5*, suggestive of a different dependence/sensitivity of advanced lesions on/to stromal Wnt/Rspo ligands. Further experiments will be needed to assess the sequence of events that fuel the progression of Gremlin-1 initiated lesions.

Importantly, treatment with a Gremlin-1 blocking antibody completely reverted the VG phenotype in early lesions (42 days). However, application of a late regime (day 150) did not completely eliminate the polyps and ECF phenotype observed in older animals. This suggest that most VG lesions remain dependent on Gremlin-1 dysregulation, but some advanced polyps acquire mechanisms, like cell-intrinsic Wnt-activating mutations, that render them independent of Gremlin-1. Based on this data, the Gremlin-1 blocking antibody could be a useful tool to prevent CRC initiation in HMPS patients, but it may not be an efficient therapeutic by itself once an advanced polyp has been established. The use of combinatorial therapy including a Gremlin-1 blocking antibody is an strategy worth exploring not only in HMPS patients, but also in CMS4 CRC, which present stromal upregulation of GREMLIN-1[132].

Based on the above evidence, a model of VG pathogenesis was constructed, which is depicted in Fig.29. According to this model, aberrant Gremlin-1 expression induces a stromal transformation, with activation of fibroblast population which generate ectopic Wnt ligands. This enables the establishment of intestinal niches in the villi compartment, which synergize with Bmp epithelial inhibition to drive the generation of ECF. Gremlin-1 inhibition completely reverts this process, whereas Porcupine inhibition can prevent the formation of polyps and ECF, but not the stromal remodelling induced by Gremlin-1. The process by which Gremlin-1 may be exerting its effects on *Foxl1+* and *Gli1+* fibroblasts as well as other stromal populations remains to be determined.