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Identification of novel human Wnt target genes using adult endodermal tissue-derived organoids

Kim Elisabeth Boonekamp [a,1], Inha Heo [a,1], Benedetta Artegiani [a], Priyanca Asra [a], Gijs van Son [a], Joep de Ligt [b], Hans Clevers [a,c,*]

[a] Oncode Institute, Hubrecht Institute, Royal Netherlands Academy of Arts and Sciences (KNAW) and University Medical Centre (UMC) Utrecht, Utrecht, the Netherlands
[b] University Medical Centre (UMC) Utrecht, Utrecht, the Netherlands
[c] Princess Máxima Centre for Paediatric Oncology, Utrecht, the Netherlands

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ABSTRACT

Canonical Wnt signaling plays a key role during organ development, homeostasis and regeneration and these processes are conserved between invertebrates and vertebrates. Mutations in Wnt pathway components are commonly found in various types of cancer. Upon activation of canonical Wnt signaling, β-catenin binds in the nucleus to members of the TCF-LEF family and activates the transcription of target genes. Multiple Wnt target genes, including Lgr5/LGR5 and Axin2/AXIN2, have been identified in mouse models and human cancer cell lines. Here we set out to identify the transcriptional targets of Wnt signaling in five human tissues using organoid technology. Organoids are derived from adult stem cells and recapitulate the functionality as well as the structure of the original tissue. Since the Wnt pathway is critical to maintain the organoids from the human intestine, colon, liver, pancreas and stomach, organoid technology allows us to assess Wnt target gene expression in a human wildtype situation. We performed bulk mRNA sequencing of organoids immediately after inhibition of Wnt pathway and identified 41 genes as commonly regulated genes in these tissues. We also identified large numbers of target genes specific to each tissue. One of the shared target genes is TEAD4, a transcription factor driving expression of YAP/TAZ signaling target genes. In addition to TEAD4, we identified a variety of genes which encode for proteins that are involved in Wnt-independent pathways, implicating the possibility of direct crosstalk between Wnt signaling and other pathways. Collectively, this study identified tissue-specific and common Wnt target gene signatures and provides evidence for a conserved role for these Wnt targets in different tissues.

1. Introduction

Wnt signaling constitutes an evolutionary highly conserved signaling pathway, shaping the body plan of all metazoans (Loh et al., 2016). Additionally, Wnt protein-mediated signals contribute to the development, homeostasis and regeneration of many epithelial tissues in adult organisms. Moreover, as a consequence of their essential role in growth regulation, mutations in genes encoding components of the Wnt pathway can directly lead to malignancy (Nusse and Clevers, 2017).

Canonical Wnt signaling can be initiated by a wide range of Wnt proteins, engaging any of the ten Frizzled receptors in conjunction with co-receptors Lrp5 or Lrp6. The pleiotropic downstream transcriptional effects of these interactions have been the subject of many studies. The central player of canonical Wnt signaling is the transcriptional co-activator β-catenin. In the absence of Wnt ligands, newly produced β-catenin is degraded by a cytosolic destruction complex (DC), ensuring low levels of the co-activator. This DC is composed of the proteins Axin-1 and Apc, and includes the kinases CK1 and GSK3β. Phosphorylated β-catenin becomes a target for βTrcP ubiquitin-dependent degradation. Binding of Wnt ligands to Frizzled/LRP5/6 receptor combinations on the plasma membrane ultimately leads to sequestration and silencing of the DC (Liet al., 2012). Subsequently, the increased concentration of β-catenin enables its nuclear import, and subsequent binding to TCF/LEF transcription factors where it acts as a transcriptional co-activator (Molenaaret al., 1996). The interplay between TCF/LEF and β-catenin allows the transcriptional activation of different sets of Wnt target genes,

* Corresponding author. Oncode Institute, Hubrecht Institute, Royal Netherlands Academy of Arts and Sciences (KNAW) and University Medical Centre (UMC) Utrecht, Utrecht, the Netherlands.
E-mail address: h.clevers@hubrecht.eu (H. Clevers).
[1] Equal contribution.

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which are strongly dominated by the context of the resident tissue.

Over the past decades, many studies have focused on the role and the identification of Wnt target genes in the epithelium of the small intestine and colon using cancer cell lines and mouse models. Inducible expression of a dominant negative version of Wnt effector transcription factor TCF4 in colon carcinoma cell line LS174T resulted in the identification of several Wnt target genes including LGR5 (van de Weteringet al., 2002). The importance of TCF4 driven transcription became more evident by Tcf4 deletion studies in mice where intestine-specific driven deletion of Tcf4 was not compatible with life due to severe defects in the intestine (van Eset al., 2012; Korineket al., 1998). During adult intestinal homeostasis, ectopic expression of Wnt inhibitor Dickkopf1 (Dkk1) in the epithelium resulted in loss of the stem cell compartment (Pinto et al., 2003). DNA microarray experiments in colorectal cancer cell lines SW480 and DLD1 in the background of β-catenin silencing combined with published data from other colorectal cancer cell lines has resulted in a 53 gene target signature in the context of deregulated Wnt/β-catenin signaling (Herbstet al., 2014). Identification of TCF4/β-catenin target genes by assessment of chromatin immunoprecipitation (ChIP) peaks for TCF4 and β-catenin in the genome provided insights in the molecular effectors expressed upon Wnt pathway activation (Schuijers et al., 2014; Watanabeet al., 2014; Hatziset al., 2008; Doumpaset al., 2019). Together experiments in genetically-engineered mice and human cell lines have expanded our understanding of the importance of TCF4/β-catenin driven target genes.

Amongst the identified target genes were AXIN2, RNF43 and ZNRF3, these genes encode for proteins that function in intrinsic negative feedback mechanisms to control the Wnt pathway (Bernkopf et al., 2015; Kooet al., 2012). LGR5 positively regulated the pathway by binding to R-spondin ligands, facilitating downregulation of RNF43 and ZRNF3. Wnt target genes and specifically Lgr5 were found to be highly expressed in cells in the crypts of the small intestine and colon. Genetic lineage tracing experiments from the Lgr5 locus subsequently showed that Lgr5 exclusively marks stem cells that contribute to tissue homeostasis in the small intestine and colon (Barkeret al., 2007), as well as in multiple other epithelial tissues (Jakset al., 2008; Barkeret al., 2010). The availability of a Lgr5-driven GFP mouse model enabled the identification of the complete Wnt target program in murine intestinal stem cells (Munozet al., 2012).

Expansion of $Lgr5^{+}$ cells in 3-dimensional culture conditions allowed for further insights into the signals responsible for the regulation of stem cells versus the induction of differentiated cell types (Satoet al., 2009). This organoid technology allows for *in vitro* expansion of primary cells in 3-dimensional structures that mimic the original tissue and cell type heterogeneity. Modulation of the signalling components of various signaling pathways - including NOTCH, MAPK, BMP and Wnt - facilitates the outgrowth of almost any adult tissue (Kretzschmar and Clevers, 2016). Under basic conditions human tissue derived organoids consist mainly of stem cells and therefore provide a good basis to study stem cell maintenance and regeneration.

Altogether, knowledge from in vivo lineage tracing experiments and organoid generation have shown that Wnt signaling is essential for stem cell maintenance and differentiation in multiple tissues such as the small intestine, colon, liver, pancreas and stomach (Barkeret al., 2007; Barkeret al., 2010; Satoet al., 2009; Huchet al., 2013a; Huchet al., 2013b). However, it is still largely unclear whether there are common Wnt target genes important in stem cell biology in various tissues. Human adult tissue derived organoids provide an optimal cellular model to study Wnt signaling *in vitro* in a wildtype setting compared to the use of cancer cell lines or genetically-engineered mice. In this study, we modulate the Wnt pathway in organoids derived from human adult tissues of endodermal origin, such as the small intestine, liver, colon and pancreas, and perform transcriptional analysis to identify tissue-specific and common Wnt target gene signatures.

2. Results

2.1. The human Wnt signature across different organs using organoids

In order to identify Wnt target genes that are potentially important for stem cell maintenance in humans, we exploited organoid technology. We first assessed responsiveness of small intestinal organoids to blocking canonical Wnt signaling. We therefore generated a human intestinal organoid line stably expressing a Wnt reporter construct using lentiviral transduction. In this reporter system, organoids express luciferase under the control of TCF/LEF transcription factor binding elements (Korineket al., 1997; Fuerer and Nusse, 2010). We next monitored changes in TCF/β-catenin driven transcription by performing luciferase assays after incubating the organoids with two different media (Loh et al., 2016) Standard Wnt activating expansion medium or (Nusse and Clevers, 2017) Wnt inhibition medium (withdrawal of Wnt3A and R-spondin-1; addition of the Porcupine inhibitor IWP2 and Wnt antagonist DKK1). Organoids were grown for three days in expansion media and subsequently medium was changed to either expansion medium or Wnt inhibition medium. Luciferase activity was recorded for 24 h following medium change (Fig. 1A). In standard expansion medium, Wnt pathway activity remained fairly stable, whilst Wnt reporter activity decreased significantly over the time course of 24 h incubation with Wnt inhibitory medium. At 6 h post Wnt withdrawal, pathway activity decreased with more than 50% and the signal was almost zero after 24 h, suggesting that Wnt inhibition medium efficiently blocked the TCF/β-catenin driven transcription in small intestinal organoids.

Based on the luciferase experiment, we proceeded with 6 h and 24 h incubation with Wnt inhibitory medium for further experiments. Given the hypothesis that Wnt target genes are highly conserved in tissues from the same embryonic origin (endoderm), we used organoids derived from adult human small intestine, colon, liver, pancreas and stomach (Barker, 2010; Huch, 2013b, 2015, Sato, 2009, 2011). After splitting we let the organoids ($n = 3$ lines per tissue) grow for 3 days and changed the medium to Wnt inhibitory medium and harvested RNA from the organoids at baseline ($t = 0$), after 6 h ($t = 6$) and after 24 h ($t = 24$) (Fig. 1B). We performed 1×75 bp ribo-zero bulk RNA sequencing to study temporal Wnt target gene dynamics in the different tissues. Hierarchical clustering and principal component analysis revealed striking differences among the organoids derived from different tissues (Fig. 1C and S1A). We performed pairwise analysis using the DESeq2 package to prevent tissue or donor specific batch effects (Love et al., 2014). We observed that well-known Wnt target genes LGR5, AXIN2, RNF43, ZNRF3 were downregulated upon Wnt pathway inhibition. The log 2 fold change (L2FC) of these genes was small at 6 h and increased at 24 h. Furthermore, we observed clear differences in transcriptional dynamics between LGR5 and AXIN2 compared to RNF43 and ZNRF3 (Fig. S1B). To capture both medium and strongly regulated Wnt target genes, we therefore determined a cut-off of L2FC 0.4 at 6 h and L2FC 1 at 24 h to ensure that differential gene analysis was sensitive enough to discover Wnt targets with different transcriptional dynamics. To better understand tissue specific expression patterns of known Wnt targets, we assessed the expression of LGR5, AXIN2, RNF43 and ZNRF3 over time in all tissues (Fig. 1D). This data emphasized tissue and gene-specific differences between well-established Wnt target genes. Altogether we showed that organoids can be used to address Wnt target gene dynamics over time and that different tissues and genes react differently to Wnt pathway inhibition.

2.2. Common Wnt target genes in small intestine, colon, liver, pancreas and stomach

To characterize a potential common Wnt target gene signature in different tissues, we first compared all significantly downregulated genes

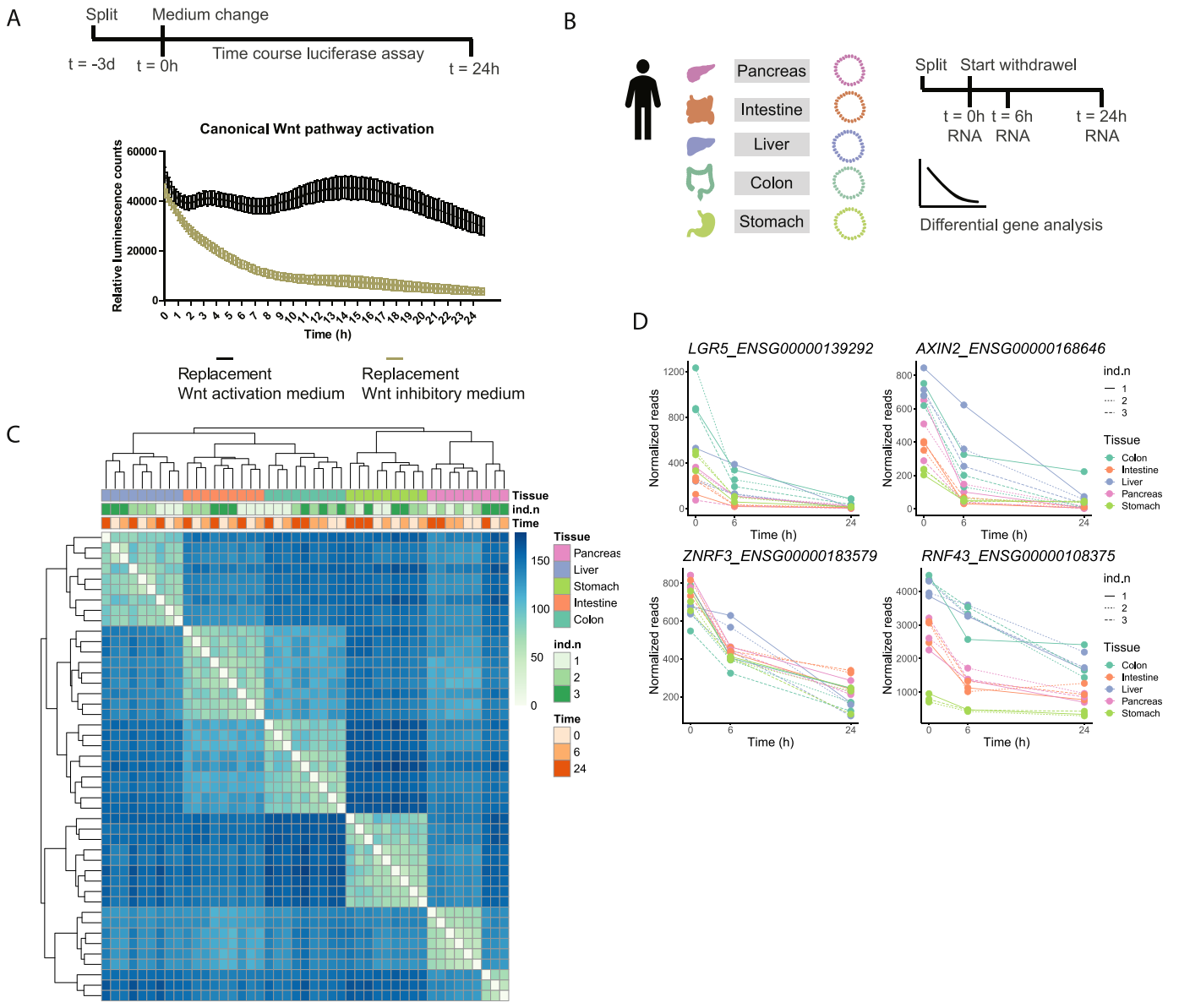

Fig. 1. Identification of novel Wnt target genes in stem cells by transcriptional analysis. (A) Organoids were split and grown in Wnt activating expansion medium for 3 days. Medium was changed to Wnt activating medium or Wnt inhibitory medium and luciferase activity was measured over the time course of 24 h. (B) Organoids were grown from primary human tissue, either pancreas, small Intestine, liver, colon and stomach, in the presence of Wnt stimulatory signals. Post splitting, organoids were grown for 3 days in medium stimulating the Wnt pathway. RNA was collected at time point 0 h and timepoint 6 h and 24 h post addition of Wnt inhibitory medium. mRNA was submitted for bulk mRNA sequencing. (C) Correlation plot representing the distance between the sequenced samples. (D) Normalized reads plotted over time for known Wnt target genes LGR5, AXIN2, RNF43 and SP5. Each color represents an organ. Dots represent individual datapoints, dotted line represents the matched data points from each donor.

at 6 h (padj < 0.01 and L2FC < 0.4) in all tested organoids (Fig. S2A). We only found two genes Pre-MRNA Processing Factor 40 Homolog B (PRPF40B) (Fig. S2B) and Transmembrane O-Mannosyltransferase Targeting Cadherins 4 (TMTC4) as significantly downregulated genes at 6 h in all 5 different organoids (Fig. S2C). Significant downregulation of PRPF40B and TMTC4 in all tissues was not maintained at 24 h indicating that these genes do not represent potential Wnt target genes. Furthermore, these genes encode for proteins involved in pre-mRNA processing and protein folding and their downregulation might be the consequence of overall decrease in transcription. These data suggested that organoids responded in a tissue dependent manner early upon Wnt pathway inhibition. We then analyzed the number of significantly differentially expressed genes per tissue at 6 h (colored) and 24 h (grey); there we found clear differences in the number of differentially expressed genes for each different tissue derived organoid type at 6 h. At 24 h the number of significantly differentially expressed genes was approximately equal for each tissue (Fig. S2F). These data show that small intestinal and colon organoids show a similar transcriptional response upon Wnt pathway inhibition compared to liver, pancreas and stomach. Based on the differences in number of differentially expressed genes between the different tissues, we reasoned that the dynamics of downregulation is strongly tissue dependent and that the 24-h timepoint allows for a better comparison.

In order to enrich for genes that were consistently downregulated upon Wnt pathway inhibition, we excluded genes that were significantly upregulated at 6 h (exclusion criteria: padj <0.01, L2FC > 0). Subsequently, we selected genes that were at least 2-fold significantly downregulated at 24 h (inclusion criteria: padj <0.01, L2FC < -1). Aiming to compare Wnt regulated genes between the different tissues we maintained these parameters over the complete analysis. As the result of these

two filtering steps, we obtained a list for each tissue highlighting genes that are significantly downregulated by Wnt inhibition. Comparison of these gene lists revealed genes that are either tissue specific, only overlap between 2, 3 or 4 organs or are regulated by Wnt in all 5 organs (Fig. 2A, S2D, S2E). At 24 h post Wnt withdrawal we observed 41 commonly downregulated genes (Table 1). Plotting the mean L2FC and standard error of the mean (SEM) for all differentially downregulated genes showed clear differences in transcriptional dynamics between the different tissues with regard to amplitude upon gene expression changes (Fig. 2B). Well-known Wnt target genes RNF43, ZNRF3, AXIN2, LGR5, SP5, EPHB3 and TFAP4 were also in the commonly regulated genes (Table 1). The novel genes in 41 commonly regulated genes showed similar changes in L2FC over time in each tissue compared to the known Wnt targets, suggesting that the novel genes would be potential Wnt targets (Fig. 2C, S2G). Plotting z-score gene expression in a heatmap confirmed lower gene expression at the 24 h time point for the commonly downregulated genes in all tissues (Fig. S2G). Additionally, we observed that the 0 h and 6 h timepoints do not cluster in contrast to the 24 h timepoint, indicative of a more robust read-out at 24 h post Wnt inhibition. Whereas we observed significant downregulation of known Wnt target genes in common between the different tissue-derived organoids at the 24 h timepoint, validating our approach.

Amongst the 41 commonly Wnt regulated genes were CDCA4 (Fig. 3A) and TEAD4 (Fig. 3B). Cell Division Cycle Associated 4 (CDCA4) encodes for a protein belonging to the E2F family of transcription factors and regulated E2F-dependent transcriptional activity and cell proliferation. Similarly, TEA Domain Transcription Factor 4 (TEAD4) is consistently downregulated over time in all organs (Fig. 3B). Transcription factors of the TEA domain/Transcription Enhancer Factor (TEAD) family bind to YAP/TAZ factors of the hippo pathway and induce translocation to the nucleus and activation of YAP/TAZ/TEAD target genes. These two target genes regulate gene expression of other genes, thereby potentially providing secondary target gene expression upon canonical Wnt pathway activation. In addition, we identified genes involved in DNA replication such as GINS Complex Subunit 3 (GINS3) and Replication Factor C Subunit 4 (RFC4). Together the list of commonly regulated Wnt target genes are implicated in various processes including DNA replication and induction of secondary target gene expression.

Table 1
Commonly Wnt regulated genes.

ENSEMBL	SYMBOL
ENSG00000003989	SLC7A2
ENSG00000034063	UHRF1
ENSG00000073111	MCM2
ENSG00000081059	TCF7
ENSG00000089351	GRAMD1A
ENSG00000090447	TFAP4
ENSG00000100350	FOXRED2
ENSG00000107719	PALD1
ENSG00000108375	RNF43
ENSG00000112394	SLC16A10
ENSG00000115318	LOXL3
ENSG00000117877	CD3EAP
ENSG00000125257	ABCC4
ENSG00000125378	BMP4
ENSG00000131153	GINS2
ENSG00000132688	NES
ENSG00000135423	GLS2
ENSG00000138316	ADAMTS14
ENSG00000138587	MNS1
ENSG00000139292	LGR5
ENSG00000143476	DTL
ENSG00000163918	RFC4
ENSG00000165244	ZNF367
ENSG00000165376	CLDN2
ENSG00000165905	LARGE2
ENSG00000167513	CDT1
ENSG00000168077	SCARA3
ENSG00000168646	AXIN2
ENSG00000170779	CDCA4
ENSG00000173894	CBX2
ENSG00000175928	LRRN1
ENSG00000181938	GINS3
ENSG00000182580	EPHB3
ENSG00000183579	ZNRF3
ENSG00000189057	FAM111B
ENSG00000196081	ZNF724
ENSG00000197905	TEAD4
ENSG00000198720	ANKRD13B
ENSG00000204335	SP5
ENSG00000204856	FAM216A
ENSG00000232445	EMSLR

2.3. Tissue specific Wnt target genes

Apart from the common Wnt target gene signature that we identified between colon, small intestine, liver, pancreas and stomach, we identified tissue-specific target gene signatures. Intestine and colon are epithelial organs in which the role of Wnt signaling has most clearly been established. The intestinal and colonic niche provide stimulatory Wnt signals to stem cells marked by LGR5 expression (Farin et al., 2012; Satoet al., 2013; Mooret al., 2016; Degirmenci et al., 2018). Activating mutations in the Wnt pathway are very frequent in colorectal cancer (Nusse and Clevers, 2017; Fearon and Vogelstein, 1990). Differential gene expression analysis in colon revealed 1031 downregulated genes and 562 upregulated genes (padj < 0.01 and L2FC < -0.4) upon 6 h of Wnt withdrawal (Fig. 4A) and 587 downregulated genes and 334 upregulated genes (padj < 0.01 and L2FC > 1) upon 24 h post withdrawal (Fig. 4B). Differential gene expression analysis in small intestinal organoids comparing 6 h of Wnt withdrawal with baseline expression showed similar numbers, namely 1326 downregulated genes and 436 upregulated genes (Fig. 4C). At 24 h post withdrawal the number of significantly downregulated genes is 1342 and 378 genes were upregulated (Fig. 4D). These results imply a large responsiveness of small intestinal and colonic human organoids to Wnt pathway inhibition. In order to look at specific genes in either colon or small intestine, we subjected the tissue specific target gene list in Fig. 2A to a subsequent filtering step and selected genes based on significantly distinct expression by performing t-tests on the mean normalized reads at $t = 0$. We identified small numbers of genes that are both Wnt regulated as well as show a distinct basal expression level compared to other tissues (Fig. 4E). Based on this analysis we have identified 11 genes including TRAF6 and PTCH1 as colon specific Wnt targets (Fig. 4F and G, Supplementary Fig. 3, Table 2). Similarly, we identified 2 genes as small intestinal specific genes TBLX1 and ARHGAP24 (Fig. 4H and I, Table 3). 85 genes are regulated in both the colon and small intestine (Fig. 2A). Subjection of this list to a filtering method where we enrich for genes that are significantly differentially expressed in either colon or small intestine or both compared to liver, pancreas and stomach this resulted in the identification of genes with expression patterns specific to the intestine and colon such as EPHB2 (Fig. 4J).

Similar analysis was performed on the data generated from the other digestive system derived organoids. Wnt signaling does not only play roles in tissue homeostasis of rapidly renewing tissues such as intestine and colon but also functions in liver zonation. $Axin2^{+}$ cells in the central vein area showed to be involved in liver homeostasis (Benhamoucheet al., 2006; Wang et al., 2015). However, the role of Wnt signaling in liver ductal cells is largely unknown. Therefore, we focused on bile duct liver organoids. Differential gene analysis in ductal liver organoids showed 65 downregulated genes and 25 upregulated genes at 6 h post Wnt withdrawal (Fig. 5A). After 24 h of Wnt withdrawal, more genes were changed namely 635 decreased and 548 increased (Fig. 5B). Similar to the small intestinal and colon organoids LGR5 was amongst the strongest downregulated genes. We identified FSTL4 (Fig. 5C) and 5 other genes as liver specific Wnt targets (Fig. S4A and Table 4).

Inactivating mutations in RNF43, which is a Wnt target gene and a negative regulator of the pathway itself, are found in pancreatic ductal

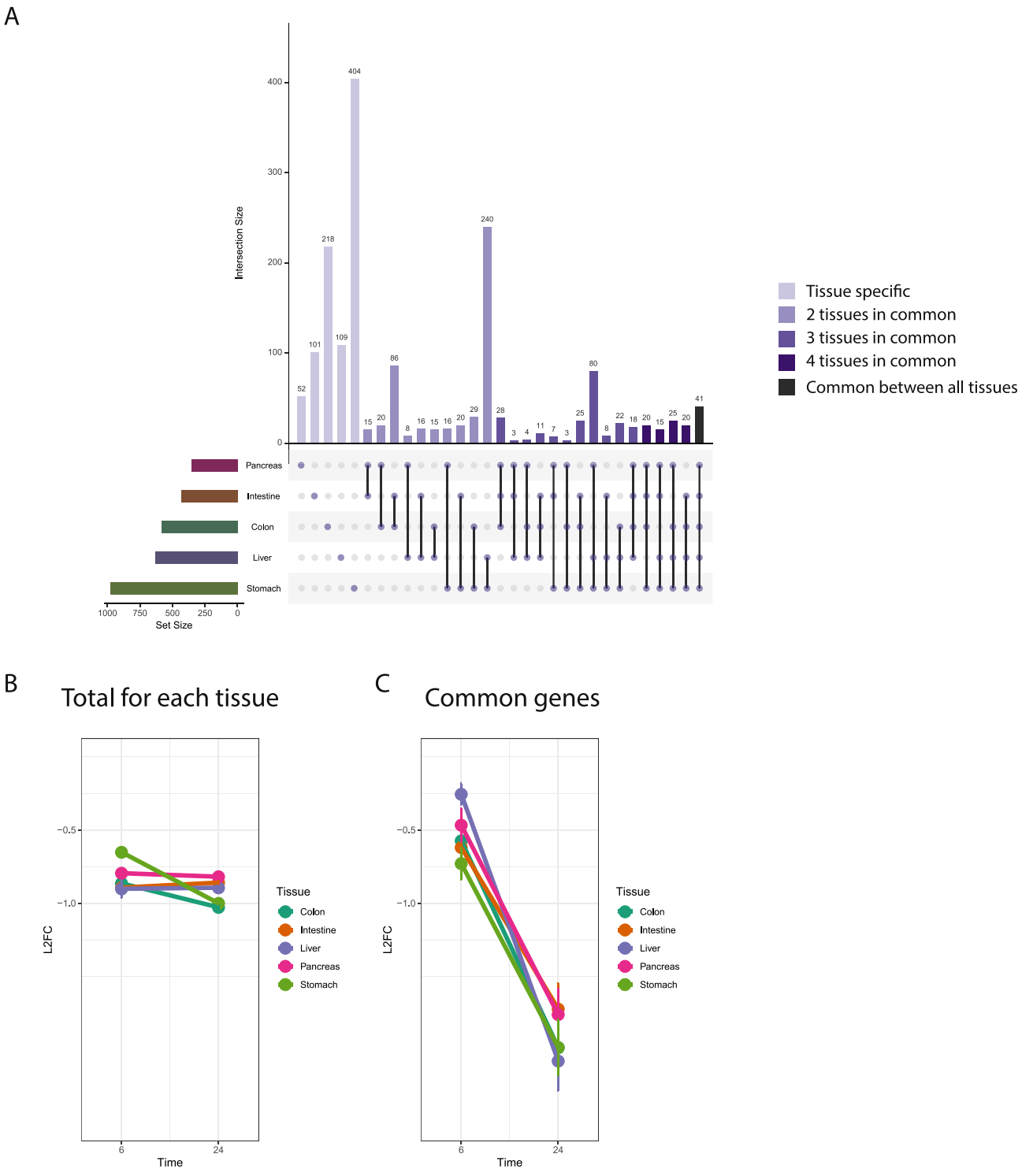

Fig. 2. Common Wnt target gene signature in Intestine, Colon, Liver, Pancreas and Stomach. (A) UpSet plot representing overlap in differentially downregulated genes LFC < -1, padj <0.01 at 24 h post Wnt withdrawal and not significantly upregulated at 6 h. (B) Average L2FC per tissue over time for all downregulated genes per tissue. Error bars represent s.e.m. (C) Average L2FC per tissue for the 41 common genes over time for all downregulated genes per tissue. Error bars represent s.e.m.

adenocarcinoma. This data provides supporting evidence for the importance of Wnt signaling in pancreatic ductal homeostasis. Although the pancreas has a limited structural hierarchy compared to the intestine and colon, researchers were still able to model the pancreatic ductal stem cell niche *in vitro* (Loomanset al., 2018; Bojet al., 2015). Differential gene analysis in pancreatic organoids showed 222 downregulated genes and 216 upregulated genes upon 6 h of Wnt withdrawal (Fig. 5D). The number of differentially expressed genes increased over time where at 24 h post withdrawal, 355 genes were downregulated and 319 upregulated (Fig. 5F). Amongst the downregulated genes at 24 h were LGR5 and TNS4 (Fig. 5F). Transcriptional analysis of pancreatic ductal adenomas (PDAs) and normal ducts in mice and humans showed increased expression of Tns4/TNS4 in PDA's (Bojet al., 2015). Although strong donor dependent differences were observed in the correlation plot of all

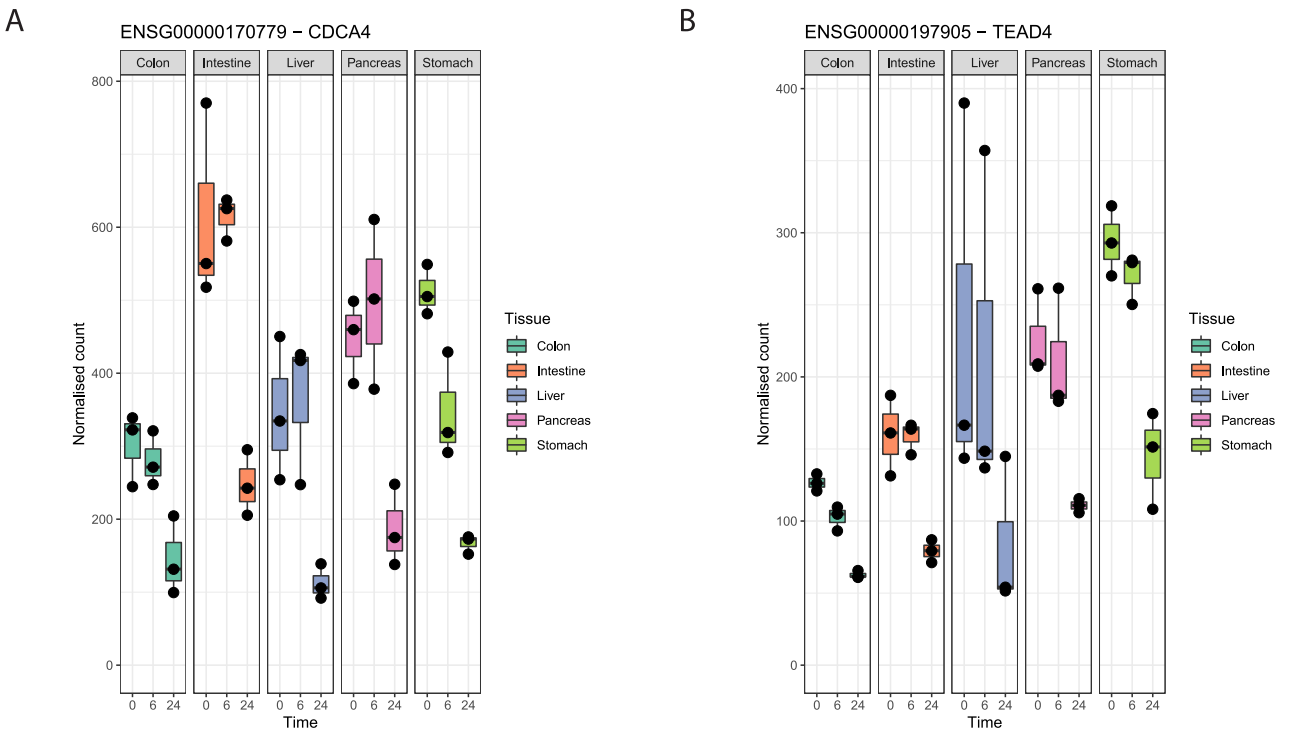

Fig. 3. Common Wnt target gene signature in Intestine, Colon, Liver, Pancreas and Stomach. (A) Normalized expression of CDCA4 plotted over time for each organ. (B) Normalized expression of TEAD4 plotted over time for each organ.

pancreas samples (Fig. 1C), paired-analysis still provided a good basis to identify known Wnt target genes such as LGR5. Filtering of the tissue specific Wnt regulated genes identified pancreas specific genes FGF9 and SLC13A5 (Fig. 5F, Fig. S4B and Table 5).

Similar to pancreatic cancers, gastric cancers have mutations in RNF43 implicating Wnt signaling needs to be tightly regulated in the stomach. Differential gene analysis showed 318 downregulated genes and 345 upregulated genes at 6 h post Wnt inhibition (Fig. 5G). After 24 h of Wnt withdrawal, more genes are changed in expression namely 975 downregulated and 852 upregulated genes (Fig. 5H). By filtering the tissue specific Wnt regulated genes, we identified 34 stomach-specific genes including SLIT3 (Fig. 5I) (Fig. S5 and Table 6). Altogether, we identified common and tissue specific Wnt signature by performing differential gene analysis upon Wnt inhibition combined with statistical analysis on basal gene expression levels.

3. Discussion

In this study we present an experimental approach to identify novel Wnt targets by applying human organoid technology. We analyzed the transcriptome of human organoids derived from small intestine, colon, liver, pancreas and stomach upon Wnt pathway inhibition. In our analysis we found bona fide Wnt target genes such as LGR5 and RNF43 which validated our experimental approach. We identified novel Wnt targets that are specific to certain organs and novel universal Wnt target genes shared in all 5 tissues. When we compared transcriptional downregulation upon Wnt pathway inhibition over colon, intestine, liver, pancreas and stomach, we found clear differences in the number of downregulated genes, implying an intrinsic difference in different tissues, potentially due to differences in either mRNA decay or signaling dynamics by itself.

In order to identify a common set of Wnt regulated genes we focused on 24 h post-withdrawal. Although one could speculate we might detect secondary targets, the main reasoning for focusing on this time point is that all different tissue derived organoids respond to WNT pathway downregulation at this point as is marked by known Wnt target genes *AXIN2* and LGR5 amongst others.

We identify a common Wnt target gene signature of 41 genes including well-known Wnt target genes LGR5, AXIN2, SP5, TFAP4, RNF43 and ZNRF3. These genes show differences in response to Wnt withdrawal with regard to the absolute L2FC, where LGR5 and AXIN2 react more strongly than RNF43 and ZNRF3. However, on average the newly identified potential Wnt target genes respond very similar to Wnt pathway inhibition as the well-known targets, providing evidence that the novel identified genes are regulated by canonical Wnt signaling. We can't exclude the possibility that secondary Wnt targets could be included in our target gene list. Further experiments such as TCF/LEF ChIP-sequencing would help to validate direct target genes.

In commonly regulated genes, transcription activating enhancer-binding protein 4 (TFAP4) has been described to activate the canonical Wnt pathway by binding of to the promotors of Wnt pathway components DVL1 and LEF1, suggestive of a potential feedback loop such as that of RNF43 and ZNRF3 in the Wnt pathway (Songet al., 2018). RNF43 and ZNRF3, related RING-type E3 ubiquitin ligases, down-regulate membrane levels of Frizzled receptors, effectively lowering signal strength (Kooet al., 2012). The actions of these stem cell-enriched ubiquitin ligases are in turn inhibited by the formation of trimeric complexes in which the exodomains of the ligases interact with R-spondins presented by Lgr5 or its Lgr4, -6 proteins homologs (de Lauet al., 2011; Haoet al., 2012). Whether some of the novel identified potential target genes in this study are also regulators of the Wnt pathway remains to be elucidated.

Another commonly regulated gene *TEAD4* is a transcription factor in the hippo pathway. The hippo pathway is reported to be essential for intestinal regeneration (Hong et al., 2016). Since both the Wnt pathway and the hippo pathway play a pivotal role for intestinal homeostasis and regeneration, it has often been pointed out that there is crosstalk between these pathways. Potentially, Wnt transcriptional co-factor β-catenin interacts with hippo transcription factor TAZ and thereby promotes its decay via β-TrCP-mediated ubiquitination (Azzolinet al., 2012; Azzolinet al., 2014). The hippo pathway has further been implicated in

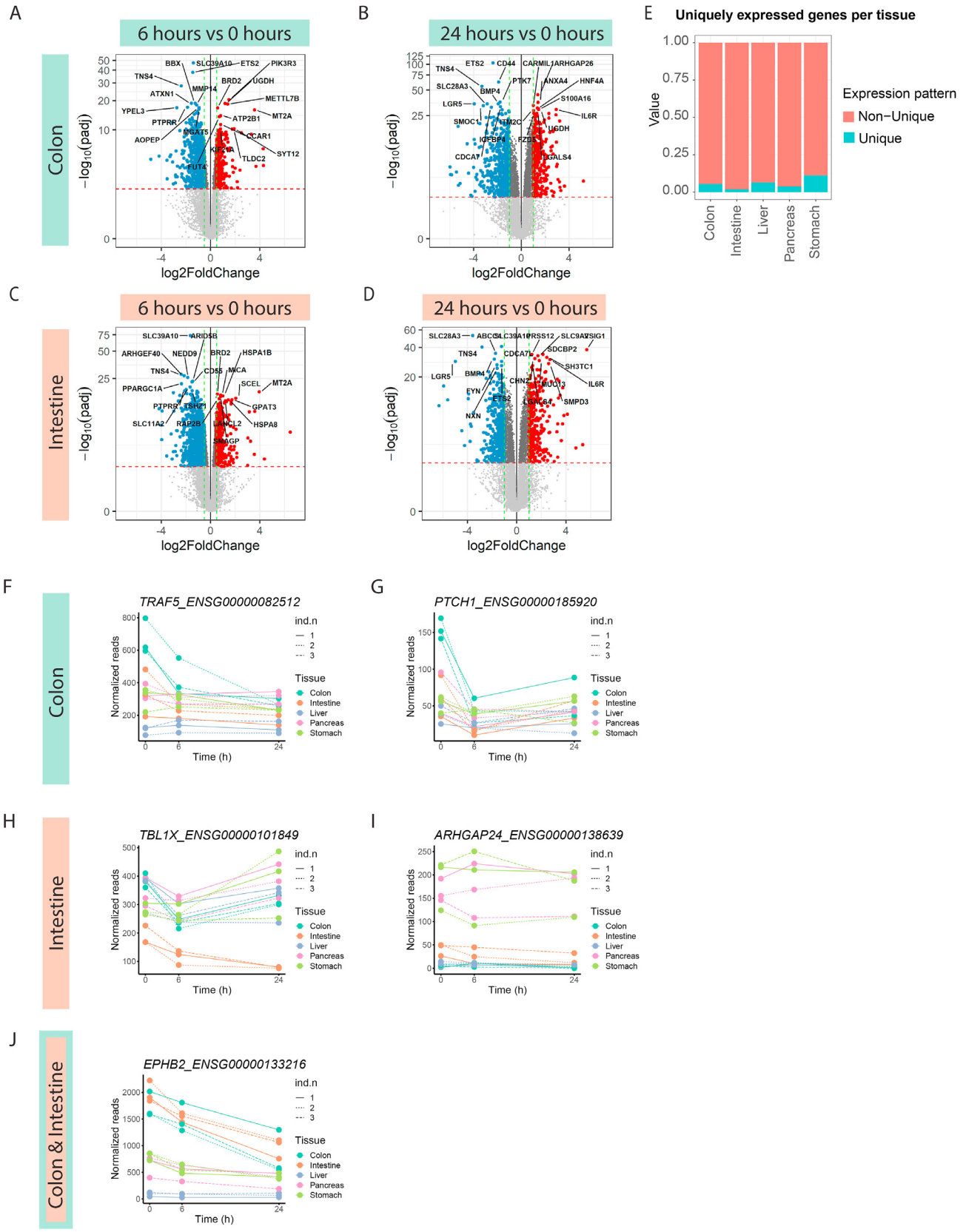

Fig. 4. Wnt target genes per tissue. (A) Volcano plot representing differentially expressed genes for colon at 6 h (A) and 24 h (B) post Wnt withdrawal. (C–D) Volcano plot representing differentially expressed genes for intestine at 6 h (C) and 24 h (D) post Wnt withdrawal. (E) Percentage of genes with a distinct expression level per tissue over the total number of Wnt regulated genes specific for that tissue. (F–G) Normalized reads plotted over time for known colon specific Wnt target gene TRAF6 (F) and PTCH1 (G). Each color represents an organ. (H–I) Normalized reads plotted over time for known intestine specific Wnt target gene TBL1X (H) and ARHGAP24 (I). Each color represents an organ. (J) Normalized reads plotted over time for known intestine and colon in common specific Wnt target gene EPHB2. Each color represents an organ. (F–J) Each color represents an organ. Dots represent individual datapoints, dotted line represents the matched data points from each donor.

Table 2
Colon specific Wnt target genes.

ENSEMBL	SYMBOL
ENSG00000059377	TBXAS1
ENSG00000082512	TRAF5
ENSG00000125864	BFSP1
ENSG00000130508	PXDN
ENSG00000146411	SLC2A12
ENSG00000165071	TMEM71
ENSG00000167107	ACSF2
ENSG00000174080	CTSF
ENSG00000185920	PTCH1
ENSG00000207864	MIR27B
ENSG00000224032	NA

Table 3
Small intestine specific Wnt target genes.

ENSEMBL	SYMBOL
ENSG00000101849	TBL1X
ENSG00000138639	ARHGAP24

tumors of the gastrointestinal tract. Experiments in mice showed that Yap inhibited differentiation of intestinal stem cells to Paneth cells and promoted cell survival and cell-renewal of Lgr5+ stem cells (Gregorieff et al., 2015). Colorectal cancers with high YAP1 expression similarly showed high levels of Wnt signaling (Choet al., 2018). In addition, we find transcription factor E2F1 to be downregulated upon Wnt pathway inhibition consistently in all different tissues. Cross-talk between Rb/E2F1 signaling has already been implicated where Rb/E2F1 drives expression of inhibitor of β-catenin and TCF4 (*ICAT*) (Wu et al., 2011). With the identification of *TEAD4* and *E2F1* as potential target genes we propose a novel mechanism of cross-talk between the hippo and E2F1 pathways and the Wnt pathway.

We also find implications for crosstalk between the Wnt and hedgehog pathway. *PTCH1* was regulated specifically in colon organoids. *PTCH1* is the receptor in hedgehog signaling and is an established hedgehog target gene. furthermore, we also found ligand Indian Hedgehog (*IHH)* gene to be upregulated upon inhibition of the Wnt pathway in human organoids of colon (1.4 fold), intestine (2.4 fold), liver (1.9 fold) and pancreas (2.1 fold) at 24 h post Wnt withdrawal. Studies of *IHH* in the mouse small intestine have shown that deletion of *IHH* results in increased proliferation and lengthening of both crypts and villi, indicating a feedback mechanism to control stem cell homeostasis (van Dopet al., 2010; Madisonet al., 2005). Further insights in the interplay between Wnt signaling and hedgehog signaling came from studies in colorectal cancer organoids, where it was shown that non-canonical hedgehog signaling is dependent on PTCH1 and sonic hedgehog (SHH) is a positive regulator of the Wnt pathway (Reganet al., 2017). These data suggested signaling pathway crosstalk between hedgehog signaling and Wnt signaling and its potential relevance for stem cell maintenance *in vitro*. Together the identification of potential Wnt target genes as direct components of other pathways potentially gives insights in why Wnt signaling plays such an important role for stem cell maintenance.

While this study was in progress, a paper was published focusing on oncogenic Wnt response in colorectal cancer organoids. Michels and colleagues compared response of normal organoid cultures to extrinsic Wnt stimulation by the culture medium and the response to intrinsic changes by mutating *APC* (Michelset al., 2019). Interestingly, of the differentially expressed genes found in their study, only a small percentage (16%), overlapped between intrinsic and extrinsic Wnt pathway activation. This data provide evidence on differences between transient Wnt target genes, modulated upon extrinsic cues which occurs during homeostasis and regeneration, and constitutive target genes, modulated upon oncogenic mutation (intrinsic). This suggests that cell lines and organoids harboring Wnt pathway activating mutations do not represent the extrinsic Wnt response well with regard to target gene expression. Furthermore, assessment of TCF/β-catenin peaks in HEK293T cells does not result in identification of all the novel Wnt target genes found in this study (Schuijers et al., 2014; Hatziset al., 2008). Altogether, we conclude based on our study that Wnt pathway assessment in human wildtype organoid lines provides a non-redundant way to identify human Wnt target genes *in vitro*.

To summarize, here we present a method to identify novel Wnt targets in primary human wild-type organoid lines. We present a robust assay that detects transcriptomic changes upon Wnt withdrawal and confirms biological preservation of Wnt pathway targets over different tissues. With this assay we provide insights on the Wnt pathway target genes in humans without the presence of oncogenic mutations found in cancer cell lines. Whether the findings in this study are directly translatable to adult human tissues or solely provide insight in stem cell maintenance and signaling in the *in vitro* context needs further elucidation.

4. Methods

4.1. In vitro organoid cultures

Organoids from human the small intestine, colon, liver ducts, pancreas and stomach were grown is expansion medium as described in earlier publications (Huchet al., 2013b; Satoet al., 2011; Huchet al., 2015; Bartfeldet al., 2015). Small intestine, colon, liver and pancreas organoids were grown in Matrigel (BD Biosciences) and stomach organoids in basement membrane extract (BME) (Cultrex). For experiments organoids were split to near a near single cell level and allowed to grow out for 3 days before harvesting $t = 0$ baseline RNA. Subsequently cultures where washed 3 times with pre-warmed wash buffer (DMEM + penicillin/streptomycin (100 U/L; Gibco)) and the expansion medium was replaced by Wnt inhibitory medium, lacking Wnt and/or R-spondin (see Table 7) and addition of DKK1 (200 ng/ml - Peprotech) and IWP2 (3 μM - Tocris). RNA was harvested at 6 h and 24 h post Wnt withdrawal. Experiments were performed in biological triplicate (n = 3 donors).

4.2. Organoid Wnt reporter assay

Human intestinal organoid cultures were transduced with Wnt reporter plasmid 7TFC, 7TFC was a gift from Roel Nusse (Addgene plasmid # 24307; http://n2t.net/addgene:24307; RRID:Addgene_24307) (Fuerer and Nusse, 2010). Flow cytometry was performed 1-week post transduction to sort for transduced lines based on expression of mCherry. Upon outgrowth of sorted single cells, organoid clones were picked and expanded. Organoid clones were split and seeded in glass bottom 96 well plates (Greiner). From 1 day before the start of the experiment, organoids were incubated with D-luciferin substrate (Biosynth). On the day of the experiment, medium was either replaced by fresh expansion medium or withdrawal medium. Wnt reporter (Luciferase) activity was read out in an environmentally controlled Tecan microplate reader for 24 h.

4.3. Bulk mRNA sequencing

RNA was harvested in RLT + β-mercaptoethanol (Sigma), frozen down on dry ice and stored at −80 °C until isolation. Subsequently, RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's outline. RNA quantity and quality was checked and measured with Bioanalyzer2100 RNA Nano 6000 chips (Agilent, Cat. 5067–1511). Libraries were prepared from 500 ng of Total RNA using the Truseq Stranded Total RNA kit with Ribo-Zero Human/Mouse/Rat set A and B by Illumina (Cat. RS-122-2201 and RS-122-2202). Prepared libraries were checked with Bioanalyzer2100 DNA High Sensitivity chips (Cat. 5067–4626) and with Qubit (Qubit dsDNA HS Assay Kit, Cat. Q32854). Subsequently, libraries were pooled in equimolar ratio to 2 nM and sequenced on the Illumina Nextseq, 2 × 75bp high output, and 1.0–1.4

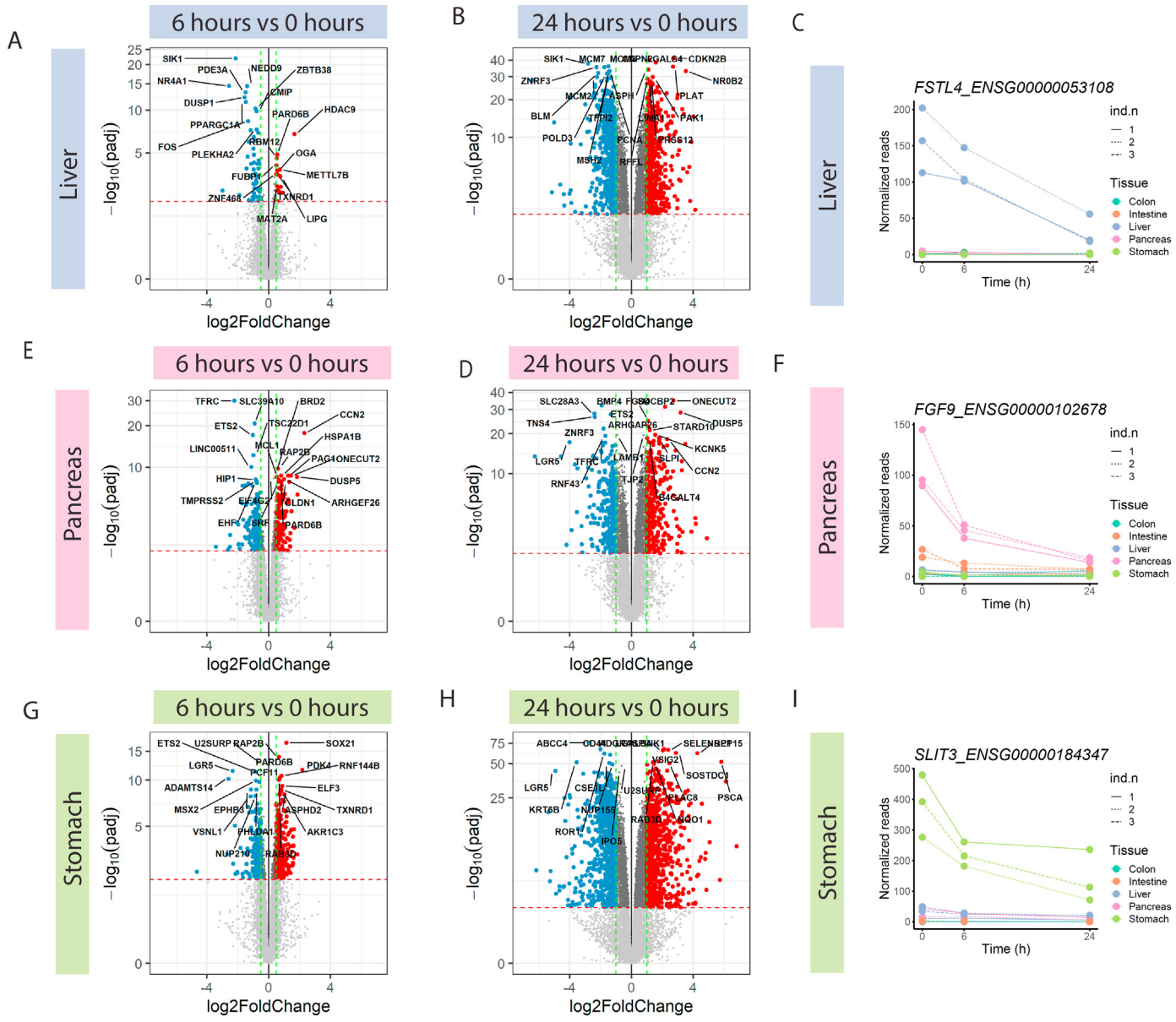

Fig. 5. Wnt target genes per tissue with unique expression patterns. (A–B) Volcanoplot representing differentially expressed genes for liver at 6 h (A) and 24 h (B) post Wnt withdrawal. (C) Normalized reads plotted over time for known liver specific Wnt target gene FSTL4. Each color represents an organ. (D–E) Volcano plot representing differentially expressed genes for pancreas at 6 h (D) and 24 h (E) post Wnt withdrawal. (F) Normalized reads plotted over time for known pancreas specific Wnt target gene FGF9. Each color represents an organ. (G–H) Volcano plot representing differentially expressed genes for stomach at 6 h (G) and 24 h (H) post Wnt withdrawal. (I–F) Normalized reads plotted over time for known stomach specific Wnt target gene SLIT3. Each color represents an organ. (C,F,I) Each color represents an organ. Dots represent individual datapoints, dotted line represents the matched data points from each donor.

Table 4
Liver specific Wnt target genes.

ENSEMBL	SYMBOL
ENSG00000053108	FSTL4
ENSG00000101665	SMAD7
ENSG00000119986	AVPI1
ENSG00000168874	ATOH8
ENSG00000172201	ID4
ENSG00000184792	OSBP2

Table 5
Pancreas specific Wnt target genes.

ENSEMBL	SYMBOL
ENSG00000102678	FGF9
ENSG00000141485	SLC13A5

pM of library pools was loaded. Samples were sequenced to average depth of 22.2 million fragments (SD 7.2 million). After sequencing quality control, read mapping and counting analyses were performed using RNAseq pipeline v2.1.0 (https://github.com/UMCUGenetics/RNASeq). Quality control was performed using FastQC (v0.11.4) after which reads were aligned to GRCh37 using STAR (v2.4.2a), read groups were assigned using Picard (v1.141). Samples passing QC were processed for counting using HTSeq-count (v0.6.1) (Anders et al., 2015) and ENSEMBL gene definitions (release 74).

Downstream bioinformatics analysis was performed using R To allow for differential gene analysis using the DESeq2 package (v1.28.1) (Love

Table 6
Stomach specific Wnt target genes.

ENSEMBL	SYMBOL
ENSG00000058866	DGKG
ENSG00000082516	GEMIN5
ENSG00000082781	ITGB5
ENSG00000084774	CAD
ENSG00000101224	CDC25B
ENSG00000105576	TNPO2
ENSG00000105722	ERF
ENSG00000112984	KIF20A
ENSG00000114767	RRP9
ENSG00000120694	HSPH1
ENSG00000123600	METTL8
ENSG00000123977	DAW1
ENSG00000128050	PAICS
ENSG00000138035	PNPT1
ENSG00000138231	DBR1
ENSG00000140511	HAPLN3
ENSG00000142207	URB1
ENSG00000144381	HSPD1
ENSG00000148843	PDCD11
ENSG00000150687	PRSS23
ENSG00000160208	RRP1B
ENSG00000164338	UTP15
ENSG00000173145	NOC3L
ENSG00000174442	ZWILCH
ENSG00000177855	NA
ENSG00000180198	RCC1
ENSG00000181472	ZBTB2
ENSG00000182389	CACNB4
ENSG00000183508	TENT5C
ENSG00000184347	SLIT3
ENSG00000185483	ROR1
ENSG00000187486	KCNJ11
ENSG00000196754	S100A2
ENSG00000197410	DCHS2

Table 7
Information on Wnt ligands and Wnt agonist in human organoid expansion medium.

	Pancreas	Liver	Colon	Intestine	Stomach
Wnt source	50% Wnt3A CM		50% Wnt3A CM	50% Wnt3A CM	50% Wnt3A CM
R-spondin	10% Rspo1 CM	10% Rspo1 CM	20% Rspo1 CM	20% Rspo1 CM	20% Rspo1 CM

et al., 2014). Differentially expressed genes were included with an absolute $\log_2$ fold change (L2FC) ≥ 0.4 for the 6 h time point and absolute LFC ≥ 1 for the 24 h timepoint. Pairwise analysis were performed between timepoints, within tissues and within donors. Genes found to be differentially expressed (adjusted p-value <0.01) between timepoints of interest and baseline were compared across the data set, the R scripts used to perform these analysis together with the relevant metadata have been made available here: https://github.com/jdeligt/WNT_paper/releases/tag/v1.

Author contributions

KEB, IH and PA performed the experiments. Data was analyzed by KEB, IH, JdL, GvS. Experiments were designed by KEB, IH, HC. BA and JdL supported in data analysis and data interpretation. KEB and HC wrote the manuscript.

Accession of data

Sequencing data generated for this study can be accessed at GSE167115 Available from the 22th of February 2021 online.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2021.01.009.

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