Original research

RNA binding protein DDX5 directs tuft cell specification and function to regulate microbial repertoire and disease susceptibility in the intestine

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ABSTRACT

Objective Tuft cells residing in the intestinal epithelium have diverse functions. In the small intestine, they provide protection against inflammation, combat against helminth and protist infections, and serve as entry portals for enteroviruses. In the colon, they have been implicated in tumourigenesis. Commitment of intestinal progenitor cells to the tuft cell lineage requires Rho GTPase Cell Division Cycle 42 (CDC42), a Rho GTPase that acts downstream of the epidermal growth factor receptor and wingless-related integration site signalling cascades, and the master transcription factor POU class 2 homeobox 3 (POU2F3). This study investigates how this pathway is regulated by the DEAD box containing RNA binding protein DDX5 in vivo.

Design We assessed the role of DDX5 in tuft cell specification and function in control and epithelial cell-specific Ddx5 knockout mice (DDX5ΔIEC) using transcriptomic approaches.

Results DDX5ΔIEC mice harboured a loss of intestinal tuft cell populations, modified microbial repertoire, and altered susceptibilities to ileal inflammation and colonic tumourigenesis. Mechanistically, DDX5 promotes CDC42 protein synthesis through a post-transcriptional mechanism to license tuft cell specification. Importantly, the DDX5-CDC42 axis is parallel but distinct from the known interleukin-13 circuit implicated in tuft cell hyperplasia, and both pathways augment Pou2F3 expression in secretory lineage progenitors. In mature tuft cells, DDX5 not only promotes integrin signalling and microbial responses, it also represses gene programmes involved in membrane transport and lipid metabolism.

Conclusion RNA binding protein DDX5 directs tuft cell specification and function to regulate microbial repertoire and disease susceptibility in the intestine.

INTRODUCTION

Intestinal epithelial cells (IECs) lining the gastrointestinal tract are essential for nutrient absorption and provide barrier protection for the host. IECs are functionally heterogeneous. One subset called tuft cells is a major sensor of microbial challenges in the intestine. They express various unique surface molecules, including receptors that bind directly to noroviruses and receptors sensing microbial metabolites, including succinate. During helminth and protist challenges, tuft cells secrete interleukin 25 (IL-25) to activate type 2 innate lymphoid cells (ILC2s). In a positive feedback circuit, activated...
ILCs2 produces IL-13 to drive tuft cell hyperplasia and ensure robust microbial clearance.5–9 Recent studies also revealed important roles of tuft cells in regulating inflammation and tumourigenesis in different intestine sections. In the ileum, reduction in tuft cell numbers is associated with elevated inflammation in the patients with Crohn’s disease (CD).10 Succinate activation of the tuft cell circuit is found to be protective against T cell mediated ileitis in a mouse model.10 In the colon, tuft cells and their unique expression of the prosurvival doublecortin-like kinase 1 (DCLK1) have been implicated in tumourigenesis.11–12 IECs have diverse turnover rates ranging from 3 to 5 days for enterocytes to 3–6 weeks for Paneth cells.13–14 They are replenished by progenitors originating from the intestinal stem cells (ISCs) residing in the crypt.13–15 Progenitor differentiation to specific IEC lineages is influenced by local nutrient availability, microbial composition, and gradients of epidermal growth factor (EGF), Wingless-related integration site (Wnt), Bone Morphogenic Protein (BMP), as well as cytokines from the immune system.16–18 Tuft cells in the small intestine (SI) and colon arise from the SOX4+ and ATOH1+ progenitors, respectively.10, 19–20 Commitment to the tuft cell lineage requires the expression of the POU domain transcription factor, POU class 2 homeobox 3 (POU2F3).21 A recent study has implicated Cell Division Cycle 42 (CDC42) in tuft cell biogenesis.22 CDC42 is a ubiquitously expressed member of the Rho GTPase family. On activation by EGF and Wnt signalling cascades,22–25 CDC42 regulates actin cytoskeleton organisation, polarity, proliferation and migration.26–27 Mutations in Cdc42 have been linked to paediatric immunodeficiency28 and severe developmental delay in patients with Takenouchi-Kosaki syndrome.29–30 Global knockout of Cdc42 in mice is embryonic lethal.31 Studies using mice where Cdc42 is specifically knocked out in IECs showed that CDC42 is required for ISCs growth and survival. Biogenesis of tuft cell, but not other secretory IEC subsets, is CDC42 dependent.22 However, little is known about how CDC42 contributes to tuft cell differentiation and upstream mechanisms involved in maintaining proper CDC42 expression in IECs. Here, we report that CDC42 promotes POU2F3 expression and tuft cell specification in the intestine, and this circuit is post-translationally regulated by the RNA binding protein called DEAD-box helicase 5 (DDX5). DDXs can directly bind to RNA substrates and use ATP hydrolysis energy to unwind RNA duplexes, facilitate RNA annealing, organise RNA-protein complex assembly32 and promote post-transcriptional processing.33 They can also partner with transcription factors to regulate gene expression.34–37 Our previous study revealed that DDX5 is the highest expressed member of the DDX family in the intestine epithelium.38 Overexpression of DDX5 predicts worse relapse-free survival in colorectal cancer (CRC) patients.39–41 and knockdown of DDX5 can significantly inhibited the growth of cancer cells in xenograft models.42, 43 We have recently reported that knocking out DDX5 in IECs protected against dextran sulfate sodium induced colitis as well as tumour formation in mice on a susceptible background.38 Given the emerging role of intestinal tuft cells in regulating intestinal inflammation and tumourigenesis, we asked whether changes in tuft cell specification and/or function may underlie the contribution of DDX5 to intestinal inflammation and tumourigenesis. Here, we report an essential role of DDX5 in maintaining CDC42-POU2F3 levels in secretory lineage progenitors to drive tuft cell specification in both small intestine and colon. In differentiated IECs, including tuft cells, DDX5 promotes antimicrobial gene programmes to regulate microbial composition and disease susceptibilities in models of ileitis and tumourigenesis.

**METHODS**

**Mice**

CS7BL/6 wild-type (Stock No: 000664) and Villin1Cre (Stock No: 021504) mice were originally obtained from the Jackson Laboratory. Previously described Ddx5 flox mice were obtained from Dr. Frances Fuller-Pace’s laboratory.44 Heterozygous mice were bred to yield 6–8 week-old Ddx5 flox/Villin1Cre (subsequently referred to as wild-type, WT) and Ddx5 flox/Villin1Cre− (referred to as DDX5/−) cohoused littersmates for experiments. For the tumour studies, Apc flox mice were obtained from Dr. Eric Fearon’s laboratory45 to generate Apc flox+/+ Ddx5 flox/Villin1Cre+ and Apc flox+/− Ddx5 flox/Villin1Cre− (referred as APC ΔIEC DDX5 WT) and Apc flox+/− Ddx5 flox+/+ Villin1Cre− (APC ΔIEC DDX5−) mice. A 3-month-old wild-type and mutant mice were given either 150 mM succinate (Alfa Aesar 41983-30) or control water (containing NaCl at 300 mM to match sodium molarity with succinate treatment) for 30 days. Colonies were harvested from 120 to 130 days old mice to assess tumour burden. Tumour measurements were determined by double-blinded analyses using ImageJ. All animal studies were approved and followed the Institutional Animal Care and Use Guidelines of the University of California San Diego. Our vivarium at UC San Diego is kept under specific pathogen-free conditions. Regular serology and PCR tests are used to monitor and ensure the absence of epizootic diarrhea of infant mouse virus (EDIM), mouse hepatitis virus (MHV), mouse parvovirus (MPV), minute virus of mice (MVM), Thielier’s murine encephalomyelitis virus (TMEV), fur mites and pinworms. Murine norovirus (MNoV) is normally present in our vivarium, litters born to MNoV+ parents naturally acquire the virus from their environment prior to weaning and were used for experiments between 8 and 12 weeks of age.

The spatial transcriptomic dataset described were performed on tissues obtained from female mice. DCLK1 immunohistochemistry experiments were performed on tissues from female mice. All other experiments reported in this study were obtained from both male and female mice in similar ratios.

**Metronidazone treatment**

For the antibiotic treatment studies, WT HEC and DDX5 HEC adult mice were treated with 1% sucrose alone or 1% sucrose containing 2.5 g/L of metronidazole in the drinking water for 2 weeks. New bedding were provided on day 7 to limit microbe reacquisition via the fecal-oral route. Mice were monitored and weighed 2–3 times per week and faecal/cecal material were collected to assess the level of protist and bacteria clearance. Pestle-homogenised faecal or cecal materials were digested at 75°C for 10 min using the KAPA Express Extract kit (Research and Development). A 1 µL of the soluble fraction was used as template for qPCR quantitation.

**Anti-CD3ε mediated model of ileitis**

WT HEC and DDX5 HEC mice were given 300 mM succinate-NaCl or equal molar of NaCl in drinking water for 2 weeks and subsequently i.p. injected 3 times with 15 µg of anti-CD3ε per mouse every other day for 5 days.46

**Histology and immunohistochemistry**

Tissues harvested from the ileum and colon were fixed overnight in 10% formalin (Research Products International) at room temperature. Paraaffin-embedded tissues were sectioned into 5 µm slices for Periodic Acid-Schiff (PAS) or immunohistochemical staining (see online supplemental table 1 for antibody information). Briefly, paraaffin sections were deparaffinised and...
rehydrated with Tris-buffered saline (TBST, pH 7.8 with 0.1% Tween-20) washes between each step. Sections were blocked first against endogenous peroxidases (immersed for 30 min in 0.3% H2O2) and then blocked against endogenous biotin using unlabeled streptavidin (Jackson ImmunoResearch, 016-000-114) and excess free biotin. Antigen retrieval was induced by heating the slide twice for 5 min in 10 mM sodium citrate buffer pH 6.0 (Sigma-Aldrich), followed by 20 min of cooling. Finally, sections were blocked against non-specific hydrophobic interactions with 1% bovine serum albumin (BSA; Biotium) in TBST. Staining was then performed with either the negative control IgG antibody or anti-DCLK1 (1:1000) antibodies (Abcam) overnight in a humid chamber at 4°C. The next day, sections were washed with TBST and then sequentially overlaid with biotinylated goat anti-rabbit (Jackson ImmunoResearch, 111-065-045) at 1:500, followed by horseradish peroxidase (HRP)-labelled Streptavidin (Jackson ImmunoResearch, 16-030-084) at 1:500. Substrate was then overlaid with 3-amino-9-ethylcarbazole from Vector labs following manufacturer directions for 30 min followed by nuclear counterstain with Mayer’s haematoxylin. Images were acquired using the AT2 Aperio Scan Scope (UCSD Moores Cancer Center Histology Core). Three intestinal regions per tissue image were randomly selected for QuPath analysis. DCLK1+ tuft cells were determined by QuPath Positive Cell Detection47 (minimum area=10 µm², maximum area=400 µm², intensity threshold=0.4). Mucin+ goblet cells were assessed similarly (minimum area=40 µm², maximum area=800 µm², intensity threshold=0.6). The average score from three regions examined in each tissue were included in the final graph.

Electron microscopy
Cardiac perfusion was performed using 5 mL Ringer’s solution (Fisher Scientific, Cat. #50-980-246), followed by 5 mL of fixation buffer containing 2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer solution (BIOTREND, Cat. # 15 960-01). Intestinal tissues were harvested and kept in the fixation buffer in room temperature for three additional hours. Fixed tissues were processed and imaged on JEOL 1400 plus at the UC San Diego Electron Microscopy Core Facility.

Intestinal epithelial and lamina propria lymphocyte harvest
Steady-state intestinal epithelial and lamina propria lymphocytes were harvested as previously described.48 To isolate IECs, intestinal tissues were first incubated in 5 mM EDTA (Invitrogen) in HBSS (Gibco) containing 1 mM DTT (Invitrogen) for 20 min at 37°C with 200 rpm agitation, and then incubated in a second wash of 5 mM EDTA in HBSS without DTT for another 20 min at 37°C with agitation. Single-cell suspensions released into the EDTA solutions were pooled and confirmed to contain over 85% non-stimulated CD3+ T cells.

Intestinal crypt isolation and organoid culture
Intestinal crypts were isolated as previously described.48 Tissues were harvested and kept in 25 mM EDTA in HBSS solution containing 10% foetal bovine serum (FBS) (Peak Serum), 1.0 mg/mL Collagenase D (Roche), 100 µg/mL DNase I (Sigma-Aldrich), and 50 U/mL dispase (Worthington Biochemical) at 37°C for 30–45 min. Lamina propria mononuclear lymphocytes were purified from the interphase of a 40:80% Percoll (Cytiva; formerly GE Healthcare Life Sciences) gradient.

Western blot
Cells were lysed in a 25 mM Tris pH 8.0 (G-Bioscience, 100 mM sodium chloride (NaCl) (G-Bioscience), 0.5% NP40 (G-Bioscience) solution) with protease inhibitor (Life Technologies) for 30 min on ice. Samples were spun down at 14,000 g for 15 min, and soluble protein lysates were harvested. The 30–50 µg protein were loaded in each lane of a SDS-PAGE gel. Blots were blocked in Odyssey Blocking buffer (Li-CoR Biosciences) and probed for DCLK1. Following incubation with IRDye secondary antibody (Li-CoR Biosciences), infrared signals on each blot were collected on the Li-CoR Odyssey CLX. The primary antibodies used in this study are listed in online supplemental table 1.
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cDNA synthesis and qPCR

Total RNA was extracted with the RNeasy Plus kit (QIAGEN) and reverse transcribed using iScript Select cDNA Synthesis Kit (Bio-Rad). Real time RT-PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad). All results were normalised to mouse Gapdh. Primers were designed using Primer-BLAST to span across splice junctions, resulting in PCR amplicons that span at least one intron. Primer sequences are listed in online supplemental table 2.

RNA-seq analysis

IEC transcriptomes from WT²⁵ and DDX5ΔIEC littermates were previously reported. Gene set enrichment analysis (GSEA) was carried out using the preranked mode of the GSEA software with default settings.¹¹ The gene list from DEseq2 was ranked by calculating a rank score of each gene as -Log$_{10}$(p value)×sign (Log, Fold Change), in which Fold Change is the fold change of gene expression in DDX5ΔIEC over that found in WT²⁵. De novo motif enrichment in the promoter regions of differentially expressed genes (log2 fold change cutoffs of ≥0.5 or ≤−0.5 and p<0.05) was performed using the HOMER (v4.10) ‘findMotifs.pl’ command line with the following parameters: ‘-nogo -start −1000 -end 500’. All other promoters of genes expressed in those particular cells were used as background. The top five motifs ranked by the lowest p-value that were found in at least 3% of the differentially expressed ‘Target’ genes were illustrated. The tumour transcriptomic sequencing data reported in this paper is available on GEO (GSE146014, reviewer access token: g4hcsouzruptyf).

For metatranscriptomic analysis of ileal associated microbial populations, reads from the DDX5ΔIEC and WT²⁵ IEC RNAseq dataset that were not mapped to the mouse genome were assigned with taxonomic labels using Kraken V.1. The standard Kraken database encompassing annotated bacterial, archaeal, and viral genomes was used for classification of sequences with the command: ‘kraken --classified-out /path/to/classified.fa --unclassified-out /path/to/unclassified.fa --db SDBNAME --paired --fastq-input pair1.fa pair2.fa > /path/to/results’. A Kraken report was generated with the the command: ‘kraken-report --SANDBNAME kraken.output’. Differential microbial counts were assessed by DEseq2 cut-off of p<0.05 with the Wald test and Log2 fold change (DDX5ΔIEC/WT²⁵)>1.5 or ≤−1.5.

Spatial transcriptomics

Female mouse ileum samples were coiled into ‘Swiss rolls’ and frozen immediately with chilled isopentane. The frozen tissues were embedded in optimal cutting temperature compound and cut in a pre-cooled cryostat at 20 μm thickness onto two 6.5 mm × 6.5 mm capture areas with 5000 oligo-barcoded spots. Frozen tissue was tested for RNA quality with RIN >7.0 (Tapestation). Slides were fixed, H&E stained, and imaged on Keyence (bx-z800) at ×20 magnification. Tissues were permeabilised for 24 min. Reverse transcription and second strand synthesis were then performed on RNAs extracted from each tissue. cDNAs were quantified by qRT-PCR using Powerup SyBr Green master mix and the Bio-Rad system. The library integrity was confirmed by the Agilent TapeStation. Pooled libraries were sequenced on NovaSeq S4 (Illumina) using 150 base-pair paired-end dual-indexed configuration. Each sample was sequenced to a depth of 100 million. Spacemaking software (V.3.1.0) from 10X Genomics was used to process, align and summarise unique molecular identifier counts against the mm10 human reference genome. Loupe Browser (V.5.0) was used for differential gene expression analysis and generation of visuals. The spatial transcriptomic sequencing data reported in this paper is available on GEO (GSE184564, reviewer access token: obaruukivrnpxad).

Ribosome pull-down assay

Small intestine IECs were lysed in polysome extraction buffer (10 mM HEPES, pH 7.4, 150 mM KCl, 5 mM MgCl₂, 1%NP40, 2 mM dithiothreitol, 80 U/mL RNaseOUT, 100 μg/mL cycloheximide and protease inhibitors). Cell extracts were subject to anti ribosome IP overnight with 2 μg anti-RPL10A antibodies (Abcam) as described previously.⁶ Level of RPL10A associated transcripts in pull-down was calculated as fraction of input for each sample.

Statistical analysis

All values are presented as mean±SD. Significant differences were evaluated using GraphPad Prism V8 software (GraphPad). The Student’s t-test was used to determine significant differences between two groups with normal distribution. A two-tailed p <0.05 was considered statistically significant in all experiments.

RESULTS

DDX5 regulates tuft cell populations in the murine small intestine and colon

In our previous report, we showed that DDX5 is highly expressed in the intestinal epithelium and regulates intestinal inflammation. However, the exact mechanism remained to be elucidated. In a recent studies activation of the tuft cell circuit is also linked to intestinal inflammation.⁵⁰ Therefore, we asked whether DDX5 may regulate intestinal inflammation at least in part by contributing to the differentiation and function of tuft cells and/or other IEC subsets. To address this question, we performed lineage-specific GSEA on our previously reported transcriptomes of steady-state IECs obtained from two matched pairs of WT²⁵ and DDX5ΔIEC male littermates. A loss of the tuft cell signature was found in both the small intestinal and colonic epithelium of DDX5ΔIEC mice (figure 1A,B and online supplemental figure 1A). IECs from independent pairs of male and female WT²⁵ and DDX5ΔIEC littermates were used to confirm significant reductions in the expressions of two tuft cell-specific transcripts, Pou2f3 and Dclkl1. In contrast, comparable expressions of select transcripts previously shown to be enriched in other IEC subsets, including Maf (enteroendocrine cells), Muc2 (goblet cells), Lyz1 (Paneth cells), Lgr5 and KIt67 (ISCs), were found in control and DDX5ΔIEC mice (online supplemental figure 1B). These results suggest that DDX5 may have a unique role in regulating tuft cell differentiation and/or function in the intestine.

Next, we investigated whether the loss of tuft cell signature in DDX5ΔIEC IECs may be due to a loss of the intestinal tuft cell population. Indeed, immunohistochemistry analysis revealed a significant reduction of DCLK1⁺ tuft cells in the DDX5ΔIEC small intestine and colon (figure 1C,D, workflow described in online supplemental figure 2A). In contrast, PAS staining of the small intestine and colon sections from control and DDX5ΔIEC mice showed comparable crypt density and goblet cell numbers (online supplemental figure 2B-D). Consistent with the histology results, flow cytometry analysis of the small intestine and colonic IECs (defined as CD45⁻CD24⁺) also confirmed a significant reduction of tuft cells (defined as Siglec-F⁻CD24⁺, similar to a previous report⁶⁰ in the DDX5ΔIEC IECs from male and female mice (figure 1E, gating strategy detailed in online supplemental figure 3A). These results demonstrate that DDX5 promotes...
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Figure 1  Epithelial DDX5 promotes the tuft cell gene programme in the intestine. (A) Heatmap summarising the GSEA results on the indicated IEC gene subsets in the RNAseq dataset obtained from the ileal (SI) and colonic IECs of two pairs of male WTIEC and DDX5ΔIEC mice (GSE123881). (B) GSEA enrichment plots of the tuft cell gene set in WTIEC and DDX5ΔIEC ileal (SI) and colonic IECs from A. (C) Representative images from immunohistochemistry analysis of DCLK1+ cells in intestinal sections from female WTIEC and DDX5ΔIEC mice. Scale bar represents 300 µm. (D) DCLK1+ tuft cell counts on ileal (SI) and colonic sections from 3 pairs of female WTIEC (black, n=3) and DDX5ΔIEC (red, n=3) mice analysed according to the automated workflow described in online supplemental figure 2A. Mice were fasted overnight to minimise the impact of varying food intake prior to tissue harvest. Each dot represents the result from one mouse. No primary antibody controls are shown in grey. *P<0.05 (unpaired t-test). (E) Summary of flow cytometry results of WTIEC and DDX5ΔIEC ileal (SI) and colonic epithelium. IECs were defined as live CD4−CD8−EpCAM+CD45lo. Proportion (%) of IECs in the tuft cell lineage defined as Siglec-F−CD24+ are graphed. See online supplemental figure 3A for gating strategy. Each dot represents the result from one mouse. *P<0.05, **P<0.01 (paired t-test). (F) Human DCLK1 expression in colonic biopsies with DDX5 expression below (low) or above (high) the mean of each respective dataset (GSE83687 and GSE75214). DDX5, DEAD-box helicase 5; GSEA, gene set enrichment analysis; IEC, intestinal epithelial cell; IHC, immunohistochemical; NES, normalised enrichment score; FDR, false discovery rate.

Figure 1 continued on page 5

impaction of tuft cell biogenesis in the murine small intestine and colon. In two human colonic IEC transcriptomic datasets previously reported,54 55 elevated DCLK1 expression was also associated with higher DDX5 expression (figure 1F), suggesting regulation of the tuft cell gene programme by DDX5 is likely evolutionarily conserved.

Impaired expression of the tuft cell commitment factor, Pou2f3, in DDX5ΔIEC IEC secretory lineage progenitors

In the small intestine, ATOH1 and SOX4 expressing IECs are progenitors of the secretory lineages, including tuft cells, goblet cells, Paneth cells and enteroendocrine cells.19 20 56 Next, we asked whether DDX5 contribute to the generation of these progenitor populations and/or their ability to express factors required for the generation of each secretory lineage, including Pou2f3-Gfi1b for tuft cells, Spdef for Goblet cells, Sox9 for Paneth cells, and Neurod1 for enteroendocrine cells. Spatial transcriptomics (10X Visium) analysis of ileal sections revealed similar number of Atoh1hi and Sox4hi spots were present in the WTIEC and DDX5ΔIEC ileal (SI) and colonic epithelium. IECs were defined as live CD4−CD8−EpCAM+CD45lo. Proportion (%) of IECs in the tuft cell lineage defined as Siglec-F−CD24+ are graphed. See online supplemental figure 3A for gating strategy. Each dot represents the result from one mouse. *P<0.05, **P<0.01 (paired t-test). (F) Human DCLK1 expression in colonic biopsies with DDX5 expression below (low) or above (high) the mean of each respective dataset (GSE83687 and GSE75214). DDX5, DEAD-box helicase 5; GSEA, gene set enrichment analysis; IEC, intestinal epithelial cell; IHC, immunohistochemical; NES, normalised enrichment score; FDR, false discovery rate.

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DDX5ΔIEC ileum showed higher levels of Neurod1, but similar levels of Gfi1, Spdef, and Sox9 compared with Atoh1Δ spots from WTIEC ileum (online supplemental figure 4A). Similar differential gene expression analysis on Lgr5Δ spots, but revealed only a relatively minor footprint of DDX5 in the ISCs (online supplemental figure 4B and online supplemental table 3), consistent with our earlier observation from our bulk IEC analysis (online supplemental figure 1B). These results suggest that DDX5 may regulate tuft cell biogenesis by promoting Pou2f3 expression in the secretory lineage progenitors.

Consistent with findings from the spatial transcriptomic studies, small intestine crypts enriched with ISC and progenitors isolated from DDX5ΔIEC mice had lower Pou2f3 mRNA abundance compared with those found in crypts obtained from WTIEC mice (Figure 2F). This suggests that DDX5 may regulate tuft cell biogenesis by modulating Pou2f3 expression in the secretory lineage progenitors.
from their WT<sup>IEC</sup> littermates (online supplemental figure 4C). In addition, DDX5<sup>IEC</sup> small intestinal crypts cultured ex vivo over 4–7 passages in the presence of the Wnt pathway agonist R-spondin (Rspo), EGF, the BMP inhibitor Noggin (as previously described,<sup>16</sup> penicillin, streptomycin, and gentamicin developed into organoids and maintained lower Pou2f3 expression compared with those derived from WT<sup>IEC</sup> mice (figure 2C,D). Similar observations were found in DDX5<sup>ΔIEC</sup> colonic organoids (figure 2E). No significant change in organoid viability and size were found between cultures obtained from control or DDX5<sup>ΔIEC</sup> crypts (online supplemental figure 5A). Furthermore, tuft cell-specific genes harbouring a promoter POU2F motif had the greatest loss in the expression in the DDX5<sup>ΔIEC</sup> tissue (figure 2F,G). Together, these results demonstrate an epithelial cell-intrinsic role of DDX5 in maintaining Pou2f3 expression in vivo and ex vivo.

**DDX5 is dispensable for IL-13 driven tuft cell hyperplasia**

Differentiation of IEC subsets, including tuft cells, is heavily influenced by the local microbial composition and immune cytokine profiles. In the presence of protozoan *Trichomonas muris*, for example, the small intestinal tuft cell population expands rapidly in an IL-13-dependent manner.<sup>3,4</sup> Therefore, we assessed whether the loss of tuft cell numbers in the DDX5<sup>ΔIEC</sup> intestine may be explained by a reduction in local IL-13 level, difference in *Trichomonas* abundance, and/or expressions of IL-13 receptors in the ISC and progenitors. At the RNA level, we found a similar abundance of transcripts encoding *Il13* expressed by immune cells in the lamina propria of cohoused WT<sup>IEC</sup> and DDX5<sup>ΔIEC</sup> littermates (online supplemental figure 6A). Furthermore, cohoused WT<sup>IEC</sup> and DDX5<sup>ΔIEC</sup> littermates acquired similar levels of *Trichomonas* species from their parents prior to weaning (online supplemental figure 6B).

To assess whether the *Trichomonas* species present in our mouse colony were implicated in the tuft cell specification, we treated cohoused WT<sup>IEC</sup> and DDX5<sup>ΔIEC</sup> littermates with metronidazole in drinking water for 14 days to deplete majority of the *Trichomonas* species together with other metronidazole sensitive bacterial species (online supplemental figure 6B). Interestingly, WT<sup>IEC</sup> mice treated with metronidazole had no significant change in the proportion of tuft cells in the small intestine and colon as measured by flow cytometry (defined as Siglec-F<sup>+</sup>CD24<sup>+</sup>). Likewise, the proportion of tuft cells and epithelial Pou2f3 transcript levels in the metronidazole-treated DDX5<sup>ΔIEC</sup> mice remained lower than levels found in WT<sup>IEC</sup> mice under similar treatment (online supplemental figure 6C). These results suggest that the metronidazole-sensitive *Trichomonas* and bacteria species present in our mouse colony were not involved in the establishment of tuft cell in the WT<sup>IEC</sup> and DDX5<sup>ΔIEC</sup> mice. It remained to be investigated whether specific metronidazole-insensitive microbe(s) and/or metabolites present in our colony contribute to the DDX5-dependent generation of tuft cell we observed.

Furthermore, we found no defect in the ability of DDX5<sup>IEC</sup> ISC and secretory lineage progenitors to express genes encoding receptors for IL-13 (online supplemental figure 7A). Therefore, we hypothesised that DDX5 is likely dispensable for tuft cell hyperplasia in response to IL-13. To test this possibility, WT<sup>IEC</sup> and DDX5<sup>ΔIEC</sup> small intestinal and colonic organoids were treated with recombinant IL-13 for 3 days. At the RNA level, both WT<sup>IEC</sup> and DDX5<sup>ΔIEC</sup> organoids upregulated Pou2f3 to a similar extent, resulting in a ≥twofold increase in tuft cell numbers compared with vehicle-treated organoids (online supplemental figure 7B–D). Together, these results suggest that DDX5 is dispensable for type 2 immune cytokine-driven tuft cell hyperplasia.

**DDX5 promotes Cdc42 translation to induce tuft cell biogenesis**

Next, we asked whether DDX5 promotes Pou2f3 expression and drives tuft cell biogenesis by directly binding to Pou2f3 mRNA and/or transcripts encoding its upstream regulators. To this end, we examined the DDX5-associated RNA interactome identified by the enhanced cross-linked immunoprecipitation (eCLIPseq)<sup>17</sup> experiment on IECs derived from the steady-state WT small intestine.<sup>35</sup> To our surprise, we did not identify any DDX5 footprint on the Pou2f3 transcript, suggesting that DDX5 likely regulates Pou2f3 expression indirectly. Of the eleven known molecules previously implicated in Pou2f3 expression and tuft cell biogenesis (*Gfi1b, Mtor, Raptor, Stat6, Ogt, Tas1r3, Sox4, Hes1, Dil1, Atoh1 and Cdc42*), only the Cdc42 transcripts were bound by DDX5 (figure 3A). CDC42 is a GTP-binding protein previously implicated in ISC survival, growth, as well as tuft cell biogenesis.<sup>25</sup> In the absence of DDX5, IECs had reduced CDC42 protein abundance (figure 3B). Spatial transcriptomics analysis revealed that Cdc42 is ubiquitously expressed across all IEC subsets, and most abundantly found in Lgr5<sup>IEC</sup> and Sox4<sup>IEC</sup> progenitors (figure 3C). Murine retrovirus carrying a CDC42 (isoform 1, V1) expression construct partially rescued CDC42 protein levels in DDX5<sup>ΔIEC</sup> small intestinal organoids (online supplemental figure 8A,B) and restored the Siglec-F<sup>+</sup>CD24<sup>+</sup> tuft cell population to levels found in WT<sup>IEC</sup> cultures (figure 3D,E). These results demonstrate that DDX5 promotes tuft cell biogenesis by regulating CDC42.

EGF and R-spondin in the organoid culture media engage EGF receptor (EGFR) and Wnt signalling pathways known to drive CDC42 activity. At the RNA level, small intestinal *Sox4*<sup>IEC</sup> progenitors abundantly expressed Egrf, Fed2 and Lrp5 (figure 3F). Therefore, we asked whether CDC42 GTPase activity may be implicated in the generation of tuft cells in the organoid culture system. Consistent with this possibility, DDX5<sup>ΔIEC</sup> small intestinal organoids transduced with the dominant negative CDC42<sup>△N7N</sup> or CDC42<sup>△V12V</sup> expression constructs failed to restore the Siglec-F<sup>+</sup>CD24<sup>+</sup> tuft cell population (figure 3D), suggesting that the GTPase activity of CDC42 is required for DDX5-mediated tuft cell biogenesis. To bypass the essential function of CDC42 in ISC reported previously,<sup>12,38</sup> established WT organoids were passaged, seeded on fresh Matrigel, and allowed to differentiate normally for 48 hours and then subjected to CDC42 inhibition by ML141. In this assay, acute ML141 treatment did not result in measurable difference in organoid survival or growth (online supplemental figure 9A). We also did not detect significant change in the abundance of *Klf4, Lyc1*, and *Lgr5* transcripts in ML141 treated organoids (online supplemental figure 9B). However, Pou2f3 expression was significantly diminished in WT organoids transiently treated with ML141 for 24 hours (figure 3G). Intriguingly, ML141 treated wild-type small intestinal and colonic organoids remained responsive to IL-13 by expanding their tuft cell population to a similar extent as control treated cultures (online supplemental figure 9C,D). Together, these results confirm that the DDX5-CDC42 axis is dispensable for IL-13-induced tuft cell expansion ex vivo.
Intestinal inflammation

**Figure 3**  DDX5 regulates Pou2f3 expression and tuft cell specification by promoting Cdc42 translation. (A) Left: schematic representation of the enhanced clip (eCLIPseq) experiment. Right: Integrative Genomics Viewer (IGV) browser view of DDX5 eCLIPseq signals at the Cdc42 loci from two independent experiments. (B) Representative Western blot for Cdc42 in whole cell lysates of small intestinal IECs from WTIEC and DDX5ΔIEC mice. Experiments were repeated three times using independent biological samples with similar results. (C) Violin plots displaying expression of Dclk1, Pou2f3, Ddx5, and Cdc42 in small intestinal stem cells (ISC, Lgr5<sup>hi</sup>), Atoh1<sup>hi</sup> and Sox4<sup>hi</sup> secretory lineage progenitors, or tuft cells from the spatial transcriptomics dataset described in figure 2A. (D) Summary of the % of SiglecF<sup>+</sup>CD24<sup>+</sup> tuft cells in WTIEC and DDX5ΔIEC small intestinal organoids 7 days post-transduction with retrovirus (marked by CD90.1<sup>+</sup>) carrying the indicated expression vectors. Each dot represents the result from an independent organoid experiment. *P<0.05, **p<0.01 (unpaired t-test). (E) Representative flow cytometry plots from D. (F) Heatmap indicating the average expression of known signalling molecules upstream of Cdc42 activation in ISCs, progenitors and tuft cells from figure 2A. (G) Fold change of Pou2f3 mRNA in WTIEC (n=6) or DDX5ΔIEC (n=5) small intestine organoids cultured in the presence of Cdc42 inhibitor ML141 (10 µM) for 24 hours relative to WTIEC cells treated with vehicle control (DMSO). Each dot represents the result from one mouse. **P<0.01, ****p<0.0001 (unpaired t-test). (H) IGV browser view of WTIEC and DDX5ΔIEC ileal IEC RNAseq-derived sample reads mapped to the Ddx5 and Cdc42 loci (GSE123881). (I) Fold change of ileal Cdc42 mRNA in DDX5ΔIEC mice (n=3) relative to values found in WTIEC mice (n=3). Each dot represents the result from one mouse. n.s. not significant (unpaired t-test). (J) Ribosome RPL10A enrichment from Cdc42 mRNA in small intestinal IECs. Results shown represent the average of two independent experiments. Each dot represents the result from one mouse. *P<0.05 (unpaired t-test). DDX5, DEAD-box helicase 5; DMSO, dimethylsulfoxide; IEC, intestinal epithelial cell; n.s, not significant.
Mechanistically, DDX5 did not alter IEC Cdc42 transcript abundance (figure 3H,1). Ribosome capture assay revealed that optimal translation machinery engagement on Cdc42 mRNA transcripts in small intestinal IECs was DDX5-dependent (figure 3J). Intriguingly, retrovirus carrying wild-type DDX5 or the helicase dead DDX5\textsuperscript{\textsc{dead}}, expression constructs were able to rescue the Siglec-F\textsuperscript{+}CD24\textsuperscript{+} tuft cell population in DDX5\textsuperscript{\textsc{ΔIEC}} small intestinal organoids to levels found in WT IEC cultures (figure 3D). This suggests that DDX5 helicase activity is dispensable for promoting Cdc42 ribosomal engagement. Together, results from our genetic, genomic and pharmacological experiments demonstrate that epithelial DDX5 binds Cdc42 transcripts and promotes CDC42 protein translation in a helicase-activity-independent manner to control tuft cell biogenesis.

**DDX5 negatively regulates tuft cell lipid and protein metabolic programmes**

Despite a significant loss of tuft cells in the DDX5\textsuperscript{\textsc{ΔIEC}} intestine, both immunohistochemistry and flow cytometry analyses demonstrated a small population of tuft cells can be generated in the absence of DDX5 (figure 1C–E). Next, we tested whether DDX5\textsuperscript{\textsc{ΔIEC}} tuft cells may be morphological and/or functional distinct from those with intact DDX5 expression. Electron microscopy revealed similar brush-like microvilli structures characteristic of elongated tuft cells in both WT IEC and DDX5\textsuperscript{\textsc{ΔIEC}} small intestine (figure 4A), suggesting that DDX5 does not influence tuft cell morphology.

Previous reports clustered the small intestinal tuft cells into two main subsets: one having higher expression of genes related to neuronal development (type 1), and the other (type 2) enriched with immune-related genes, such as the protein tyrosine phosphatase receptor Type C gene encoding the pan-rectum cell marker CD45.\textsuperscript{1,5} Of the few DDX5\textsuperscript{\textsc{ΔIEC}} tuft cells present in the small intestine, their proportion of CD45 low and mid-subpopulations were similar to those found among WTI EC tuft cells (online supplemental figure 10A). GSEA of the small intestinal IEC transcriptomes\textsuperscript{38} also confirmed that both type 1 and 2 programmes were significantly impaired in the DDX5\textsuperscript{\textsc{ΔIEC}} epithelium (online supplemental figure 10B).

To fully elucidate IEC subset-specific DDX5-dependent gene programmes, we performed differential gene analysis on the spatial transcriptomic datasets obtained from the WTI EC and DDX5\textsuperscript{\textsc{ΔIEC}} small intestinal sections discussed in figure 2A. We uncovered 17.5% of the DDX5-induced transcripts and 3.8% of the DDX5-repressed genes were shared among tuft, enteroendocrine, goblet, and Paneth cells (online supplemental figure 11A,B and online supplemental table 4). The numbers of DDX5-regulated genes in goblet and enterendocrine cells is lower than those found in tuft and Paneth cells. Notably, DDX5\textsuperscript{\textsc{ΔIEC}} small intestinal tuft cells had reduced expression of genes involved in microbial responses (figure 4B–D), including a loss of C/\textsc{dt}300l\textsubscript{f} transcripts encoding a surface molecule used by the Murine norovirus (MNoV) as a key docking site for host entry\textsuperscript{2} (online supplemental figure 12A). As a result, DDX5\textsuperscript{\textsc{ΔIEC}} mice raised by MNoV\textsuperscript{+} parents had significantly fewer MNoV transcripts in their small intestinal epithelium compared with their cohoused wild-type littermates (online supplemental figure 12A). In addition, meta-transcriptomic analyses of the WTI EC and DDX5\textsuperscript{\textsc{ΔIEC}} intestinal epithelium transcriptomes revealed significantly lower mucosal-associated reads mapping to viral and bacterial genomes from the DDX5\textsuperscript{\textsc{ΔIEC}} small intestine and colon, respectively (online supplemental figure 12B,C). In particular, species of Helicobacters and Prevotella were significantly reduced in the DDX5\textsuperscript{\textsc{ΔIEC}} colon (online supplemental figure 12D and online supplemental table 5).

While none of the DDX5-dependent genes involved in microbial responses harbour a DDX5\textsuperscript{ECLIPSEQ} footprint, ten of the genes involved in transmembrane transport and protein and lipid metabolism that were upregulated in DDX5\textsuperscript{\textsc{ΔIEC}} tuft cells were direct targets of DDX5 (indicated with a blue asterisk on figure 4E). These results reveal a novel role of DDX5 as a repressor of transmembrane transport and protein and lipid metabolic programmes in tuft cells of the small intestine. Together, these results extend the role of DDX5 in shaping IEC subset gene programmes and modulating the microbial community in the intestine.

**Succinate-Induced tuft cell hyperplasia protects against ileitis and restores colon tumourigenic potential in DDX5\textsuperscript{\textsc{ΔIEC}} mice**

Small intestinal tuft cells have been recently reported to provide protection against T-lymphocyte mediated ileitis on anti-CD3ε challenge.\textsuperscript{10} Therefore, we hypothesised that the reduced tuft cell numbers in the small intestine of DDX5\textsuperscript{\textsc{ΔIEC}} mice may result in enhanced susceptibility to ileitis. Consistent with our hypothesis, 50% of the DDX5\textsuperscript{\textsc{ΔIEC}} mice challenged in this model succumbed to the disease by day 18 (figure 5A). Of the ones that survived, mononuclear immune cells from their ileal lamina propria had elevated transcripts encoding the inflammatory cytokine TNF (figure 5B). Preadministration of succinate, a microbial-derived metabolite known to promote tuft cell hyperplasia in an IL-13 dependent manner,\textsuperscript{4,6} in drinking water was able to partially restore Delk1 expression in the small intestine epithelium of DDX5\textsuperscript{\textsc{ΔIEC}} mice (figure 5C), similar to previous reports,\textsuperscript{3,10} dampened Tnf expression, and significantly protected them against mortality from the anti-CD3ε challenge (figure 5A,B).

In the colon, multiple IEC subsets, including DCLK1\textsuperscript{+} tuft cells, have been reported to harbour tumourigenic potential.\textsuperscript{11,12} We recently reported that DDX5\textsuperscript{\textsc{ΔIEC}} mice on the intestinal tumour-susceptible Apc\textsuperscript{\textsc{ΔIEC}} background\textsuperscript{45} harboured fewer intestinal tumours.\textsuperscript{38} Transcriptomic analysis of control and DDX5 deficient tumours revealed pathways as well as a subset of DDX5-dependent genes that were similarly regulated in tuft cells (online supplemental figure 13A–C and online supplemental table 6). Therefore, we tested whether succinate supplementation can also promote colonic tuft cell programmes, including Delk1 expression, and restore the tumourigenic potential of the Apc\textsuperscript{\textsc{ΔIEC}}DDX5\textsuperscript{\textsc{ΔIEC}} mice. Immunohistochemistry analysis confirmed partial restoration of the DCLK1\textsuperscript{+} population in succinate treated DDX5\textsuperscript{\textsc{ΔIEC}} mice (figure 5D). In the tumour susceptible background Apc\textsuperscript{\textsc{ΔIEC}} succinate treated DDX5\textsuperscript{\textsc{ΔIEC}} mice had higher colonic tumour counts than those treated with vehicle control alone (figure 5E). Altogether, these results highlight the critical roles of epithelial DDX5 in protecting against ileal inflammation yet contributing to colonic tumourigenesis (modelled in figure 5F).

**DISCUSSION**

Tuft cells in the small intestine and colon arise from the SOX4\textsuperscript{+} and ATOH1\textsuperscript{+} progenitors respectively.\textsuperscript{10,19,20} Commitment to the tuft cell lineage requires the expression of the POU domain transcription factor, POUF3.\textsuperscript{2,24} Induction of Pon2β3 is best characterised in response to IL-13 and IL-4 stimulation during helminth and protist challenges.\textsuperscript{2,5,9} However, the molecular mechanisms that control Pon2β3 expression in the absence of IL-13 and IL-4 stimulation remain elusive. Here, we report that
CDC42 and its GTPase activity is essential for Pou2f3 expression. Consistent with our findings, epithelial specific knockout of CDC42 resulted in a loss of intestinal tuft cells, but no change to other secretory IEC subsets. In the small intestine, we show that Cdc42 is the abundantly expressed in ISCs and secretory lineage progenitor cells. Optimal translation of the Cdc42 transcript rely on the RNA binding protein DDX5 in a helicase-activity independent manner. Knocking out DDX5 specifically in IECs results in reduced CDC42 protein abundance and loss of tuft cell populations in both the small intestine and colon. Ex vivo, retroviral expression of wild-type CDC42 rescued the tuft cell biogenesis defect in small intestinal DDX5ΔIEC organoids. Surprisingly, the DDX5-CDC42 axis is dispensable for tuft cell hyperplasia in response to IL-13. WT organoids cells treated with the CDC42 inhibitor and DDX5ΔIEC organoids remain capable of responding to IL-13, upregulating Pou2f3 expression, and inducing tuft cell expansion. Future studies will be needed to investigate whether these parallel DDX5-CDC42 and IL-13-induced pathways recruit shared or distinct transcription factors and/or machineries to drive Pou2f3 expression in the secretory lineage progenitors. Compared with the secretory lineage progenitors, DDX5 has a limited transcription footprint on ISCs. We also did not find abnormalities in growth and survival in the DDX5ΔIEC crypts containing ISCs and progenitors, unlike
those observed in cells from the CDC42ΔIEC mice. We speculate that this likely suggests that the remaining CDC42 levels in the DDX5ΔIEC epithelium is sufficient to maintain ISC growth and survival and/or CDC42 expression in ISC may be DDX5-independent. Future studies that genetically ablate Ddx5 in specific IEC progenitor and/or IEC subsets will be needed to more definitively address whether the contribution of DDX5 to tuft cell differentiation and function may be cell-autonomous and/or indirect.

In the small intestine, tuft cells protect against ileal inflammation in a mouse model and a loss of tuft cells is identified in CD patients. As expected, DDX5ΔIEC mice with reduced intestine tuft cell biogenesis were more susceptible to disease in a model of ileitis. Administration of succinate partially restored tuft cell numbers in DDX5ΔIEC mice and allowed for greater resilience during ileitis. Compared with the small intestine, the colon epithelium harboured fewer tuft cells and their physiologic and pathological functions are not well understood. Results from this study suggest that the DDX5-CDC42 axis is also implicated in colonic tuft cell biogenesis. In contrast to the anti-inflammatory role of DDX5 in the small intestine IECs, we previously reported that DDX5 in the colonic IECs promotes inflammation in a DSS-induced model of colitis. Regional-specific roles of DDX5 in intestine inflammation may be related to distinct microbe–host interactions and/or distinct gene programmes regulated by DDX5 in colonic IECs. Future single-cell transcriptomic studies will be needed to fully elucidate the mechanisms underlying these specificities. Development of

Figure 5  Succinate-induced tuft cell hyperplasia protects DDX5ΔIEC mice against exacerbated ileitis and restores tumourigenic potential in the DDX5ΔIEC colon. (A) Survival plot of mice treated with succinate-NaCl (Succ., WTIEC, n=6; DDX5ΔIEC, n=7) or equal molar of NaCl (Veh., WTIEC, n=4; DDX5ΔIEC, n=6) in drinking water for 2 weeks and subsequently challenged by 15 µg of anti-CD3ε per mouse for 5 days. *P<0.05 (Mantel-Cox test). (B) Normalised mRNA expression of Tnf in small intestinal lamina propria (LP) mononuclear cells isolated from mice from A. on day 19. Each dot represents the result of one mouse. *P<0.05 (paired t-test). (C) Normalised mRNA expression of Dclk1 in the small intestinal IECs isolated from mice from A. on day 19. One dot represents the result of one mouse. *P<0.05 (unpaired t-test). (D) Representative images of DCLK1 staining in colon from mice treated with NaCl (Veh.) or succinate-NaCl in drinking water for 30 days. Scale bar represents 100 µm. (E) Left: representative images of colonic tumours from 120 days old APCΔIEC DDX5WT and APCΔIEC DDX5ΔIEC mice treated with succinate-NaCl or equal molar of NaCl in drinking water on day 90 for 30 days. Right: summary of colonic tumour counts from treated APCΔIEC DDX5WT and APCΔIEC DDX5ΔIEC mice. Each dot represents the result from one mouse. *P<0.05 (unpaired t-test). Scale bar represents 1 cm. (F) Working model. DDX5-CDC42-POU2F3 axis regulating progenitor commitment toward the tuft cell lineage (left), DDX5 dependent gene programmes in differentiated tuft cells (middle) and intestinal tumours (right). DDX5, DEAD-box helicase 5; IEC, intestinal epithelial cell; n.s. not significant.
additional IEC-subset specific knockout models will be needed for definitively attributing specific DDX5 regulated programmes to the various phenotypes observed here.

In summary, this study uncovered an epithelial cell intrinsic pathway involving the RNA binding protein DDX5 and its ability to regulate the CDC42-POU2F3 axis critical for tuft cell biogenesis that is parallel but distinct from the IL-13 induced hyperplasia responses and can serve as new molecular targets for the restoration of mucosal homeostasis in disease settings.

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Contributors TL and NA designed and performed the mouse studies. TL and IMS performed the small intestine and colon organoid assays. YL performed the bioinformatics analyses on the RNAseq datasets. JEH performed the crypt and goblet cell analyses. BAY performed the eCLIPseq analyses. GWY directed the eCLIPseq studies and edited the manuscript. PG and SD directed the organoid studies and edited the manuscript. SM initiated the spatial transcriptomic studies and TL completed the studies with help from AF. PT provided resources for the spatial transcriptomic and organoid imaging studies. TL wrote the manuscript with input from WIMH, NA and JEH. WIMH is the guarantor.

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Competing interests G.W.Y. is a co-founder, a member of the Board of Directors, a scientific advisor, an equity holder and a paid consultant for Locanbio and Eclipse Biotechnologies. G.W.Y. is a visiting professor at the National University of Singapore. G.W.Y.’s interests have been reviewed and approved by the University of California San Diego, in accordance with its conflict-of-interest policies. The authors declare no other competing interests.

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