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ARTICLE

Cellular and Molecular

Transcriptomic and functional analyses reveal a tumour-promoting role for the IL-36 receptor in colon cancer and crosstalk between IL-36 signalling and the IL-17/ IL-23 axis

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BACKGROUND: The interleukin (IL)-36 cytokines are a sub-family of the IL-1 family which are becoming increasingly implicated in the pathogenesis of inflammatory diseases and malignancies. Initial studies of IL-36 signalling in tumorigenesis identified an immune-mediated anti-tumorigenic function for these cytokines. However, more recent studies have shown IL-36 cytokines also contribute to the pathogenesis of lung and colorectal cancer (CRC).

METHODS: The aim of this study was to investigate IL-36 expression in CRC using transcriptomic datasets and software such as several R packages, Cytoscape, GEO2R and AnalyzeR. Validation of results was completed by qRT-PCR on both cell lines and a patient cohort. Cellular proliferation was assessed by flow cytometry and resazurin reduction.

RESULTS: We demonstrate that IL-36 gene expression increases with CRC development. Decreased tumoral IL-36 receptor expression was shown to be associated with improved patient outcome. Our differential gene expression analysis revealed a novel role for the IL-36/IL-17/IL-23 axis, with these findings validated using patient-derived samples and cell lines. IL-36γ, together with either IL-17a or IL-22, was able to synergistically induce different genes involved in the IL-17/IL-23 axis in CRC cells and additively induce colon cancer cell proliferation.

CONCLUSIONS: Collectively, this data support a pro-tumorigenic role for IL-36 signalling in colon cancer, with the IL-17/IL-23 axis influential in IL-36-mediated colon tumorigenesis.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common, and second leading cause of cancer-related death worldwide. Globally, the incidence of newly diagnosed CRC cases has been predicted to increase to an annual total of up to 3.2 million, with a current incidence of 1.93 million cases detected per annum [1]. Although there have been vast improvements in the detection of colorectal cancer in the last 50 years, the increased detection has also highlighted that this cancer type occurs in younger cohorts than previously thought [2]. Whilst the 5-year survival rate of ~64% is relatively high for the average CRC case, metastatic CRC (mCRC) shows a significantly worse prognosis with survival rates as low as 12% amongst patients [3]. Although complete surgical excision remains integral to curative treatment, conventional chemotherapy and radiation therapies can result in dose-limiting toxicities and acquired cancer-cell resistance to treatment. More recent improvements in our understanding of the pathways promoting colonic carcinogenesis have helped to develop targeted therapies based on the molecular profile of the tumour e.g., the epidermal growth factor receptor (EGFR) status of the tumour [4]. This has also been closely followed by the emergence of immunotherapies with the recent approval of immune checkpoint blockade (ICB) targeting therapies proving beneficial to patient outcomes in mCRC, although this response is seen in a small minority of patients [5], with ICBs currently proving ineffective for the majority of patients with CRC.

The interplay between inflammation and cancer has long been established, with chronic inflammation now recognised as a hallmark of cancer [6]. The importance of inflammation in the development of CRC is an area of intense study, and this is particularly evident in cases of colitis-associated cancer [7]. The role of inflammation in sporadic and hereditary cancer has been more recently highlighted with the use of clinical molecular subtyping, which helps to describe the inflammatory background of each of these CRC types [8]. Increasing evidence suggests that inhibition of this sustained inflammatory background may be beneficial in the prevention of, or simply the delay in, the development of hereditary and sporadic cases of CRC [9]. Upon development of CRC, however, a pro-inflammatory tumour microenvironment (TME) is generally considered a positive prognostic factor, promoting tumour rejection by infiltration and activation of anti-tumour immune populations such as $CD8^+$ tumour-infiltrating lymphocytes (TILs) [10].

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One of the most influential families of cytokines in the inflammatory process is the IL-1 superfamily, which plays a vast role in many diverse tissues in both homoeostasis and disease [11]. The integral role played by IL-1 family members in cancer was exemplified by the CANTOS trial, where patients receiving the IL-1 receptor-inhibiting antibody showed a dramatic decrease in lung cancer incidence and mortality [12]. Despite this significant finding, the pleiotropic nature of IL-1 family members make them very complex cytokines to study, with varying roles across multiple tissues and pathologies [13]. The IL-36 cytokines are a recently described subset of the IL-1 family of cytokines [11]. The three agonistic members of this family, IL-36α, IL-36β, and IL-36γ, share the same receptor complex, which is composed of the IL-36 receptor (IL-36R/IL1RRP2/IL1RL1) and the IL-1 receptor accessory protein (IL-1RAcP). A biological inhibitor to this complex has been identified, the IL-36R antagonist (IL-36Ra). Recently, this family has been shown to play an important role in the pathogenesis of chronic inflammatory diseases, in particular psoriasis [14, 15], inflammatory bowel diseases [16, 17], respiratory diseases [18] and several malignancies [11]. However, the role of IL-36 signalling in the context of cancer remains unclear, with evidence of divergent pro- and anti-tumorigenic phenotypes being reported, similar to that seen with other IL-1 family members [19]. Many studies have focused on the immunostimulatory potential of IL-36 signalling in order to augment the anti-tumour immune response, with IL-36 administration resulting in improved activation and proliferation of cells such as $CD8^+$ TILs and natural killer (NK) cells [20–25]. More recently, however, several reports have highlighted the pro-tumorigenic effects of IL-36 signalling on IL-36 receptor (IL-36R)-expressing tumour cells, in particular in both lung cancer and CRC [26–28].

The aim of this study was to investigate the pluripotent role of the IL-36 family of cytokines in CRC using multiple publicly available transcriptomic datasets as well as in vitro assay validation. In this study, we demonstrate that IL-36 family member expression is significantly increased in CRC, associated with decreased 5-year survival rates and show IL-36 signalling can synergistically induce chemokine expression with IL-17a or IL-22 as well as augmenting cancer cell proliferation in combination with IL-17a.

MATERIALS AND METHODS

Study populations

The study protocol, including all procedures and study populations, has been previously described [20]. In brief, the study was approved by the University College Cork Clinical Research Ethics Committee of the Cork Teaching Hospitals (ECM (3) P 3 September 2013). All samples were obtained during surgery at the Mercy University Hospital Cork following informed consent. For patient cohort 1, 24 fresh samples of human colon cancer and paired normal tissues were collected in RNAlater and stored at −20 °C until processing.

Cell line maintenance

HT29 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin solution in a 37 °C, 5% CO_2 humidified incubator unless otherwise stated.

Quantitative real-time PCR (qRT-PCR)

Total cellular RNA was isolated using the GenElute Mammalian Total RNA Mini Kit (Sigma-Aldrich, Dorset, UK) according to the manufacturer's instructions. cDNA was synthesised using the Bioline kit (London, UK). RT-PCR was performed using the LightCycler480 System (Roche, West Sussex, UK). Individual PCR primer pairs and probes were designed using the Roche Universal Probe Library Assay Design Centre (www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp) or primer probe pairs were obtained from Integrated DNA Technologies (CA, USA). Primer sequences and probe combinations are provided in Supplementary File 1. All samples were run in triplicate, and relative quantitation was calculated using the $2^{-\Delta\Delta Ct}$ method. Transcript levels were normalised to the amount of β-actin mRNA, and expression levels are shown as fold induction relative to untreated.

Cellular proliferation assays

HT29 cells (2.5×10^3/well) were seeded in 96-well plates and serum starved for 24 h. Fresh media was then added along with the indicated stimulant/control. Twenty-four hours later, cells were washed, media supplemented with 44 μM resazurin was added, and resazurin reduction to resorufin measured fluorometrically using a GENios plate reader (TECAN, Grodig, Austria) and Xfluor spreadsheet software. The results obtained were normalised relative to the untreated cells. Experiments were repeated three times and each experimental condition was performed in triplicate. Alternatively, cell proliferation was measured using flow cytometry and the CellTrace CFSE Cell Proliferation assay was completed as per manufacturers guidelines, using 1×10^5 cells/mL in 24-well plate format over 96 h.

Pan-cancer and normal tissue gene expression

Gene expression comparison of IL1RL2 expression was assessed using the 'Cancer Exploration' suite of the TIMER2.0 web tool [29].

Sample stratification and survival analysis

Samples were stratified according to their quartile range, with values in the 1st quartile (top 25%) designated as 'High' and values in the 4th quartile (bottom 25%) designated as 'Low'. Incomplete samples were not included. For IL-36α analysis, each of two large datasets with similar samples types (GSE17536 and GSE39582, both bulk tissue samples and analysed by the same microarray platform) were individually stratified by levels of target gene expression, with top and bottom quartiles then combined for survival analysis. The R package "survival" was utilised to visualise the Kaplan–Meier survival curves and perform log-rank testing using the TCGA-COAD (Colorectal adenocarcinoma) dataset.

Differential Gene expression determination

GEO2R, a data processing tool on GEO (Gene expression omnibus) was used to identify differentially expressed genes (DEGs) [30]. Four datasets were used to compare colon cancer tissue and normal colonic tissue; selection criteria pertained to the selection of datasets containing bulk tumour tissue and normal bulk tissue samples, transcriptomic analysis by microarray and number of samples per dataset ($N > 20$). Each dataset was individually analysed, the DEG's then compiled and compared after analysis. In each dataset, cancer samples were stratified into two groups; (A) IL-36R high (upper 25% of IL-36R expressing tumours) and (B) IL-36R low (lower 25% of IL-36R expressing tumours). Upregulated genes (fold change (FC) > 1.5 and P value <0.05) were compared across datasets and only genes that were present in all four were proceeded with. Downregulated genes (FC < 1.5 and P value <0.05) were compared similarly. IL-36R high and IL-36R low upregulated genes were then compared, with genes exclusive to either group then proceeded with for subsequent analysis (e.g., 12 genes upregulated in IL-36R high tumours, 4 genes upregulated in IL-36R low tumours). The same was completed for downregulated genes in both tumour groups.

Protein–protein interaction, clustering and module analysis

Search Tool for the Retrieval of Interacting Genes (STRING) [31] database was used to investigate protein–protein interactions of DEGs. The interactions include direct (physical) and indirect (functional) associations, which are based on computational predictions using the medium confidence setting with strictly query genes used in the mapping. Protein–protein interaction (PPI) networks were visualised using Cytoscape software, a visualisation tool for PPI networks [32]. The Molecular Complex Detection (MCODE) plug-in was then used to identify gene modules of interconnected proteins by Markov clustering [33]. Modules identified were then separated as individual networks, and STRING enrichment was performed for functional annotation of gene clusters with top Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) annotations used. Default confidence scores were applied with no additional nodes added for data exploration.

Correlation matrix

A correlation matrix of genes from the KEGG *'IL-17 signalling pathway'* was made using the NCBI GEO dataset GSE39582. The dataset GSE39582 was used to investigate this as this dataset set contains a large number of CRC samples from a multi-institutional study previously used for the Consensus Molecular Subtype [34]. IL-36 family members and associated genes were included using the R package 'corrplot'.

Gene expression correlation in normal and cancer tissue

The web tool 'AnalyzeR' was used to investigate gene expression of IL1RL2, CXCL-1 and LCN2 in normal and cancerous intestinal tissue [35].

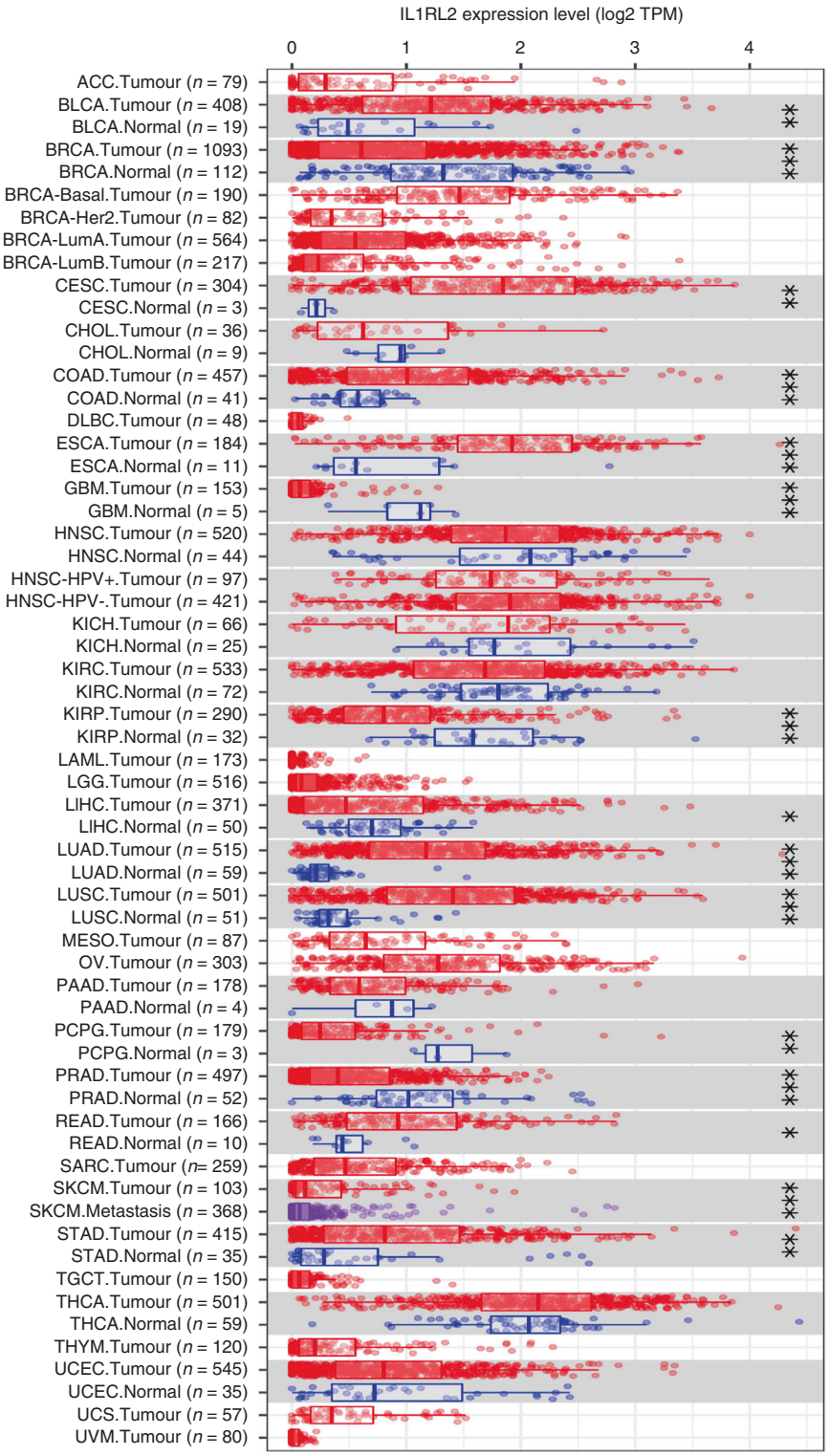


Fig. 1 Expression of IL-36R is increased in multiple malignant tissues relative to normal adjacent tissue. IL-36R (IL1RL2) expression was investigated using the 'Cancer Exploration' suite of the TIMER2.0 to examine differences in gene expression between normal healthy and malignant tissues in similar anatomical locations. The statistical significance computed by the Wilcoxon test is annotated by the number of stars (*P value <0.05; **P value <0.01; ***P value <0.001).

Statistical analysis

The statistical analysis of parameters was assessed by various methods as outlined per figure legend. P values are presented as follows unless otherwise stated; *P value <0.05; **P value <0.01; ***P value <0.001. Wilcoxon or Student's T tests were used as indicated in figure legends.

RESULTS

Expression of IL-36 family members is increased in multiple tumour types, with expression also increasing during the development of CRC

Initially, the expression of the IL-36R across various cancer types was examined and compared to normal adjacent tissue, using the

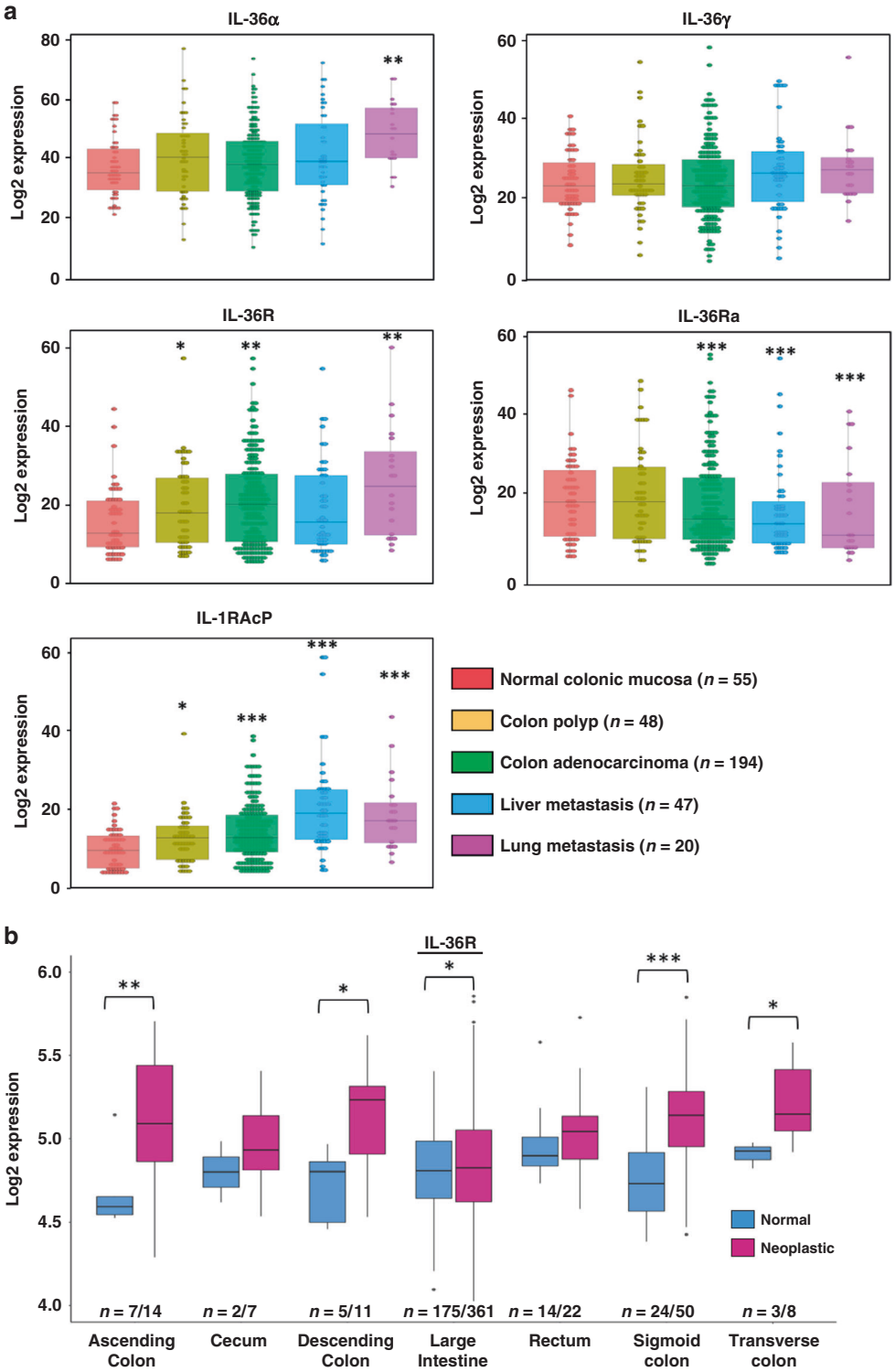


Fig. 2 IL-36 family member expression is altered in CRC disease development with expression of the IL-36R increased in neoplastic tissue relative to normal tissue in multiple anatomical sites across the intestine. a Expression of IL-36 family members (IL-36α, IL-36γ, IL-36R, IL-36RN and IL-1RAcP) were investigated across tissue types in CRC progression using the dataset GSE68468. **b** Changes in IL-36R expression in anatomical sites of the colon/rectum were investigated in normal and malignant tissue using a meta-dataset published by Rohr et al. [37].

web tool TIMER2.0 to assess RNAseq expression data from multiple cancer types [29]. Transcript levels (Log2 transcripts per millions detected) across cancer types varied greatly according to the tissue site, with normal tissue showing both decreased and increased levels of IL-36R expression relative to the corresponding cancer type. The most significant increases in IL-36R expression were detected in epithelium-originating cancers such as colon adenocarcinoma (COAD), oesophageal adenocarcinoma (ESCA), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC) and stomach adenocarcinoma (STAD) (Fig. 1). Conversely, a more diverse array of cancers showed decreased IL-36R expression relative to healthy tissue including breast invasive carcinoma

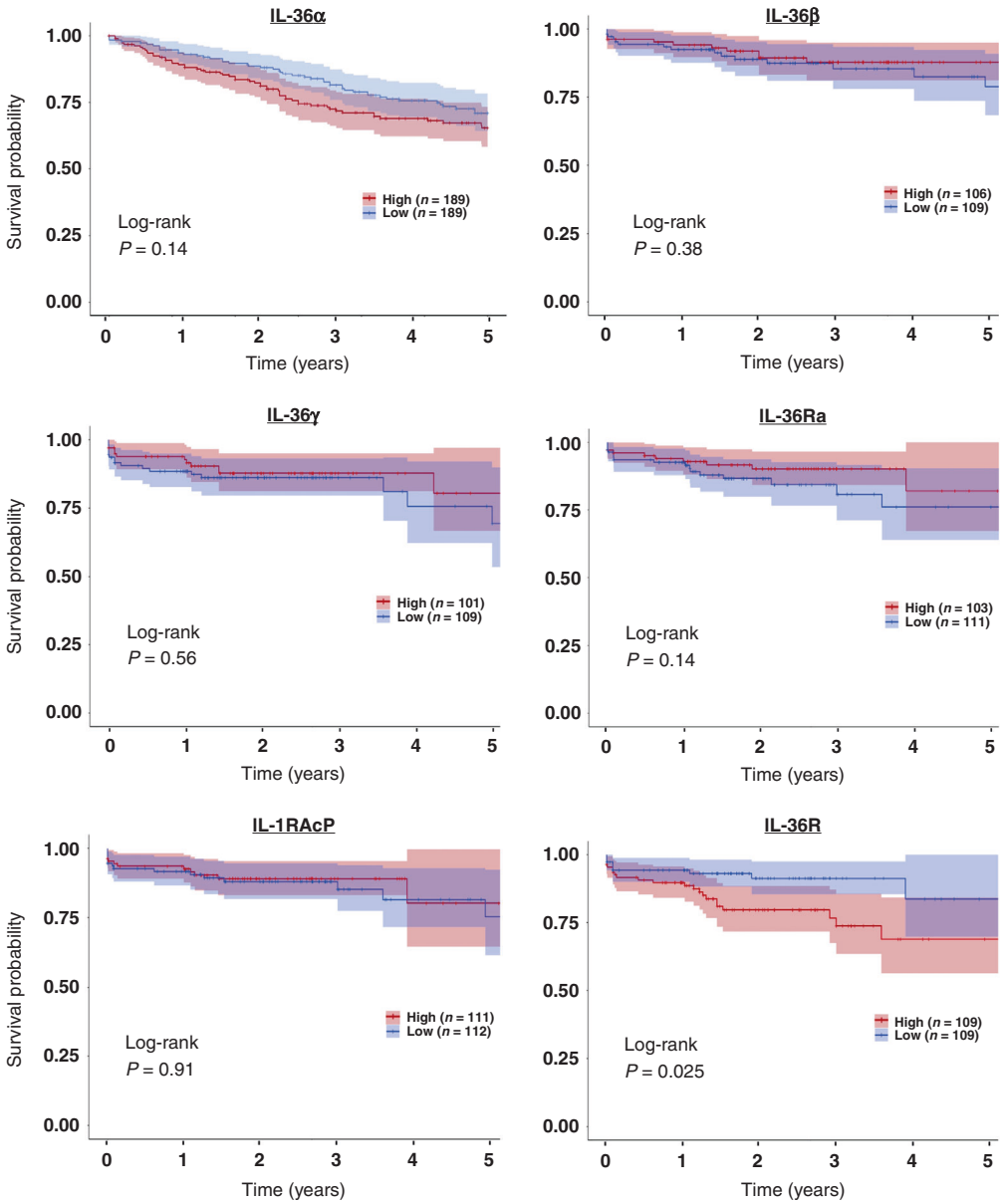


Fig. 3 High expression of IL-36R is associated with a poorer patient 5-year survival rate in CRC. Samples were stratified according to their quartile range, with values in the 1st quartile (top 25%) designated as 'High' and values in the 4th quartile (bottom 25%) designated as 'Low'. Incomplete samples were not included. For IL-36α analysis, each of two large datasets with similar sample types (GSE17536 and GSE39582, both bulk tissue samples and analysed by the same microarray platform) were individually stratified by levels of target gene expression, with top and bottom quartiles then combined for survival analysis. The relationship between IL-36 family member expression in CRC and clinical prognosis was analysed by Kaplan–Meier analysis. Differences in survival curves were determined by the log-rank test.

(BRCA), glioblastoma (GBM), kidney renal papillary cell carcinoma (KIRP) and prostate adenocarcinoma (PRAD). Given the increasing evidence of involvement of IL-36 in CRC [11, 27, 36] and the significant increase in IL-36R expression detected in TCGA adenocarcinoma datasets which implicate IL-36 signalling in tumorigenesis, it was decided to investigate IL-36 family member expression in the adenoma–carcinoma sequence of CRC. The dataset GSE68468 was used to assess this due to the histological description of tissue samples across a large dataset throughout the adenoma–carcinoma sequence. The expression of IL-36 family members, with the exception of IL-36RN, the gene encoding the IL-36Ra, showed a trend of increased expression from normal colonic tissue through to colonic adenocarcinoma and subsequent lung and liver metastases (Fig. 2a). The largest increases in gene expression were observed for the IL-36R ($P = 0.0019$) and IL-1RAcP ($P < 0.001$). Of note, the largest increase in IL-36 family member gene expression was detected in lung metastases when compared to normal colonic tissue. In addition, IL-36RN gene expression showed a significant decrease in expression from normal colonic tissue to adenocarcinoma ($P < 0.001$) and subsequent metastases ($P < 0.001$). As this dataset did not include IL-36β, it is unknown whether this cytokine would also have been increased during CRC development.

Given the important prognostic value of the anatomical location within the colon/rectum of cancers, a meta-dataset [37] was used to investigate the expression of the IL-36R in different anatomical sites of the colon in both normal and neoplastic tissue (Fig. 2b). In all anatomical sites, the expression of the IL-36R was significantly increased in the tumour tissue, with the exception of the rectum. This increase in expression was particularly apparent in the

Table 1. Details of the datasets used for transcriptomic analyses.

Dataset	Platform	Normalisation	Used in paper for;	Source	Sample type
The cancer genome atlas (TCGA)	Illumina HiSeq	Limma	Malignant vs normal tissue expression multiple cancer types	TIMER2.0	Bulk tissue
GSE68468	Affymetrix Human Genome U133A Array (HG-U133A-GPL96)	RMA	Colon tissue gene expression comparison, differential gene expression (DEG) analysis	NCBI GEO	Bulk tissue
GSE39582	Affymetrix Human Genome U133 Plus 2.0 Array [HG-U133_Plus_2	RMA	Correlation matrix	NCBI GEO	Bulk tissue
GSE86362	Affymetrix Human Genome U133 Plus 2.0 Array [HG-U133_Plus_2	fRMA	Immune cell gene expression	NCBI GEO	Bulk tissue
TCGA-COAD	Illumina HiSeq	Limma	Survival analysis	TCGA	Bulk tissue
E-MTAB-10089 [37]	Affymetrix Human Genome U133 Plus 2.0 Array [HG-U133_Plus_2	fRMA	Normal and neoplastic colonic tissue gene expression	ArrayExpress	Bulk tissue
GSE37364	[HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	Mas5	DEG analysis	NCBI GEO	Bulk tissue
GSE23878	[HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	PLIER	DEG analysis	NCBI GEO	Bulk Tissue
GSE25070	Illumina HumanRef-8 v3.0 expression beadchip	RSN	DEG analysis	NCBI GEO	Bulk tissue
Multicohort (GSE17536, GSE39582)	Affymetrix Human Genome U133 Plus 2.0 Array [HG-U133_Plus_2	fRMA	IL-36α survival analysis	NCBI GEO	Bulk tissue

proximal colon, which is a site associated with a poor patient survival rate [38]. These findings further implicate IL-36 cytokines as important molecules in colon cancer tumorigenesis and progression.

Increased tumoral expression of the IL-36R is associated with a decreased 5-year survival rate

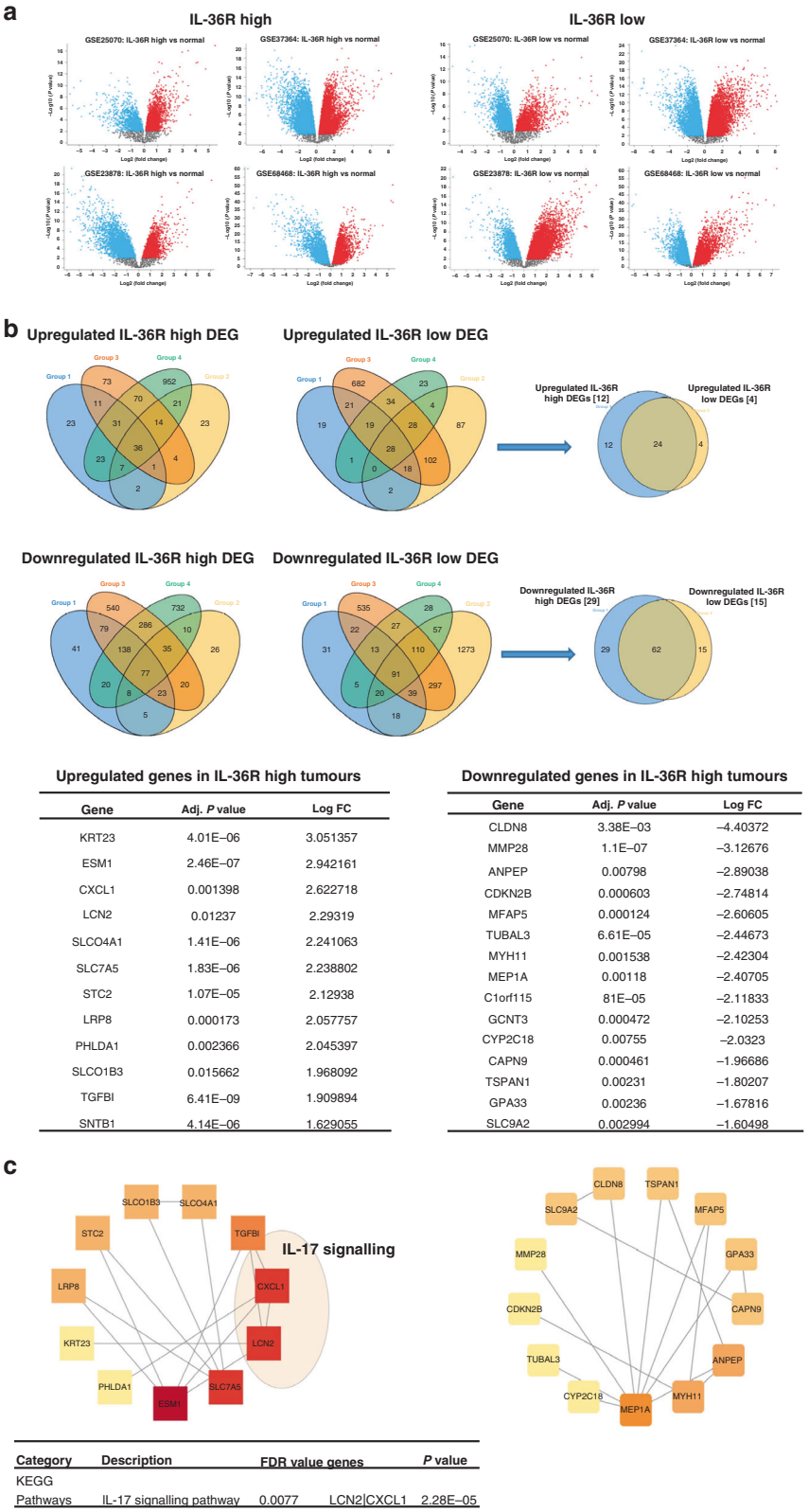
In order to investigate the clinical outcome of dysregulated IL-36 signalling in CRC tumours, the 5-year survival rate of patients with tumours containing high versus low expression of IL-36 family members, was investigated (Fig. 3). All family members, with the exception of IL-36α were investigated using the TCGA-COAD dataset. This dataset was selected as it contained a large number of bulk tumour samples and used standardised processing with sufficient numbers to quartile data which still contained large sub-cohorts for survival analysis. As this dataset did not contain IL-36α, a multicohort dataset (Table 1) was used to investigate the 5-year survival rate of patients with IL-36α high expressing versus IL-36α low expressing tumours. These datasets were selected based on samples being bulk tumour samples and transcriptomic analysis carried out by the same platforms and normalisation method, as described in Table 1. No significant difference in the 5-year survival rate was observed for any family member with the exception of the IL-36R, with patients whose tumours had high expression of the IL-36R having a significantly reduced 5-year survival ($P = 0.025$), particularly in the first 4 years of the disease.

Differential gene expression shows increased tumoral IL-36R expression is associated with increased IL-17 signalling

A total of four independent datasets were selected to identify DEGs in IL-36R high and IL-36R low expressing tumours in comparison to normal colonic tissue. Datasets were selected based on the availability of adjacent normal tissue samples, transcriptomic analysis type (microarray), sample numbers ($n > 20$) and sample types (bulk RNA from tissue). Independent datasets were favoured over one large individual dataset to minimise technical bias. A total of 12 upregulated and 15 downregulated genes were identified, which were unique to IL-36R high-expressing tumours, when compared to normal colon tissue (Fig. 4a, b). Both sets of genes underwent STRING protein–protein interaction (PPI) analysis in order to identify clusters of closely associated genes. Functional enrichment revealed only one small cluster, consisting of LCN2 and CXCL-1 genes, which were annotated by KEGG analysis to be involved in IL-17 signalling (Fig. 4c). No significant clusters were identified in downregulated genes in IL-36R high tumours. These data suggest that upregulated IL-17 signalling may be an associated factor contributing to the pathogenesis of CRC, in particular with regard to IL-36R high-expressing tumours.

IL-36 family members show a positive correlation with genes involved in IL-17 signalling in CRC

In order to further investigate the IL-36/IL-17 crosstalk in the context of CRC, a correlation matrix was completed for IL-36 family members and IL-17 signalling annotated genes from KEGG pathway analysis. Hierarchal clustering showed a positive, correlative relationship to exist between IL-17 family genes and IL-36 family members, as well as chemokines and cytokines involved in the IL-17 signalling pathway (Fig. 5a). Given the pro-tumorigenic functions identified for CXCL-1 in CRC [39, 40] and the PPI findings shown in Fig. 5, CXCL-1 expression was further investigated in the context of expression of the IL-36R gene (IL1RL2). Using the AnalyzeR web tool (Fig. 5b), CXCL-1 was shown to have a positive correlation with IL1RL2 expression in normal intestinal tissue ($R = 0.37$). Furthermore, this correlation was stronger in intestinal cancer tissue in comparison to normal intestinal tissue ($R = 0.61$). LCN2 gene expression was shown to



Upregulated genes in IL-36R high tumours

Gene	Adj. *P* value	Log FC
KRT23	4.01E−06	3.051357
ESM1	2.46E−07	2.942161
CXCL1	0.001398	2.622718
LCN2	0.01237	2.29319
SLCO4A1	1.41E−06	2.241063
SLC7A5	1.83E−06	2.238802
STC2	1.07E−05	2.12938
LRP8	0.000173	2.057757
PHLDA1	0.002366	2.045397
SLCO1B3	0.015662	1.968092
TGFBI	6.41E−09	1.909894
SNTB1	4.14E−06	1.629055

Downregulated genes in IL-36R high tumours

Gene	Adj. *P* value	Log FC
CLDN8	3.38E−03	−4.40372
MMP28	1.1E−07	−3.12676
ANPEP	0.00798	−2.89038
CDKN2B	0.000603	−2.74814
MFAP5	0.000124	−2.60605
TUBAL3	6.61E−05	−2.44673
MYH11	0.001538	−2.42304
MEP1A	0.00118	−2.40705
C1orf115	81E−05	−2.11833
GCNT3	0.000472	−2.10253
CYP2C18	0.00755	−2.0323
CAPN9	0.000461	−1.96686
TSPAN1	0.00231	−1.80207
GPA33	0.00236	−1.67816
SLC9A2	0.002994	−1.60498

Category	Description	FDR value genes		*P* value
KEGG Pathways	IL-17 signalling pathway	0.0077	LCN2|CXCL1	2.28E−05

have a similar change in increased positive correlation in intestinal cancer tissue ($R = 0.81$) in comparison to normal intestinal tissue ($R = 0.44$). To validate these in silico findings, CXCL-1, LCN2 and IL-36R expression was first quantified in a patient cohort consisting of paired adjacent normal and malignant colon tissue (Fig. 5c). Expression of each of these genes was significantly increased in CRC tissue compared to the respective control samples. To further examine the relationship between IL-36R expression and identified IL-17 signalling genes, human HT29 colon cancer cells were stimulated with IL-36γ (50 ng/mL). IL-36γ significantly increased

Fig. 4 High expression of IL-36R in CRC is associated with IL-17 signalling pathway genes. a Using GEO2R, DEG analysis was performed on the four datasets (GSE68468, GSE37364, GSE23878 and GSE25070) to identify significantly altered gene expression in comparison to normal healthy colonic tissue. In each dataset, cancer samples were stratified into two groups; IL-36R high (upper 25% of IL-36R expressing cancer samples) and IL-36R low (lower 25% of IL-36R expressing tumours). **b** DEG's present in all four datasets assessed were identified in IL-36R high and IL-36R low tumours and were grouped according to being upregulated (Log2 fold change >1.5, $P < 0.05$) or downregulated (Log2 fold change <1.5, $P < 0.05$). Upregulated or downregulated genes were checked for overlapping genes between IL-36R high and low tumours in order to identify DEGs unique to IL-36R high tumours (i.e., 12 upregulated genes). **c** Protein–protein interactions were identified using the STRING database, PPI networks visualised using Cytoscape. Gene modules of interconnected proteins were identified by Markov clustering. Functional gene enrichment (KEGG) was used to identify and annotate gene clusters in upregulated and downregulated genes in IL-36R high tumours.

the expression of both identified DEG genes associated with IL-17 signalling, namely CXCL-1 and LCN2 (Fig. 5d) in these cells.

IL-36γ, in combination with either IL-17 or IL-22, can synergistically induce chemokine expression in CRC cells in vitro, whilst only IL-17 can augment IL-36-induced proliferation

To further investigate the relationship between IL-36 and IL-17 signalling, HT29 cells were stimulated with IL-36γ (50 ng/mL) or IL-17a (50 ng/mL), either alone or in combination, and expression of key genes in these signalling pathways investigated. While the IL-17 receptor was more highly expressed than the IL-36R receptor (Fig. 6a), IL-36γ alone was shown to strongly induce CXCL-1, CCL20, LCN2 and IL-23 whereas IL-17a alone had no effect (Fig. 6b). A synergistic induction of the myeloid and lymphoid chemoattractants, CXCL-1 and CCL20, respectively, was shown when cells were stimulated with both IL-36γ and IL-17a. Given the strong proliferative capacity of IL-36 signalling, which has been previously reported [27, 41], the effect of IL-17 and IL-36 co-stimulation on HT29 cell proliferation was investigated. CFSE cell trace assays showed IL-36γ and IL-17a could additively increase the number of times HT29 cells had undergone cell division (Fig. 6c). Approximately 39% of untreated cells had undergone two or more cellular divisions over four days in culture, whilst 49% of IL-17a and 63% of IL-36γ-treated cells had completed at least two cellular divisions. Cells co-stimulated with IL-36γ and IL-17a showed a total of 68% of cells had undergone at least two cellular divisions. This was reflected in resazurin reduction assays, which showed that IL-17 could further augment the proliferative effect of IL-36γ on HT29 cells (Fig. 6d).

Given the close association between IL-17 and IL-22 [42], the above assays were repeated with IL-22 in place of IL-17 stimulation. Similar to the IL-17R, the IL-22R was more highly expressed in these cells than the IL-36R (Fig. 6e), although cells were more responsive to IL-36γ stimulation alone (Fig. 6f). IL-22 acted synergistically with IL-36γ to induce CXCL-1 and LCN2 gene expression, indicating a novel role for these cytokines in the induction of these genes in CRC cells. Once more, cellular division and proliferation were assessed by CFSE cell trace assays and resazurin reduction following stimulation of cells with IL-36γ and/or IL-22. Unlike IL-17a, IL-22 did not augment IL-36γ-mediated cellular proliferation (Fig. 6g/h). This data strongly indicates that crosstalk exists between the IL-36 and the IL-17/IL-22 signalling pathways, which can induce gene expression of discrete cytokines and chemokines expression, as well as additively modulate colon cancer cell proliferation.

DISCUSSION

Given the recently described divergent roles for the IL-36 family in cancer [11], the aim of this study was to utilise transcriptomic analyses to identify the potential roles of IL-36 signalling in CRC. A diverse array of transcriptomic data was processed and analysed in order to gain insights into the pleiotropic nature of IL-36 cytokines in CRC. We have shown a significant increase in IL-36 family member expression from normal colonic mucosal tissue to tumour tissue to distant metastasis. TCGA-COAD derived Kaplan–Meier graphs showed an association between decreased IL-36R expression and improved patient outcomes. Furthermore, by DEG analysis, PPI analysis and Markov clustering, we have shown that tumours that express high levels of the IL-36R are associated with increased IL-17 signalling, which may contribute to disease pathogenesis. We have confirmed several of these in silico findings using both cell lines and a patient cohort. Moreover, we have also shown that IL-36 signalling acts synergistically with IL-17 and IL-22 to induce key chemokines involved in the IL-17/IL-22/IL-23 axis. Collectively our analysis indicates tumoral IL-36 signalling to be an important therapeutic target for investigation.

Oncomine analysis has been previously utilised to show that IL-36γ expression is decreased in metastatic melanoma tissue in comparison to primary melanoma and melanoma pre-cursor tissue [21]. Similarly, IL-36γ expression has been shown to be reduced in hepatocellular carcinoma (HCC) in comparison to paired adjacent tumour samples, whilst IL-36α expression is reduced in moderately and poorly differentiated HCC patient tissues [43, 44]. In contrast, our analysis of the colorectal cancer dataset, GSE68468, showed no significant changes in the expression of IL-36γ, whilst IL-36α expression was seen to increase in CRC metastatic tissue, indicating that expression of IL-36 agonists appear to be differentially regulated across different cancer types. Indeed, the most significant changes observed in the datasets assessed here were in the IL-36R and the IL-36RN, with these being significantly increased and decreased, respectively. Furthermore, analysis of IL-36 family members showed increased expression of IL-36R in nearly all anatomical colonic locations in malignant tissue in comparison to normal adjacent. As both pro- and anti-tumorigenic roles for IL-36 have now been reported across different cancer types, it is possible that such changes in expression levels may be indicative of a tissue-specific role for the IL-36 family members. Of note, in both melanoma and HCC, only anti-tumorigenic roles for IL-36 cytokines have been reported and these tumours both showed decreased expression of IL-36 cytokines. In contrast, both pro- and anti-tumorigenic roles have been reported for IL-36 in CRC. Further analysis of the expression levels of the IL-36 family members across multiple tumour types will facilitate a greater understanding of the tissue-specific roles of IL-36 family members in tumorigenesis.

Recent studies investigating IL-36 signalling have shown that IL-36Ra and IL-38, another IL-36R natural antagonist, can inhibit the ability of IL-36R signalling to increase tumour cell migration and proliferation [26, 27]. Previous work by our group has shown that both IL-36β and IL-36γ can potently drive the migration and invasion of colon cancer cells in vitro [27]. Other recent studies have shown that IL-36γ and IL-36Ra may modulate cell–matrix adhesion molecules and Wnt signalling, two key components to the metastatic potential of primary tumours and stemness. Our analysis of transcriptomic datasets further suggests a potential pro-metastatic role for IL-36R tumoral signalling in CRC, with both an increase in IL-36R and a converse decrease in IL-36RN gene expression shown in lung metastasis compared to normal colonic tissue. These data support the concept that IL-36 cytokine

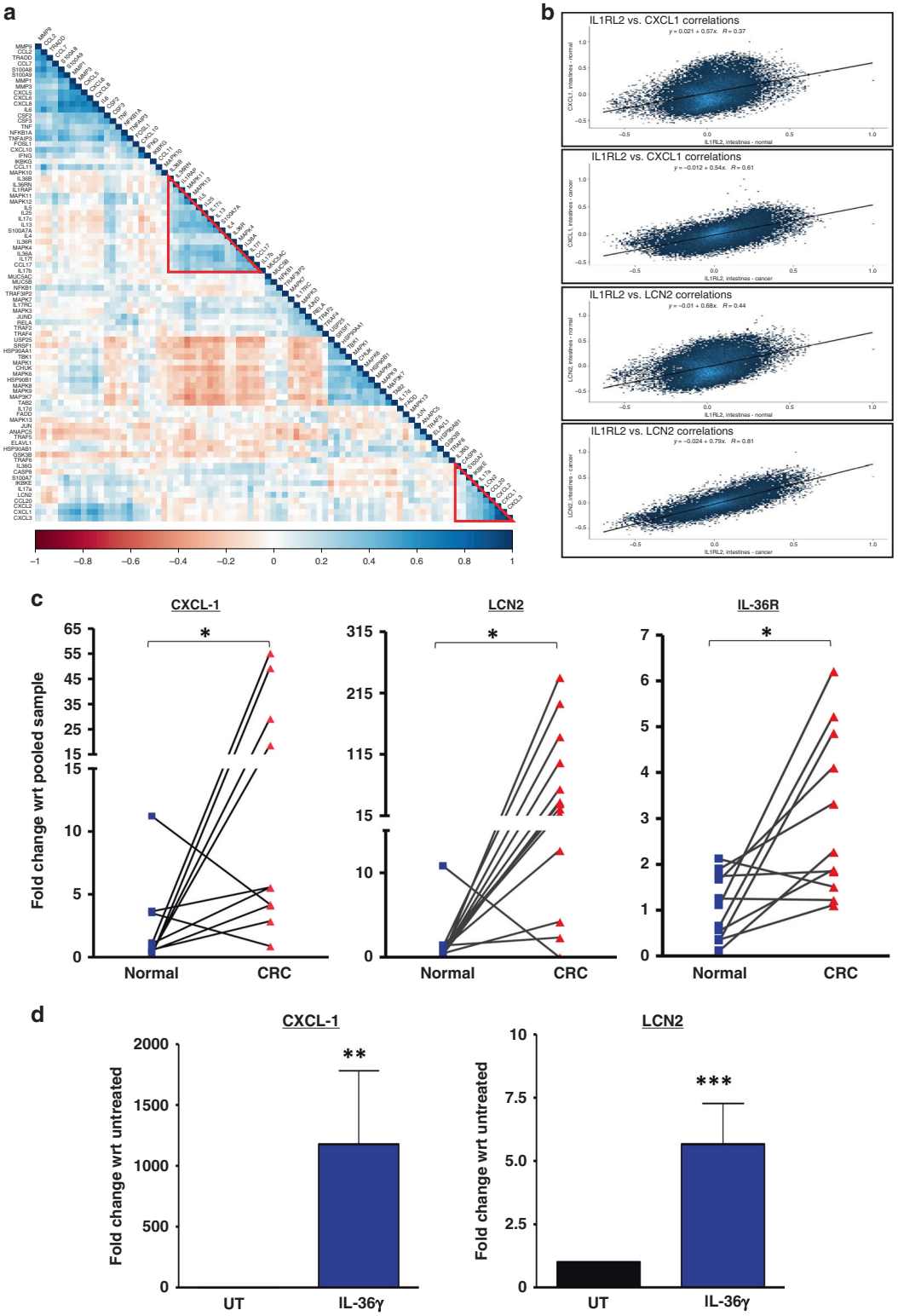
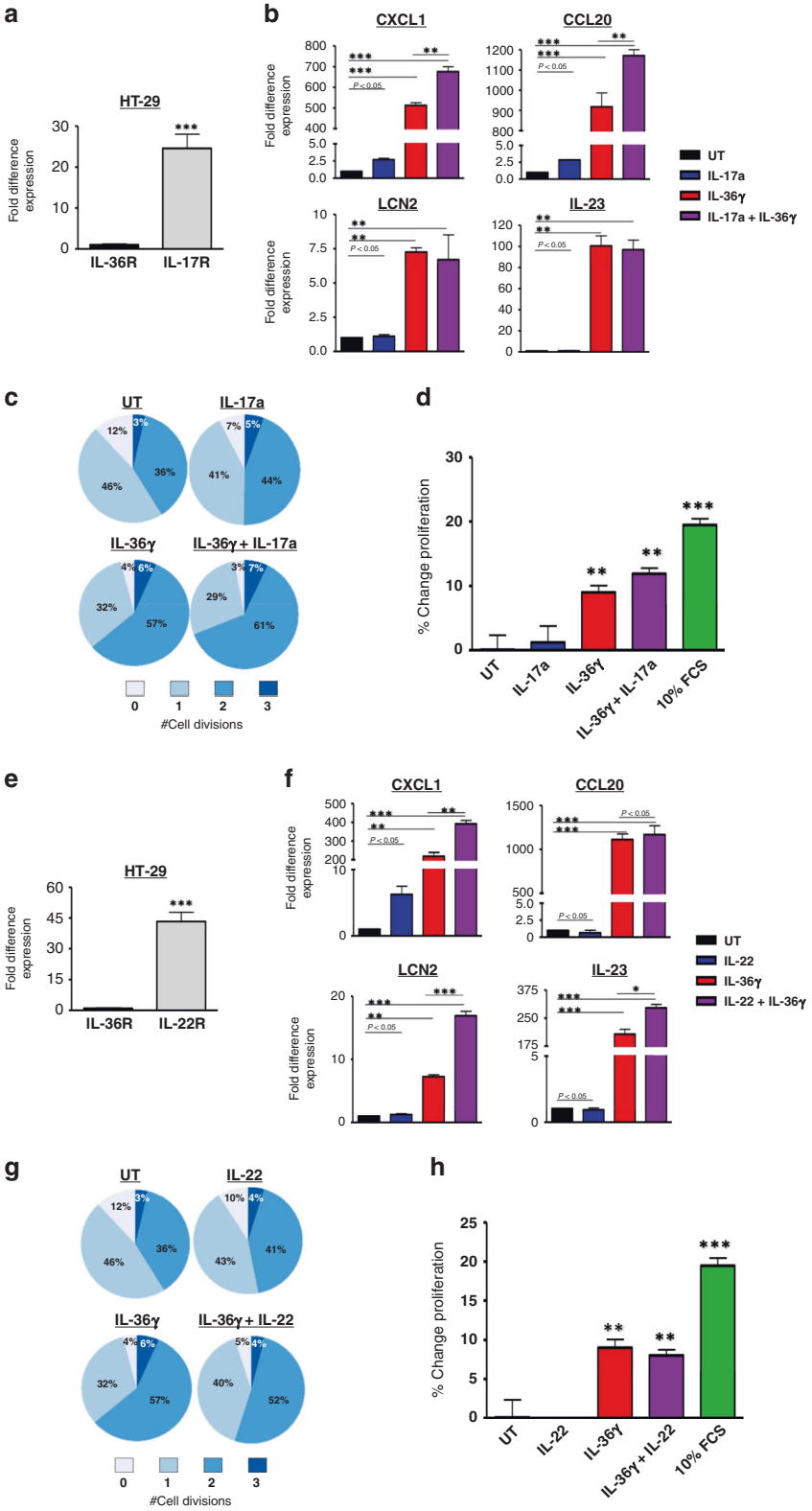


Fig. 5 IL-36 and IL-17 signalling genes are associated in vitro and ex vivo. a A correlation matrix was graphed comparing IL-36 family member gene expression and KEGG pathway 'IL-17 signalling pathway' genes using the dataset GSE39582. **b** Correlation plots between cluster genes identified from DEG analysis and IL-36R expression were analysed using the AnalyzeR web tool in normal and malignant intestinal tissue. **c** Gene expression (mRNA) in human colon cancer and normal tissue was investigated in CXCL-1, LCN2 and IL-36R. **d** HT29 cells were stimulated with 50 ng/mL IL-36γ. CXCL-1 and LCN2 gene expression changes were detected by qRT-PCR after 4 h relative to untreated cells. The statistical significance computed by paired Student's *T* test is annotated by the number of stars (**P* value < 0.05; ***P* value <0.01; ****P* value <0.001).



signalling, most likely on tumour cells, may contribute to the metastatic niche of colon cancer cells.

Findings from this study indicate that increased tumoral expression of the IL-36R is associated with poorer patient outcomes. Contrasting findings have been published in previous studies with regards to IL-36 family member expression and patient survival across different tumour types. Increased IL-36α has been shown to be associated with improved patient survival in HCC, with poorer prognosis being associated with a decrease in IL-36α expression [44]. A similar association for IL-36γ has also been demonstrated in HCC, with increased IL-36γ associated with improved patient outcomes [43]. Similarly, a decrease in the

Fig. 6 IL-36 cytokines, in combination with IL-17 or IL-22, can synergistically induce chemokine expression in CRC cells. a The relative expression of the IL-36R and the IL-17 receptor by HT29 cells was assessed by qRT-PCR. **b** HT29 cells were stimulated with IL-36γ (50 ng/mL), IL-17a (50 ng/mL) or a combination of both (50 ng/mL of each cytokine) and expression of several chemokines involved in the IL-17 signalling pathway assessed by qRT-PCR. Cellular division and proliferation were assessed in HT29 cells by (**c**, **g**) CFSE cell division assay and by (**d**, **h**) resazurin reduction assays in response to similar stimulants as described for the above gene expression analysis, with 10% FCS used as a positive control for cellular proliferation. Cellular division assays are represented by pie charts indicating the percentage of cells having undergone increasing cell divisions following the indicated stimulant(s) over 96 h. **e** Gene expression of the IL-22 receptor was assessed by qRT-PCR in HT29 cells, as well as several chemokines involved in the IL-17 signalling pathway following stimulation with (**f**) IL-36γ (50 ng/mL), IL-22 (50 ng/mL) or a combination of both (50 ng/mL of each cytokine). Graphs are representative of three independent experiments completed in technical duplicate. The statistical significance computed by one-way ANOVA is annotated by the number of stars (*P value < 0.05; **P value <0.01; ***P value <0.001).

expression of IL-36α has been associated with poorer patient outcomes in epithelial ovarian cancer [45]. Little has been reported to date regarding expression of IL-36 family members and patient prognosis in CRC, with one study reporting that IL-36α expression was associated with improved patient CRC survival, although the same report demonstrated that lower levels of IL-36γ was also associated with improved patient survival [46]. Our analysis did not observe any association for either IL-36α or IL-36γ with patient outcome in CRC, but did observe a clear association between improved patient outcome and lower expression of the IL-36R. Further multivariate analysis of IL-36R expression and clinicopathological features, extending beyond AJCC staging, may reveal roles for IL-36R signalling in CRC. The contrasting findings observed between different cancer types for different IL-36 family members may be influenced by the TME composition, given the direct effects IL-36 signalling may have on tumour cells and immune cells, which can result in both anti-tumour and pro-tumour signalling [28, 47].

Direct signalling of IL-36 on tumour cells and crosstalk with other pathways has not been extensively investigated in colon cancer. The majority of epithelial cell IL-36 signalling findings have been produced whilst investigating inflammatory diseases such as psoriasis and IBD, which show downstream induction of many chemokines, particularly myeloid chemoattractants [48]. In these and other diseases, activation of the IL-36 signalling pathway has been closely associated with activation of the IL-17/IL-23 signalling axis, an association which has not, to date, been reported in cancer [49–51]. Our DEG findings suggest crosstalk may exist in CRC between IL-36 and the IL-17/IL-22/IL-23 axis. Hierarchal clustering showed positive correlations of gene expression between IL-17 pathway genes and IL-36 family genes. Furthermore, analysis of IL-36R high DEGs, CXCL-1 and LCN2, revealed a stronger correlation with IL1RL2 (IL-36R) gene expression in neoplastic tissue in comparison to normal intestinal tissue, suggesting this axis may be most active during malignancy. These in silico findings are reflected in our own patient cohort expression data, showing CXCL-1, LCN2 and IL-36R expression to all be increased in tumour tissue relative to adjacent normal tissue. It has been shown that as well as being closely linked with IL-36 signalling, the IL-17 signalling pathway is well characterised in the direct pathogenesis of CRC via STAT3 activation, resulting in the expression of genes such as matrix metalloproteases (MMPs) and anti-apoptotic genes [52].

Furthering our transcriptomic findings, we have identified that IL-17, and the closely associated cytokine IL-22, can act synergistically with IL-36γ to induce key chemokines involved in the IL-17 signalling pathway in CRC. Both IL-17a and IL-22 synergistically induced the myeloid cell chemoattractant, CXCL-1, which can recruit macrophages and neutrophils to tissue sites [40]. Both of these cell types have been previously described as important sources of IL-36 cytokines and IL-36 cleaving proteases [53], respectively, which may therefore potentially propagate a pro-tumorigenic feedback loop via IL-36/IL-17/22 signalling. Co-stimulation of cells with IL-36γ and IL-22 synergistically induced IL-23, a key cytokine for the activation of Th17 and Th22 cell secretion of their respective chemokines, indicating an additional means of augmenting this IL-36 and IL-17/22 pathway [51]. A role for IL-36 signalling in IL-23 induction has previously been reported in intestinal tissue, however, this was shown to drive classical IL-23 production from IL-36-stimulated dendritic cells [54]. Here, we show that IL-23 expression in colon cancer cells can be strongly driven by IL-36γ which may thereby contribute to Th17 and Th22 secretion, although this will require further experimentation to prove these interactions between cell types. Collectively, our gene expression induction data indicates that IL-36 stimulation of colon cancer cells may lead to CXCL-1-modulated myeloid cell infiltration to provide further IL-36 cytokines and IL-36 activating proteases, CCL20-modulated T-cell recruitment and direct induction of IL-23 expression for activation of infiltrating T cells. These mechanisms may potentially result in a positive feedback loop propagating this IL-36/IL-17/IL-22 signalling axis, as similarly described in other inflammatory pathologies [51, 55]. Further co-culture assays will be capable of elucidating and confirming this novel colon cancer cell and immune infiltrate crosstalk.

IL-17 has been shown to synergistically induce epithelial cellular proliferation in combination with IL-36 in keratinocytes [55]. Indeed IL-17 and IL-36 have both previously been shown to be capable of directly inducing intestinal epithelial cell proliferation, with reports of IL-17 also being capable of indirectly inducing cancer cell proliferation via fibroblast activation [56, 57]. Here, we have identified a similar direct response in colon cancer cells to IL-17 and IL-36 signalling, albeit in an additive manner, further implicating IL-36 and IL-17 signalling to contribute to colon cancer tumorigenesis. These signalling pathways activate the NF-κB and STAT3 transcription factors which can result in the upregulation of LCN2, a gene identified in this study as a key upregulated gene in IL-36R high tumours. IL-36-mediated induction of LCN2 has been previously reported however conflicting roles for LCN2 in colon cancer have been described [58, 59]. Reflective of recent IL-36 signalling findings in CRC, it has been reported that LCN2 may contribute to carcinogenesis via anti-apoptotic pathways resulting in enhanced cancer cell proliferation [60]. Contrasting this, it has also been reported that LCN2 can negatively regulate colon cancer cell proliferation via metabolic alterations [61], highlighting the requirement to delineate the role of LCN2 in IL-36-mediated CRC cell proliferation.

In conclusion, our analysis of transcriptomic datasets further supports the recent identification of a pro-tumorigenic function for the IL-36R in CRC. Our findings suggest that inhibition of this signalling pathway on cancer cells may benefit overall patient survival and that IL-36 interplay with the IL-17/IL-23 axis may contribute to colon carcinogenesis, and targeting this axis may be a beneficial therapeutic avenue to investigate.

DATA AVAILABILITY

The datasets analysed during the current study are available publically, as outlined in Table 1. The data underlying this article will be shared on a reasonable request to the corresponding author.

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AUTHOR CONTRIBUTIONS

KJB conceived and designed work, performed both transcriptomic analysis and experimental work and was involved in the writing of the manuscript. EB and AH conceived and designed work, undertook the data analysis and writing of the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the University College Cork Clinical Research Ethics Committee of the Cork Teaching Hospitals (ECM (3) P 3 September 2013). All samples were obtained during surgery at the Mercy University Hospital Cork following informed consent.

CONSENT TO PUBLISH

Not applicable.

ADDITIONAL INFORMATION

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