ORIGINAL RESEARCH

Therapeutic potential of FLANC, a novel primate-specific long non-coding RNA in colorectal cancer

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► Additional material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/gutjnl-2019-318903).

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Received 16 April 2019
Revised 21 November 2019
Accepted 24 December 2019
Published Online First 27 January 2020

To cite: Pichler M, Rodriguez-Aguayo C, Nam SY, *et al*. *Gut* 2020;**69**:1818–1831.

ABSTRACT

Objective To investigate the function of a novel primate-specific long non-coding RNA (lncRNA), named FLANC, based on its genomic location (co-localised with a pyknon motif), and to characterise its potential as a biomarker and therapeutic target.

Design FLANC expression was analysed in 349 tumours from four cohorts and correlated to clinical data. In a series of multiple in vitro and in vivo models and molecular analyses, we characterised the fundamental biological roles of this lncRNA. We further explored the therapeutic potential of targeting FLANC in a mouse model of colorectal cancer (CRC) metastases.

Results FLANC, a primate-specific lncRNA feebly expressed in normal colon cells, was significantly upregulated in cancer cells compared with normal colon samples in two independent cohorts. High levels of FLANC were associated with poor survival in two additional independent CRC patient cohorts. Both in vitro and in vivo experiments demonstrated that the modulation of FLANC expression influenced cellular growth, apoptosis, migration, angiogenesis and metastases formation ability of CRC cells. In vivo pharmacological targeting of FLANC by administration of 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine nanoparticles loaded with a specific small interfering RNA, induced significant decrease in metastases, without evident tissue toxicity or pro-inflammatory effects. Mechanistically, FLANC upregulated and prolonged the half-life of phosphorylated STAT3, inducing the overexpression of VEGFA, a key regulator of angiogenesis.

Conclusions Based on our findings, we discovered, FLANC as a novel primate-specific lncRNA that is highly upregulated in CRC cells and regulates metastases formation. Targeting primate-specific transcripts such as FLANC may represent a novel and low toxic therapeutic strategy for the treatment of patients.

Significance of this study

What is already known on this subject?

- Primate-specific lncRNAs represent a newly discovered class of transcripts in need for functional characterisation in human carcinogenesis.
- RNA-based targeting strategies have entered clinical trials in humans in different diseases.

What are the new findings?

- Using data about genomic patterns of DNA motifs in cancer-associated regions, we were able to identify a novel primate-specific lncRNA, that we termed FLANC.
- FLANC is upregulated in CRC tissue and poorly expressed in normal colon cells, and possesses prognostic potential in CRC.
- FLANC influences cellular growth, migration, anchorage-independent growth, angiogenesis and metastases formation in vivo.
- Mechanistically, FLANC induces angiogenesis via STAT3/VEGFA pathway.
- Targeting FLANC by small interfering RNA carrying nanoparticles reduces angiogenesis and represents a potential novel cancer therapeutic approach with higher specificity for malignant cells.

How might it impact on clinical practice in the foreseeable future?

- The primate-specific lncRNA FLANC represents a novel CRC driver, a prognostic factor and a therapeutic target.

INTRODUCTION

Cancer mortality represents a huge medical issue,[1] in spite of major advances in the characterisation of the genomic alterations in most cancers types[2]

and the development of a plethora of biomarkers[3] and targeted therapies.[4] Colorectal cancer (CRC) is the third most common malignancy in high-income countries[5] and a substantial number of patients initially present or sequentially develop distant metastases, leading to a poor 5-year cancer-specific survival rate of about 10%–20%.[6] Treatment options for metastatic CRC are limited, and include combination cytotoxic chemotherapy with 5-fluorouracil, irinotecan and/or oxaliplatin, as well as the addition of antiangiogenic (antibodies against vascular endothelial growth factor (VEGF) and/or its receptor) or epidermal growth factor receptor (EGFR)-directed drugs.[7]

Within the last decade, non-coding (nc)RNA alterations have been identified to be involved in the progression from adenoma-to-carcinoma in colorectal carcinogenesis.[8 9] Several ncRNAs, which include long non-coding RNAs (lncRNAs) and short microRNAs (miRNAs), have recently been functionally characterised.[10–14] Because of their cancer-specific expression pattern, lncRNAs carry tremendous potential for novel targeted treatment approaches.[15 16] Although the number of lncRNAs, including the primate-specific/human-specific ones,[17–22] probably will surpass the number of coding genes,[23 24] at present time only few of the former are proved to be involved in cancers,[25] as the main focus of the translational research nowadays is on genes (coding or non-coding) highly conserved in evolution.[8 13 26] Recently, we reported that multiple genomic non-coding loci harbouring human-specific/primate-specific motifs, named pyknons[27], are differentially transcribed between CRC and normal colon and between poor versus good prognosis chronic lymphocytic leukaemia (CLL), and the expression levels correlate with patients' overall survival.[25] We further dissected the function of one of these transcripts in CRC, the lncRNA N-BLR, and we observed that it plays a key role in metastases. Herein, we functionally characterised a novel primate-specific lncRNA from one of these regions and we demonstrated the therapeutic potential of targeting this transcript, through RNA-based strategies, without evident tissue toxicity or pro-inflammatory effects in vivo.

METHODS

Patient samples

Four different patient cohorts were studied with the staging performed by Dukes classification: cohorts A[28] and B[25] for the evaluation of FLANC expression in CRC tissue, compared with matched normal colon, and two larger CRC cohorts, C (n=170) and D (n=126), for testing the prognostic value of FLANC (online supplementary table 1 to 3). The patients' clinicopathological data were collected from medical records at the same institutions. All cases were reviewed based on pathology reports and histological slides. Written informed consent was obtained from each patient for these four cohorts.

In vivo treatment of colorectal cancer metastases

Male athymic nude mice were purchased from Taconic Farms (Hudson, New York, USA). All animals were aged 6–8 weeks at the time of injection. Metastatic liver models of CRC were developed as described previously.[29] For all animal experiments, HCT116 cells (labelled with a stable expression luciferase gene) were used. For all therapeutic experiments, the dose of FLANC small interfering RNA (siRNA) was 200 µg/kg, as described previously.[30] The oligos were incorporated into neutral 1,2-dio leoyl-sn-glycero-3-phosphatidylcholine (DOPC) nanoliposomes. These were administered via intravenous injection twice weekly beginning 2 weeks after tumour cell injection and continued for 4 weeks. All mouse studies were approved and supervised by the MD Anderson Cancer Center Institutional Animal Care and Use Committee.

Additional methods are presented in the online supplementary methods section.

RESULTS

A novel primate-specific long non-coding RNA resides within the first intron of *CELSR1*

Following our initial report about enrichment of primate-specific motifs in ncRNAs involved in CRC,[25] we identified a novel transcript located at chromosome 22q13.31 in the first intron of the cadherin EGF LAG seven-pass G-type receptor 1 (*CELSR1*) gene (online supplementary figure 1A). The protein encoded by this gene is a member of the flamingo subfamily of the cadherin superfamily[31] and has been described to be associated with the Wnt signalling pathway in gastrointestinal (GI) malignancies and leukaemia.[32 33] According to its location, we named this novel transcript *fla*mingo *n*on-*c*oding RNA (FLANC). We studied FLANC following the workflow presented in figure 1A. By strand-specific qRT-PCR, we proved that FLANC is transcribed in the antisense direction compared with *CELSR1* (online supplementary figure 1B), being therefore an independent transcript from the *CELSR1* messenger RNA or primary RNA.

By using Rapid Amplification of cDNA Ends (RACE) on RNA isolated from the HCT116 cell line, we cloned a mono-exonic 873 nucleotides (nts) transcript from the *CELSR1* intron 1 (online supplementary figure 2A). Using in silico analysis, we were only able to identify the transcript in primates, with 98% homology with *Pan troglodytes* and 87% homology with *Gorilla* genomes. Further analysis of the sequence and conservation of this genomic region suggested that the evolution of this gene started within the class of mammalians and further evolved by the introduction of two Alu elements in primates (online supplementary figure 2B).

We next analysed the protein coding potential of FLANC using multiple methods. First, an in vitro transcription-translation assay suggested lack of protein coding potential (online supplementary figure 3A). We further used mass spectrometry for the identification of large proteins or small micropeptides (<100 amino acids long), and could not identify any of the peptides predicted by an open reading frame analysis of this transcript (online supplementary figure 3B). All these data support the lack of protein-coding potential of FLANC.

FLANC expression in colorectal cancer correlates with patients' overall survival

We first measured the expression of FLANC in a panel of eight CRC cell lines (online supplementary table 4). In five of them, FLANC expression was significantly higher than in five pooled normal colon tissues ($p<0.05$, online supplementary figure 1C). Additionally, we measured the level of FLANC in seven different organs and peripheral blood mononuclear cells (PBMCs) from healthy controls. FLANC was not detectable in healthy brain and liver tissues, was expressed at very low levels in normal colon, ovary, spleen and PBMCs tissues and was expressed at low levels in lung and testicular tissue (online supplementary figure 1D). Hence, we concluded that FLANC is a CRC-specific transcript.

To evaluate the levels of FLANC and its tissue location in CRC, we performed in situ hybridisation (ISH) on a commercially available tissue microarray. Significantly higher levels of FLANC were observed in cancerous (primary adenocarcinoma and metastatic tumours) compared with normal colon tissues

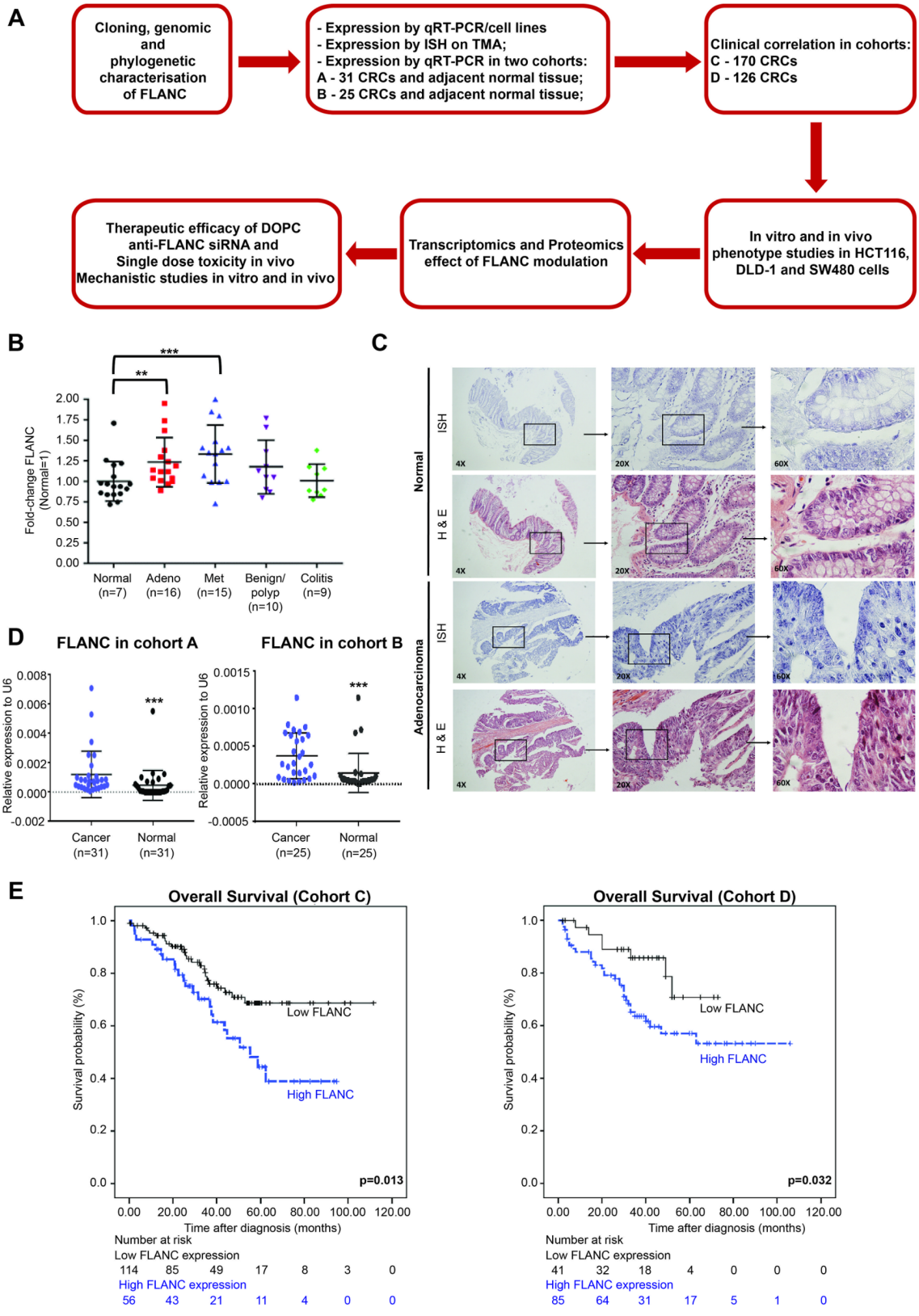

Figure 1 FLANC is overexpressed in colorectal cancer (CRC) and correlates with patients' overall survival. (A) Schematic workflow of the identification and comprehensive functional characterisation of FLANC in colorectal carcinogenesis. (B–C) Expression levels of FLANC in normal colon mucosa, adenocarcinoma of the colon, metastases of CRC, benign colon polyp and IBD, specifically detected by in situ hybridisation (ISH) on a tissue microarray. Overall, in adenocarcinoma ($p<0.01$) and metastatic tissue ($p<0.001$), FLANC is significantly upregulated in comparison to normal, benign polyps and inflamed colon tissue. The corresponding tissue slides indicate a nuclear and cytoplasmic upregulation and location of FLANC in epithelial cancer cells. (D) Using two independent cohorts, cancer tissue contained significantly higher expression levels of FLANC than corresponding normal colon tissue. (E) In addition, two large cohorts of patients with CRC were analysed and high expression levels of FLANC were associated with poor survival in both independent cohorts (log-rank test). Data are presented as means±SD (**$p<0.01$; ***$p<0.001$).

(metastatic CRC vs normal colon, p<0.001, primary adenocarcinoma vs normal colon, p<0.01). In contrast, there were no significant differences between colitis and benign/polyp lesions versus normal tissue, suggesting that the upregulation of FLANC probably occurs in epithelial malignant cells, and not in stroma cells, premalignant or in inflammatory lesions (figure 1B,C and online supplementary figure 4). This observation is supported by the fact that FLANC has very low expression in healthy tissues and PBMCs (online supplementary figure 1D), but further clarification by performing microdissection studies on CRC specimens is needed. Epithelial CRC cells of adenocarcinoma and metastatic tissue showed an enriched localisation of FLANC in both cytoplasm and nucleus when compared with non-malignant cells (figure 1C and online supplementary figures 4-5). Biochemical separation of nuclear and cytoplasmic RNA fractions confirms the ISH findings of expression in both cell compartments (online supplementary figure 5A, B). In line with the results from ISH analysis, FLANC was significantly upregulated, measured by qRT-PCR, in CRC tissue compared with paired normal colon tissue using two independent cohorts (p<0.01, A and B, see 'Methods' section). Of note, most of the normal tissues show no expression or very low expression of FLANC (figure 1D).

To test the value of FLANC as a prognostic biomarker, we performed clinical correlation in two additional independent CRC cohorts (C and D, see 'Methods' section and an overview about the two cohorts in online supplementary table 1): high expression levels of FLANC were associated with high pathological tumour stage in both cohorts and with vessel invasion in cohort C (in cohort D, information about vessel invasion was not available) (online supplementary tables 2 and 3). In the screening cohort C (n=170), high expression of FLANC was associated with poor overall survival using a Kaplan-Meier curve (p=0.013, log-rank test, figure 1E, left). Using univariate and multivariate Cox proportional models, FLANC expression represented an independent prognostic factor even after adjustment for other well-known prognostic factors including tumour differentiation and stage (table 1). For independent confirmation, we measured the FLANC expression in a validation cohort D (n=126) and confirmed the association of poor survival with high FLANC expression (figure 1E, right, p=0.032), which prevailed again in multivariate analyses (table 1).

Knock-down of FLANC inhibits cell proliferation, migration and induces apoptosis in colorectal cancer cells

In order to identify the biological roles of FLANC in colorectal carcinogenesis, we established two independent siRNAs targeting FLANC (plus strand, see online supplementary figure 1A). A ~40%–50% knock-down efficiency was obtained at 96 hours, whereas the host *CELSR1* gene expression (located on the minus strand) was not significantly affected by this treatment (HCT116 cell line, online supplementary figure 6A–D). Afterwards, we evaluated the biological effect of transient FLANC knock-down in a panel of three CRC cell lines expressing high levels of FLANC: the microsatellite instability (MSI) positive HCT116 and DLD-1 cells and the microsatellite stable (MSS) SW480 cells (online supplementary table 4 and online supplementary figure 1C). Reduction of FLANC expression induced a significant decrease in cellular growth in all tested CRC cell lines after 120 hours; this was verified by CCK8 assay (figure 2A). To confirm the ability of FLANC to regulate cell growth, we used a clonogenic assay as a second independent method. We measured a significant reduction in the number of colonies by FLANC knock-down after 10–14 days (about 40% for HCT116 (p<0.01), and 50%–70% reduction of colonies in SW480 (p<0.001) and DLD-1 cells (p<0.05), figure 2B,C). In contrast, in HT-29 cells, which contained almost undetectable levels of endogenous FLANC and were regarded as a negative control, FLANC knock-down did not affect cell growth ability and colony formation (online supplementary figure 7A,B). Next, we proved that the anchorage-independent growth ability of CRC cells substantially decreased when FLANC levels were reduced by siRNA (p<0.001 for all three cell lines, figure 2D). Moreover, the number and size of tumour spheres were significantly reduced in HCT116 and DLD-1 cells (p<0.001, online supplementary figure 7C,D). Because SW480 cells did not form tumour spheres under the selected conditions, we could not generate data for this cell line. In addition, knock-down of FLANC decreased migration of CRC cells (reduction between 40% and 50% for both, the HCT116 and SW480 cells, p<0.05, respectively, online supplementary figure 7E,F).

We further explored whether reduced levels of FLANC can induce apoptosis. We performed a multiparametric apoptosis assay which evaluated the different steps in the apoptotic cascade (ie, mitochondrial depolarisation, caspase 3/7 activation, phosphatidylserine exposure on the external leaflet of the plasma membrane and chromatin condensation) after siRNA-mediated knock-down. Reduction of FLANC levels induced an increase of apoptosis in HCT116 cells, as measured by the increase of the apoptotic markers evaluated (figure 3A and online supplementary figure 8). In order to confirm these findings with a second alternative assay, we established a second multiparametric apoptosis assay using fluorescence dye-based single cell high-resolution microscopy (online supplementary figure 9). Using this approach, we could confirm the activation of all steps of apoptosis induced by siRNA-mediated knock-down of FLANC, (χ^2=341.4, p<0.0001 figure 3B and online supplementary figure 10). Finally, we performed a third alternative assay (caspase activity) and we found that reduced levels of FLANC induced

Table 1 Univariate and multivariate analyses for predictors of overall survival in cohorts C and D

	Cohort C (n=170)						Cohort D (n=126)					
	Univariate			Multivariate			Univariate			Multivariate		
Variables	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value
Gender (female vs male)	0.94	0.53 to 1.66	0.828	0.97	0.53 to 1.78	0.933	1.22	0.63 to 2.35	0.549	1.02	0.50 to 2.09	0.941
Age (≧68 (median) vs <68)	0.59	0.33 to 1.04	0.071	1.77	0.97 to 3.23	0.059	0.78	0.41 to 1.48	0.450	1.20	0.59 to 2.44	0.600
Location (colon vs rectum)	0.87	0.49 to 1.55	0.648	0.95	0.53 to 1.72	0.888	1.35	0.69 to 2.64	0.370	1.44	0.69 to 2.96	0.323
Histological type (differentiated vs undifferentiated)	3.88	1.98 to 7.62	**<0.001**	4.27	2.09 to 8.73	**<0.001**	1.85	0.89 to 3.83	0.097	2.77	1.27 to 6.03	**0.010**
Stage classification (stage I–III vs stage IV)	6.76	3.83 to 11.93	**<0.001**	7.65	4.26 to 13.7	**<0.001**	7.47	3.9 to 14.30	**<0.001**	9.97	4.82 to 20.6	**<0.001**
FLANC expression (low vs high)	2.00	1.14 to 3.50	**0.015**	2.08	1.17 to 3.70	**0.012**	2.37	1.05 to 5.39	**0.038**	2.38	1.02 to 5.55	**0.044**

In bold—p<0.05.

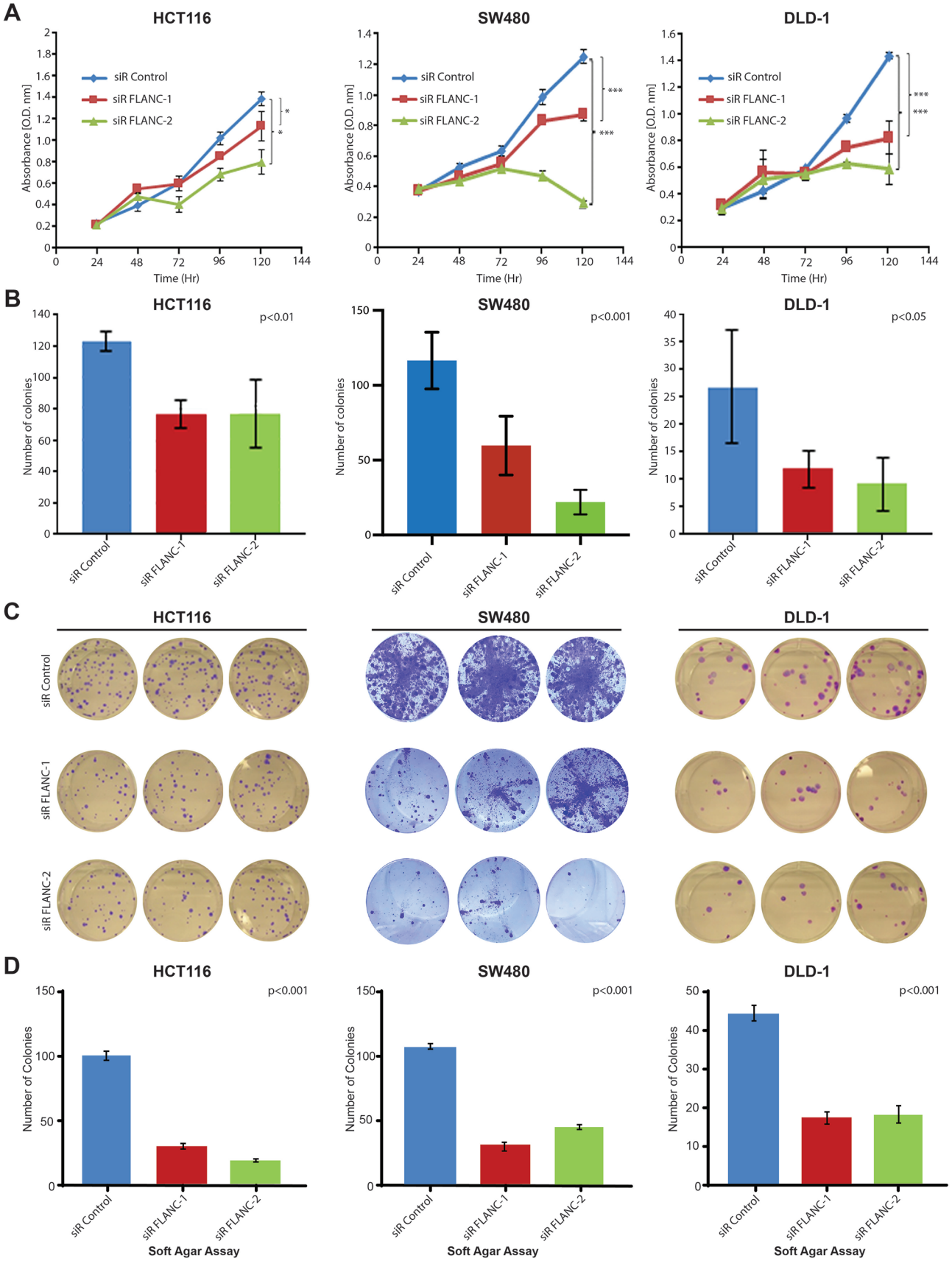

Figure 2 FLANC is an oncogene in CRC cell lines. (A) Cellular growth assays (CCK8) demonstrate that knock-down of FLANC leads to decreased proliferation in three independent CRC cell lines, which (B–C) is confirmed by lower numbers of colonies in an independent clonogenic growth assay. Analysis of variance test was used to test for differences through all groups. (D) Knock-down of FLANC decreased the ability of these three CRC cell lines to grow under anchorage-independent conditions in soft agar. Data are presented as means±SD (*p<0.05; ***p<0.001).

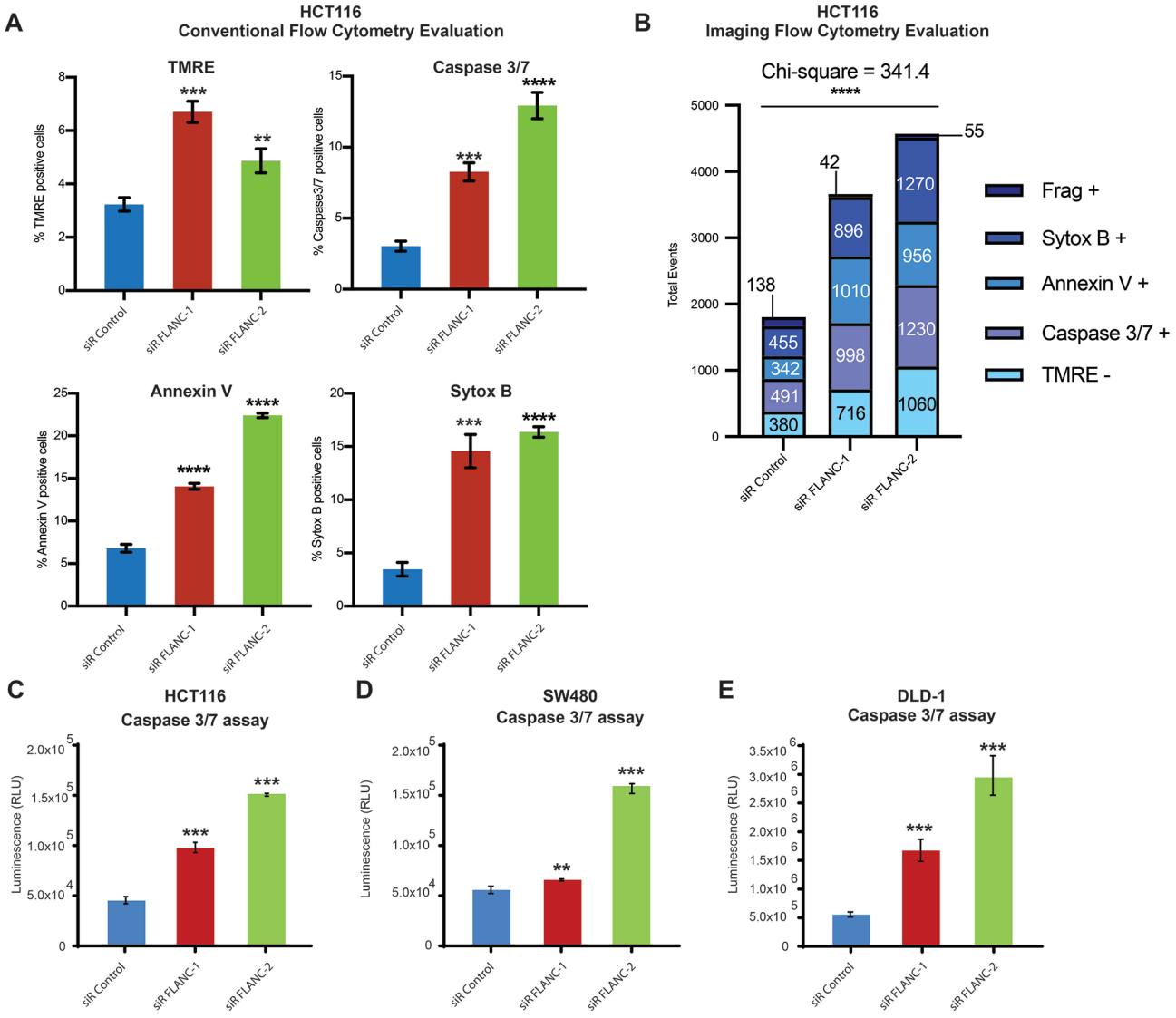

Figure 3 FLANC has an anti-apoptotic effect in colorectal cells (CRC). (A) A multiparametric apoptosis marker FACS assay shows that knock-down of FLANC increased all indicators of apoptosis, (B) which is confirmed by single cell high-resolution multiparametric apoptosis assay. Chi-square test was used to calculate differences between groups (C) caspase 3/7 assays confirmed the pro-apoptotic phenotype after knock-down of FLANC in all three CRC cell lines. Data are presented as means±SD (Student's t-test: **p<0.01; ***p<0.001; ****p<0.0001).

activation of caspase 3 and caspase 7 in HCT116, SW480 and DLD-1 cells (figure 3C–E). Our findings clearly indicated that knock-down of FLANC has a pro-apoptotic effect in CRC cells.

FLANC induces growth and metastatic spread of colorectal cancer in vivo

To evaluate the role of FLANC in vivo, we generated HCT116 clones which had either a stable knock-down of FLANC by two independent short hairpin RNAs (shRNA FLANC-1 and shRNA FLANC-2) or stable overexpression of FLANC transcript. The stable expression of shRNA targeting FLANC reduced FLANC levels by about 60%–80% compared with the empty vector control (online supplementary figure 11A). We performed cell growth, clonogenic growth and caspase 3/7 assays to confirm the results we observed after transient knock-down of FLANC by siRNAs. As shown in online supplementary figure 11B–D, cell growth rate and number of colonies decreased significantly in shRNA FLANC-1 and shRNA FLANC-2 clones, whereas apoptosis increased significantly. Conversely, HCT116 cells stably overexpressing FLANC (online supplementary figure 12A) had significantly increased proliferation rates (confirmed by two independent methods), increased ability to form colonies and decreased apoptosis (online supplementary figure 12B–E). To confirm these data in a second cell line, we generated FLANC overexpressing HT-29 cells. Here, we measured a significant increase in cellular growth (online supplementary figure 12F,G).

For in vivo confirmation of the observed phenotype, we subcutaneously injected 1×10^6 HCT116 stably expressing cells with: shRNA FLANC-1, shRNA control, FLANC overexpressing and empty vector control to the flank of male nude mice and measured the tumour volume for 7 weeks. The shRNA-mediated knock-down of FLANC led to a significant decrease in tumour volume in comparison with shRNA control (median/25th–75th percentiles: 295/282–341 mm^3 and 458/369–825 mm^3, p<0.01, figure 4A). Conversely, FLANC overexpression generated larger tumours compared with empty vector control (median/25th–75th percentiles: 766/497–1001 mm^3, 631/523–794 mm^3, respectively, p=ns, online supplementary figure 13A).

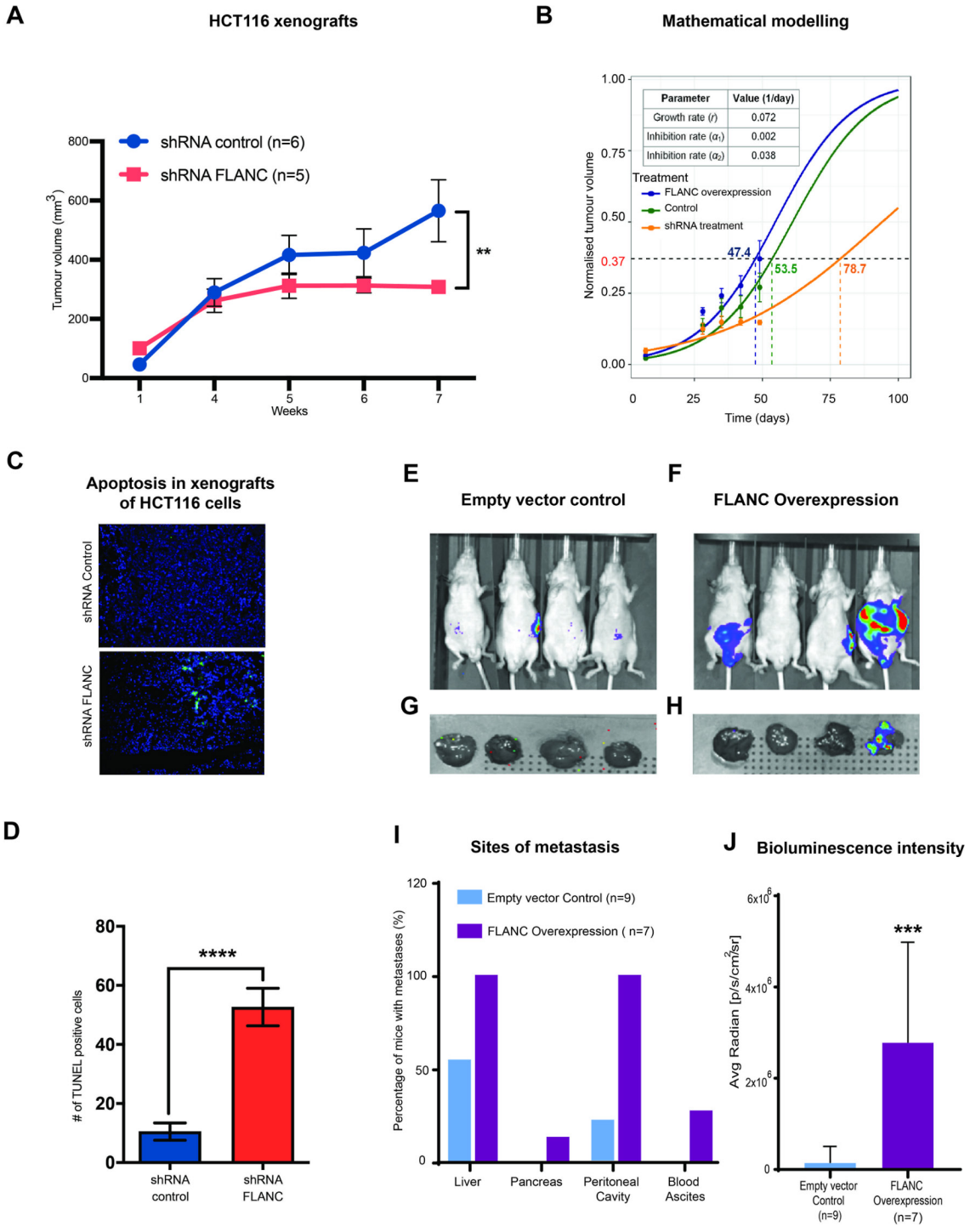

Figure 4 In vivo xenograft colon cancer model for the role of FLANC in tumour growth. (A) Mice injected with HCT116-shRNA-FLANC cells showed significantly smaller tumours than the shRNA control. (B) Prediction of tumour volume in different experimental conditions using a mathematical model (see supplementary materials for modelling details). Tumour growth rate was first estimated (r=0.072/day), and then drug inhibition rates for the control (α_1=0.002/day) and the shRNA-mediated knock-down of FLANC groups (α_2=0.038/day) were estimated. With these estimated parameters, the model successfully predicts experimentally measured tumour volume change over time. It can be observed that the model predicts the outcome with acceptable accuracy in all three conditions: FLANC overexpression (blue), control (green) and shRNA treatment (orange). (C–D) The TUNEL assay (marks apoptotic bodies) suggests that knock-down by shRNA-FLANC is able to induce apoptosis, compared with shRNA control. CRC metastases formation was assessed by applying intrasplenic injection of FLANC-expression manipulated HCT116 cells (E–J). Representative chemiluminescent images from whole body nude mice of empty control (E), FLANC overexpression (F) and from removed livers of the empty control (G) and FLANC overexpression cells (H) after approximately 7 weeks of intrasplenic injection with empty vector and FLANC overexpressing HCT116 clones are shown. (I) Overall numbers of mice positive by macroscopical exploration of different sites (liver, pancreas, intracavity of peritoneum and formation of bloody ascites). (J) The higher number of metastasis correlated with significantly higher intensity of bioluminescence signal in the livers of the group of FLANC overexpressing mice compared with those expressing empty vector control. Data are presented as means±SD (Student's t-test: **p<0.01; ***p<0.001; ****p<0.0001). shRNA, shorthairpin RNA.

Next, we modelled the tumour response to drug treatment based on our prior work on mathematical modelling of cancer.[34–36] We first fit the model (where the inhibition rate (α)=0) to the data for the FLANC overexpression group in order to determine the tumour growth rate (r). We then fit the model to drug treatment data to obtain tumour inhibition rate α for the control group and the shRNA FLANC group. The model successfully predicted treatment outcome (change of tumour volume over time) and reproduced experimentally obtained values with acceptable accuracy in all three conditions: FLANC overexpression, control, and shRNA FLANC (figure 4B). According to this model, to reach a tumour volume of 775 mm^3 at the end time point (corresponding to normalised value of 0.37), it takes 47.4 days for the FLANC overexpression group, 53.5 days for the control group, and 78.7 days for the shRNA FLANC treatment group (note these are model-predicted values).

Microscopic examination showed that tumours with FLANC knock-down induced higher number of apoptotic cells compared with shRNA control ($p<0.0001$) (TUNEL assay, figure 4C,D), but no difference could be observed between FLANC overexpression and empty vector control (p=ns) (online supplementary figure 13B,C).

Furthermore, we tested whether FLANC expression influences metastases formation in vivo. We performed intra-splenic injection of 1×10^6 HCT116 cells (empty vector control and FLANC overexpressing cells) and monitored metastases formation by luciferase intensity imaging and pathological exploration (figure 4E–H). After 6 weeks, we observed a higher number of macro-metastases detected by anatomical exploration of the metastatic sites in mice injected with HCT116 cells overexpressing FLANC compared with HCT116 cells expressing empty vector control (figure 4I). The higher number of metastasis correlated with significantly higher intensity of bioluminescence signal in the livers of the group of FLANC overexpressing mice compared with those expressing empty vector control (figure 4J). Overall, our data support that moderately increased levels of FLANC (HCT116 shRNA control vs shRNA FLANC) regulate tumour growth and highly increased levels (HCT116 FLANC overexpression vs FLANC empty vector control), regulate metastatic spread in vivo and the effects can be modelled for improving therapeutic efficacy.

FLANC expression modulation has genome-wide effects on transcripts and protein expression

The mechanisms of action of ncRNAs are numerous and still poorly understood.[8 13] Therefore, we performed gene expression analyses (GEA) from HCT116 clones with either downregulation (shRNA) or upregulation (overexpression) FLANC (GEO accession#: GSE127785 and GSE127786) (figure 5A–D) and combined the results with reverse phase protein array (RPPA) data (online supplementary figure 14A–D). On GEA, we identified 25 cancer-related pathways with negative correlation between the two types of clones; the most significantly dysregulated signalling pathways include the WNT signalling pathway and transcriptional regulation by TP53 (figure 5B–D), both well-known drivers of colon tumourigenesis.[37 38] We investigated the protein expression changes by RPPA[39] (online supplementary figure 14A). We detected 34 proteins to be upregulated and 43 downregulated in HCT116 FLANC overexpressing cells when compared with empty vector control (online supplementary table 5). The FLANC upregulated proteins were grouped into pathways and the top 20 stimulated pathways are shown in online supplementary figure 14B. Correspondingly, pathway analysis was also performed for the proteins inhibited by FLANC and the top 20 inhibited pathways are represented in online supplementary figure 14C. In order to visualise the proteins regulated by FLANC, we characterised the molecular network controlled by FLANC. According to the highest p values, the top five inhibited and top five activated pathways, with the corresponding proteins, are shown in online supplementary figure 14D. When combining the RPPA data with GEA data (online supplementary figure 15A), we identified 11 signalling pathways in common between the various types of profiling performed, with the most significantly abnormal being 'integrated cancer pathways', 'DNA damage response', 'retinoblastoma in cancer' and 'transcriptional regulation by TP53' (online supplementary figure 15B). All these data proved that the deregulation of FLANC has a widespread effect both on gene expression and protein regulation levels.

siRNA therapeutics targeting FLANC leads to decreased proliferation and metastasis in vivo

To study FLANC as a potential therapeutic target, we evaluated whether siRNA-mediated targeting of FLANC might result in effective antitumour activity. We determined the effects of systemic delivery of FLANC siRNA #2 on metastasis formation using FLANC siRNA incorporated into DOPC nanoliposomes. This vehicle is currently used for small RNAs delivery in clinical trials of patients with cancer (ClinicalTrials.gov identifier: NCT01591356). For this experiment, we injected HCT116 cells into the spleen of mice (n=10 each group) and monitored liver metastases formation by in vivo imaging. After 2 weeks, mice were randomly assigned to the following groups: untreated, treated with scrambled siRNA control and treated with FLANC siRNA, and we started administering the treatments twice a week (200 µg of siRNA/kg per injection). After 6 weeks, the body weight of mice treated with FLANC siRNA was significantly higher when compared with mice treated with the scramble siRNA (median/(25th–75th percentiles) body weight: 25.3/(24.41–27.76) g (control siRNA) vs 28.92/(25.75–29.86) g (FLANC siRNA, p=0.045), respectively. Regarding tumour growth-related parameters, we observed a significant decrease in bioluminescence signals in the whole mice (figure 6A), as well as in the isolated livers (figure 6B), in the FLANC siRNA group compared with siRNA scramble control (figure 6C). Overall, 5 out of 10 mice in the untreated group, 5 out of 10 mice in the siRNA scramble control group and 4 out of 8 mice in the siRNA FLANC group showed macroscopically visible liver metastases. The macroscopically countable liver metastases were 26 in the untreated mice, 21 in the siRNA control group and 6 in the siRNA FLANC group ($p<0.05$, analysis of variance test), respectively (figure 6D,E). The successful knock-down of FLANC was confirmed in vivo by performing ISH for FLANC (online supplementary figure 16). We next examined the effects of the treatment on proliferation (Ki67 staining) and apoptosis (TUNEL assay) in vivo. Administration of siRNA FLANC resulted in reduced tumour cell proliferation (figure 6F,G). Significant apoptosis induction was measured after the treatment with siRNA FLANC ($p<0.05$) compared with the corresponding siRNA scramble control (figure 6F and H). In summary, the treatment with siRNA FLANC reduced the number of macrometastases in

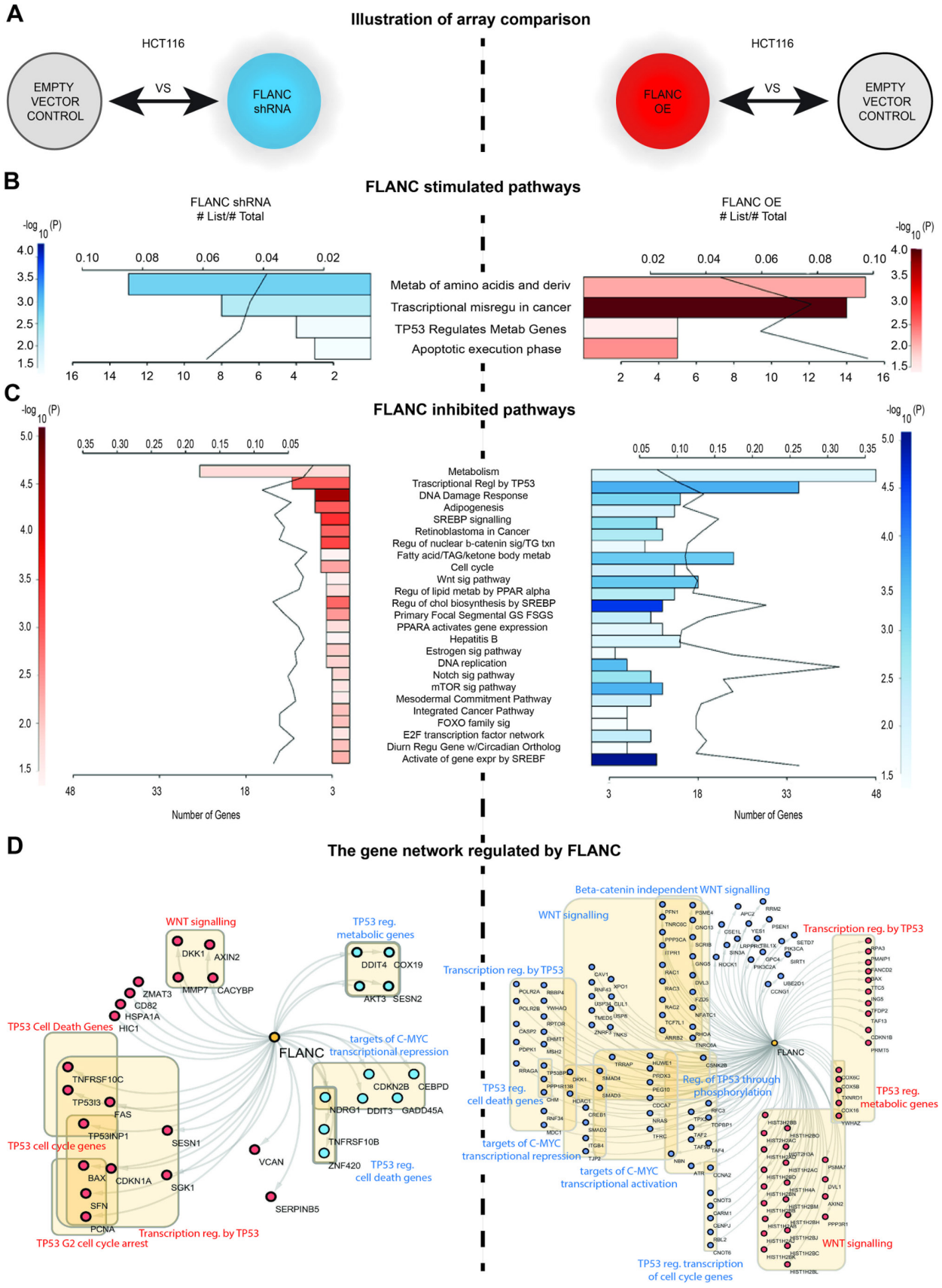

Figure 5 Genome-wide gene expression variation after FLANC levels modulation. (A) Illustrative representation of the HCT116 clones used for the array comparison. (B) FLANC stimulated signalling pathways negatively correlated in FLANC knock-down (blue=inhibited) and overexpression clones (red=activated) of HCT116 cells. (C) FLANC inhibited signalling pathways negatively correlated in FLANC knock-down (red=activated) and overexpression clones (blue=inhibited) of HCT116 cells. (D) The genes regulated by FLANC are key components of the TP53, WNT and C-MYC pathway, which negatively correlate in FLANC knock-down vs overexpression clones (red=activation, blue=inhibition).

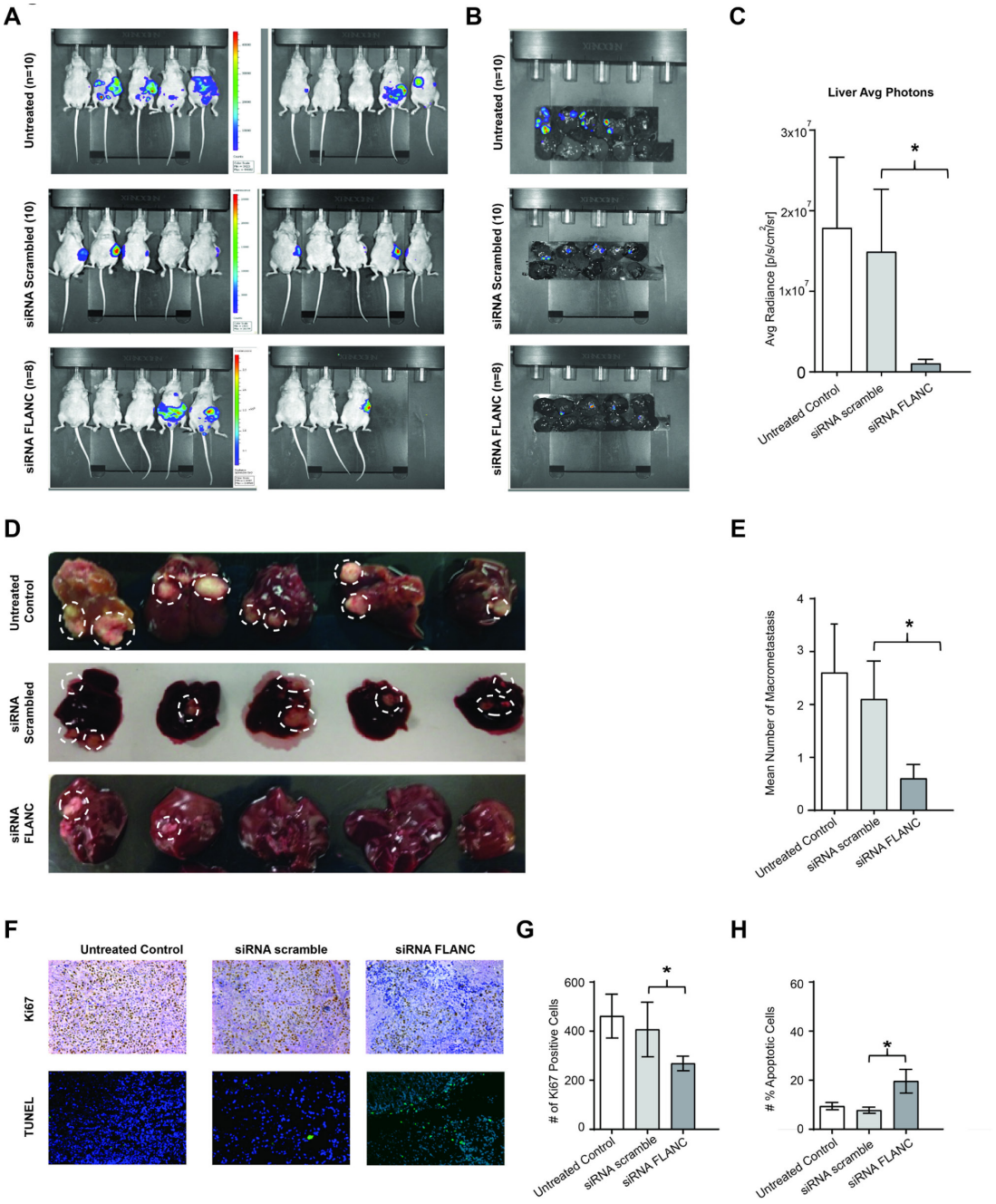

Figure 6 Therapeutically in vivo intrasplenic colon cancer model. (A) Representative chemiluminescent images from nude mice of untreated, siRNA scramble and siRNA-FLANC groups, and (B) liver metastases after approximately 7 weeks of intrasplenic injection with HCT116 cells are shown. (C) Weekly imaging was performed using the Xenogen IVIS spectrum system within 12 min following injection of D-Luciferin (150 mg/mL). Living image 4.1 software was used to determine the regions of interest (ROI), and average photon RADIANCE (p/s/cm^2/sr) was measured for each mouse. (C–E) Representative images from liver macrometastasis and quantification of macrometastasis. (F) Immunohistochemistry for Ki67 (proliferation marker) and the TUNEL assay (marks apoptotic bodies) suggest that knock-down FLANC is able to reduce cell proliferation, and to induce apoptosis. (G–H) Quantification analysis of Ki67 positive cells and number of positive apoptotic cell. Data were log-transformed before analysis, n=10. Data are presented as means±SD (Student's t-test: *p<0.05).

the liver, tumour burden in the liver and the tumour cell proliferation and apoptosis.

Absence of acute tissue toxicity or inflammatory response after anti-FLANC therapy

Additionally, we studied the effect of therapy on physiological parameters and evaluated the possible acute toxicity of the treatment (eg, impaired organ function or inflammatory cytokine responses). Male C57BL/6J mice were either untreated or treated with single intravenous injections of either siRNA scramble control or FLANC siRNA and serum was collected after 72 hours. As shown in online supplementary figure 17A, there were no differences in parameter measuring liver (aspartate aminotransferase) and kidney function (blood urea nitrogen) or high cell turnover parameters (lactate dehydrogenase). In addition, we did not detect significant differences in levels of 11 pro-inflammatory cytokines (IFN-γ,

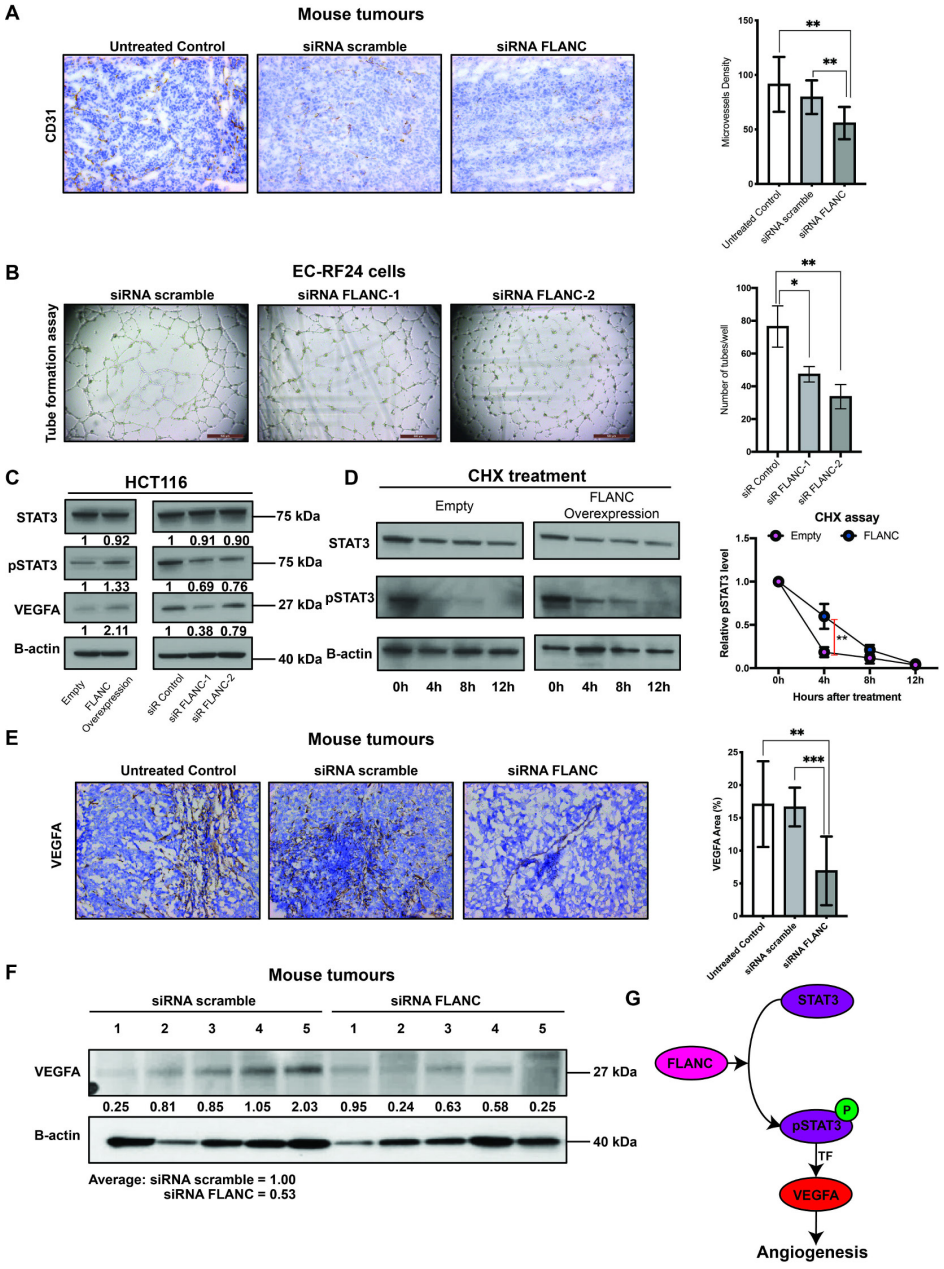

Figure 7 FLANC induces angiogenesis in vitro and in vivo. (A) Immunohistochemistry for CD31 (endothelial cell marker) suggested that knock-down of FLANC is able to reduce angiogenesis in vivo. (B) Endothelial cell tube formation assay showing that siRNA FLANC-1 and siRNA FLANC-2 decreased the number of tubes compared with siRNA scramble in vitro. (C) Immunoblotting analysis showed that FLANC overexpression compared with empty vector control increases the protein level of phosphorylated STAT3 Tyr 705 (pSTAT3) and VEGFA, but not of total STAT3 (left panel). The revers was observed after siRNA mediated knock-down of FLANC, both siRNA FLANC-1 and siRNA FLANC-2 decrease the level of pSTAT3 and VEGFA compared with siRNA control (right panel). (D) Cycloheximide Chase assay suggested that the half-life of pSTAT3, but not of total STAT3 in HCT116 cells with FLANC overexpression was prolonged compared with empty control vector (left panel). ImageJ quantification analysis showed that 4 hours after adding cycloheximide to the culture media the protein expression of pSTAT3 was higher in FLANC overexpression clones (right panel). (E) Immunohistochemistry expression of VEGFA in mouse tumours proved that siRNA FLANC treatment reduces the protein expression of VEGFA compared with siRNA scramble and untreated control. (F) Immunoblotting analysis showing that VEGFA protein level in mouse tumours is on average higher in the siRNA scramble group (average intensity=1) compared with the siRNA FLANC group (average intensity=0.53). (G) Schematic representation of the pathway regulated by FLANC. FLANC increases the expression level of pSTAT3 by prolonging its half-life and pSTAT3 is an established transcription factor for VEGFA, which in turn induces neovascularisation of the tumour. Data are presented as means±SD (Student's t-test: *p<0.05; **p<0.01; ***p<0.001). siRNA, small interfering RNA.

MIP-2, KC, interleukin (IL)-10, IL-6, IL-12, IL-4, IL-13, MCP-1, tumour necrosis factor-α, VEGF), using a Luminex assay (online supplementary figure 17B). Tissue samples were also obtained for postmortem histopathology studies (brain, spleen, liver and kidney). H&E staining of the various tissues were analysed by Histopathology Core's veterinary pathologist and no inflammatory changes were observed in the tissues studied (online supplementary figure 18). In summary, the administration of FLANC siRNA resulted in an effective decrease of metastatic disease without any signs for acute toxicity or inflammatory response.

FLANC promotes angiogenesis in vitro and in vivo

We also checked the effect of FLANC inhibition on angiogenesis by analysing the microvessel density (CD31 expression) in fresh frozen specimens from the therapeutic in vivo mouse study. Administration of FLANC siRNA induced a significant reduction of endothelial cells compared with siRNA scramble or untreated control ($p<0.01$) (figure 7A). In vitro, we observed that siRNA mediated knock-down of FLANC in EC-RF24 cells determined a reduction in endothelial tube formation ($p<0.05$ for siRNA FLANC-1 and $p<0.01$ for siRNA FLANC-2) (figure 7B). These data corroborated with the GEA data, made us focus on VEGFA, which is one of the top downregulated molecules in the shRNA FLANC versus shRNA control cell lines and a key regulator of angiogenesis.[40] Of note, the RPPA antibody panel did not include anti-VEGFA.

Indeed, we confirmed by western blot analysis that VEGFA protein expression is positively correlated with FLANC modulation (figure 7C). Because STAT3 is an established transcription factor for VEGFA,[41] we hypothesised that FLANC regulates VEGFA via STAT3. Western blot analysis data showed that FLANC overexpression induced a higher level of the phosphorylated STAT3 (pSTAT3 at Tyr 705), the active form that induces VEGFA transcription,[42 43] but not of total STAT3. FLANC knock-down confirmed the reverse effect (figure 7C). It is known that lncRNAs are capable to alter the half-life of proteins.[44] Therefore, we checked the half-life of STAT3 and pSTAT3 in empty vector control and FLANC overexpression clones. We observed that high levels of FLANC prolonged the half-life of pSTAT3, but not total STAT3 (figure 7D).

In vivo IHC data showed that siRNA FLANC treatment induces a downregulation of VEGFA in tumour tissue compared with siRNA scramble ($p<0.001$) and untreated control ($p<0.01$) (figure 7E). These results were confirmed by western blot analysis of in vivo tumours where despite samples heterogeneity, a tendency of downregulation of VEGFA by siRNA FLANC treatment was observed (average density of scramble siRNA=1 and of siRNA FLANC=0.53) (figure 7F). In summary, FLANC induces angiogenesis via STAT3/VEGFA axis (figure 7G).

DISCUSSION

Our study addresses an important novel therapeutic concept that could be exploited to kill malignant cells in vivo with less toxicities: targeting a primate-specific lncRNA, expressed at very low levels in normal cells, but at significantly higher levels in tumour cells. We identified and functionally characterised FLANC, a novel primate-specific lncRNA that is located in the first intron and expressed in the antisense direction of the *CELSR1* protein coding gene. FLANC expression levels do not influence considerably the host gene expression levels, as we saw no significant difference in *CELSR1* expression after FLANC knock-down. This suggests that FLANC is not an antisense exonic transcript acting in *cis* to regulate the sense host gene transcript. This novel approach has the potential to become a mainstream of cancer gene targeting. Recent studies identified about 11 000 primate-specific lncRNAs by analysing the expression patterns in tetrapods[45] and found that about 20% of the human long intergenic ncRNAs are not expressed beyond closely related primates such as chimpanzee or rhesus monkey.[46] Several of such hominid-specific genes have a coordinated expression under the effect of extracellular factors involved in cancers, such as oestrogens.[47]

In order to identify therapeutically actionable primate-specific transcripts, we used a category of very short (16 nts) DNA motifs with species-specific sequences named 'pyknons'.[27] We proved earlier that pyknons can serve as 'proxies' for transcripts that comprise them and can be functionally consequential.[25] The majority of the >209 000 pyknons reported for the human genome[27 48] are specific to humans/primates. This suggests the possibility that a vast spectrum of primate-specific lncRNAs exist, which are important for our understanding of human diseases and still remain uncharacterised. The absence of sequence conservation suggests that pyknon-containing transcripts can potentially reveal human-specific biology that the use of mouse models cannot uncover.

FLANC has a useful characteristic for therapeutic RNA targeting: it is expressed at very low levels in normal colonic cells, and therefore the cell death induced by RNA-targeting agents will be restrained mainly to the cancer cells with low toxicity. To test the therapeutic potential of FLANC, we used an intrasplenic injection approach commonly used as a CRC liver metastases model.[49] As ncRNAs are involved in all steps of immune system and inflammatory cell fate,[50] and a previous first-in-human miRNA restoration trial exhibited toxicity due to pro-inflammatory side effects,[51] we further evaluated cellular or immunological changes for the proposed treatment. Although we did not find any toxicities, further dose-response studies need to be carried out to determine the optimal pharmacological therapeutic window to optimise the efficacy. Because previously we reported that pyknons can also differentiate ZAP-70 positive from ZAP-70 negative CLL,[25] we do not exclude the hypothesis that FLANC or other primate-specific transcripts could be overexpressed also in other cancer types and could represent ideal therapeutic targets.

In summary, in this study we described a novel treatment approach in CRC and beyond, based on the identification of a novel primate-specific transcript FLANC, which is involved in colorectal carcinogenesis. Mechanistically, we observed that FLANC regulates the STAT3/VEGFA axis being involved in angiogenesis. FLANC function cannot be studied by genetically engineered mouse models of colorectal carcinogenesis,[49] as FLANC is primate-specific and thus not conserved in rodents. Because of the wide effect of FLANC on transcription and translation, we consider that other pathways involved in cancer are regulated by FLANC and need to be characterised. Due to the large number of potential primate-specific/human-specific transcripts that define the 'humanity' fingerprint of cancers, this approach could represent a future mainstream of therapeutics, a concept further discussed in a recent review .[52]

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Correction notice This article has been corrected since it published Online First. The funding statement has been updated.

Contributors Conception and design: MP, CR-A, SYN, MPD, GAC. Development of methodology: MP, CR-A, SYN, MPD, GAC. Acquisition of data (provided animals, acquired and treated patients, provided facilities, etc): MP, CR-A, SYN, MPD, RBa, SA, EK, EF-M, SKL, HL, GH, TCI, LH, YO, HK, AT, EB, RBh, PA, WRH, AMT, PV, DLB, XZ, CI, SK, BL, RG, ZW, SMH, MBE, GL, OS, AG, IR. Analysis and interpretation of data (eg, statistical analysis, biostatistics, computational analysis, mathematical analysis): MP, SYN, MPD, CI, ZW, VC, IR, GAC. Writing, review and/or revision of the manuscript: MP, CR-A, SYN, PA, SA, EK, MPD, IR, GAC. Administrative, technical or material support (ie, reporting or organising data, constructing databases): MP, CR-A, SYN, PA, SA, EK, MPD, GAC. Study supervision: GL-B, GAC.

Funding GAC is the Felix L. Endowed Professor in Basic Science. Work in GAC's laboratory is supported by National Institutes of Health (NIH/NCATS) grant UH3TR00943-01 through the NIH Common Fund, Office of Strategic Coordination (OSC), the NCI grants 1R01 CA182905-01 and 1R01CA222007-01A1, an NIGMS 1R01GM122775-01 grant, a U54 grant #CA096297/CA096300 – UPR/MDACC Partnership for Excellence in Cancer Research 2016 Pilot Project, a Team DOD (CA160445P1) grant, a Ladies Leukemia League grant, a Chronic Lymphocytic Leukemia Moonshot Flagship project, a Sister Institution Network Fund (SINF) 2017 grant and the Estate of C. G. Johnson Jr. GH was supported by China scholarship Council. CR-A was supported by the NIH through the Ovarian SPORE Career Enhancement Program, the NCI grants FP00000019. MP was supported by an Erwin Schroedinger Scholarship of the Austrian Science Funds (No. J3389-B23). ZW and VC were supported from the National Science Foundation Grant DMS-1930583, the NIH Grants 1U01CA196403, 1U01CA213759, 1R01CA226537, 1R01CA222007 and U54CA210181. GL-B is the John Q. Gaines Professor of Cancer Research. AG's work was supported by the grants CA72851, CA181572, CA184792 and CA187956 from the National Cancer Institute, National Institute of Health. IR is the Richard Hevner Professor in Computational Medicine at Thomas Jefferson University. IR's work was partially supported by a William M. Keck Foundation grant and by Institutional Funds. The Functional Proteomics RPPA Core facility and the Flow Cytometry and Cellular Imaging Core Facility (FCCICF) are supported by NCI Cancer Center Support Grant P30CA16672. AMT was supported by the CPRIT Research Training Program (RP170067).

Competing interests None declared.

Patient consent for publication Not required.

Ethics approval This study was approved by the Institutional Review Board of UT MDACC Texas LAB07-0734; Ethics Committee of University of Ferrara, Italy; Ethics Committee of Mie University Graduate School of Medicine, Japan; Ethics Committee of Comprehensive Cancer Care, Masaryk Memorial Cancer Institute, Czech Republic Ethics Committee of Croatian Human Tumor Bank.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available in a public, open-access repository. (i) Genome-wide microarray expression profiling to study the genetic landscape alterations induced by the knock-down of FLANC by shRNA/overexpression of FLANC in HCT-116 cells. (ii) Gene expression omnibus. (iii) Data are publicly available starting with 27 December 2019. As per your request, we have released the data for public access: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE127785; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE127786.

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REFERENCES

1 Siegel RL, Miller KD, Jemal A, *et al*. Cancer statistics, 2017. *CA Cancer J Clin* 2017;67:7–30.
2 Punt CJA, Koopman M, Vermeulen L, *et al*. From tumour heterogeneity to advances in precision treatment of colorectal cancer. *Nat Rev Clin Oncol* 2017;14:235–46.
3 Anfossi S, Babayan A, Pantel K, *et al*. Clinical utility of circulating non-coding RNAs - an update. *Nat Rev Clin Oncol* 2018;15:541–63.
4 Dang CV, Reddy EP, Shokat KM, *et al*. Drugging the 'undruggable' cancer targets. *Nat Rev Cancer* 2017;17:502–8.
5 Siegel RL, Miller KD, Fedewa SA, *et al*. Colorectal cancer statistics, 2017. *CA Cancer J Clin* 2017;67:177–93.
6 Kuipers EJ, Grady WM, Lieberman D, *et al*. Colorectal cancer. *Nat Rev Dis Primers* 2015;1:15065.
7 Puccini A, Lenz H-J. Colorectal cancer in 2017: Practice-changing updates in the adjuvant and metastatic setting. *Nat Rev Clin Oncol* 2018;15:77–8.
8 Ling H, Vincent K, Pichler M, *et al*. Junk DNA and the long non-coding RNA twist in cancer genetics. *Oncogene* 2015;34:5003–11.
9 Fearon ER, Vogelstein B, *et al*. A genetic model for colorectal tumorigenesis. *Cell* 1990;61:759–67.
10 Carotenuto P, Fassan M, Pandolfo R, *et al*. Wnt signalling modulates transcribed-ultraconserved regions in hepatobiliary cancers. *Gut* 2017;66:1268–77.
11 Almeida MI, Nicoloso MS, Zeng L, *et al*. Strand-Specific miR-28-5p and miR-28-3p have distinct effects in colorectal cancer cells. *Gastroenterology* 2012;142:886–96.
12 Ling H, Pickard K, Ivan C, *et al*. The clinical and biological significance of miR-224 expression in colorectal cancer metastasis. *Gut* 2016;65:977–89.
13 Ferdin J, Nishida N, Wu X, *et al*. HINCUTs in cancer: hypoxia-induced noncoding ultraconserved transcripts. *Cell Death Differ* 2013;20:1675–87.
14 Dragomir MP, Knutsen E, Calin GA, *et al*. Snapshot: unconventional miRNA functions. *Cell* 2018;174:1038–1038.e1.
15 Adams BD, Parsons C, Walker L, *et al*. Targeting noncoding RNAs in disease. *J Clin Invest* 2017;127:761–71.
16 Shah MY, Ferrajoli A, Sood AK, *et al*. microRNA therapeutics in cancer — an emerging concept. *EBioMedicine* 2016;12:34–42.
17 Awan HM, Shah A, Rashid F, *et al*. Primate-Specific long non-coding RNAs and microRNAs. *Genomics Proteomics Bioinformatics* 2017;15:187–95.
18 Durruthy-Durruthy J, Sebastiano V, Wossidlo M, *et al*. The primate-specific noncoding RNA HPAT5 regulates pluripotency during human preimplantation development and nuclear reprogramming. *Nat Genet* 2016;48:44–52.
19 Széll M, Danis J, Bata-Csörgő Z, *et al*. PRINS, a primate-specific long non-coding RNA, plays a role in the keratinocyte stress response and psoriasis pathogenesis. *Pflugers Arch - Eur J Physiol* 2016;468:935–43.
20 Liu J, Li Y, Lin B, *et al*. HBL1 is a human long noncoding RNA that modulates cardiomyocyte development from pluripotent stem cells by counteracting miR1. *Dev Cell* 2017;43:372.
21 Li D, Cheng M, Niu Y, *et al*. Identification of a novel human long non-coding RNA that regulates hepatic lipid metabolism by inhibiting SREBP-1c. *Int J Biol Sci* 2017;13:349–57.
22 Zhou Q, Yu B, Anderson C, *et al*. LncEGFL7OS regulates human angiogenesis by interacting with max at the EGFL7/miR-126 locus. *eLife* 2019;8.
23 Pertea M, Salzberg SL, *et al*. Between a chicken and a grape: estimating the number of human genes. *Genome Biol* 2010;11:206.
24 Abascal F, Juan D, Jungreis I, *et al*. Loose ends: almost one in five human genes still have unresolved coding status. *Nucleic Acids Res* 2018;46:12194.
25 Rigoutsos I, Lee SK, Nam SY, *et al*. N-BLR, a primate-specific non-coding transcript leads to colorectal cancer invasion and migration. *Genome Biol* 2017;18:98.
26 Calin GA, Trapasso F, Shimizu M, *et al*. Familial Cancer Associated with a Polymorphism in *ARLTS1*. *N Engl J Med* 2005;352:1667–76.
27 Rigoutsos I, Huynh T, Miranda K, *et al*. Short blocks from the noncoding parts of the human genome have instances within nearly all known genes and relate to biological processes. *Proc Natl Acad Sci U S A* 2006;103:6605–10.
28 Spaventi R, Pecur L, Pavelic K, *et al*. Human tumour bank in Croatia: a possible model for a small bank as part of the future European tumour bank network. *Eur J Cancer* 1994;30:419.
29 Lu J, Ye X, Fan F, *et al*. Endothelial cells promote the colorectal cancer stem cell phenotype through a soluble form of Jagged-1. *Cancer Cell* 2013;23:171–85.
30 Nishimura M, Jung E-J, Shah MY, *et al*. Therapeutic synergy between microRNA and siRNA in ovarian cancer treatment. *Cancer Discov* 2013;3:1302–15.
31 Tatin F, Taddei A, Weston A, *et al*. Planar cell polarity protein Celsr1 regulates endothelial adherens junctions and directed cell rearrangements during valve morphogenesis. *Dev Cell* 2013;26:31–44.

32 Katoh M, Katoh M. Comparative integromics on non-canonical Wnt or planar cell polarity signaling molecules: transcriptional mechanism of PTK7 in colorectal cancer and that of SEMA6A in undifferentiated ES cells. *Int J Mol Med* 2007;20:405–9.
33 Kaucka M, Plevova K, Pavlova S, *et al*. The planar cell polarity pathway drives pathogenesis of chronic lymphocytic leukemia by the regulation of B-lymphocyte migration. *Cancer Res* 2013;73:1491–501.
34 Pascal J, Bearer EL, Wang Z, *et al*. Mechanistic patient-specific predictive correlation of tumor drug response with microenvironment and perfusion measurements. *Proc Natl Acad Sci U S A* 2013;110:14266–71.
35 Wang Z, Kerketta R, Chuang Y-L, *et al*. Theory and experimental validation of a spatio-temporal model of chemotherapy transport to enhance tumor cell kill. *PLoS Comput Biol* 2016;12:e1004969.
36 Brocato TA, Brown-Glaberman U, Wang Z, *et al*. Predicting breast cancer response to neoadjuvant chemotherapy based on tumor vascular features in needle biopsies. *JCI Insight* 2019;4.
37 Shen P, Pichler M, Chen M, *et al*. To Wnt or lose: the missing non-coding LiNc in colorectal cancer. *Int J Mol Sci* 2017;18:2003.
38 Carethers JM, Jung BH, *et al*. Genetics and genetic biomarkers in sporadic colorectal cancer. *Gastroenterology* 2015;149:1177–90.
39 Lu Y, Ling S, Hegde AM, *et al*. Using reverse-phase protein arrays as pharmacodynamic assays for functional proteomics, biomarker discovery, and drug development in cancer. *Semin Oncol* 2016;43:476–83.
40 Apte RS, Chen DS, Ferrara N, *et al*. Vegf in signaling and disease: beyond discovery and development. *Cell* 2019;176:1248–64.
41 Niu G, Wright KL, Huang M, *et al*. Constitutive STAT3 activity up-regulates VEGF expression and tumor angiogenesis. *Oncogene* 2002;21:2000–8.
42 Chen S-H, Murphy D, Lassoued W, *et al*. Activated STAT3 is a mediator and biomarker of VEGF endothelial activation. *Cancer Biol Ther* 2008;7:1994–2003.
43 Zhao J, Du P, Cui P, *et al*. Lncrna PVT1 promotes angiogenesis via activating the STAT3/VEGFA axis in gastric cancer. *Oncogene* 2018;37:4094–109.
44 Shah MY, Ferracin M, Pileczki V, *et al*. Cancer-associated rs6983267 SNP and its accompanying long noncoding RNA *CCAT2* induce myeloid malignancies via unique SNP-specific RNA mutations. *Genome Res* 2018;28:432–47.
45 Necsulea A, Soumillon M, Warnefors M, *et al*. The evolution of lncRNA repertoires and expression patterns in tetrapods. *Nature* 2014;505:635–40.
46 Washietl S, Kellis M, Garber M, *et al*. Evolutionary dynamics and tissue specificity of human long noncoding RNAs in six mammals. *Genome Res* 2014;24:616–28.
47 Lin C-Y, Kleinbrink EL, Dachet F, *et al*. Primate-Specific oestrogen-responsive long non-coding RNAs regulate proliferation and viability of human breast cancer cells. *Open Biol* 2016;6:150262.
48 Tsirigos A, Rigoutsos I. Human and mouse introns are linked to the same processes and functions through each genome's most frequent non-conserved motifs. *Nucleic Acids Res* 2008;36:3484–93.
49 Romano G, Chagani S, Kwong LN, *et al*. The path to metastatic mouse models of colorectal cancer. *Oncogene* 2018;37:2481–9.
50 Mehta A, Baltimore D. Micrornas as regulatory elements in immune system logic. *Nat Rev Immunol* 2016;16:279–94.
51 van Zandwijk N, Pavlakis N, Kao SC, *et al*. Safety and activity of microRNA-loaded minicells in patients with recurrent malignant pleural mesothelioma: a first-in-man, phase 1, open-label, dose-escalation study. *Lancet Oncol* 2017;18:1386–96.
52 Dragomir MP, Kopetz S, Ajani JA, *et al*. Non-Coding RNAs in Gi cancers: from cancer hallmarks to clinical utility. *Gut* 2020. Epub ahead of print: 2020.