The Journal of Pathology: Clinical Research
J Pathol Clin Res January 2023; **9:** 73–85
Published online 8 November 2022 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/cjp2.300

ORIGINAL ARTICLE

Olfactomedin 4 associates with expression of differentiation markers but not with properties of cancer stemness, EMT nor metastatic spread in colorectal cancer

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Abstract

Tumor stem cells play a pivotal role in carcinogenesis and metastatic spread in colorectal cancer (CRC). Olfactomedin 4 (OLFM4) is co-expressed with the established stem cell marker leucine-rich repeat-containing G protein-coupled receptor 5 at the bottom of intestinal crypts and has been suggested as a surrogate for cancer stemness and a biomarker in gastrointestinal tumors associated with prognosis. Therefore, it was the aim of the present study to clarify whether OLFM4 is involved in carcinogenesis and metastatic spread in CRC. We used a combined approach of functional assays using forced OLFM4 overexpression in human CRC cell lines, xenograft mice, and an immunohistochemical approach using patient tissues to investigate the impact of OLFM4 on stemness, canonical Wnt signaling, properties of metastasis and differentiation as well as prognosis. OLFM4 expression correlated weakly with tumor grade in one patient cohort (metastasis collection: $p = 0.05$; pooled analysis of metastasis collection and survival collection: $p = 0.19$) and paralleled the expression of differentiation markers (FABP2, MUC2, and CK20) ($p = 0.002$) but did not correlate with stemness-associated markers. Further analyses in CRC cells lines as well as xenograft mice including forced overexpression of OLFM4 revealed that OLFM4 neither altered the expression of markers of stemness nor epithelial–mesenchymal transition, nor did OLFM4 itself drive proliferation, migration, or colony formation, which are all prerequisites of carcinogenesis and tumor progression. In line with this, we found no significant correlation between OLFM4 expression, metastasis, and patient survival. In summary, expression of OLFM4 in human CRC seems to be characteristic of differentiation marker expression in CRC but is not a driver of carcinogenesis nor metastatic spread.

Keywords: OLFM4; olfactomedin 4; stemness; stem cells; colorectal cancer

Received 18 May 2022; Revised 6 October 2022; Accepted 13 October 2022

No conflicts of interest were declared.

Introduction

Metastasis is the primary reason for the decrease in survival and the major cause of death in colorectal cancer (CRC) patients [1]. Advances in genomic and transcriptomic analyses have led to better understanding of the molecular underpinnings of CRC regarding tumor initiation and progression. In line with this,

focus has shifted toward tumor biology, addressing intra- and intertumor heterogeneity, to personalize patient management and optimize individual treatment and outcome in a precision medicine approach [2]. In this respect, identification of molecular biomarkers that correlate with tumor initiation (e.g. *KRAS*/*BRAF* mutations, deficient DNA mismatch repair system), predict metastasis at an early stage, inform on prognosis, guide therapeutic decisions, and represent potential targets for targeted therapies is highly advantageous [3].

In line with this, we highlighted the importance of stemness-associated markers in metastatic spread and prognosis in CRC in previous work. We could show that the combination of microsatellite stable status with high expression of CD133 and β-catenin, which are markers associated with stem cell features of tumor cells, is significantly associated with hepatic spread and prognosis in CRC [4]. Of interest, further analysis revealed that the expression of stem cell markers (CD44 and CD133) even correlated with different patterns of metastatic spread [5–7]. The importance of stem cell associated markers in metastasis development was further highlighted by the fact that we identified SOX2, a regulator of β-catenin, to be associated with distant spread [5,8].

In this respect, potentially underpinning the above-described processes, olfactomedin 4 (OLFM4) has been proposed as a surrogate marker for stemness and has been identified as a prognostic biomarker in a variety of gastrointestinal cancers [9–14]. OLFM4 is co-expressed at the bottom of the intestinal crypt, the niche of adult stem cells, with leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), a strong cancer stem cell marker in CRC [15–17]. Proteins of the olfactomedin family including OLFM4 have been shown to modulate Wnt signaling, a pathway that is essential to maintain the adult stem cell niche [18–23]. Aberrant activation of the Wnt pathway in CRC stem cells initiates tumor growth and drives colorectal carcinogenesis [24,25]. As a pivotal downstream target of the respective pathway, β-catenin promotes CRC progression through activation of epithelial–mesenchymal transition (EMT), a key program that enables stationary epithelial cells to lose their cell–cell adherence and acquire mesenchymal properties that are essential for invasion and metastasis [26]. OLFM4 has been suggested to be a negative regulator of the Wnt/β-catenin and NF-κB pathways that inhibit colon cancer development [22]. At the same time, OLFM4 was reported in well- and moderately differentiated tumors as well as adenomas and early stages of CRC and to significantly correlate with better tumor-specific survival (TSS) [27–29].

Overall, the role of OLFM4 with regard to tumor growth and progression in CRC is still unclear. This study aimed to investigate whether OLFM4 carries a functional role in the maintenance of cancer stemness, thereby acting as a driving force in the process of CRC progression, modulating Wnt signaling and potentially underpinning the previously described processes of our study group including β-catenin and c-MET driven EMT, proliferation and migration, or whether it is only a passenger. In this respect, we used a combined approach of functional assays using forced OLFM4 overexpression in human CRC cells and xenograft mice and an immunohistochemical approach using different collections focusing on survival, metastasis, or β-catenin driven EMT at the invasive front to understand the role of OLFM4 on human colorectal carcinogenesis.

Materials and methods

Cell culture

CRC cell lines DLD1, HT29, HCT116, LOVO, and SW480 (DSMZ, German collection for microorganisms and cells, Braunschweig, Germany) were cultivated in DMEM (10% FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin), primary tumor cell lines in StemPro® hESC SFM medium (0.01 µg/ml FGFb) (all Life Technologies) in Ultra-Low Attachment Surface culture flasks (Corning Life Science) in a humidified atmosphere at 5% CO_2. Cell lines were regularly tested for mycoplasma contamination using the Mycoplasma Test Kit from Applichem (Darmstadt, Germany). The identity of cell lines was recurrently confirmed by in-house short tandem repeat profile analysis.

Functional cell culture experiments

To assess the functional role of OLFM4 in the regulation of cancer-specific characteristics as it was proposed to regulate Wnt activity [18,22], we interfered with the endogenous expression levels of OLFM4 in cultivated CRC cell lines to see whether stemness-related properties of the tumor cells like activity of the Wnt pathway, expression of stemness markers, EMT, migration, colony formation (transformation, anchorage independent growth), or proliferation were influenced by OLFM4 expression. For the selection of appropriate cell lines, we investigated 14 long-term cultivated CRC cell lines (CaCo2, Colo320, DLD1, HCT115, HCT116, HT29, LOVO, LS174T, RKO, SW1222, SW403, SW620, SW480, and T84) for their

endogenous expression of *OLFM4* mRNA. LS174T and to a much lesser extent SW1222 (26-fold) produced reasonable amounts of OLFM4 compared with the other investigated cells (supplementary material, Figure S1). Therefore, a strategy of forced OLFM4 expression was chosen for the experimental part of the work. The five cell lines DLD1, HCT116, HT29, LOVO, and SW480 were stably transduced using a lentiviral expression system encoding either an OLFM4-V5 tag or as the control a chloramphenicol acetyltransferase (CAT)-V5 tag fusion cDNA. The bacterial CAT gene was chosen to exclude interference with the host's metabolism as much as possible.

Transfer vectors

OLFM4 encoding cDNA was amplified from RNA of LS174T cells and subcloned via BamHI and NotI sites via pEF-ENTR A-vector in frame into pLenti X1-DEST-PGK-Puro, which contains a C-terminal V5 tag 45 using Gateway® Technology following the user's manual [30]. As a control, CAT-V5 expression clones were subcloned in parallel using pcDNA3-CAT (Life Technologies, Darmstadt, Germany) as the template. Cloning with a special focus on the junction sites was verified by Sanger-sequencing (Eurofinns, Ebersberg, Germany).

Virus production, conditional expression

For packing expression vectors into lentiviral particles, 293T cells were co-transfected with 3.68 µg expression vector and 3.68 µg pCMV8.9 as well as 0.74 µg pVSV.G plasmids together with Fugene6 (Roche, Basel, Switzerland) in 60 mm ∅ dishes. Exactly 48 h after transfection, cell culture supernatants were filtered (0.45 µm), and 1 ml was used for infecting CRC cell lines. For selection, cells were treated with 1–2.5 µg/ml puromycin (Sigma-Aldrich, Hamburg, Germany).

Aldefluor assay

For the separation of aldefluor-positive (ALDH+) and -negative (ALDH−) cells, ALDEFLUOR™ kits (STEMCELL Technologies, Cologne, Germany) were used as recommended by the manufacturer. Five percent of the cells with the highest (ALDH+) or the lowest ALDH1-activity (ALDH−) respectively were fluorescence activated cell sorting (FACS) sorted (FACS Aria® Cell Sorter; Becton Dickinson, Hamburg, Germany). The effects of forced OLFM4 overexpression on ALDH activity were measured by applying ALDEFLUOR™ kits in combination with an Accuri C6 flow cytometer (Becton Dickinson) and C-Flow Plus Software. For the exclusion of dead cells, 1 µg/ml propidium iodide (Sigma-Aldrich) was added. Cutoff values for aldefluor were determined by addition of the ALDH1-inhibitor diethylamino-benzaldehyde.

Chemoresistance

LS174T cells were incubated for 5 days in six-well plates in the presence or absence (control) of 5-fluorouracil (5-FU; 40 and 50 µM in DMSO, Sigma-Aldrich). This assay was repeated three times at independent time points.

Proliferation—cell viability

About $1.5–3 \times 10^3$ cells were seeded in 96-cluster well plates together with thiazolyl blue tetrazolium bromide (MTT) tests (CellTiter 96® Non-Radioactive Cell Proliferation Assay; Promega, Waldorf, Germany) following the manufacturer's instructions. Extinction was measured at 570 nm employing a Varioskan reader (Thermo Fisher Scientific, Dreieich, Germany).

RT-qPCR, immunofluorescence, luciferase activity, protein expression by western blotting, *in situ* hybridization, immunohistochemistry, and expression analysis were performed as previously described in detail using whole slides [4,31–34] (supplementary material, Figure S2).

Wound-healing assay

Exactly 5×10^4 cells were seeded in triplicates in ibidi chambers (ibidi, Gräfelfing, Germany) in the presence of 10 µg/ml mitomycin C (Sigma-Aldrich) for 3 h. After removing the chambers, cells were washed twice in PBS and kept in DMEM with FCS. The resulting gap between the cells was photographed at 0, 24 (DLD1 and HCT116), and 48 h (HT29). Images were analyzed by applying ImageJ software (NIH, https://imagej.nih.gov/ij/).

Colony formation

Colony formation or transformation was measured with the help of methyl cellulose assays. Five hundred cells were mixed in 1 ml 0.01% methyl cellulose (Sigma-Aldrich), seeded in quadruplicates in 35 mm ∅ Petri dishes for 12–16 days. Then 200 µl MTT solution (Sigma-Aldrich) was added. The next day photos were taken and analyzed by counting the blue-colored colonies applying ImageJ software (NIH).

Tumor growth in mice

Mouse experiments were reviewed and approved by the government of Upper Bavaria, and mice were housed in special pathogen-free cages. SW480 colon cancer cells expressing OLFM4 or CAT were injected in increasing numbers (10^3–10^6 cells) subcutaneously into the right (OLFM4) and left (CAT) flank of age- and gender-matched 6–8-week-old NOD/SCID mice for xenograft formation (five mice per group). Primary tumor size was measured for 7 weeks at 3 days intervals using an external caliper. Tumor volumes were calculated using the modified ellipsoid formula: $V = \frac{W^2 \times L}{2}$ (V: tumor volume, W: width, L: length of tumor). Mice were sacrificed, tumors removed, and either formalin-fixed or paraffin-embedded for histology and immunostaining.

Tumor collections

The metastasis collection (Table 1) has been described previously. In brief, it contains 106 patients with and without distant metastasis. The collection is powered as a case–control study thus consisting of pairs of patients with highly comparable characteristics [4,7]. The survival collection consists of 252 patients with CRC (UICC stage II, T3/4, N0, M0) with intended curative surgical resection (treated between 1995 and 2007 at the University Hospital of LMU Munich) and 5 years follow-up. Patients dying within 6 months after resection were excluded to reduce surgery-related side effects. Fifty-five patients (21.8%) died from CRC within the follow-up period. Survival data of 197 patients (78.2%) were censored when the follow-up was discontinued or patients died of reasons other than CRC (Table 2). Fifty-two events of cancer-specific death and 77 of tumor progression within 10 years were within the expected range [35]. The detection of relative risks was 2.2 (TSS) or 1.9 (progression-free survival [PFS]) [36]. For analyses of co-expressions, 10 moderately differentiated CRC with budding-related invasive front were randomly selected from both collections [37].

The study was performed according to the recommendations of the local ethics committee of the Medical Faculty of LMU München.

Immunohistochemistry score

Immunohistochemistry was evaluated independently by two observers (JN and SJ). OLFM4 expression was scored similarly as published [28]. In this respect, the percentage of positively stained cells was divided into four grades: <30% (score 0); 30–50% (score 1); 51–70% (score 2); and >70% (score 3). The two lower and higher categories were summarized as low (score 0 and 1) or high (score 2 and 3) expression, respectively. With regard to β-catenin expression, the staining score for nuclear expression of β-catenin was based on the proportion of stained tumor nuclei throughout the whole tumor. The scoring system was as follows: 0: negative; 1+: <30%; 2+: 30–60%; 3+: >60% positive cells. Additionally, the cases were classified into low- (score 0 and 1) and high-grade (score 2 and 3) extent [4]. Co-expression was determined either by analysis of serial sections or immunohistochemically double-staining for OLFM4 and CK20 (cytoplasmic staining) or β-catenin and Ki67

Table 1. Patient characteristics of the metastasis collection and correlation with expression of OLFM4 as well as clinicopathological parameters. This collection contains patients ($N = 106$) with or without distant metastasis. This collection was powered as a case–control study thus consisting of pairs of patients with highly comparable characteristics except for distant metastasis. Therefore, all frequencies in the collection are even numbered. OLFM4 expression was scored either as low or high based on immunohistochemical stains applying the analysis score. In this collection, OLFM4 expression significantly correlated with tumor grade ($p = 0.043$, compare Table 2), *BRAF* status and dMMR, but not with age, gender, T (tumor), N (nodal status), or M (distant metastasis) as the main endpoint of this collection

	OLFM4			
Characteristic	Total	Low	High	*P* value
All patients	106 (100)	58 (54.7)	48 (45.3)	
Age group				
≤66	52 (49.1)	26 (24.5)	26 (24.5)	0.338
≥67	54 (50.9)	32 (30.2)	22 (20.8)	
Gender				
Male	53 (50)	32 (30.2)	21 (19.8)	0.242
Female	53 (50)	26 (24.5)	27 (25.5)	
T-stage (UICC)				
T1	2 (1.9)	0 (0)	2 (1.9)	0.169
T2	10 (9.4)	4 (3.8)	6 (5.7)	
T3	78 (73.6)	47 (44.3)	31 (29.2)	
T4	16 (15.1)	7 (6.6)	9 (8.5)	
Nodal status				
N0	50 (47.2)	27 (25.5)	23 (21.7)	0.889
N+	56 (52.8)	31 (29.2)	25 (23.6)	
Distant metastasis				
M0	53 (50)	32 (30.2)	21 (19.8)	0.242
M1	53 (50)	26 (24.5)	27 (25.5)	
Tumor grade (WHO)				
Low grade	38 (35.8)	16 (15.1)	22 (20.8)	0.05
High grade	68 (64.2)	42 (39.6)	26 (24.5)	
KRAS				
Wild type	70 (66)	35 (33.0)	35 (33.0)	0.174
Mutant	36 (34)	23 (21.7)	13 (12.3)	
BRAF				
Wild type	90 (84.9)	45 (42.5)	45 (42.5)	0.021
Mutant	16 (15.1)	13 (12.3)	3 (2.8)	
dMMR				
MSS	84 (79.2)	41 (38.7)	43 (40.6)	0.017
MSI	22 (20.8)	17 (16.0)	5 (4.7)	

Table 2. Patient characteristics of the survival collection and correlation with the expression of OLFM4 as well as clinicopathological parameters. This collection contains patients ($N = 252$) in UICC stage II thus without lymph node (N) or distant metastases (M). For all cases OLFM4 expression was scored on the basis of immunohistochemical stains into one of the two groups (low or high). No correlation was found for PFS (see Figure 5C), TSS (see Figure 5D), or for the other given clinicopathological parameters

	OLFM4			
Characteristic	Total	Low	High	*P* value
All patients	252 (100)	111 (44.0)	141 (56.0)	
Age group				
≤68.6	120 (47.6)	49 (19.4)	71 (28.2)	0.327
>68.6	132 (52.4)	62 (24.6)	70 (27.8)	
Gender				
Male	138 (54.8)	61 (24.2)	77 (30.6)	0.956
Female	114 (45.2)	50 (19.8)	64 (25.4)	
T-stage (UICC)				
T3	206 (81.7)	85 (33.7)	121 (48.1)	0.059
T4	46 (18.3)	26 (10.3)	20 (7.9)	
Tumor grade (WHO)				
Low grade	151 (59.9)	67 (26.6)	84 (33.3)	0.899
High grade	101 (40.1)	44 (17.5)	57 (22.6)	
KRAS				
Wild type	157 (62.3)	67 (26.6)	90 (35.7)	0.573
Mutant	95 (37.7)	44 (17.5)	51 (20.2)	

(nuclear staining) resulting in four categories of expression: (1) co-expression of both, (2) expression of one or (3) the other marker, and (4) no expression. For statistical analysis, the mean of each category was calculated.

Statistics

Significance of correlations was calculated applying χ^2- and Student's *t*-models. Survival was calculated by employing the Kaplan–Meier method, and significance was tested by the log-rank procedure. For analysis of concordance, Cohen's κ-coefficient algorithm was applied. For all tests, a two-sided α-error lower than 5% ($p \leq 0.05$) was considered statistically significant. All analyses were performed using SPSS software (SPSS v. 21.0, IBM Inc., New York, USA).

Results

Expression of OLFM4 and markers of cancer stemness do not correlate

Cell culture

To assess the expression of OLFM4 in correlation with markers indicative for cancer stemness or terminal differentiation of CRC cells, levels of *OLFM4* mRNA were compared with those of the cancer stemness markers *ALDH1A1* (aldehyde dehydrogenase 1A1),

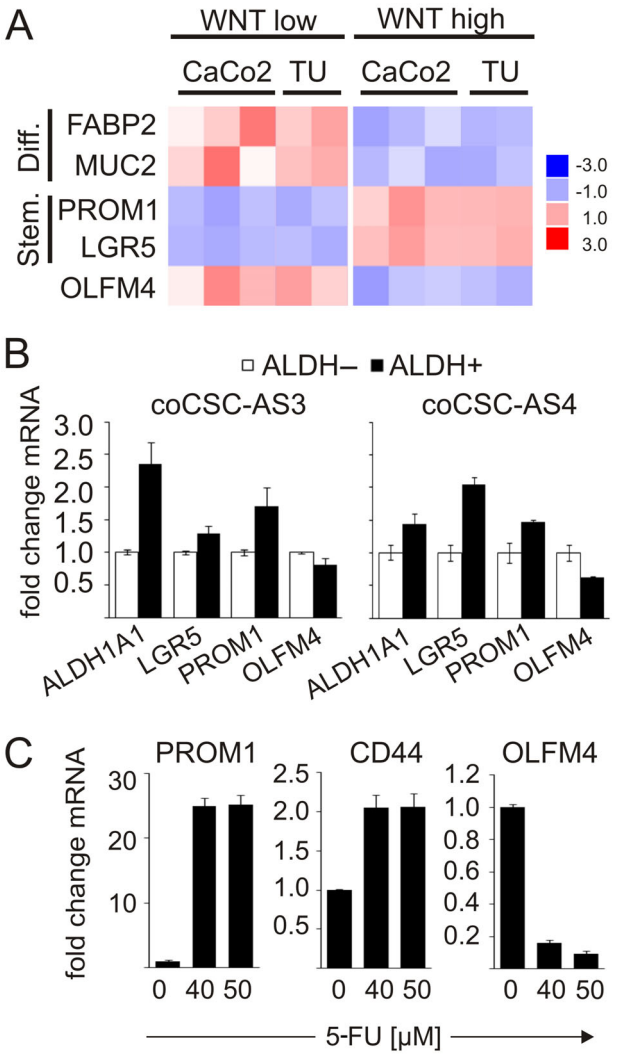


Figure 1. *OLFM4* expression does not correlate with the expression of markers of cancer stemness. (A) *OLFM4* mRNA expression parallels the expression profile of the markers of differentiation *FABP2* and *MUC2* but not of the stemness-associated markers *PROM1* (CD133) and *LGR5* both in the cultivated colorectal cell line CaCo2 as well as human colorectal primary tumor (TU). (B) Subpopulations of CRC cell lines characterized by the expression of higher amounts of ALDH1 (ALDH+) using the aldefluor-assay express higher mRNA levels of the stemness markers *ALDH1A1*, *LGR5*, and *PROM1* but lower levels of *OLFM4* mRNA compared to the ALDH− subpopulation. (C) The colorectal tumor line LS174T was treated with the chemotherapeutic drug 5-FU (40, 50 μM) for 5 days. The remaining cells expressed higher mRNA levels of the stemness markers *PROM1* and *CD44* but lower amounts of *OLFM4* mRNA compared with the untreated population (0 μM) respectively.

CD44, *CD133* (prominin1, PROM1), and *LGR5* [15,38–41] as well as the differentiation markers *FAB2* (fatty acid binding protein 2) and *MUC2* (mucin 2) [42]. The respective expression levels were compared

Table 3. Colorectal tumor cells do not show co-expression of nuclear β-catenin and OLFM4. Serial sections of 358 patients (252 – survival collection, 106 – metastasis collection) were stained for the expression of β-catenin and OLFM4. The expression was categorized for both markers as either high or low. No significant correlation was found applying a χ^2-model

	OLFM4 low		OLFM4 high		
	N	%	*N*	%	*P* value
β-Catenin					
Survival collection ($N = 252$)					
High	66	26.2	93	36.9	0.289
Low	45	17.9	48	19.0	
Metastasis collection ($N = 106$)					
High	19	17.9	16	15.1	0.950
Low	39	36.8	32	30.2	

in subpopulations of tumor cells displaying either high- or low activity of the canonical Wnt pathway as this indicates CRC cells with properties of cancer stem cells [43]. In this respect, we used a dataset based on xenograft tumors of CaCo2 as well as primary colon cancer cells [44]. Cells with high activity of the canonical Wnt pathway displayed increased amounts of *PROM1* and *LGR5* but low amounts of *FABP2* and *MUC2* [45]. The expression of *OLFM4* did not parallel the expression of the stemness markers, especially *LGR5* [15]. Instead, *OLFM4* expression correlated with that of the differentiation markers *FABP2* and *MUC2* (Figure 1A). Next, two primary cell lines coCSC-AS3 and coCSC-AS4 with higher activity of ALDH (ALDH+) were selected and enriched applying the aldefluor assay in combination with FACS [38]. ALDH+ subpopulations also expressed higher amounts of ALDH1A1, thereby confirming the success of the selection process. Expression of stemness markers *LGR5* and *PROM1* was found to be elevated, but that of *OLFM4* again was lower in the ALDH+

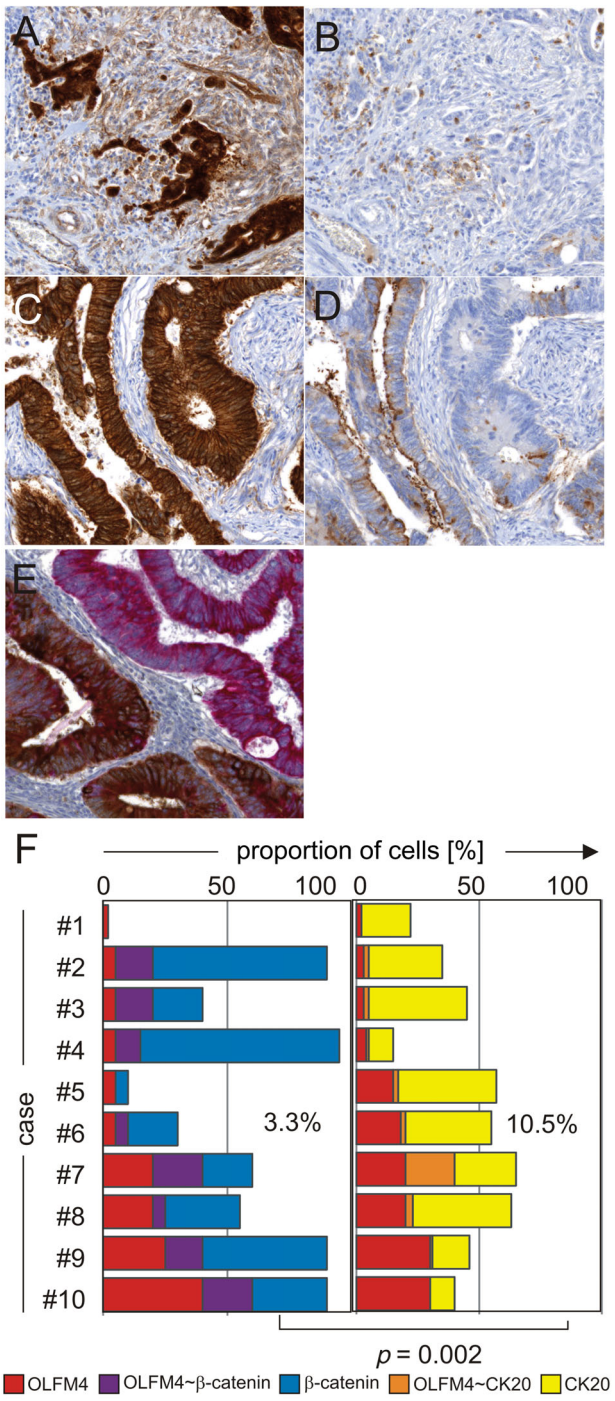
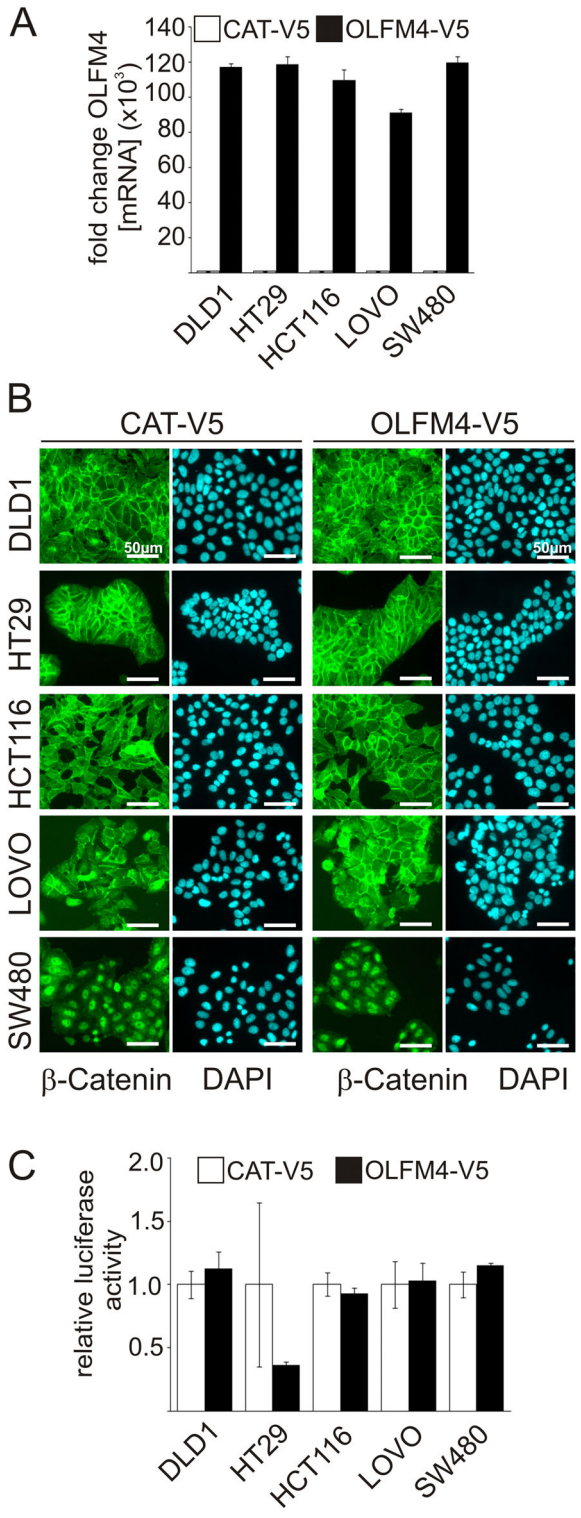


Figure 2. OLFM4 and CK20 co-expression correlate in human CRCs. (A) Tumor cells at the invasive front displaying strong nuclear β-catenin expression (B) did not co-express OLFM4. A comparable situation was seen in (C) areas with absence of nuclear expression of β-catenin which lacks (D) co-expression of OLFM4. (E) In contrast, a substantial amount of co-expression of OLFM4 (brown chromogen) and CK20 (red chromogen) was seen in double stains. (F) Counting of areas with co-expression of either nuclear β-catenin or CK20 with OLFM4 demonstrated that only 3.3% of tumor cells co-expressed nuclear β-catenin and OLFM4 in contrast to 10.5% of CK20/OLFM4 co-expressing tumor cells. This difference was statistically highly significant ($p = 0.002$) applying the χ^2-model. Total magnifications: ×100.

Figure 2. Legend on next page.

population, thus running antiparallel with the expression of the investigated stemness markers in both primary cell lines (Figure 1B). Furthermore, we used the chemotherapeutic agent 5-FU to enrich for tumor cells with characteristics of cancer stemness [46]. The CRC cell line LS174T was treated with two different concentrations of 5-FU (40 and 50 μM) for 5 days, which was escorted by massive cell death. The remaining resistant cells expressed higher mRNA amounts of the stemness markers *PROM1* and *CD44* but lower levels of *OLFM4* (Figure 1C).



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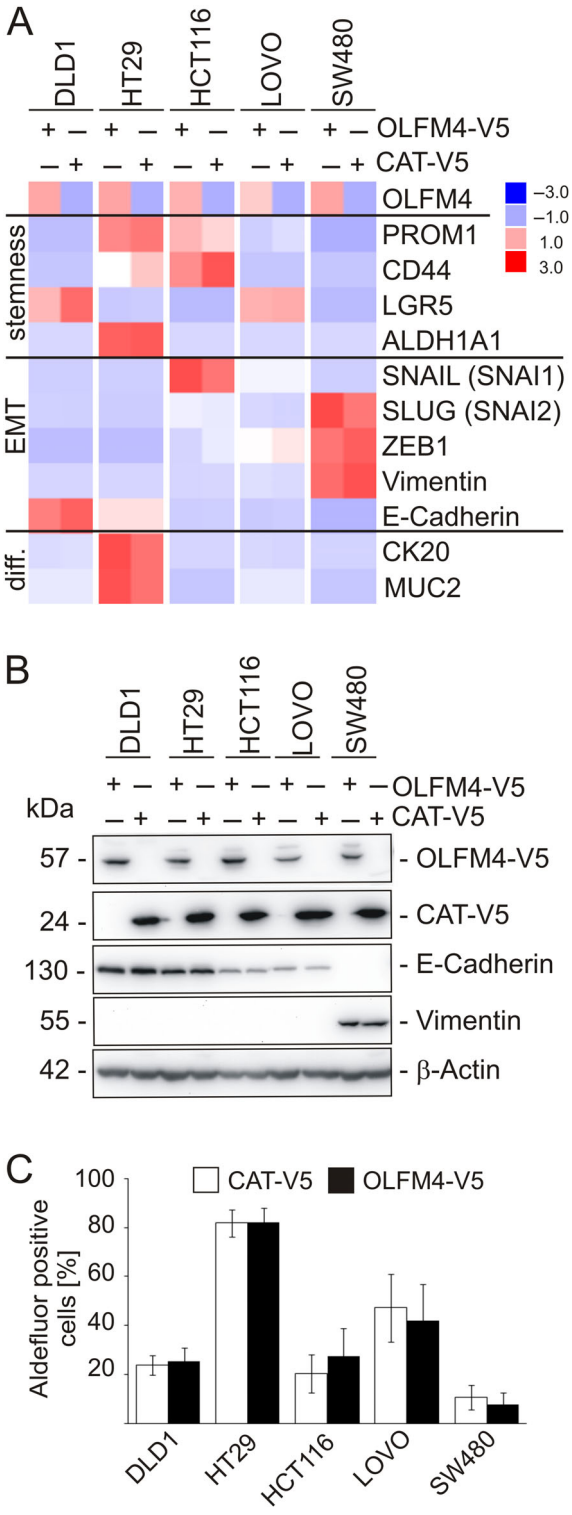
Human cohorts

In a second set of investigations, these results were translated into human CRC. In a collection of CRCs with prototypic tumor budding at their invasive fronts characterized by nuclear expression of β-catenin ($N = 10$) [37,47], the expression of OLFM4 was correlated with the nuclear localization of β-catenin as it is known that tumor cells expressing nuclear β-catenin indicate tumor cells with properties of cancer stemness, whereas cells without nuclear expression of β-catenin are mostly characterized by the co-expression of CK20 (cytokeratin 20) indicative for differentiated CRC cells [37,40,41,48]. Tumor cells characterized by nuclear localized β-catenin seldom co-expressed OLFM4 and, if so, to a lesser degree compared with tumor cells without nuclear expression of β-catenin (Figure 2A–D,F and supplementary material, Figure S1). This finding was supported by results from subgroups of two larger collections of CRCs. In both the survival ($N = 252$, Table 2) and the metastasis collection ($N = 106$, Table 1), no significant correlation was found between nuclear expression of β-catenin and OLFM4 (survival collection, $p = 0.289$; metastasis collection $p = 0.950$) (Table 3) [4]. In contrast however, OLFM4 and CK20 were frequently co-expressed (Figure 2E), which was significant compared with the co-expression of nuclear β-catenin and OLFM4 (10.5% versus 3.3%, $p = 0.002$; Figure 2F).

Figure 3. Forced overexpression of OLFM4 did not result in a change of the subcellular localization of β-catenin nor activity of the canonical Wnt pathway. (A) Stable transduction of the colorectal cell lines DLD1, HT29, HCT116, LOVO, or SW480 demonstrated a robust overexpression of OLFM4-V5 at the mRNA level (for protein compare Figure 4B). (B) OLFM4 overexpression did not result in a change of the subcellular localization of β-catenin which was mostly nuclear for SW480 and to a lesser extend in LOVO cells. DLD1, HT29, and HCT116 cells expressed β-catenin mostly at the membrane. (C) In support of these data, OLFM4-V5 overexpression did not result in a change of canonical Wnt signaling activity measured as ratio of TOP- and FOP-flash activities.

In summary, expression of OLFM4 seems to be characteristic for terminally differentiated CRC cells indicated by the co-expression of CK20, FABP2, or MUC2. By contrast, tumor cells that show expression of markers indicating stemness such as nuclear β-catenin, CD44, LGR5, or PROM1 did either not co-express OLFM4 or when expressed then at an inferior level.



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OLFM4 has no driving properties for colorectal carcinogenesis

Functional cell culture experiments

To investigate the functional role of OLFM4 in the regulation of cancer-specific characteristics, the effect of forced OLFM4 expression in five cell lines (DLD1, HCT116, HT29, LOVO, and SW480) was assessed with respect to stemness-related properties of the tumor cells such as activity of the Wnt pathway, expression of stemness markers, EMT, migration, colony formation (transformation, anchorage independent growth), or proliferation. After stable transduction and selection, bulk cultures were obtained, which expressed reasonable amounts of *OLFM4-V5* or *CAT-V5* mRNA (Figures 3A and 4A), or protein (Figure 4B).

First, OLFM4-V5 expression did not result in a change in the subcellular localization of β-catenin as the driving force of canonical Wnt activity (Figure 3B), which was confirmed by the absence of changes in TOP/FOP-flash activity (Figure 3C) [43]. Second, OLFM4-V5 expression influenced the mRNA expression levels of neither the stemness markers *ALDH1A1*, *CD44*, *LGR5*, and *PROM1* nor EMT markers *E-cadherin*, *SLUG* (SNAI2), *SNAIL* (SNAI1), *vimentin*, or *ZEB1* nor the differentiation markers *CK20* and *MUC2* (Figure 4A). This was also verified for the EMT markers E-cadherin and vimentin at the protein level (Figure 4B). Third, OLFM4-V5

Figure 4. Forced overexpression of OLFM4 did not alter the proportion of tumor cells with properties of cancer stem cells, EMT or terminal differentiation. (A) Stably transduced colorectal cell lines DLD1, HT29, HCT116, LOVO, or SW480 overexpressing either OLFM4-V5 or CAT-V5 as the control did not show differences in the mRNA expression levels of stemness indicating markers *ALDH1A1*, *CD44*, *LGR5*, or *prominin1* nor EMT indicative markers *E-cadherin*, *SLUG* (SNAI2), *SNAIL* (SNAI1), *vimentin*, and *ZEB-1*, nor *CK20* or *MUC2* markers specific for terminal differentiation. (B) OLFM4-V5 and CAT-V5 expression were verified at the protein level. In support of the results from the measurements of mRNA no differences were seen for the expression of the EMT markers E-cadherin and vimentin at the protein level as indicated by western blotting. (C) OLFM4-V5 overexpression did not change the proportion of tumor cells with high expression of ALDH as indicated by aldefluor activity.

expression did not influence the proportion of aldefluor-positive cells as determined by employing the aldefluor assay (Figure 4C, supplementary material, Figure S3).

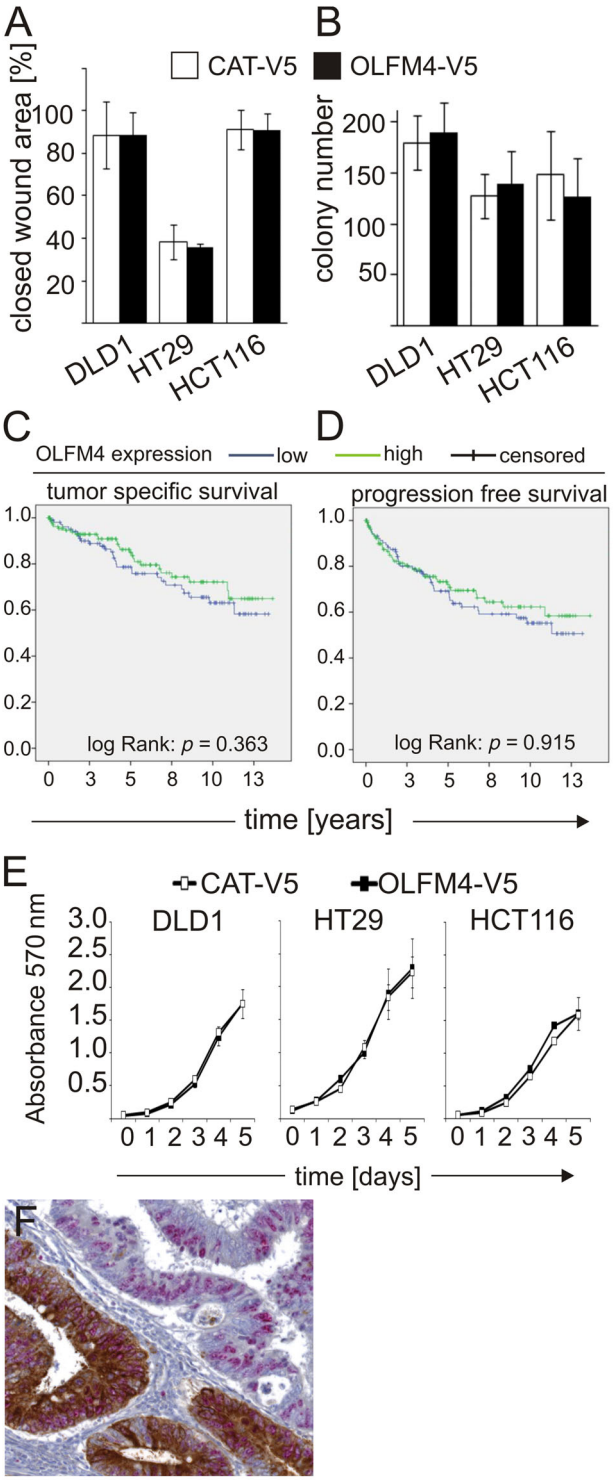

Figure 5. Legend on next page.

Fourth, OLFM4-V5 expression did not influence the capacity of transduced cells for migration as measured by the ability of tumor cells to close a defined wound/gap (Figure 5A and supplementary material, Figure S4A). Fifth, OLFM4-V5 overexpression did not change colony formation under conditions of anchorage independent growth when cultivating OLFM4-V5 and CAT-V5 overexpressing tumor cells in methyl cellulose mimicking a situation that tumor cells encounter in the blood stream (Figure 5B and supplementary material, Figure S4B). As both migration and colony formation are two essential pre-requirements for metastases, one would not expect a correlation of OLFM4 with survival or metastasis on the basis of these experimental results from the cell culture [6].

Human cohorts

Sixth, we thus translated this hypothesis into human CRC using two collections: (1) a survival collection consisting of UICC stage II patients ($N = 252$, Table 2) and (2) a collection powered for distant metastasis consisting of 53 pairs of patients with or without distant metastasis in the form of a case–control study (Table 1) [4]. The cases of these collections were immunohistochemically stained for OLFM4, the expression levels categorized into the two groups high and low expression, and these finally correlated with both TSS (Figure 5C) and PFS (Figure 5D), using the Kaplan–Meier model. In both cases, no significant correlation between survival and OLFM4 expression was found (TSS – $p = 0.363$, PFS – $p = 0.915$). Comparably, OLFM4 expression also did not correlate with the development of distant metastasis (Table 1, $p = 0.242$). In both collections, no correlation with other clinicopathological parameters like age, gender, and T-stage was found applying the χ^2-model. Only MSI status ($p = 0.017$), *BRAF* mutation status

Figure 5. Forced expression of OLFM4-V5 did not alter migration, colony formation or proliferation. (A) Stably transduced colorectal cell lines DLD1, HT29, and HCT116 overexpressing OLFM4-V5 did not show a higher rate of migration compared with CAT-V5 expressing control cells (see also supplementary material, Figure S4A). (B) The same was seen for colony formation of these cell lines in methyl cellulose (see also supplementary material, Figure S4B). OLFM4 expression correlated with neither (C) TSS nor (D) PFS in UICC stage II CRCs. (E) OLFM4-V5 overexpression also did not influence the proliferation of the transduced tumor cells DLD1, HT29 nor HCT116. (F) In human CRCs OLFM4 (brown chromogen) was co-expressed with the proliferation marker Ki67 (red chromogen).

Table 4. OLFM4 does not drive tumor growth in mice. SW480 cells with forced OLFM4 or CAT expression were injected in different quantities (10^3–10^6 cells) into the rear flanks of mice. Tumor growth was observed over a period of 7 weeks and eventually the number of tumors that had grown was counted. There was no significant difference with regard to tumor count between mice with forced OLFM4 or CAT expression ($p = 0.8588$)

Cell number	CAT	OLFM4
1,000	1	1
10,000	4	3
100,000	5	5
1,000,000	5	5

($p = 0.021$), and tumor grade ($p = 0.05$) correlated significantly with OLFM4 expression in the metastasis collection (Table 1). However, in a pooled analysis, tumor grade (that correlated weakly with OLFM4 in the metastasis collection) did no longer correlate with OLFM4 expression. Seventh, OLFM4-V5 overexpression did not influence the proliferation rate of the cultivated tumor cells DLD1, HT29, and HCT116 when OLFM4-V5 and CAT-V5 overexpressing clones were compared (Figure 5E) on the basis of MTT assays. As OLFM4 was described to be co-expressed with LGR5 1 that defines a population of highly proliferating cells [17], it can be concluded that OLFM4 is not causatively involved in the regulation of proliferation.

Xenograft models

Finally, we investigated the functional role of OLFM4 with regard to colorectal carcinogenesis *in vivo* using an experimental xenograft mouse model. There was no significant difference in size nor numbers of tumors expressing OLFM4 or CAT (control arm) ($p = 0.8588$) (Table 4).

Taken together, expression of OLFM4 seems to be characteristic for terminally differentiated CRC cells as verified by our immunohistochemical analyses. Furthermore, forced expression of OLFM4 did not drive proliferation, migration, colony formation *in vitro* nor tumor initiation or tumor growth *in vivo*.

Discussion

CRC incidence is on the rise, especially in the young, often presenting at an advanced stage. In this respect, and with CRC being one of the leading causes of cancer-related death worldwide, there is a pressing need not only to understand the underlying mechanisms of tumor growth and progression but also to identify biomarkers that allow assessment of prognosis and guide therapeutic decisions [49]. While anatomic staging and grading is still a strong pillar of therapeutic and prognostic decisions, precision oncology aims to identify molecular biomarkers that allow better risk stratification, administration of tailored treatments that reflect individual molecular patient characteristics, refinement of screening strategies and follow-up care, and prediction of prognosis [2]. We have previously shown the significance of stemness-associated markers involving Wnt-pathway activation and β-catenin dependent signaling with regard to metastatic spread and prognosis in CRC [4-6,8]. In this context, others proposed OLFM4 as a useful marker for stem cells as it is co-expressed with LGR5, a specific cancer stem cell marker, in the human intestine and might modulate Wnt signaling [15-17]. Furthermore, OLFM4 has been reported to be overexpressed in CRC but downregulated in later stages, while at the same time being associated with better patient survival [22,27-29]. Thus, we sought to investigate the functional role of OLFM4 in CRC and elucidate whether OLFM4 acts as a driver of colorectal carcinogenesis, similar to LGR5, potentially underpinning the previously described processes of tumor initiation, proliferation, invasion, and EMT of our study group [5,7,8,50].

In the present study, we examined the role of OLFM4 in CRC with regard to stemness, canonical Wnt signaling, properties of metastasis and differentiation as well as prognosis. We found that OLFM4 expression correlated with the expression of differentiation markers but did not correlate with stemness-associated markers. Moreover, functional analyses in CRC cell lines and xenograft mice including forced overexpression of OLFM4 revealed that OLFM4 neither altered the expression of markers of stemness nor EMT, neither did OLFM4 itself drive proliferation, migration, and colony formation, which are all prerequisites of carcinogenesis and tumor progression. In line with this, we found no significant correlation between OLFM4 expression, metastasis, and patient survival.

A variety of studies have reported OLFM4 mRNA and protein to be overexpressed in early-stage CRC but reduced or lost in advanced stages [27,28,51-53]. Furthermore, a marked increase in OLFM4 expression has been demonstrated in well-differentiated tissues, corresponding to the early stages of tumor development, as opposed to poorly differentiated tissues exhibiting low levels of OLFM4 [10,54]. Additionally, a recent study using patient-derived organoids showed that OLFM4 expression correlated with tumor

differentiation but was dispensable for metastatic spread [55]. This is in alignment with the results of our study that showed that OLFM4 paralleled the expression of differentiation markers but did not have the capacity to initiate differentiation or alter the expression of those markers.

Concerning the functional role of OLFM4 with regard to tumorigenesis including tumor initiation and proliferation in CRC, data are sparse. Few studies report tumor-suppressive effects of OLFM4. Downregulation or deletion of OLFM4 was shown to induce CRC in *APC OLFM4* double-mutant mice [22]. In line with this, upregulation of OLFM4 in HT29 cells led to inhibition of cell migration [14]. One study found that forced OLFM4 expression in CRC did not change cell proliferation, but decreased cell adhesion and migration in CRC cell lines [14]. To our knowledge, we are the first to investigate the functional role of OLFM4 both *in vitro* and *in vivo* as well as in human CRC. In this respect, we could demonstrate that OLFM4 overexpression did not initiate tumor growth in mice nor promote proliferation or migration in CRC cell lines. As our results from functional cell culture experiments highly correlated with the results from xenograft models and were highly significant, we did not extend our study to knockdown experiments, which could be of interest to further investigate in a future study.

Complex interactions between OLFM4 and other signaling intermediates, with OLFM4 being part of an intricate network and multi-directory crosstalk, might explain different mechanisms of action and differing activities of OLFM4 as part of a bigger picture. In this respect, OLFM4 expression might be part of feedback loops and regulated at a transcriptional as well as translational level [18,23]. Furthermore, OLFM4 function might additionally be orchestrated by the microenvironment that has previously been suggested to play an active role in CRC progression, enabling the growth of an environmental CRC type through specific signals, dominating in well-differentiated colon carcinomas as opposed to a genetic type in less differentiated CRC, thereby reflecting the high heterogeneity of CRC. In this respect, OLFM4 has been suggested to be involved in NF-κB signaling. However, this study did not test for inflammation dependency; thereby a co-stimulation by inflammatory signals cannot be excluded and is a limitation of this study [22]. Further data with regard to OLFM4 and interactions with the tumor microenvironment and immune system are missing and at this point only speculative but should be addressed in future studies [37,56].

A further limitation of this study is the biased human cohort. Although the metastasis collection is a well-characterized cohort that is very homogenous due to its design as a matched case–control study (including CRC with and without distant spread), it only includes CRC of the right hemicolon as opposed to the survival collection (only CRC without distant spread) that includes CRC of the whole colon. In this respect, a bias when interpreting the clinical correlation of OLFM4 expression and clinicopathological characteristics (e.g. grading, dMMR, and *BRAF*) cannot be excluded.

Overall, studies investigating cellular properties such as stemness, proliferation, EMT, migration, and adhesion have proposed several roles for OLFM4. However, the functional role of OLFM4 with regard to tumor progression remains controversial. The results of our study including functional tests investigating the effect of OLFM4 on Wnt signaling and EMT suggest that OLFM4 expression is associated with expression of differentiation markers in CRC but is not associated with properties of cancer stemness and is not a driver of colorectal carcinogenesis nor metastatic spread but rather a passenger.

Author contributions statement

SJ designed the study and collected and analyzed data. EP analyzed and interpreted data, wrote the manuscript, and performed the literature search. JN collected and analyzed data and edited the manuscript. AS, MS, JK and SS collected and analyzed data. MA edited the manuscript. FRG designed the study, analyzed and interpreted data and provided funding. LB collected data. FK edited the manuscript and provided funding. DH collected, analyzed, and interpreted data. TK designed the study, interpreted data, and provided funding. AJ designed the study, analyzed and interpreted data, provided funding, and edited the manuscript.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPLEMENTARY MATERIAL ONLINE

Figure S1. *OLFM4* expression profiles of cultures human colorectal cell lines

Figure S2. Validation of OLFM4-specific immunohistochemistry

Figure S3. Effect of forced OLFM4 expression on aldefluor activity

Figure S4. Effect of forced OLFM4 expression on migration and colony formation