Histol Histopathol (2016) 31: 469-478

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Review

FHL2: A scaffold protein of carcinogenesis, tumour-stroma interactions and treatment response

Laurine Verset[1], Lynn Feys[2], Anne-Laure Trépant[1], Olivier De Wever[2] and Pieter Demetter[1]

[1]Department of Pathology, Erasme University Hospital, Université Libre de Bruxelles, Bruxelles and [2]Laboratory of Experimental Cancer Research, Department of Radiation Oncology and Experimental Cancer Research, Ghent University, Ghent, Belgium

Summary. Four-and-a-half LIM-domain protein 2 (FHL2) is a multifunctional scaffolding protein regulating signalling cascades and gene transcription. It shuttles between focal adhesions and the nucleus where it signals through direct interaction with a number of proteins including β-catenin. The multiplicity of molecular pathways affected by FHL2 suggests an important role in several physiological and pathological events. The function of FHL2 in cancer is particularly intriguing, since it may act as an oncoprotein or as a tumour suppressor in a tissue-dependent fashion.

In this review we present the current knowledge on the role of FHL2 in carcinogenesis, with emphasis on the digestive tract. We discuss the overexpression of FHL2 in colorectal, gastric and pancreatic cancer, the downregulation in hepatocellular carcinoma and the role of FHL2 in epithelial-mesenchymal transition. We briefly look at the potential role of FHL2 in the tumoural microenvironment and discuss how FHL2 expression and function might influence cancer treatment. Before implementation of FHL2 as a biomarker by pathologists, antibody validation should, however, be carried out.

Key words: FHL2, Carcinogenesis, Digestive tract

Offprint requests to: Pieter Demetter, MD, PhD, Department of Pathology, Erasme University Hospital, Route de Lennik 808, 1070 Brussels, Belgium. e-mail: pieter.demetter@erasme.ulb.ac.be

DOI: 10.14670/HH-11-709

Introduction

Four-and-a-half LIM-domain protein 2 (FHL2) is the second member of the FHL-subfamily which is a part of the LIM-only proteins family (Kleiber et al., 2007). A LIM domain is a cysteine-rich motif and is formed by two zinc fingers that each coordinate the binding of one Zn^{2+} ion (Johannessen et al., 2006). FHL2 was first identified by Genini et al. who demonstrated FHL2 downregulation in rhabdomyosarcoma cells compared to normal myoblasts (Genini et al., 1997). In 1998, Chan et al. carried out molecular cloning of FHL2 using human fetal heart in order to describe the full length cDNA and the nucleotide sequence of FHL2 protein which contains 279 amino acids and which is located at chromosome 2q12-q13 (Chan et al., 1998).

FHL2 is a multifunctional scaffolding protein regulating signaling cascades and gene transcription. By forming a protein complex with integrins and focal adhesion kinase, FHL2 supports the clustering of integrins and integrin-driven assembly of matrix proteins. By interaction with cytosolic proteins, FHL2 is involved in regulation of NF-ϰB and MAPK signalling cascades. In addition to modulating signalling molecules, FHL2 shuttles between the cytosol and nucleus, acting as a cofactor of transcriptional activity.

The multiplicity of molecular pathways affected by FHL2 suggests an important role in several physiological events. High level of FHL2 expression is found in heart, and FHL2 is a key role protein throughout embryonic heart development and in adults (Chu et al., 2000). FHL2 plays an antihypertrophic role and its expression is reduced in human heart failure

(Friedrich et al., 2014). FHL2 seems also to be an important regulator of bone formation. FHL2 controls the osteogenic differentiation of mesenchymal stromal cells, enhances bone formation and bone mass through the modulation of Wnt molecules (Brun et al., 2013). Furthermore, FHL2 mediates osteogenic differentiation in mesenchymal stem cells induced by dexamethasone through activation of Wnt/beta-catenin signaling-dependent Runx2 expression (Hamidouche et al., 2008). FHL2 is also essential to allow wound healing. During the normal wound healing process, FHL2 is upregulated in myofibroblasts and the α-SMA expression is regulated by FHL2 through interaction with serum response factors. FHL2 deficiency impairs wound healing due to a defective collagen contraction and a decrease of *FHL2*$^{-/-}$mesenchymal stem cell motility (Wixler et al., 2007). Although FHL2 deficiency does not lead to a lethal phenotype, *FHL2*$^{-/-}$ deficient mice display impairment of cutaneous wound healing (Wixler et al., 2007) and intestinal healing (Kirfel et al., 2008), develop osteopenia (Brun et al., 2013) and present a hypertrophic response of the heart after β-adrenergic stimulation (Kong et al., 2001). FHL2 is also implicated in cell cycle and apoptosis (Johannessen et al., 2006).

In recent years, the number of publications studying a role for FHL2 in cancer is increasing. This review aims to discuss the current knowledge on the role of FHL2 in carcinogenesis, tumour-stroma interactions and cancer treatments as summarised in Fig. 1, with emphasis on the digestive tract. The emerging impact of FHL2 in cancer may warrant the use of FHL2 as biomarker.

Colorectal cancer

In human colorectal cancer, an overwhelming majority of cases carry mutations in Wnt pathway components including the adenomatous polyposis coli gene (Apc), axin and β-catenin. All the mutations lead to constitutive activation of the Wnt signalling pathway characterised by the formation of constitutive nuclear β-catenin/TCF complex. In a murine model, introduction of a biallelic deletion of *FHL2* into mutant *Apc*$^{\Delta 14/+}$ mice substantially reduces the number of intestinal adenomas but not tumour growth, suggesting a role of FHL2 in the initial steps of tumourigenesis (Labalette et al., 2010).

Study of FHL2 expression by immunoblotting and immunohistochemistry revealed a higher level of FHL2 in colorectal adenocarcinoma than in normal colonic mucosa (Wang et al., 2007). Recently, we demonstrated that immunohistochemical expression of FHL2 is related to a poor overall and metastasis free survival in patients

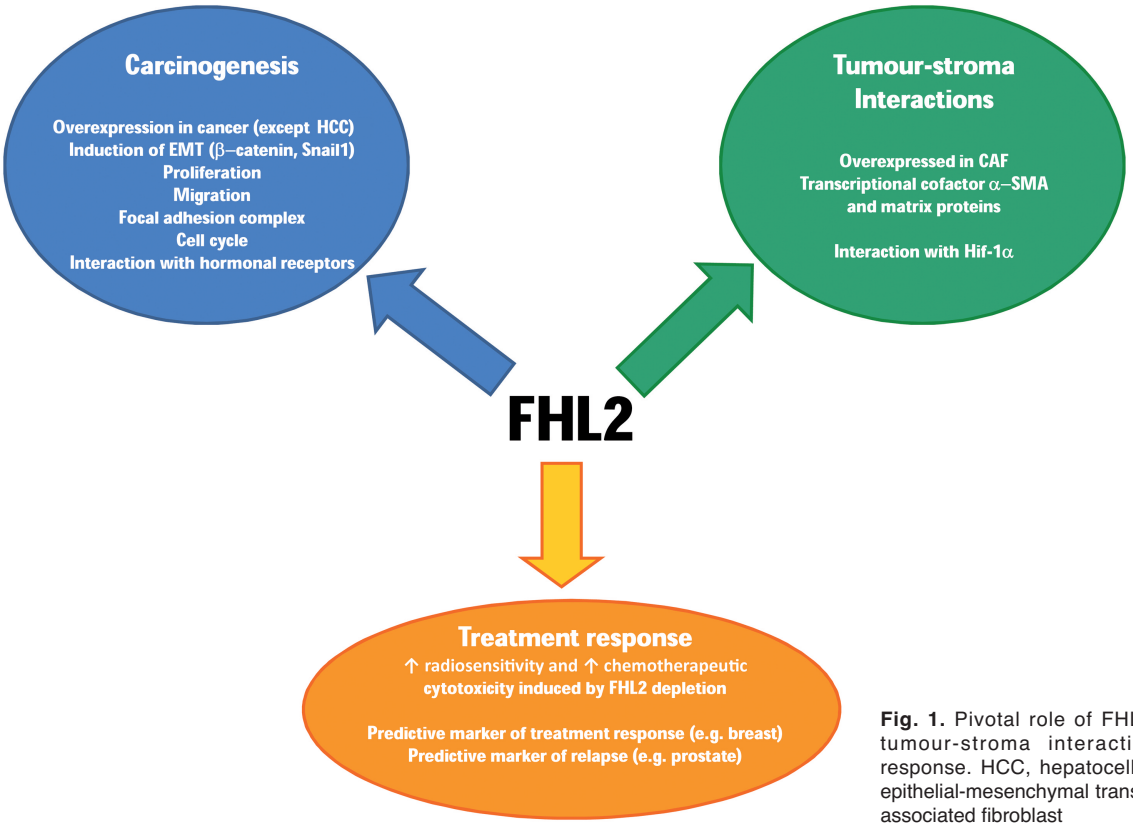

Fig. 1. Pivotal role of FHL2 in carcinogenesis, tumour-stroma interactions and treatment response. HCC, hepatocellular carcinoma; EMT, epithelial-mesenchymal transition; CAF, carcinoma-associated fibroblast

operated for colorectal adenocarcinoma (Verset et al., 2013). Another group demonstrated that nuclear expression of FHL2 in cancer cells is associated with lymphatic metastasis in sporadic but not in HNPCC-associated colon cancer (Al-Nomani et al., 2015). This result could be explained by the pivotal role exerted by FHL2 in epithelial-mesenchymal transition (EMT).

EMT is a multi-step, reversible program leading to a phenotypic switch of epithelial cells into mesenchymal cells either partially or fully (Derynck et al., 2014). Transforming growth factor-beta (TGFβ), a well-known inducer of EMT, increases FHL2 and vimentin expression and reduces E-cadherin expression in DLD1 cells treated with TGFβ1 for 48 hours (Zhang et al., 2010). TGFβ1 stimulates Krüppel-like factor (KLF) 8, and KLF8-induced FHL2 activation has been identified recently as a critical mechanism underlying colorectal (and breast) cancer invasion and metastasis (Yan et al., 2015).

The destruction of cellular junctions is one of the first steps of EMT, and loss of E-cadherin function contributes to cancer progression by increasing invasion (De Craene and Berx, 2013). FHL2 siRNA transfected SW480 cells present high levels of E-cadherin and low levels of vimentin, MMP-9, twist and Snail. The authors obtained the inverse results when they induced an overexpression of FHL2 in DLD1 (Zhang et al, 2010). These results are intriguing because transforming growth factor-β receptor II gene (*TGFβR2*) is frequently mutated in colorectal cancer with DNA microsatellite instability (MSI) (Markowitz et al., 1995) and the DLD1 colon cancer cell line is known as a MSI cell line (Woodford-Richens et al., 2001). Furthermore, Pino et al. studied TGFβ1 treatment on microsatellite stable (MSS) and on MSI colon cancer cells lines. For this purpose, they used two MSS cell lines (SW480 and HT29) and two MSI cell lines (DLD1 and HCT116). They demonstrated that TGFβ1 induced EMT in MSS colon cancer cell lines with wildtype *TGFβR2*. E-cadherin and vimentin expression did not change in TGFβ1-treated DLD1 which is known to have a mutated TGFβRII (Pino et al., 2010).

The transcription factor Snail1 is a master regulator of the EMT program, strongly represses E-cadherin and is overexpressed in several tumour types (De Craene and Berx, 2013). By *in vitro* studies, Zhang et al. demonstrated that FHL2 interacts with Snail1, induces Snail accumulation into the nucleus and, finally, modulates E-cadherin transcription in DLD1 and SW480 cells (Zhang et al., 2011).

FHL2 is also able to interact with β-catenin (Martin et al., 2002; Wei et al., 2003) and, in colon cancer cells, FHL2 is able to induce nuclear accumulation of β-catenin by preventing its phosphorylation-mediated degradation (Zhang et al., 2010). Furthermore, in FHL2 transfected DLD1 cells, the plasma membrane-associated E-cadherin/β-catenin complex is dissolved while the presence of these complexes is obvious after transfection with FHL2 siRNA in HCT116 (Zhang et al., 2010).

Overexpression of FHL2 in colorectal cancer cells seems essential to maintain their malignant phenotype: FHL2 suppression in Lovo cancer cells is characterised by morphotypic changes which are characteristic for differentiated cells. This effect is presumably mediated through inhibition of several oncogenes (*survivin, cox-2, hTERT, c jun*) leading to a reduction in cancer cell growth and, finally, a lower tumourigenicity in *in vivo* models (Wang et al., 2007). However, when over-expression of FHL2 was established in HT29, which is a well differentiated colon cancer cell line with low levels of endogenous FHL2, the authors observed an increased E-cadherin expression compared to the control, and an inhibition of the proliferation of FHL2 expressing cells (Amann et al., 2010).

Gastric cancer

FHL2 expression is higher in human gastric cancer compared to matched normal tissue (Wang et al., 2007). In agreement, Kato-III gastric cancer cell lines exhibit high levels of FHL2. As demonstrated in Lovo cancer cells, FHL2 suppression leads to an inhibition of oncogenes like *survivin, c jun* and *hTERT* (Wang et al., 2007). In AGS cancer cell lines, FHL2 interacts with XAF1 (XIAP associated factor-1) which is known as a tumour suppressor. XAF1 is able to reduce the nuclear FHL2 level and to increase the mitochondrial amount of FHL2. XAF1 tends to attenuate FHL2 transactivity by decreasing the promoter activity of the TCF/β-catenin transcription complex (Zhang et al., 2011).

Pancreatic cancer

FHL2 is overexpressed in pancreatic ductal adenocarcinoma. FHL2 mRNA expression is higher in pancreatic cancer compared to normal pancreatic tissue. Immunohistochemistry demonstrated that FHL2 presents mainly cytosolic and nuclear localization; in pancreatic cell lines, however, FHL2 is clearly located in the focal adhesion complex (Zienert et al., 2015).

Mutations in KRAS are commonly seen in pancreatic ductal adenocarcinoma (Reid et al., 2014; Heestand and Kurzrock, 2015); data on interaction of FHL2 with KRAS signalling are, however, lacking.

Hepatocellular carcinoma

While FHL2 is overexpressed in colorectal, prostate, breast and ovarian cancer, the expression of FHL2 is lower in hepatocellular carcinoma compared to matched normal adjacent liver tissue. Similarly, WRL68, a normal hepatocyte cell line, exhibits high levels of FHL2 compared to three cancer cell lines (Huh7, HepG2 and Hep3B) (Ng et al., 2011). The establishment of a FHL2 stable transfectant Hep3B results in lower protein levels of cyclin D1 and higher protein levels of p21 and p27 in transfectants than in controls. Furthermore, FHL2

overexpression causes inhibition of cell progression from G1 to S phase, decreased cell motility and inhibition of apoptosis, in particular a resistance to doxorubicin-induced apoptosis in FHL2 stable transfectants (Ng et al., 2011). This antiproliferative property of FHL2 was also demonstrated by Ding et al. in HepG2 cells (Ding et al., 2009).

While FHL2 exhibits antiproliferating characteristics in hepatic cancer cells, the situation is different in normal tissue. In FHL2 transgenic mice (Apo-FHL2 model displaying an increase of FHL2 transcripts by 11- to 17-fold in the liver), the enforced hepatic expression of FHL2 accelerates the hepatocyte turn-over by inducing proliferation and apoptosis without detectable injury. In this study, the stimulated proliferation was accompanied by an up-regulation of cyclin D1 while the induction of apoptosis depended on p53 activation. In this mouse model, FHL2 is critical for early stages of liver regeneration and activates the proliferation in answer to liver injury like partial hepatectomy (Nouët et al., 2012).

Interestingly, FHL2 upregulation in human liver specimens shows significant association with increasing inflammation and cirrhosis. While Apo-FHL2 mice develop no tumours, the FHL2 transgene enhances hepatocarcinogenesis induced by liver-specific deletion of the adenomatous polyposis coli gene and aberrant Wnt/β-catenin signaling in Apclox/lox animals (Nouët et al., 2012).

Other cancer types

FHL2 interacts with the androgen receptor (AR) and is a co-activator of AR-dependent transcription in an agonist and activation function-2 (AF-2) dependent manner (Müller et al., 2000). In the normal prostate, FHL2 is almost confined to the plasma membrane but an intense cytoplasmic staining is detected by immunohistochemistry in the basal cell compartment while a strong nuclear and cytoplasmic expression is observed in undifferentiated prostate carcinoma (Müller et al., 2002). In high grade localized prostate carcinoma, the distribution of FHL2 is nuclear and associated with cancer relapse following radical prostatectomy (Kahl et al., 2006).

The nuclear translocation of FHL2 in prostate cancer could be launched through different mechanisms. Firstly, stimulation of the Rho pathway induces the translocation of FHL2 into the nucleus generating subsequent activation of FHL2- and androgen receptor-dependent genes (Müller et al., 2002). Secondly, calpain cleavage of filamin seems particularly important for nuclear FHL2 localisation in castrate-resistant prostate cancer (McGrath et al., 2013). Filamin promotes the formation of actin fibers in the cytoplasm, the cellular motility and the subsequent invasiveness of prostate cancer cells (Bedolla et al., 2009). Filamin is a substrate for Ca^{2+}-dependent calpain proteases; the cleavage of filamin results in nuclear FHL2 storage and increased AR coactivation in prostate cancer cell lines (McGrath et al., 2013). Furthermore, in castrate resistant prostate cancer, the authors demonstrated a ligand-independent activation of AR by FHL2 (McGrath et al., 2013). While FHL2 activates androgen receptor dependent genes, androgens induce FHL2 expression ensuring AR transcription and this effect is mediated by an indirect serum-response-factor (SRF) dependent mechanism (Heemers et al., 2007).

An immunohistochemical study showed that patients with breast cancer expressing low amounts of FHL2 are characterised by a significantly better survival compared to those with high intratumoural FHL2 expression. Furthermore, the authors showed that patients receiving tamoxifen and presenting a tumour expressing high amounts of FHL2 exhibit an improved survival rate (Gabriel et al., 2006). Martin et al have confirmed the high expression of FHL2 in breast cancer. In this study, they evaluated FHL2 expression by western blotting in normal breast tissue, ductal carcinoma *in situ* and invasive breast carcinoma. They found an absence of FHL2 in normal tissue while low levels and high levels were highlighted in DCIS and invasive carcinoma, respectively. They also demonstrated high expression of FHL2 in T47D, SKBR3 and MDA-MB 231 breast cancer cells while moderate levels were retrieved in BT549, EFM-192A and EFM-192B; no detectable level of FHL2 protein was found in MCF7-, DU4475-, MDA-MB 435- and BT474 cells (Martin et al., 2007).

FHL2 is capable of interacting specifically with the BRCT2 domain of BRCA1 and the FHL2 transactivation is enhanced by BRCA1 (Yan et al., 2003). FHL2 induces p21 expression in MDA-MB 231 breast cancer cells independent of p53 but dependent on MAP kinase signaling (Martin et al., 2007). Furthermore, the authors demonstrated that FHL2 contributes to MAPK-induced transcriptional activation of AP1-dependent target genes like interleukin-6 and -8 in breast cancer cells (Martin et al., 2007). This was, however, not the first study demonstrating the role of FHL2 in the MAPK signaling pathway. FHL2 is a transcriptional coactivator of c-jun in monkey kidney cells (Morlon and Sassone-Corsi, 2003) and interacts with phosphorylated ERK2 in cardiomyocytes (Purcell et al., 2004). FHL2 participates in a transcriptional regulatory network including c-fos and FRA1 essential to control the ERK signaling pathway through a negative feedback loop for MCF-7 cell differentiation (Saeki et al., 2009).

FHL2 has been identified as a protein interacting with the oestrogen receptor (ER) alpha (Kobayashi et al., 2004). Another study confirms this result and shows that FHL2 functionally interacts with ER alpha and beta, and that FHL2 overexpression is related to a reduced ER transcriptional activity in breast cancer cells. This result is the consequence of a synergistic inhibition created by FHL2 and SMAD4 (Xiong et al., 2010).

FHL2 is overexpressed in ovarian cancer and interacts with pp125FAK, a cytoplasmic tyrosine kinase, involved in anchorage-independent growth of tumour

cells (Gabriel et al., 2004).

While FHL2 is more and more studied in carcinomas, few data have been reported about the role of FHL2 in brain tumours, particularly in gliomas. To our knowledge, only one study investigated the role of FHL2 in glioma (Li et al., 2008). This study showed that FHL2 mRNA is upregulated in the majority of human high-grade glioma samples as compared to normal brain tissues. Furthermore, FHL2 mRNA and protein expression was detected in the majority of the conventional glioma cell lines studied.

Concerning the functional roles of FHL2 in glioblastoma, this study showed that overexpression of FHL2 increases glioblastoma cell proliferation and migration *in vitro*. The authors supported their results by FHL2 knockdown by short hairpin RNA which inhibited glioblastoma cell proliferation and migration. Furthermore, by subcutaneous injection of glioblastoma cells in nude mice the authors demonstrated that overexpression of FHL2 promotes glioblastoma tumourigenesis *in vivo*.

The SKI oncoprotein enhances the activation of *FHL2* and/or *β-catenin*-regulated gene promoters in melanoma cells. Furthermore, transient overexpression of SKI and FHL2 in *ski*$^{-/-}$ melanocytes synergistically enhances cell growth (Chen et al., 2003). Recently, an immunohistochemical study demonstrated that high expression of FHL2 is correlated with poor survival in human melanoma (Westphal et al, 2015).

Data on FHL2 in lung cancer are lacking.

Potential role of FHL2 in the tumoural micro-environment

Carcinoma-associated fibroblasts (CAFs) guide tumoural invasion and metastasis by providing an appropriate soil (De Wever et al., 2008, 2014). FHL2 deficiency in fibroblasts leads to an impaired assembly of the extracellular matrix (Park et al., 2008), and to a less efficient wound healing process (Wixler et al., 2007). FHL2 is highly expressed in myofibroblasts in wound healing (Wixler et al, 2007) but also in CAFs in colorectal cancer (Gullotti et al., 2011; Verset et al., 2013) (Fig. 2). FHL2 acts as a cofactor in the transcription of αSMA (alpha smooth muscle actin) and some matrix proteins, but inhibits the expression of matrix metalloproteinases (Alnajar et al., 2013). Gullotti et al. demonstrated that FHL2 overexpression in CAFs correlates to lymph node metastasis in sporadic colon cancer but not in hereditary non-polyposis colon cancer; they showed that FHL2 expression in CAFs could be due to TGF-β1 production by tumoural cells (Gullotti et

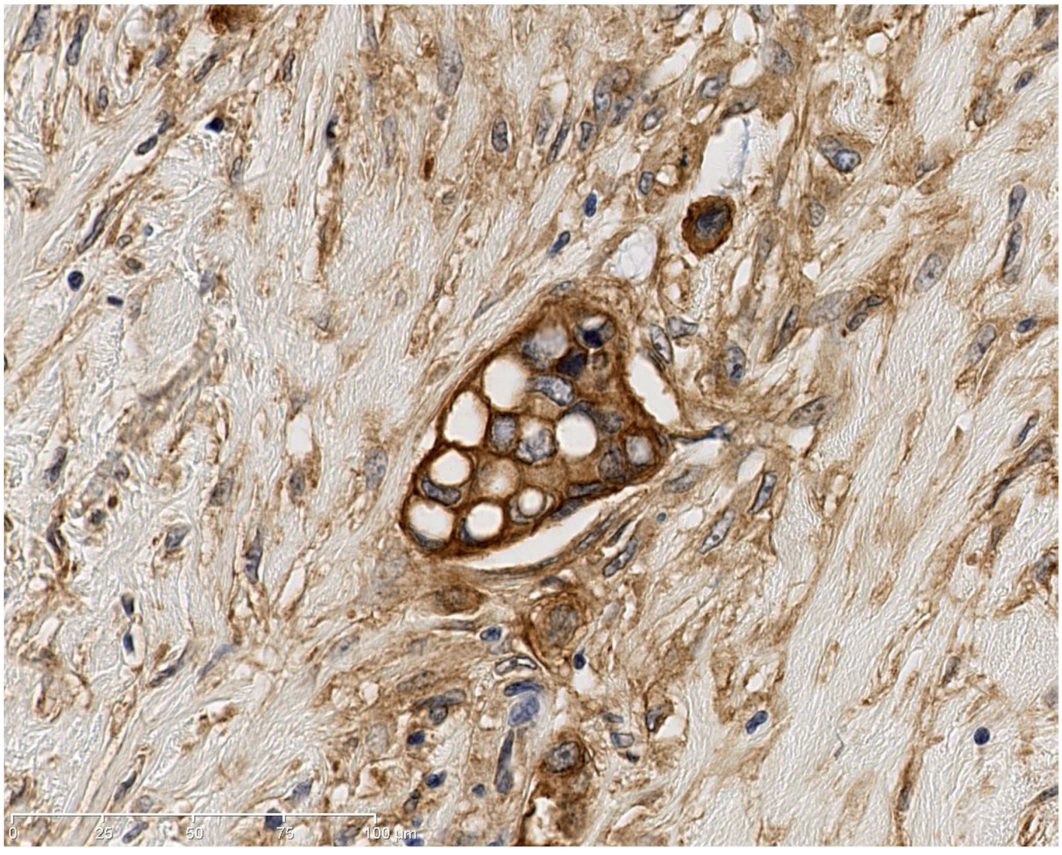

Fig. 2. FHL2 expression in neoplastic epithelium and in cancer-associated fibroblasts in colorectal cancer.

al., 2011).

FHL2 could also be implicated in angiogenesis. FHL2 interacts with Hif-1α and inhibits Hif-1 transcriptional activity (Hubbi et al., 2012; Lin et al., 2012). Furthermore, deletion of *FHL2* in mice decreases chemical-induced corneal angiogenesis (Chu et al., 2008).

The role and impact of FHL2 in cancer treatment

Sporadic reports describe an impact of FHL2 on cancer treatment protocols or vice versa, an impact of the treatment on FHL2 expression or localisation. Irradiation of 3D organised pancreatic cell cultures showed that FHL2 depletion stimulates radiosensitisation. Knocking down the expression of FHL2 by siRNA in pancreatic cancer cells with high FHL2 expression levels sensitises the cancer cells to radiation comparable to the radiosensitivity of cancer cells with low expression levels of FHL2 (Zienert et al., 2015). The mechanism of radiosensitisation may involve the cell cycle inhibitor $p21^{Cip/Waf1}$.

Martin et al. found that the expression of the $p21^{Cip/Waf1}$ is dependent on FHL2 in MDA-MB-231 breast cancer cells. In response to DNA damage there is an upregulation of FHL2 which is paralleled by $p21^{Cip/Waf1}$ upregulation in FHL2 positive cells but there is no upregulation either of FHL2 or $p21^{WAF1}$ in FHL2 negative cells. So, FHL2 depletion of the breast cancer cells enhances cytotoxicity by the DNA damaging agent doxorubicin (Martin et al., 2007). Interestingly, in the tumour environment, not only cancer cells may show upregulation of FHL2 after DNA-damaging therapy but also stromal fibroblasts. Gamma irradiation provoked elevated FHL2 mRNA levels and elevated p21Cip/wAF1 levels in p53 +/+ primary mouse myo- and fibroblasts (Scholl et al., 2000). $p21^{Cip/Waf1}$ is a well-known cyclin/CDK inhibitor; it inhibits cell progression from G1 to S phase by inhibiting cyclin/CDK activities and thereby maintains the hypophosphorylated repressor state of Rb. Moreover, $p21^{Cip/Waf1}$ can also associate with transcription factors such as E2F or c-Myc to suppress cell cycle progression. So, elevated levels of $p21^{Cip/Waf1}$ keep cells alive after DNA damage in order to repair the damage (Delavaine and La Thangue, 1999; Abbas and Duta, 2009). Moreover, p21Cip/Waf1 overexpression has been described to prevent apoptosis by binding and inhibiting the p34cdc-2/CDK-1 complex, which plays an important role in resistance to apoptosis induced by paclitaxel in breast cancer cells (Shen et al., 1998; Yu et al., 1998). In contradiction, an immunohistochemical-based retrospective correlation study on paraffin-embedded tissues from breast cancer patients revealed that adjuvant tamoxifen treatment gives a significantly improved survival rate for patients receiving tamoxifen and being diagnosed with a tumour expressing high amounts of FHL2. This might indicate that tamoxifen is at least partially capable of reversing the negative prognostic impact of high FHL2 expression (Gabriel et al., 2006). FHL2 is a predictive marker; for some treatments it may provide cancer cells a survival advantage but for others a high presence may benefit treatment.

FHL2 can serve as predictive biomarker for

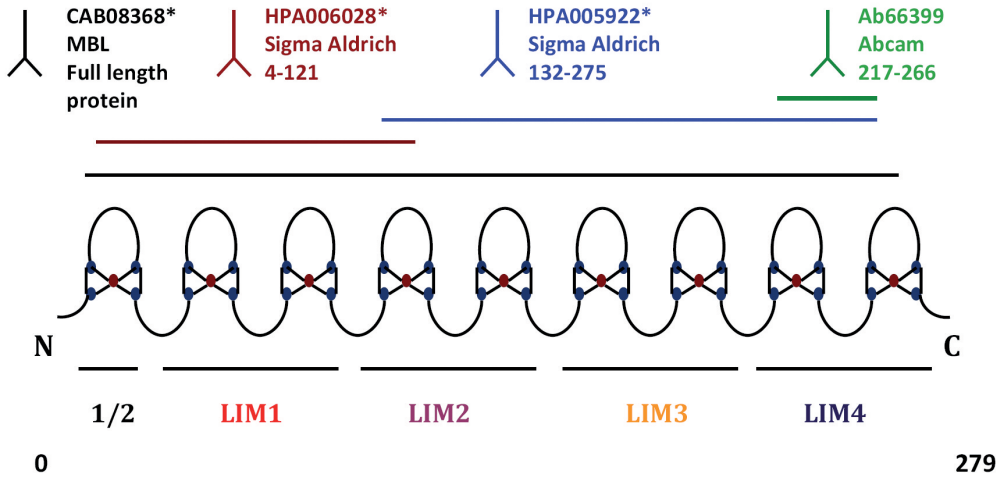

Fig. 3. Four commercial anti-FHL2 antibodies and their target epitopes. FHL2 consists of 279 amino acids arranged in four LIM domains and a half at the N terminus. The amino acid sequence is detailed at the bottom of the figure, the specific sequence of each LIM domain is highlighted by different colours (www.uniprot.org). Four commercial antibodies are represented at the top of the figure with their target epitope. Asterisk marked antibodies have been assessed in different normal and tumoural tissues and referred by the Human Protein Atlas (www.proteinatlas.org). CAB08368 is synthesised based on the recombinant human full length FHL2 protein. Figure adapted from the review of Kleiber et al. (Kleiber et al., 2007).

treatment response in prostate cancer. FHL2 localises to the nucleus in high grade, localised prostate cancer, correlating with cancer recurrence following radical prostatectomy (Kahl et al., 2006; McGrath et al., 2013). Also, FHL2 was found out of 2400 known human genes, to be a possible biomarker for ionising radiation exposure. 89% upregulation was seen in the peripheral blood lymphocytes after a single exposure of 1Gy. A linear dose-response relationship between 0.5 and 4Gy was observed 12 h after irradiation, suggesting that relative expression levels of FHL2 in peripheral blood lymphocytes may give a good estimation of total radiation exposure (Kang et al., 2003).

FHL2: Use as a potential biomarker in cancer?

The spatial localisation and the expression level of FHL2 in several malignancies suggest the use of FHL2 as a prognostic marker. Taking into account the impact of cancer treatment protocols on FHL2 expression/localisation, FHL2 could potentially be used as a biomarker in cancer to guide new targeted therapy or to predict recurrence. Appropriate antibodies need, however, to be established to perform reproducible (interlab) immunostainings. Table 1 summarises the different immunohistochemical studies using anti-FHL2 antibodies, thereby highlighting the discrepancies

Table 1. Immunohistochemical studies with different anti-FHL2 antibodies and observed staining patterns.

Reference	Techniques	Antibody Company		Staining pattern in epithelial cells
Colorectal cancer				
Wang et al., 2007	IHC	MM MBLI	N	No expression detected
			T	Nuclear expression
Zhang et al., 2010	IHC	R Abcam	N	No expression detected
			T	Strong immunoreactivity in the cytoplasm and in the nuclei
Zhang et al., 2011	IHC	R Abcam	N	Higher expression of FHL2 in tumoural tissue compared to adjacent normal tissue, no description of subcellular distribution in human tissues.
			T	
Gullotti et al., 2011	IHC, IF	R* Schüle Lab.	N	Not studied
			T	Strong membranous but nearly absent FHL2 nuclear expression in HNPCC associated cancer
Verset et al., 2013	IHC	RP Abcam	N	not studied
			T	Cytoplasmic and membranous immunoreactivity. No nuclear staining.
Al-Nomani et al., 2015	IHC, IF	R* Schüle Lab.	N	Membranous staining of enterocytes without nuclear staining
			T	Nuclear staining particularly in poorly differentiated cancer cells
Pancreatic cancer				
Zienert et al., 2015	IHC	RP Sigma Aldrich	N	Not studied
			T	Predominant cytosolic and nuclear staining
Prostate cancer				
Müller et al., 2000	IHC	MM*	N	Cytoplasmic and nuclear FHL2 expression detected in the secretory epithelium of the prostate
			T	Not studied
Müller et al., 2002	IHC	RP*	N	Intense cytoplasmic expression in basal cells
			T	Strong nuclear and cytoplasmic expression in undifferentiated prostate cancer
Kahl et al., 2006	IHC	MM*	N	Cytoplasmic expression in basal cells but not in luminal cells
			T	More nuclear staining in higher Gleason score cancer
McGrath et al., 2013	IHC	R**	N	Cytoplasmic expression in luminal and basal cells
			T	Cytoplasmic expression in low grade localized prostate cancer and strong nuclear FHL2 expression in castrate resistant prostate cancer
Breast cancer				
Gabriel et al., 2006	IHC	RP*	N	Not studied
			T	Cytoplasmic expression
Ovarian cancer				
Gabriel et al., 2004	IHC	RP*	N	Strong expression in epithelial cells
			T	Strong cytoplasmic and membranous expression. No nuclear staining
Melanoma				
Westphal et al., 2015	IHC, IF	R* Schüle Lab.	N	Membranous expression in keratinocytes. No expression in melanocytes.
			T	Cytoplasmic and/or membranous expression. No nuclear staining

* non-commercial antibody **clone and company not precised. HNPCC, hereditary non-polyposis colorectal cancer; IF, immunofluorescence; IHC, immunohistochemistry; MM, mouse monoclonal; N, normal; R, rabbit; RP, rabbit polyclonal; T, tumoural.

between studies. As well as in the "human protein atlas" (www.proteinatlas.org), the three verified antibodies displayed different immunohistochemical patterns. Antibody validation should be carried out using standard guidelines (Howat et al., 2014) before implementation of FHL2 as a new biomarker in cancer. Fig. 3 illustrates four commercially available antibodies with the corresponding epitopes, possibly resulting in different immunohistochemical staining patterns.

References

Abbas T. and Dutta A. (2009). p21 in cancer: intricate networks and multiple activities. Nat. Rev. Cancer 9, 400-414.

Alnajar A., Nordhoff C., Schied T., Chiquet-Ehrismann R., Loser K., Vogl T., Ludwig S. and Wixler V. (2013). The LIM-only protein FHL2 attenuates lung inflammation during bleomycin-induced fibrosis. PLoS One 8, e81356.

Al-Nomani L., Friedrichs J., Schüle R., Büttner R. and Friedrichs N. (2015). Tumoral expression of nuclear cofactor FHL2 is associated with lymphatic metastasis in sporadic but not in HNPCC-associated colorectal cancer. Pathol. Res. Pract. 211, 171-174.

Amann T., Egle Y., Bosserhoff A.K. and Hellerbrand C. (2010). FHL2 suppresses growth and differentiation of the colon cancer cell line HT-29. Oncol. Rep. 2010; 23, 1669-1674.

Bedolla R.G., Wang Y., Asuncion A., Chamie K., Siddiqui S., Mudryj M.M., Prihoda T.J., Siddiqui J., Chinnaiyan A.M., Mehra R., de Vere White R.W. and Ghosh P.M. (2009). Nuclear versus cytoplasmic localization of filamin A in prostate cancer: immunohistochemical correlation with metastases. Clin. Cancer Res. 15, 788-796.

Brun J., Fromigué O., Dieudonné F.X., Marty C., Chen J., Dahan J., Wei Y. and Marie P.J. (2013). The LIM-only protein FHL2 controls mesenchymal cell osteogenic differentiation and bone formation through Wnt5a and Wnt10b. Bone 53, 6-12.

Chan K.K., Tsui S.K., Lee S.M., Luk S.C., Liew C.C., Fung K.P., Waye M.M. and Lee C.Y. (1998). Molecular cloning and characterization of FHL2, a novel LIM domain protein preferentially expressed in human heart. Gene 210, 345-350.

Chen D., Xu W., Bales E., Colmenares C., Conacci-Sorrell M., Ishii S., Stavnezer E., Campisi J., Fisher D.E., Ben-Ze'ev A. and Medrano E.E. (2003). SKI activates Wnt/beta-catenin signaling in human melanoma. Cancer Res. 63, 6626-6634.

Chu P.H., Ruiz-Lozano P., Zhou Q., Cai C. and Chen J. (2000). Expression patterns of FHL/SLIM family members suggest important functional roles in skeletal muscle and cardiovascular system. Mech. Dev. 95, 259-265.

Chu P.H., Yeh L.K., Lin H.C., Jung S.M., Ma D.H., Wang I.J., Wu H.H., Shiu T.F. and Chen J. (2008). Deletion of the FHL2 gene attenuating neovascularization after corneal injury. Invest. Ophthalmol. Vis. Sci. 49, 5314-5318.

De Craene B and Berx G. (2013). Regulatory networks defining EMT during cancer initiation and progression. Nat. Rev. Cancer 13, 97-110.

Delavaine L. and La Thangue N.B. (1999). Control of E2F activity by p21Waf1/Cip1. Oncogene 18, 5381-5392.

Derynck R., Muthusamy B.P. and Saeteurn K.Y. (2014). Signaling pathway cooperation in TGF-β-induced epithelial-mesenchymal transition. Curr. Opin. Cell Biol. 31C, 56-66.

De Wever O., Demetter P., Mareel M. and Bracke M. (2008). Stromal myofibroblasts are drivers of invasive cancer growth. Int. J. Cancer 123, 2229-2238.

De Wever O., Van Bockstal M., Mareel M., Hendrix A. and Bracke M. (2014). Carcinoma-associated fibroblasts provide operational flexibility in metastasis. Semin. Cancer Biol. 25, 33-46.

Ding L., Wang Z., Yan J., Yang X., Liu A., Qiu W., Zhu J., Han J., Zhang H., Lin J., Cheng L., Qin X., Niu C., Yuan B., Wang X., Zhu C., Zhou Y., Li J., Song H., Huang C. and Ye Q. (2009). Human four-and-a-half LIM family members suppress tumor cell growth through a TGF-beta-like signaling pathway. J. Clin. Invest. 119, 349-361.

Friedrich F.W., Reischmann S., Schwalm A., Unger A., Ramanujam D., Münch J., Müller O.J., Hengstenberg C., Galve E., Charron P., Linke W.A., Engelhardt S., Patten M., Richard P., van der Velden J., Eschenhagen T., Isnard R. and Carrier L. (2014) FHL2 expression and variants in hypertrophic cardiomyopathy. Basic Res. Cardiol. 109, 451.

Gabriel B., Mildenberger S., Weisser C.W., Metzger E., Gitsch G., Schüle R. and Müller J.M. (2004). Focal adhesion kinase interacts with the transcriptional coactivator FHL2 and both are overexpressed in epithelial ovarian cancer. Anticancer Res. 24, 921-927.

Gabriel B., Fischer D.C., Orlowska-Volk M., zur Hausen A., Schüle R., Müller J.M. and Hasenburg A. (2006). Expression of the transcriptional coregulator FHL2 in human breast cancer: a clinicopathologic study. J. Soc. Gynecol. Invest. 13, 69-75.

Genini M., Schwalbe P., Scholl F.A., Remppis A., Mattei M.G. and Schäfer B.W. (1997). Subtractive cloning and characterization of DRAL, a novel LIM-domain protein down-regulated in rhabdomyosarcoma. DNA Cell. Biol. 16, 433-442.

Gullotti L., Czerwitzki J., Kirfel J., Propping P., Rahner N., Steinke V., Kahl P., Engel C., Schüle R., Buettner R. and Friedrichs N. (2011). FHL2 expression in peritumoural fibroblasts correlates with lymphatic metastasis in sporadic but not in HNPCC-associated colon cancer. Lab. Invest. 91, 1695-1705.

Hamidouche Z., Haÿ E., Vaudin P., Charbord P., Schüle R., Marie P.J. and Fromigué O. (2008). FHL2 mediates dexamethasone-induced mesenchymal cell differentiation into osteoblasts by activating Wnt/beta-catenin signaling-dependent Runx2 expression. FASEB J. 22, 3813-3822.

Heemers H.V., Regan K.M., Dehm S.M. and Tindall D.J. (2007). Androgen induction of the androgen receptor coactivator four and a half LIM domain protein-2: evidence for a role for serum response factor in prostate cancer. Cancer Res. 67, 10592-10599.

Heestand G.M. and Kurzrock R. (2015). Molecular landscape of pancreatic cancer: implications for current clinical trials. Oncotarget 6, 4553-4561.

Howat W.J., Lewis A., Jones P., Kampf C., Pontén F., van der Loos C.M., Gray N., Womack C. and Warford A. (2014). Antibody validation of immunohistochemistry for biomarker discovery: recommendations of a consortium of academic and pharmaceutical based histopathology researchers. Methods 70, 34-38.

Hubbi M.E., Gilkes D.M., Baek J.H. and Semenza G.L. (2012). Four-and-a-half LIM domain proteins inhibit transactivation by hypoxia-inducible factor 1. J. Biol. Chem. 287, 6139-6149.

Johannessen M., Møller S., Hansen T., Moens U. and Van Ghelue M. (2006). The multifunctional roles of the four-and-a-half-LIM only protein FHL2. Cell. Mol. Life. Sci. 63, 268-284.

Kahl P., Gullotti L., Heukamp L.C., Wolf S., Friedrichs N., Vorreuther R., Solleder G., Bastian P.J., Ellinger J., Metzger E., Schüle R. and

Buettner R (2006). Androgen receptor coactivators lysine-specific histone demethylase 1 and four and a half LIM domain protein 2 predict risk of prostate cancer recurrence. Cancer Res. 66, 11341-11347.

Kang C.M., Park K.P., Song J.E., Jeoung D.I., Cho C.K., Kim T.H., Bae S., Lee S.J. and Lee Y.S. (2003). Possible biomarkers for ionizing radiation exposure in human peripheral blood lymphocytes. Radiat. Res. 159, 312-319.

Kirfel J., Pantelis D., Kabba M., Kahl P., Röper A., Kalff J.C. and Buettner R. (2008). Impaired intestinal wound healing in Fhl2-deficient mice is due to disturbed collagen metabolism. Exp. Cell Res. 314, 3684-3691.

Kleiber K., Strebhardt K. and Martin B.T. (2007). The biological relevance of FHL2 in tumour cells and its role as a putative cancer target. Anticancer Res. 27, 55-61.

Kobayashi S., Shibata H., Yokota K., Suda N., Murai A., Kurihara I., Saito I. and Saruta T. (2004). FHL2, UBC9, and PIAS1 are novel estrogen receptor alpha-interacting proteins. Endocr. Res. 30, 617-621.

Kong Y., Shelton J.M., Rothermel B., Li X., Richardson J.A., Bassel-Duby R. and Williams R.S. (2001). Cardiac-specific LIM protein FHL2 modifies the hypertrophic response to beta-adrenergic stimulation. Circulation 103, 2731-2738.

Labalette C., Nouët Y., Levillayer F., Colnot S., Chen J., Claude V., Huerre M., Perret C., Buendia M.A. and Wei Y. (2010). Deficiency of the LIM-only protein FHL2 reduces intestinal tumorigenesis in Apc mutant mice. PLoS One 5, e10371.

Li M., Wang J., Ng S.S., Chan C.Y., Chen A.C., Xia H.P., Yew D.T., Wong B.C., Chen Z., Kung H.F. and Lin M.C. (2008). The four-and-a-half-LIM protein 2 (FHL2) is overexpressed in gliomas and associated with oncogenic activities. Glia 56, 1328-1338.

Lin J., Qin X., Zhu Z., Mu J., Zhu L., Wu K., Jiao H., Xu X. and Ye Q. (2012). FHL2 family members suppress vascular endothelial growth factor expression through blockade of dimerization of HIF1α and HIF1β. IUBMB Life 64, 921-930.

Markowitz S., Wang J., Myeroff L., Parsons R., Sun L., Lutterbaugh J., Fan R.S., Zborowska E., Kinzler K.W. and Vogelstein B. (1995). Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. Science 268, 1336-1338.

Martin B., Schneider R., Janetzky S., Waibler Z., Pandur P., Kühl M., Behrens J., von der Mark K., Starzinski-Powitz A. and Wixler V. (2002). The LIM-only protein FHL2 interacts with beta-catenin and promotes differentiation of mouse myoblasts. J. Cell Biol. 159, 113-122.

Martin B.T., Kleiber K., Wixler V., Raab M., Zimmer B., Kaufmann M. and Strebhardt K. (2007). FHL2 regulates cell cycle-dependent and doxorubicin-induced p21Cip1/Waf1 expression in breast cancer cells. Cell Cycle 6, 1779-1788.

McGrath M.J., Binge L.C., Sriratana A., Wang H., Robinson P.A., Pook D., Fedele C.G., Brown S., Dyson J.M., Cottle D.L., Cowling B.S., Niranjan B., Risbridger G.P. and Mitchell C.A. (2013). Regulation of the transcriptional coactivator FHL2 licenses activation of the androgen receptor in castrate-resistant prostate cancer. Cancer Res. 73, 5066-5079.

Morlon A. and Sassone-Corsi P. (2003). The LIM-only protein FHL2 is a serum-inducible transcriptional coactivator of AP-1. Proc. Natl. Acad. Sci. USA 100, 3977-3982.

Müller J.M., Isele U., Metzger E., Rempel A., Moser M., Pscherer A., Breyer T., Holubarsch C., Buettner R. and Schüle R. (2000). FHL2, a novel tissue-specific coactivator of the androgen receptor. EMBO J. 19, 359-369.

Müller J.M., Metzger E., Greschik H., Bosserhoff A.K., Mercep L., Buettner R. and Schüle R. (2002). The transcriptional coactivator FHL2 transmits Rho signals from the cell membrane into the nucleus. EMBO J. 21, 736-748.

Ng C.F., Ng P.K., Lui V.W., Li J., Chan J.Y., Fung K.P., Ng Y.K., Lai P.B. and Tsui S.K. (2011). FHL2 exhibits anti-proliferative and anti-apoptotic activities in liver cancer cells. Cancer Lett. 304, 97-106.

Nouët Y., Dahan J., Labalette C., Levillayer F., Julien B., Jouvion G., Cairo S., Vives F.L., Ribeiro A., Huerre M., Colnot S., Perret C., Nhieu J.T., Tordjmann T., Buendia M.A. and Wei Y. (2012). The four and a half LIM-only protein 2 regulates liver homeostasis and contributes to carcinogenesis. J. Hepatol. 57, 1029-1036.

Park J., Will C. Martin B., Gullotti L., Friedrichs N., Buettner R., Schneider H., Ludwig S. and Wixler V. (2008). Deficiency in the LIM-only protein FHL2 impairs assembly of extracellular matrix proteins. FASEB J. 22, 2508-2520.

Pino M.S., Kikuchi H., Zeng M., Herraiz M.T., Sperduti I., Berger D., Park D.Y., Iafrate A.J., Zukerberg L.R. and Chung D.C. (2010). Epithelial to mesenchymal transition is impaired in colon cancer cells with microsatellite instability. Gastroenterology 138, 1406-1417.

Purcell N.H., Darwis D., Bueno O.F., Muller J.M., Schule R. and Molkentin J.D. (2004). Extracellular signal-regulated kinase 2 interacts with and is negatively regulated by the LIM-only protein FHL2 in cardiomyocytes. Mol. Cell Biol. 24, 1081-1095.

Reid M.D., Saka B., Balci S., Goldblum A.S. and Adsay N.V. (2014). Molecular genetics of pancreatic neoplasms and their morphologic correlates: an update on recent advances and potential diagnostic applications. Am. J. Clin. Pathol. 141, 168-180.

Saeki Y., Endo T., Ide K., Nagashima T., Yumoto N., Toyoda T., Suzuki H., Hayashizaki Y., Sakaki Y. and Okada-Hatakeyama M. (2009). Ligand-specific sequential regulation of transcription factors for differentiation of MCF-7 cells. BMC Genomics 10, 545.

Shen S.C., Huang T.S., Jee S.H. and Kuo M.L. (1998). Taxol-induced p34cdc2 kinase activation and apoptosis inhibited by 12-O-tetradecanoylphorbol-13-acetate in human breast MCF-7 carcinoma cells. Cell Growth. Differ. 9: 23-29.

Scholl F.A., McLoughlin P., Ehler E., de Giovanni C. and Schäfer B.W. (2000). DRAL is a p53-responsive gene whose four and a half LIM domain protein product induces apoptosis. J. Cell Biol. 151, 495-506.

Verset L., Tommelein J., Moles Lopez X., Decaestecker C., Mareel M., Bracke M., Salmon I., De Wever O. and Demetter P. (2013). Epithelial expression of FHL2 is negatively associated with metastasis-free and overall survival in colorectal cancer. Br. J. Cancer 109, 114-120.

Wang J., Yang Y., Xia H.H., Gu Q., Lin M.C., Jiang B., Peng Y., Li G., An X., Zhang Y., Zhuang Z., Zhang Z., Kung H.F. and Wong B.C. (2007). Suppression of FHL2 expression induces cell differentiation and inhibits gastric and colon carcinogenesis. Gastroenterology 132, 1066-1076.

Wei Y., Renard C.A., Labalette C., Wu Y., Lévy L., Neuveut C., Prieur X., Flajolet M., Prigent S. and Buendia M.A. (2003). Identification of the LIM protein FHL2 as a coactivator of beta-catenin. J. Biol. Chem. 278, 5188-5194.

Westphal P., Mauch C., Florin A., Czerwitzki J., Olligschläger N., Wodtke C., Schüle R., Büttner R. and Friedrichs N. (2015). Enhanced FHL2 and TGF-β1 expression is associated with invasive

growth and poor survival in malignant melanomas. Am. J. Clin. Pathol. 143, 248-256.

Wixler V., Hirner S., Müller J.M., Gullotti L., Will C., Kirfel J., Günther T., Schneider H., Bosserhoff A., Schorle H., Park J., Schüle R. and Buettner R. (2007). Deficiency in the LIM-only protein Fhl2 impairs skin wound healing. J. Cell Biol. 177, 163-721.

Woodford-Richens K.L., Rowan A.J., Gorman P., Halford S., Bicknell D.C., Wasan H.S., Roylance R.R., Bodmer W.F., Tomlinson I.P. (2001). SMAD4 mutations in colorectal cancer probably occur before chromosomal instability, but after divergence of the microsatellite instability pathway. Proc. Natl. Acad. Sci. USA 98, 9719-9723.

Xiong Z., Ding L., Sun J., Cao J., Lin J., Lu Z., Liu Y., Huang C. and Ye Q. (2010). Synergistic repression of estrogen receptor transcriptional activity by FHL2 and Smad4 in breast cancer cells. IUBMB Life 62, 669-676.

Yan J., Zhu J., Zhong H., Lu Q., Huang C. and Ye Q. (2003). BRCA1 interacts with FHL2 and enhances FHL2 transactivation function. FEBS Lett. 553, 183-189.

Yan Q., Zhang W., Wu Y., Wu M., Zhang M., Shi X., Zhao J., Nan Q., Chen Y., Wang L., Cheng T., Li J., Bai Y., Liu S. and Wang J. (2015). KLF8 promotes tumorigenesis, invasion and metastasis of colorectal cancer cells by transcriptional activation of FHL2. Oncotarget 6, 25402-25417.

Yu D, Jing T, Liu B, Yao J, Tan M, McDonnell TJ, Hung MC. (1998). Overexpression of ErbB2 blocks Taxol-induced apoptosis by upregulation of p21Cip1, which inhibits p34Cdc2 kinase. Mol. Cell. 2, 581-591.

Zhang W., Jiang B., Guo Z., Sardet C., Zou B., Lam C.S., Li J., He M., Lan H.Y., Pang R., Hung I.F., Tan V.P., Wang J. and Wong B.C. (2010). Four-and-a-half LIM protein 2 promotes invasive potential and epithelial-mesenchymal transition in colon cancer. Carcinogenesis 31, 1220-1229.

Zhang W., Wang J., Zou B., Sardet C., Li J., Lam C.S., Ng L., Pang R., Hung I.F., Tan V.P., Jiang B. and Wong B.C. (2011). Four and a half LIM protein 2 (FHL2) negatively regulates the transcription of E-cadherin through interaction with Snail1. Eur. J. Cancer 47, 121-130.

Zhang W., Yang Y., Jiang B., Peng J., Tu S., Sardet C., Zhang Y., Pang R., Hung I.F., Tan V.P., Lam C.S., Wang J. and Wong B.C. (2011). XIAP-associated factor 1 interacts with and attenuates the trans-activity of four and a half LIM protein 2. Mol. Carcinog. 50, 199-207.

Zienert E., Eke I., Aust D. and Cordes N. (2015). LIM-only protein FHL2 critically determines survival and radioresistance of pancreatic cancer cells. Cancer Lett. 364, 17-24.

Accepted December 17, 2015