Gene Expression and Protein Array Studies of Folliculin-regulated Pathways

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Abstract. *The familial cancer syndrome Birt-Hogg-Dube syndrome is characterised by the development of skin (fibrofolliculomas) and renal tumours (and lung cysts) and is caused by mutations in the FLCN tumour suppressor gene. Though the FLCN gene product (folliculin) has been linked to the regulation of a variety of signalling pathways (e.g. the mTOR, AMPK, TGFbeta and hyoxia-responsive genes) the precise function of the folliculin protein is not well-defined. In order to identify potential novel pathways linked to folliculin function we analysed paired isogenic folliculin-deficient and folliculin-expressing cell lines by gene expression and protein (Kinexus) arrays. Gene expression microarray analysis in the folliculin +/- non-renal cancer line (FTC133), revealed 708 differentially expressed targets (fold change >2 and p<0.001) with enrichment of genes in the cadherin and Wnt signalling pathways. Comparison of the differentially expressed genes in the FTC133 datasets and previously reported gene expression data for a folliculin-deficient renal tumour and the UOK257 renal cell carcinoma cell line, revealed that RAB27B was dysregulated in all three datasets (increased expression in folliculin-deficient cells). The Kinexus protein array analysis suggested 73 candidate, differentially expressed, proteins and further investigation by western blot analysis of 5 candidates that were also differentially expressed in the FTC133 gene expression microarray data, revealed that EIF2AK2 (PKR) and CASP1 were reduced and PLCG2 was increased in folliculin-deficient FTC133 cells and in a BHD renal tumour. In view of the role of CASP1 in apoptosis we investigated whether other apoptosis-related proteins might be regulated by folliculin and found increased levels of SMAC/Diablo and HtrA2 in folliculin-expressing FTC133 cells. These findings identify novel pathways and targets linked to folliculin tumour suppressor activity.*

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Kidney cancers account for about 2% of all cancers and, worldwide, more than 200,000 new cases are diagnosed each year (1). When detected early, surgical resection can be curative but though treatment with targeted-antiangiogenic therapies have improved survival in RCC, the outcome of patients with metastatic disease remains poor. Familial forms of RCC are infrequent (about 3% of all cases), but the elucidation of the molecular basis of rare familial forms of kidney cancer has provided seminal insights into the pathogenesis of the common non-familial forms of RCC. Thus, the identification of the gene (*VHL*) for the rare syndromic form of inherited RCC, known as von Hippel-Lindau disease, provided the basis for the discovery that somatic inactivation of the *VHL* tumour suppressor gene occurred in most sporadic clear-cell RCC (2-6). Furthermore, subsequent investigations demonstrated that *VHL* inactivation leads to stabilisation of hypoxia-inducible factor (HIF)-1 and HIF-2 transcription factors and activation of the hypoxic response gene pathway (7,8 and references within) and that HIF-mediated RCC growth may be antagonised by antiangiogenic multi-tyrosine kinase inhibitors, which are now widely used in the treatment of metastatic kidney cancer (9).

Birt-Hogg-Dube syndrome is a syndromic form of inherited RCC caused by mutations in the *FLCN* gene that encodes the folliculin tumour suppressor protein (10-12).

Individuals with germline FLCN mutations are also predisposed to develop skin (fibrofolliculomas) and colorectal tumours and lung cysts (13-15). The precise function of the folliculin tumour suppressor protein is not well-defined although two, folliculin interacting proteins (FNIP1 and FNIP2) were linked to the mTOR and AMPK signalling pathways (16-18) and mice with kidney-targeted homozygous inactivation of Flcn developed renal tumours and cysts that demonstrated mTOR activation (19-20). Interestingly, though, mTOR inhibitors have shown promise as treatments for metastatic RCC (21), some studies have suggested that the effect of folliculin on mTOR signaling may not be straightforward and can be context-dependent (22). Recently, folliculin has been reported to regulate TGF-beta signaling and hypoxia-response pathways (23-25). In the knowledge that many tumour suppressors (*e.g.* pVHL) have multiple functions we analysed matched folliculin-deficient and folliculin-expressing cell lines by gene expression and protein arrays, in order to determine which signaling pathways might be regulated by the folliculin tumour suppressor protein.

Materials and Methods

Cell culture. A matched pair of folliculin-deficient and -expressing cell lines was studied. FTC133 is a metastatic thyroid carcinoma cell line that harbours a somatic *FLCN* gene mutation (c.1285delC). FTC133 cell line has been described previously (26) but in brief FTC133 cells were obtained from ECACC (Salisbury,UK) and grown in a mixture (1:1) of Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Steinheim, Germany) and Ham's F-12 (Sigma-Aldrich) and supplemented with 10% FBS (Sigma-Aldrich), 2mM l-glutamine and penicillin/streptomycin. In order to obtain cells stably-expressing FLCN, FTC133 cells were stably transfected with pFLAG-FLCN construct or a construct containing an empty pFLAG-CMV vector (26). Transfected cells were then grown in a selective medium containing 0.5 mg/mL G418.

RNA extraction and gene expression microarray analysis. Total RNA was extracted from exponentially growing FTC133 cells with folliculin-null and -expressing cells (>2×10^6 cells) using the RNeasy kit (Qiagen, Crawley, UK). RNAs were extracted from triplicate plates of each cell line, labelled and analysed using GeneChip Human Exon 1.0 ST Array (Affymetrix, High Wycombe, UK) following standard Affymetrix protocols. Gene level analysis of the array data was performed using Affymetrix Expression Console with the default settings of "Extended: RMA-Sketch". Differentially expressed genes between different treatment groups were identified using the limma package (27) with a threshold of $p<0.001$ and fold change >2. Heatmaps were generated using dChip (http://www.dchip.org/) with the default settings.

Kinexus antibody array. FTC133 folliculin-transfected and folliculin-null cells (>2×10^6 cells) were washed twice with ice-cold PBS, trypsinized and pelleted by centrifugation and stored at –80˚C. The cell pellets were sent to Kinexus Bioinformatics Corporation (Vancouver, Canada) www.kinexus.ca/ for Kinex TM KAM-1.3 chip antibody array. Signal median was subtracted by background median and normalized by its total on each array.

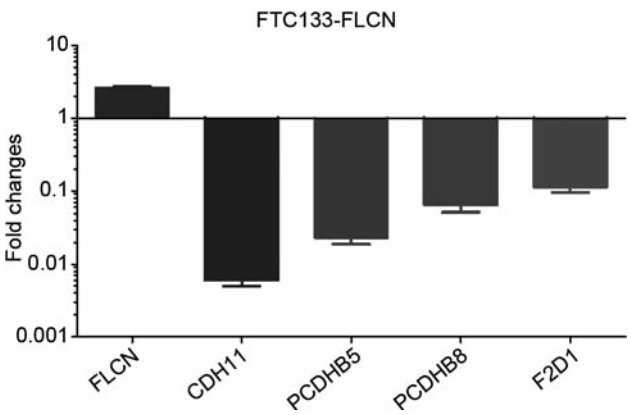

Figure 1. *Validation of differentially expressed genes in Cadherin and Wnt signaling in FTC133-FLCN-expressing compared to FLCN-null cell line, by real-time qPCR. The experiment was performed in triplicate and the standard deviation was calculated.*

Apoptotic antibody arrays. Analysis of samples on arrays was as per manufacturer's recommendation (R&D systems Abingdon, UK.). In short, 250ugs total cellular proteins were spotted in duplicate onto a nitrocellulose membrane array with apoptotic capture antibodies. Following a 16-h incubation unbound proteins were washed away and a second incubation with streptavidin-HRP linked detection antibodies. Chemiluminescence detection produced a spot corresponding to the amount of bound protein. Peak intensity of the bound protein was calculated and values were obtained on a syngene analysis system.

Immunoblotting. FTC133 cells were lysed in RIPA buffer (Tris pH 7.4, NaCl 150 mM, EDTA 0.5 mM, 1% Triton) containing a protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN) and the supernatant removed following centrifugation. The protein concentration of cell lysates was estimated using the DC protein assay kit, according to the manufacturer's instructions (Bio-Rad Laboratories Ltd, Hertfordshire, UK). Equal amounts of total cellular protein were separated on a 7% to 12.5% SDS-PAGE gel and the primary antibodies used were as follows: PLCG2, STK33, β-Actin, Tubulin, EIF2AK2(PKR), SMAC/Diablo and HtrA2 (Abcam), Casp1 and FAK (PTK2) (Cell Signaling Technology).

Real-time qRT-PCR. Total RNA was extracted from FTC-133 cells (>5×10^6 cells) using the RNeasy kit (Qiagen Inc, Valencia, CA) and converted into cDNA using Superscript III reverse transcriptase. TaqMan Universal PCR Master Mix and the validated probes for the genes of interest were purchased from Applied Biosystems. The PCR conditions were denaturation at 95˚C for 10 min followed by 40 cycles of denaturation at 95˚C for15 sec and annealing and extension at 60˚C for 1 min. Each gene was analysed in triplicate and normalized to GAPDH mRNA levels.

Results

Gene expression profiles of FLCN null and FLCN restored FTC133. mRNA extracted from paired isogenic FTC-133 cell lines stably transfected with Flag-FLCN or an empty Flag vector

Table I. *Pathways de-regulated in FTC133-FLCN-null versus FLCN-restored cell line.*

Pathway	No of genes	*P*-value	Fold enrichment
PANTHER_P00012: Cadherin signalling pathway	19	6.4-4	2.4
PANTHER_P00057: Wnt signalling pathway	24	6.2E-2	1.4
PANTHER_P00034: Integrin signalling pathway	17	7.7E-2	1.5
PANTHER_P00019: Endothelin signalling pathway	9	9.5E-2	1.9

Table II. *Genes de-regulated in Cadherin and Wnt pathways in FTC133-FLCN-null versus FLCN-restored cell line.*

Gene	Representative public ID	Fold change FTC133FLCN+/-	Wnt signalling pathway	Cadherin signalling pathway
Cadherin 11 (*CDH11*)	NM_001797	–5.09	+	+
Cadherin 6 (*CDH6*)	NM_004932	–2.11	+	+
Cadherin 9 (*CDH9*)	NM_016279	–4.12	+	+
Frizzled homolog 1 (*Fzd1*)	NM_003505	–2.85	+	+
Protocadherin 18 (*PCDH18*)	NM_019035	–2.68	+	+
Protocadherin beta 10 (*PCDHB10*)	NM_018930	–4.46	+	+
Protocadherin beta 11 (*PCDHB11*)	NM_018931	–4.18	+	+
Protocadherin beta 12 (*PCDHB12*)	NM_018932	–5.28	+	+
Protocadherin beta 13 (*PCDHB13*)	NM_018933	–4.38	+	+
Protocadherin beta 14 (*PCDHB14*)	NM_018934	–3.60	+	+
Protocadherin beta 16 (*PCDHB16*)	NM_020957	–4.01	+	+
Protocadherin beta 2 (*PCDHB2*)	NM_018936	–6.96	+	+
Protocadherin beta 5 (*PCDHB5*)	NM_015669	–20.95	+	+
Protocadherin beta 6 (*PCDHB6*)	NM_018939	–6.20	+	+
Protocadherin beta 7 (*PCDHB7*)	NM_018940	–2.56	+	+
Protocadherin beta 8 (*PCDHB8*)	NM_019120	–14.35	+	+
Solute carrier family 6 (*SLC6A6*)	NM_003043	–2.14	+	+
v-yes-1 Yamaguchi sarcoma viral related oncogene homolog (*LYN*)	NM_002350	3.57	–	+
SMAD family member 4 (*SMAD4*)	NM_005359	2.31	+	–
Cyclin D2 (*CCND2*)	NM_001759	5.46	+	–
Dapper, antagonist of beta-catenin (*DACTI*)	NM_016651	–10.72	+	–
Excision repair cross complementing rodent repair deficiency complementation group 6 (*ERCC6*)	NM_000124	–3.75	+	–
Low-density lipoprotein receptor related protein 5 (*LRP5*)	NM_002335	–2.16	+	–
Secreted frizzled-related protein 4 (*SFRP4*)	NM_003014	–2.38	+	–

was hybridised to Affymetrix Human Exon 1.0 ST Arrays. 458 and 250 genes were >2-fold up- and down-regulated respectively in the folliculin expressing FTC-133 cell line, compared to folliculin null cell line (supplementary Table I; supplementary tables can be obtained from E.R.Maher@bham.ac.uk). We validated the results of the microarray expression analysis by real time PCR analysis for selected differentially expressed genes (*FZD1*, *CDH11*, *BCDHB5* and *BCDHB8*). Each of the genes was confirmed to be down-regulated in a folliculin expressing FTC133 cell line (Figure 1).

Pathway analysis of folliculin-dependent differentially expressed genes. Pathway analysis of the 708 differentially expressed genes (fold change >2 and $p<0.001$) in folliculin-expressing and null FTC-133 cells, using David bioinformatics program (28, 29) showed abnormal regulation of the known oncogenic pathways including cadherin and the Wnt pathway (Table I and Figure 1). There was a considerable overlap between these two pathways with 17 common genes (Table II). Thus 18 genes were de-regulated in the cadherin signalling pathway (17 down-regulated and 1 up-regulated by folliculin) whilst 23 genes belonging to Wnt signalling were de-regulated (21 down-regulated and 2 up-regulated by folliculin) (Table II). *PCDHB5* and *PCDHB8* showed the greatest down-regulation (20- and 14-fold respectively).

Previously Hong *et al*. (23) reported gene expression microarray data for folliculin-expressing and null UOK257 renal cancer cell lines and Klomp *et al*. (30) described gene

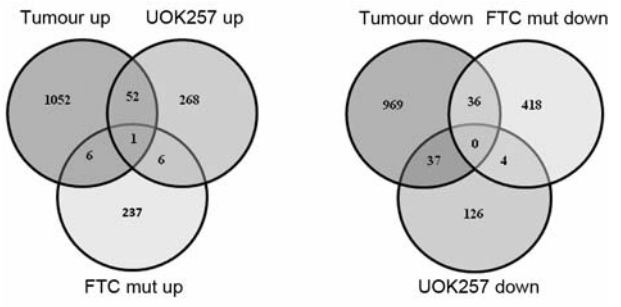

Figure 2. *Genes down- and up-regulated by FLCN in three datasets. FTC mut up or down=genes up-or down-regulated in FTC133 FLCN-null cell line. Tumour up or down=genes up- or down-regulated in BHD tumours (30); UOK257 up or down=genes up-or down-regulated in the UOK257 FLCN-null renal cancer cell line (23).*

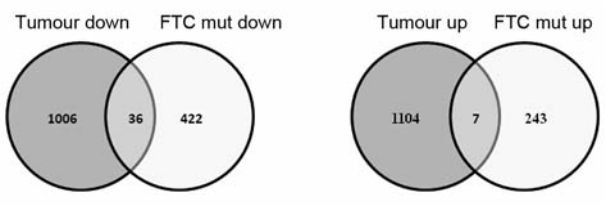

Figure 3. *Genes down- and up-regulated by FLCN in combined FTC133 and BHD tumour datasets. FTC mut up or down=genes up-or down-regulated in FTC133-FLCN-null cell line. Tumour up or down=genes up- or down-regulated in BHD tumours (30).*

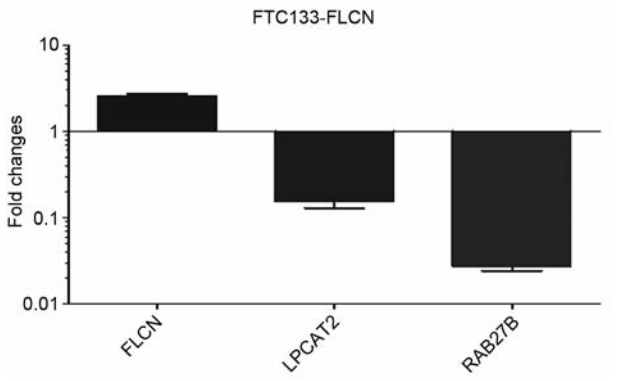

Figure 4. *Validation of expression levels of LPCAT2 and Rab27B in folliculin-expressing and folliculin-null FTC133 cell lines by real-time qPCR. The experiment was performed in triplicate and the standard deviation was calculated.*

expression microarray data for 6 kidney tumours from patients with BHD using Affymetrix Human Genome U133 Plus 2.0 Arrays. Comparison of the differentially expressed genes in the paired FTC133 cell lines and those described by Hong *et al*. (23) and Klomp *et al*. (30) identified just one gene that demonstrated altered expression in all three datasets (Figure 2). Thus *RAB27B* 2 (Rab27B, member of RAS oncogene family) was down-regulated by folliculin (Figure 2). However, a combined analysis of microarray data for FTC133 and 6 BHD-associated kidney tumours revealed 7 genes (*TSPAN7, PCDH18, LPCAT2, CD36, RAB27B, KIT* and *NCKAP5*), down-regulated and 36 genes up-regulated by folliculin in both datasets (Figure 3).

Down-regulation of the expression of *Rab27B* and *LPCAT* in folliculin-expressing FTC133 cell line was confirmed by real-time qRT-PCR (Figure 4).

Protein array expression profiles of FLCN-null and FLCN-restored FTC-133 lines. The Kinex™ KAM-1.2 microarray-platform was utilised to identify differentially expressed proteins in paired isogenic FTC-133 folliculin-null and FLCN-restored cell lines.

The Kinex™ protein array interrogates 800 proteins (approximately 500 antibodies assess protein expression and 300 phosphoantibodies detect for phosphorylation-specific proteins). 73 targets were differentially expressed in FTC-133 folliculin null and expressing cell lines (fold change >1.5) (supplementary Table II; supplementary tables can be obtained from E.R.Maher@bham.ac.uk).

Pathway analysis of antibody array data using David bioinformatics program (28) revealed de-regulation of proteins participating in apoptosis and MAPK signaling pathways in FTC-133 folliculin-null compared to folliculin-restored cell line. However, as previous studies have suggested that the protein array analysis can lead to false-positive findings, we compared the results of the antibody array and gene expression microarray in the paired FTC-133 cell lines in order to prioritise the validation of differentially expressed proteins. Eight candidate folliculin targets were differentially expressed in both experimental systems (PTK2

Table III. *Combined analysis of the Kinexus antibody array and gene expression array in FTC133-FLCN compared to FTC133-FLCN-null cell lines.*

Gene symbol	Protein fold change	mRNA fold change
EIF2AK2 (PKR)	2.588704	1.518611
MAP3K1	1.605497	1.500888
PLCG2	2.130971	3.227447
PTK2 (FAK)	1.733409	1.999118
SGK3	1.817855	3.594169
SLK	1.656782	1.500281
STK33	2.643266	1.518217
CASP1	1.59	2.82

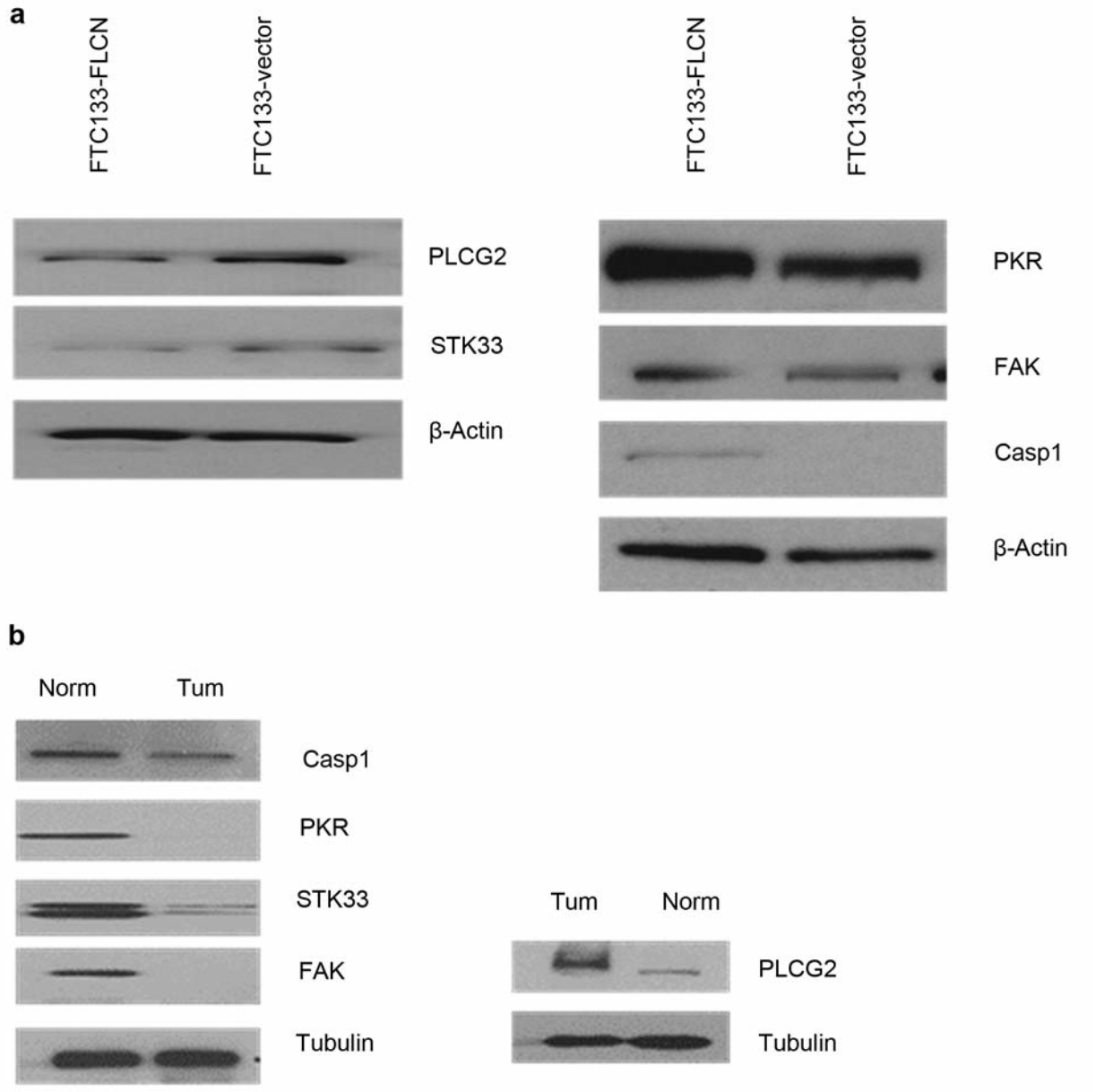

Figure 5. *Validation of differentially expressed proteins. A) Expression levels of STK33, PKR, PLCG2, FAK and Casp1 in FTC33 folliculin-null cell line and FTC133 folliculin-expressing (FTC133-FLCN) cell line. B) Expression levels of the same proteins in BHDS-associated renal tumour compared to normal kidney. Equal amounts of protein were separated on a 7% to 12.5% SDS-PAGE gel and probed for PLCG2, STK33, EIF2AK2(PKR), Casp1 and PTK2(FAK). β-Actin or Tubulin was used as a loading control. Blots were exposed to photographic film and scanned using a flat bed scanner. Tum=BHDS associated tumour tissue. Norm=matched normal kidney tissue.*

(FAK), MAP3K1, EIF2AK2 (PKR), PLCG2, SGK3, SLK, STK33 and Casp1) (see Table III). Western blot analysis of the cell lines was undertaken for 5 targets, for which suitable antibodies were available and revealed that, compared to folliculin-null cells, folliculin-expressing cells expressed increased EIF2AK2 (PKR) and Casp1 but lower levels of PLCG2 and STK33 (no significant difference in expression of FAK could be detected) (Figure 5). We then performed western blot analysis of renal tumour removed from a patient with BHD (with a germline FLCN missense mutation Arg239Cys and loss of the wild-type allele in the tumour) for EIF2AK2 (PKR), Casp1, PLCG2 and STK33. This demonstrated reduced levels of PKR and Casp1 and increased levels of PLCG2 in the tumour tissue, compared

to the matched normal tissue (i.e. similar to the folliculin-null/expressing cell lines) but STK33 levels (that were reduced in folliculin-expressing cell lines) were reduced/absent in BHD-associated kidney tumour (compared to matched normal kidney tissue) (Figure 5).

In view of the role of CASP1 in apoptosis we investigated FTC-133-null and -expressing cell lines for the expression of other proteins involved in apoptosis using apoptotic protein arrays (R & D Systems Abingdon, UK). Two different FLCN-expressing FTC-133 cell lines displayed increased levels of SMAC/Diablo, HtrA2 compared to FTC-133 folliculin-null cell line (Figure 6) supporting the role of folliculin in apoptosis.

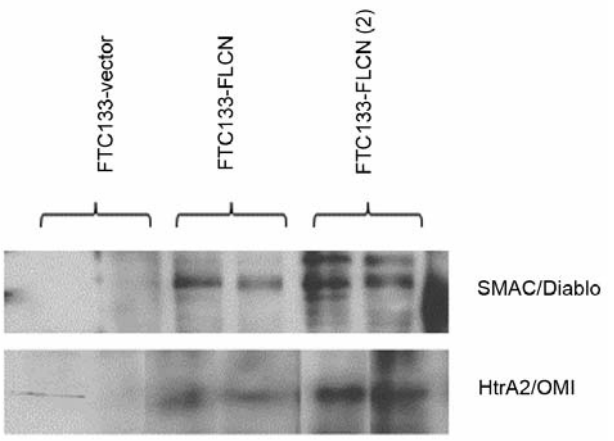

Figure 6. *Expression levels of SMAC/Diablo and HtrA2/OMI in FTC133 folliculin-null and -expressing cell lines.*

Discussion

We investigated paired folliculin-expressing and -deficient cancer cell lines using gene expression microarrays and Kinex™ antibody arrays to detect differentially expressed transcripts and proteins. Previously gene expression analysis of folliculin-null and -replete UOK257 RCC cells was reported by Hong *et al*. (23) and of 6 BHD renal tumours (in comparison to normal renal tissue) by Klomp *et al*. (30), but the gene expression microarrays we utilised interrogated a larger number of transcripts and we report on the first analysis of non-renal folliculin-deficient cells. We found that folliculin regulates the cadherin and Wnt pathways. Both of these pathways have been implicated in a wide diversity of human cancer types and de-regulation of cadherin signalling in the folliculin-null UOK257 cell line was also reported by Hong *et al*. (23). Specific microarrays detected changes in the cadherin and Wnt signal pathways that were confirmed independently, thus, folliculin suppressed the expression of *CDH11, PCDHB5, PCDHB8* and *FZD1*. Though CDH11 (cadherin 11) has been reported to have tumour suppressor properties (31, 32), CDH11 has also been reported to be overexpressed in a metastatic prostate cell line and to increase invasiveness (33, 34). *RAB27B* was found to be de-regulated in both folliculin-deficient renal and non-renal carcinoma cell lines and also in BHD renal tumours. Previously, *RAB27B* has been linked to oncogenesis in non-BHD-related tumours and reported to enhance breast cancer growth and invasiveness (35).

The Kinexus protein array has not been previously used to analyse folliculin-deficient cell lines. In order to prioritise the large number of potential "hits" we prioritised those targets that were also dysregulated in the gene microarray dataset. Though this strategy might overlook proteins that were dysregulated by post-transcriptional mechanisms, even among those targets that were apparently dysregulated at both transcript and protein level, some targets could not be validated by western blotting. Previous studies have shown that the Kinexus antibody array is more sensitive than western blotting and so a proportion of false positive results are expected (36). Three proteins (EIF2AK2 and Casp1 and PLCG2) were also confirmed to be dysregulated in a BHD renal tumour. EIF2AK2/PKR has been reported to have tumor suppressor activity (37-39) and de-regulation of PLCG2, has been noted in Wilms tumour (40). Caspase 1 (CASP1) expresssion was reduced in folliculin-deficient cancer cell lines and renal tumour. CASP1 is a member of the cysteine-aspartic acid protease family that induces cell apoptosis when activated. The observation that CASP1 levels were reduced by folliculin deficiency suggested that folliculin might promote apoptosis. Moreover, increased levels of Smac/Diablo and HtrA2/OMI were detected in the presence of folliculin. Smac (Second mitochondria-derived activator of caspase) protein, also known as Direct Inhibitor of Apoptosis-Binding protein with LOw pI (DIABLO) and high temperature requirement protein A2 HtrA2/OMI participate in the mitochondrial apoptotic pathway where these proteins are released from the mitochondrial inter-membrane space in response to various stimuli (41). Smac/DIABLO has been shown to be down-regulated in RCC and the absence of Smac/DIABLO expression was predictive of worse prognosis (42). Recently Cash *et al*. (24) also linked folliculin to apoptosis, by reporting that folliculin deficiency resulted in resistance to apoptosis and reduced levels of the pro-apoptotic protein Bim. Hence our findings further contribute to the elucidation of the mechanisms of folliculin tumour suppressor activity.

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