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**Article:**  
Journal of Biological Chemistry . ISSN 0021-9258

https://doi.org/10.1074/jbc.RA117.000975

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Ankyrin repeat and single KH domain 1 (ANKHD1) drives renal cancer cell proliferation via binding to and altering a subset of miRNAs

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Running title: ANKHD1 drives renal cancer cell proliferation via miRNAs

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Keywords: ANKHD1, miRNA-binding, miR-29a, proliferation, renal cell carcinoma, clear cell, ccRCC

ABSTRACT

Clear cell Renal Cell Carcinoma (ccRCC) represents the most common kidney cancer worldwide. Increased cell proliferation associated with abnormal microRNA (miRNA) regulation are hallmarks of carcinogenesis. Ankyrin repeat and single KH domain 1 (ANKHD1) is a highly conserved protein found to interact with core cancer pathways in Drosophila, however its involvement in RCC is completely unexplored. Quantitative PCR studies coupled with large-scale genomics data analyses demonstrated that ANKHD1 is significantly upregulated in kidneys of RCC patients when compared to healthy controls. Cell cycle analyses revealed that ANKHD1 is an essential factor for RCC cell division. To understand the molecular mechanism(s) utilized by ANKHD1 to drive RCC cell proliferation we performed bioinformatics analyses which revealed that ANKHD1 contains a putative miRNA-binding motif. We screened 48 miRNAs with tumour-enhancing or suppressing activities, and found that ANKHD1 binds to and regulates three tumour-suppressing miRNAs (i.e. miR-29a, miR-205, and miR-196a). RNA-immunoprecipitation assays demonstrated that ANKHD1 physically interacts with its target miRNAs via a single K-Homology (KH)-domain, located in the c-terminus of the protein. Functionally we discovered that ANKHD1 positively drives ccRCC cell mitosis via binding to and suppressing mainly miR-29a and to a lesser degree via miR-196a/205, leading to an upregulation in pro-proliferative genes such as CCND1. Collectively, these data identify ANKHD1 as a new regulator of ccRCC proliferation via specific miRNA interactions.

Introduction

Renal cell carcinomas (RCCs) represent one of the commonest diagnosed cancers worldwide, with clear cell Renal Cell Carcinoma (ccRCC) accounting for approximately 80% of all RCCs. Molecularly, the tumour suppressor von Hippel-Lindau (VHL) is frequently inactivated by mutations (1), leading to increased proliferation, enhanced angiogenesis and de-differentiation of tumour cells. ccRCC pathogenesis is linked to abnormal regulation of certain microRNAs (miRNAs) (2,3). The latter are a class of ~22 nucleotide-long, single-stranded non-coding, nucleic acid molecules involved in the regulation of proliferation (4,5). In ccRCC, the tumour-suppressor miR-29a is strongly downregulated and predicts metastasis (6). MiR-29a, expressed by the kidney, is responsible for the regulation of extracellular matrix (7, 8) in addition to acting as a core regulator of cellular proliferation (9). The mechanisms and proteins that govern the control of the levels of miR-29a in ccRCC are currently unknown.
Ankyrin repeat and KH domain-containing protein 1 (ANKHD1), is a K homology (KH) containing protein first discovered in *Drosophila* as a genetic regulator of the EGF/MAPK pathway (10). It was later shown to regulate additional signalling cascades including the cancer-associated HIPPO (11-14) and PINK/Parkin in *Drosophila* (15). Clinical epidemiological studies suggested that ANKHD1 is elevated and may be involved in acute leukaemia (16) and prostate cancer (11). Despite these insights, the molecular mechanisms employed by ANKHD1 to exert these pleiotropic cancer-associated functions are as-yet-unknown.

ANKHD1 contains a single KH domain, a motif predicted to bind single-stranded nucleic acids. However, the function of the KH, including its potential ability to bind nucleic acids, has not been experimentally tested. Here, we report that ANKHD1 is elevated in patients with ccRCC and contributes to cancer cell proliferation via binding to and suppressing a number of tumour-suppressor miRNAs.

**Results**

**ANKHD1 is overexpressed in renal cell carcinoma (RCC).**

Given that ANKHD1 regulates some cancer-associated biochemical pathways we first set out to examine the expression of ANKHD1 in normal and RCC human kidneys. Using a commercially available rabbit polyclonal antibody, we found that anti-ANKHD1 antibodies are strongly bound in tubular epithelial, parietal epithelial and renal microvascular cells with no detectable signal in fibroblasts nor glomerular cells in the normal mouse kidney (representative pictures shown in SI Figure 1A, n=5 animals). Similar findings were observed in normal human kidney sections stained with an anti-ANKHD1 antibody (Figure 1A, left). To examine if ANKHD1 is expressed by kidney cancer cells we analysed its expression in 20 different RCC cancer patients (Figure 1A, right panel and SI Figure 1B). Having established that ANKHD1 is expressed at the protein level in kidney cancer, we next wished to study whether kidneys of patients with renal cell carcinoma had altered ANKHD1 mRNA levels. We found a statistically significant elevation of ANKHD1 in RCC samples when compared to healthy control kidney tissues (Figure 1B). Furthermore, ANKHD1 mRNA levels were not significantly different between early (I/II - non-metastatic) and late stages (III/IV - metastatic) of cancer, suggesting that ANKHD1 is up-regulated early in the carcinogenesis process (Figure 1C). Consistent with our findings, bioinformatics analyses of the NIH cancer genome atlas using cBioPortal (17,18) showed that 33% of all ccRCC patients (of 538 patients) had significantly overexpressed *ANKHD1* mRNA levels, with 16% of patients exhibiting elevated ANKHD1 due to locus amplification (http://www.cbioportal.org/index.do?session _id=59e0b593498e5df2e2969c52&show_sa mples=false&); thus providing an explanation as to why ANKHD1 is elevated early in a large proportion of these patients.

Taken together, these studies demonstrate that ANKHD1 is frequently upregulated in RCC when compared to healthy non-cancer renal tissues and may contribute to this disease.

**ANKHD1 controls cancer cell proliferation**

To better understand the function of ANKHD1 and examine whether it promotes or inhibits cancer-associated phenotypes, we initially tested its role in two independent renal cancer lines derived from ccRCC patients (RCC4 and 786-0). Two independent siRNAs targeting ANKHD1 mRNA were used and their efficiency tested by qPCR and western blotting (SI Figure 2A-B). Reduction of ANKHD1 by 70% or greater was used in all subsequent experiments. Given that ccRCCs are characterised by their proliferative nature, we decided to study whether ANKHD1 controls the ability of RCC cells to divide using Propidium-iodine stained cells by flow cytometry. In both renal cell lines tested and in each independent experiment, ANKHD1 silencing resulted in a significant reduction in the number of cells in G2-M phase, fewer cells in S phase, and a statistically significant increase in G1 phase (Figure 2A). Ultimately, for cancer cells to divide and propagate they need to undergo
mitosis, therefore to investigate whether ANKHD1 knockdown reduces mitosis, we studied phospho-serine10 Histone H3 (19) (subsequently referred to as pH3), which is a specific marker of mitosis. Loss of ANKHD1 function, using two independent siRNAs in multiple experiments, resulted in a statistically significant reduction in the number of pH3 positive cells in both RCC4 cells (Figure 2C and E) and 786-O cells (Figure 2D). Taken together, these results indicate that ANKHD1 is a positive regulator of proliferation in RCC-derived cancer cells in vitro, thus suggesting it contributes to disease.

**ANKHD1 reduces the expression of a subset of miRNAs**

We next set out to determine the molecular mechanisms underlying the pro-proliferative activity of ANKHD1. ANKHD1 contains a single K-Homology (KH) domain from amino acids 1694 to 1764. Given that other KH domain-containing proteins KSRP (20), RCF3 (21) and Bicc1 (22) were previously shown to bind to miRNAs, we set out to test whether ANKHD1 similarly controls proliferation via an interaction with miRNAs. To identify ANKHD1-relevant miRNAs, initially we measured the levels of 48 cancer-associated miRNAs using a hybridization array in cancer cells with and without ANKHD1 silencing. We found ANKHD1 siRNA resulted in enhanced expression of five miRNAs by greater than 2-fold (Figure 3A, red box); while 4 miRNAs (namely, -342, 1, 223 and -182) showed more than 2-fold decreased when ANKHD1 was silenced (Figure 3A, black box).

We then carried out qPCR analysis of miR-29a-3p, miR-196a, miR-143, miR-122 and miR-205 and found that silencing of ANKHD1 resulted in a significant upregulation of all miRNAs identified in the array expect for miR-122 (Figure 3B). miR-122, whilst it showed a 11-fold increase in the array (Figure 3A), it did not change significantly in our independent qPCR validation experiments (Figure 3B), suggesting that it is likely to represent a false positive in the initial assay, due to a technical artefact. As a control, we also tested miR-15a (Figure 3B), which did not show a significant change, as expected from the array results (Figure 3A, green box). Collectively, our findings indicate that ANKHD1 controls expression of a subset of miRNAs, including miR-29a-3p.

**ANKHD1 physically associates with miR-29a-3p, -196a, and -205 via its KH domain.**

ANKHD1 contains two clusters of ankyrin repeats, which have previously been shown to mediate protein-protein interactions, and a single KH domain - a motif predicted to bind to single-stranded nucleic acids (23). Therefore, theoretically, if ANKHD1 interacts with miRNAs this interaction may be mediated via its KH domain, or alternatively, ANKHD1 may associate with a miRNA-binding protein complex via its ankyrin domains. In the first instance, we tested whether endogenous full-length ANKHD1 is capable of binding miRNAs by undertaking endogenous RNA immunoprecipitations (RIP). The efficiency of the ANKHD1 pull-down was checked by Western blotting indicating that ANKHD1 is pulled down effectively when an anti-ANKHD1 antibody is used by not with IgG control, the identity of ANKHD1 was also validated by a single siRNA against ANKHD1 (SI Figure 2C). We found a statistically significant enrichment of miR-29a-3p, miR-196a and miR-205 in ANKHD1 pull-downs when compared to IgG control antibodies (Figure 4A), indicating that ANKHD1 physically associates with these miRNAs. While, not surprisingly, ANKHD1 antibodies were not able to pull-down miR-122, one of the miRNAs with false positive results (SI Figure 2D), confirming the specificity of the RNA-immunoprecipitation approach. In order to test whether the full-length ANKHD1 molecule binds to miRNAs directly, via its KH domain, or indirectly, via its ankyrin repeats, we generated and overexpressed flag-tagged constructs. A full-length flagged-tagged wild type ANKHD1 construct (FL-WT) was used to generated a truncation construct which we termed KH-WT, this construct lacked the N-terminal region of the protein but maintained the C-terminus with an intact KH domain (KH-WT) (Figure 4B). We transfected these constructs into cells and performed RIP assays which showed that the KH-WT was able to...
physically associate with miR-29a-3p (Figure 4E) and miR-196a (Figure 4F) to a level comparable to that observed with FL-WT ANKHD1 (compare bar 2 with 4), thus indicating that the N-terminal portion of the protein (which contains the ankyrin repeats) is not required for this interaction. Having shown that the ankyrin domains are redundant for the miRNA interaction we next examined whether there was a functional unit within the KH domain that directly mediated the nucleic acid binding. Previously a GxxG loop within KH domains of other proteins was identified to be critical for mediating miRNA interactions (24). We searched the KH sequence of ANKHD1 and found a canonical GxxG loop which exhibited 100% amino acid conservation between humans, mouse and Drosophila (SI Figure 2E), suggesting an important biological role. We next went on to create a 2-amino acid mutation within this loop, which generated a GDDG structure (FL-Mut), previously shown to exhibit reduced nucleic acid binding ability without compromising protein stability (24,25), we confirmed the mutation by sequencing (Figure 4C). We, next overexpressed both FL-WT and FL-Mut versions of ANKHD1 and performed RIP assays. We found that the FL-Mut construct possessed reduced RNA binding ability (Figure 4D-E), thus suggesting that ANKHD1 binds its targets miRNAs via the GxxG loop of its KH domain.

Functionally, to test whether an intact ANKHD1-GxxG loop is required to mediate the proliferative functions of ANKHD1, we forced expressed FL-WT or mutant FL-Mut ANKHD1 in RCC4 cells. Consistent with previous knock-down experiments, forced expression of FL-WT was sufficient to increase the proportion of cells undergoing mitosis, while transfection with the FL-Mut reduced proliferation compared to empty vector (EV) (Figure 4F), thus suggesting that ANKHD1 mediates its pro-proliferative actions via its GxxG loop.

Taken together, these data demonstrate that ANKHD1 physically associates with its target miRNAs via its single KH domain and that mutations in this region are capable to abolish miRNAs binding and further abolish the ability of ANKHD1 to enhance proliferation of ccRCC cells.

**ANKHD1 regulates proliferation of cancer cells via miR-29a-3p.**

Given the significant alterations in miRNA levels mediated by ANKHD1, we hypothesized that ANKHD1 may exert its pro-proliferative effects via regulating one or more of the miRNAs identified above. Firstly, to test whether miR-29a is significantly reduced in RCC, as previously suggested, we performed qPCR analysis which confirmed that miR-29a is indeed downregulated in RCC (SI Figure 2F). As a starting point, we searched the MirTarbase database for experimentally validated mRNA targets of miR-29a-3p, miR-196a and miR-205 (SI Table 1). We then queried the annotation of these targets using the David functional annotation tool and found that ‘regulation of cell proliferation’ was a statistically significantly enriched gene ontology (GO) term for miR-29a-3p (9 target genes) and miR-205 (4 target genes) but not for miR-196a (SI Table 1). Since miR-29a-3p is associated with the greatest number of proliferation-regulating genes, we started by testing whether it may mediate the proliferative effects of ANKHD1. We firstly knocked-down ANKHD1 in RCC4 cells transfected with either a miScript microRNA inhibitor targeting miR-29a-3p or an empty vector control (Figure 5A). As expected, silencing of ANKHD1 reduced the proportion of pH3 positive RCC4 when compared to non-target RNAi treated cells (NT si) (Figure 5B). Importantly transfection of a miR-29a-3p inhibitor did not affect the proliferation of RCC4 cells on its own (Figure 5B), however, co-transfection of the miR-29a-3p inhibitor together with knocking out ANKHD1 was capable of completely relieving the block in proliferation mediated by silencing of ANKHD1, thus restoring cell division to basal levels (Figure 5A-B). This suggested that miRNA-29a-3p suppression is required for ANKHD1 to promote mitosis in ccRCC cells. To check whether ANKHD1 regulates proliferation via miR-29a-3p in an additional RCC-derived cell-line, 786.O cells were transfected either with ANKHD1 siRNA on its own or co-transfected with an miR-29a-3p inhibitor. Consistent with
previous observations in RCC4 cells, co-transfection of miR-29a-3p inhibitor in 786.O cells was sufficient to enhance proliferation when ANKHD1 was concomitantly silenced, suggesting that the suppression of miR-29a-3p is a key mechanism employed by ANKHD1 to drive proliferation in RCC-derived cancer cells.

In addition to miR-29a-3p, ANKHD1 regulates miR-205 and miR-196a too. To examine if the proliferative effects of ANKHD1 are only via miR-29a-3p or whether these other miRNAs also play a role, we silenced ANKHD1 and transfected either with miR-196a inhibitor or miR-205 inhibitor. We found that miR-205 / miR-196a inhibition caused a marginal increase in proliferation (Figure 5C). These results collectively suggest that the main proliferative mechanism employed by ANKHD1 is suppression of miR-29a-3p and to a lesser degree miR-205/196a.

Finally, to test how silencing of ANKHD1 may be driving a reduced proliferation of RCC cells, we silenced ANKHD1 using two-independent siRNAs and tested by qPCR the expression of CCND1, VEGFA and KLF4, three well-characterised targets of miR-29a-3p. We found that ANKHD1 loss-of-function led to a statistically significant reduction of each of these genes, a phenomenon that could be restored by inhibiting miR-29a-3p (Figure 5D).

These data together suggest that ANKHD1 is a positive regulator of proliferation in renal cancer cells via suppressing primarily miR-29a-3p and to a lesser degree via miR-205/miR-196a, thus altering a number of known proliferation altering genes such as CCND1.

DISCUSSION

Here we presented the first study assigning control of cancer cell proliferation as a molecular function of ANKHD1 in ccRCC. We showed that ANKHD1 controls renal cancer cell proliferation by engaging with and altering the levels of a subset of tumour-suppressing miRNAs. We demonstrated that ANKHD1 physically forms a complex with three target miRNAs (namely miR-29a-3p, miR-205 and miR-196a) via its KH domain, which leads to a significant reduction in the levels of these miRNAs. Indeed, silencing of ANKHD1 leads to an increase of these miRNAs and inhibition of these restores the proliferative potential of cancer cells. Finally, we provide the first demonstration that ANKHD1 mRNA and protein are significantly enhanced in human ccRCC when compared to control, and may play an important role, in human ccRCC.

Previous studies have convincingly demonstrated that miR-29a, miR-196a and miR-205 possess tumour-suppressor activities and are frequently, and significantly, down regulated in RCCs (6,26-31), a finding that correlates with the upregulation of ANKHD1, demonstrated here. Furthermore, given that single inhibition of miRNA-29a is sufficient to restore the proliferation of RCC cells lacking ANKHD1, we suggest that miR-29a-targeted genes are likely to be responsible for the ANKHD1-mediated proliferation of ccRCCs. miR-29a has been previously shown to downregulate many proliferation-regulating genes including BCL2, CDC42, CCND1, CCNT2, CDK2, CDK6, NASP, VEGFA and KLF4 (32-37). Indeed, we were able to show that ANKHD1 regulates CCND1, VEGFA and KLF4 via miR-29a-3p further implicating suppression of miR-29a-3p as the main mechanism employed by ANKHD1 to control proliferation of renal cancer cells. CDC42 did not change significantly (SI Figure 1E).

The other two miRNAs identified as binding partners of ANKHD1 are miR-196a and miR-205. We found that ANKHD1 silencing leads to an increase in miR-196a and 205, and it physically interacts with these miRNAs. One of the main functional roles of ANKHD1, uncovered by this study, is the control of renal cancer cell-cycle. Yet, our experimental data only found a marginal role for miR-196a/205 in the control of proliferation. This is consistent with our bioinformatics functional annotation of miR-196a/205, using validated targets, which did not reveal a major proliferation-regulating role for these miRNAs. Interestingly, miR-196a was previously shown to have a role in autophagy instead (38). This is particularly interesting because ANKHD1 was previously shown to
control autophagy in Drosophila (15). Thus, we speculate that ANKHD1 may have important, but yet unidentified, additional molecular functions in mammalian cells, including ccRCC, one of which could be the control of autophagy. We therefore hypothesize that these additional molecular functions of ANKHD1 may be mediated via the suppression of miRNA molecules, some of which are identified in this study (e.g. miR-196a/205).

Despite a clear role in the regulation of miRNA levels, the mechanism via which ANKHD1 leads to the potent downregulation of its target miRNAs is currently unclear. Although a simple ‘trapping’ model may appear attractive, we would expect that miRNAs tightly bound to ANKHD1 in an inactive complex would still be detectable in Trizol-derived total RNA extracted from whole cells. Rather, the accumulation of miRNAs following ANKHD1 knockdown together with the evidence that ANKHD1 physically interacts with some of its targets suggests that ANKHD1 may promote destruction of the identified miRNAs or alternatively control their level of transcription — however the mechanism underlying this process is as-yet-unknown.

Interestingly ANKHD1 has been shown to physically interact with factor inhibiting HIF (FIH) (39), a molecule that alters hypoxia inducible factor (HIF) transcriptional activity; HIF is involved in the development of ccRCC (40,41). Moreover, HIF controls cell cycle in hypoxia partly via controlling p21 amongst other targets (42). Interestingly ANKHD1 has been shown to interact with p21 (43), a finding which we confirmed in our study (SI Figure 1C-D). Therefore, ANKHD1 may alter proliferation and other related cancer phenotypes via multiple mechanisms; including the regulation of miRNAs and also potentially via control of HIF signalling.

In conclusion, this study combines functional, biochemical and bioinformatics approaches to collectively demonstrate that ANKHD1 controls proliferation of renal cell carcinoma cells via binding to and modulating a specific subset of ccRCC-relevant tumour-suppressor miRNAs. Further studies using miRNA modulating agents (mimics or inhibitors) and ANKHD1 knockout animal models are required to determine the genetic role of ANKHD1 in this cancer.

**Experimental procedures**

**Cell culture and transfections**

Human renal cancer cells 786.O were purchased from ATCC while human renal clear cell carcinoma cells (RCC4) with empty vector were purchased from Sigma and maintained in Dulbecco modified Eagle medium supplemented with 10% foetal calf serum, 2mM GlutaMAX without antibiotics at 37°C in a humidified incubator with 5% CO2. Cells were trypsinised and reversed transfected with 20pmol RNAi with RNAi MAX transfection reagent following manufacturer’s instructions (Invitrogen). Two independent siRNAs were used for ANKHD1, with the following catalogue numbers: D-014405-01 and D-014405-03. In every siRNA experiment control cells were treated with non-target RNAi complexes (D-001210-01). Anti-miR29a-3p inhibitor was purchased from Qiagen (3p, MIN0000086) and cells were transfected using 20pmol inhibitor with RNAi MAX transfection reagent following manufacturer’s instructions.

**RNA immunoprecipitation (RIP)**

RIPs were performed using Magna RIP kit (upstate, 70-700), following a modified version of the manufacturer’s instructions. Briefly, around 2.5x10⁷ cells were used per immunoprecipitation, after washing the cell monolayer, cells were collected by centrifugation and lysed in RIP lysis buffer containing RNase inhibitor and incubated on ice for 5 min, cells were then placed at -80°C to allow cell lysis to take place. Immunoprecipitation using either rabbit anti-ANKHD1 antibody (sigma, HPA008718) or anti-flag M2 antibody (sigma, A2220) or relevant rabbit or mouse IgG was used in a pull-down experiment, for 4 hours at 4°C with rotation. Then, after thoroughly washing of the beads, total RNA was extracted using TriZol and reversed transcribed immediately. Any remaining RNA was stored at -80°C.
Western blotting, Immunoprecipitation and antibodies

Following specific treatment, a confluent monolayer of cells was washed twice in ice-cold Tris-phosphate-buffered saline (1xTBS). TBS was aspirated and cells were lysed using ice-cold Lysis Buffer (50mM Tris (pH 7.4) 250mM NaCl, 0.3% Triton X-100, 1mM EDTA) supplemented with protease inhibitor cocktail (Roche). Cells were then subjected to freeze-thaw and mild sonication. Whole cell lysates were then incubated with appropriate antibodies or isotype specific IgG control for 4 hours at 4°C followed by overnight incubation with Dynabeads Protein G (Life Technologies). Beads were then washed four times in cold Lysis Buffer and boiled in 2x Laemmli sample buffer for 5 minutes. Samples eluted from the beads or whole cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and transferred using the Mini-PROTEAN system (Bio-Rad). Primary antibodies were rabbit polyclonal anti-ANKHD1 (HPA008718, Sigma), and mouse monoclonal anti-β-actin (ab8226, Abcam).

Flow cytometry

Cells were reverse transfected with 20nM of either ANKHD1 siRNAs or non-target siRNA and grown in 6 well plates for 72 hours. On the day of the assay cells were lifted off the 6 well plate using Accutase solution (A6964, Sigma UK), re-suspended in DMEM with 10% FBS and centrifuged at 200g for 5 minutes, followed by addition of 1ml of ice-cold 100% methanol (kept at -20°C), cells were then immediately placed in the freezer and left for 30 minutes to allow adequate fixation. Following fixation cells were washed twice in 1xTBST by pipetting up and down at least 5 times, followed by addition of 50µl of 1xTBST by pipetting up and down at least 5 times, followed by addition of 1ml of ice-cold 100% methanol (kept at -20°C), cells were then immediately placed in the freezer and left for 30 minutes to allow adequate fixation. Following fixation cells were washed twice in 1xTBST and then either isotype control antibody (9078, Cell Signalling Technology), no antibody, or phosphorylated histone H3-pacific blue conjugated antibody (1:125) (8552, Cell Signaling Technology) were added to cells and left to incubate in the dark at room temperature for 60 minutes. After this incubation cells were transferred into flow cytometry tubes and were analysed using LSRII running BD FACS Diva software. 10,000 events were recorded in the forward and side scatter and from these a gate was placed on single cells to eliminate doublets and clumps from analysis. From these a clear positive population could be seen and percentage of positive cells obtained by gating this population. Percentage of positive cells out of the 10,000 events was plotted on Prism 6.0 from three different biological replicates and a student t-test was carried out. P values of 0.05 or lower were considered significant.

Quantitative PCR

Total RNA was extracted using TRIZOLR reagent (Invitrogen) following manufacturer’s instructions. Total RNA was then reverse transcribed using miScript RT kit, Qiagen, 218193. qPCR was carried out using and iQ SYBR green supermix reagents (Bio-Rad) following manufacturer’s instructions on a CFX-96 or CFX-384 Touch Real-time PCR detection system (Bio-Rad). Change in expression levels was calculated relative to either β-actin housekeeping gene (for mRNA) or U6 (for miRNA) using the \( \Delta\Delta^{Ct} \) method. Primers used are:

ANKHD1: forward 5’ AGCGGTACGGGCGGAG 3’, reverse: 5’ AAATAATGATTCAACCTCGGACAC 3’;

β-actin: forward 5’ ATCATTGCTCCTCTGAGCGG 3’, reverse: 5’ GACAGCGAGGCCAGGATG 3’.

MiRNA hybridization plate and human RCC cancer tissue arrays

To identify targets of ANKHD1 a miRNA hybridization plate array was used (Signosis, MA-1001). 20µg of RNA (either from cells with non-target RNA or siRNA against ANKHD1) was added to each well and incubated overnight, the array was then analysed using manufacturer’s instructions. To study the mRNA levels of ANKHD1 or miR29a in human RCC patients and healthy controls a tissue scan Kidney Cancer Tissue qPCR array was used (Origene, HKRT102), and qPCR was performed following manufacturer’s instructions (using primers stated above). To study protein levels of ANKHD1 in RCC an array of 20 independent
cases of RCC and 60 cores (each case repeated three times) was utilised (BC07013, US biomax). Tissues were deparaffinised and IHC performed as previously described, these studies abide by the Declaration of Helsinki principles and tissues were collected under HIPAA approved protocols (44).

Statistics

All experiments were repeated at least three times unless otherwise stated. GraphPad prism 6 was used to input data and either non-parametric, two-tailed, Mann-Whitney tests were performed or for two sample comparison a student t-test was used. P values less than 0.05 were recorded as significant.

Acknowledgements: The authors would like to thank Paul C Evans for carefully reading the manuscript and providing critical feedback and Lindsay Farrell for technical support. This work was supported by and a Kidney Research UK fellowship and a Thomas-Berry and Simpson Fellowship to Dr Fragiadaki and a Cancer Research UK Senior Fellowship to Dr Zeidler.

Conflict of interest: None

Author contribution: Maria Fragiadaki contributed to conception, designed, acquisition, analysis and interpretation of data, drafted and revised the manuscript and approved the final version. Martin Zeidler, conceived the study, analysed and interpreted all data, revised the manuscript and approved final version.
References


Figure legends:
Figure 1: ANKHD1 is expressed by renal epithelial cells and is overexpressed in renal cell carcinoma patients

A. A human array of kidney tissues with 20 cases of RCC and 3 control non-cancer tissues were stained with an anti-ANKHD1 antibody and microscopy performed using an upright Olympus microscope. B. Haematoxylin and Eosin (H&E) staining of the above matching tissues can be seen. C. qPCR was performed for ANKHD1 normalised to β-actin for non-cancer kidney tissue when compared to renal cell carcinoma. D. Sub-group analysis of the ANKHD1 expression in the RCC patient population was unable to identify any differences in the expression in the early (I/II) versus late stages of disease (III/IV). Error bars show mean and standard error of the mean. There is a significant difference in expression in ANKHD1 message in RCCs when compared to healthy control subjects (B) while there is no difference in expression between early and later stages of cancer (C), when analysed by a student t-test.
Figure 2: ANKHD1 is required for renal cancer cell proliferation.

A. Either 786.O (left) or RCC4 (right) cells transfected with ANKHD1 siRNA or non-targeting RNAi control were fixed and stained with Propidium iodine, the numbers of cells in the different stages of the cell cycle were counted by performing flow cytometry cell-cycle based analyses. Three independent transfection experiments are shown with standard error of the mean indicated.

B. Flow cytometry analysis of 786.O and RCC4 cells transfected with ANKHD1 siRNA or non-targeting RNAi control.

C. Flow cytometry analysis of RCC4 cells transfected with ANKHD1 siRNA or non-targeting RNAi control.

D. Graph showing the percentage of cells in the G2-M phase for 786.O cells transfected with ANKHD1 siRNA or non-targeting RNAi control.

E. Graph showing the percentage of cells in the G2-M phase for RCC4 cells transfected with ANKHD1 siRNA or non-targeting RNAi control.

Figure 2: ANKHD1 is required for the proliferation of 786.O and RCC4 renal cancer cells.

A. Either 786.O (left) or RCC4 (right) cells transfected with ANKHD1 siRNA or non-targeting RNAi control were fixed and stained with Propidium iodine, the numbers of cells in the different stages of the cell cycle were counted by performing flow cytometry cell-cycle based analyses. Three independent transfection experiments are shown with standard error of the mean indicated.
mean. There is a statistically significant increase in G1 and a decrease in S/M phase of the cell cycle when the data were analysed by a one-way Anova with Bonferroni corrections. B-C. Either 786.O cells (B) or RCC4 (C) were treated with indicated siRNAs and stained with antibody that recognises p-serine10 histone H3 (pH3). pH3 positive cells were gated and percentage of positive cells identified. Isotype fluorotope matched control was used as a negative control. A statistically significant reduction in mitotic cells is observed in both 786.O cells (D) and RCC4 cells (E), all experiments were performed three times and P values of less than 0.05 were considered significant, using a one-way-Anova.
Figure 3 – ANKHD1 regulates a subset of tumour-suppressing miRNAs

A

miRNA array

B

miR-29a-3p

miR-196a

qPCR (fold change)

NT si

ANKHD1 si

P=0.00373

P=0.00342

miR-143

miR-122

qPCR (fold change)

NT si

ANKHD1 si

P=0.0180

NS

miR-205

miR-15a

qPCR (fold change)

NT si

ANKHD1 si

P=0.0101

NS
Figure 3: ANKHD1 regulates expression of a subset of tumour-suppressing miRNAs.

A. RNA from cells treated with either a non-targeting siRNA or ANKHD1 siRNA was hybridized in a 48-miRNA hybridization plate, the levels of these miRNAs are shown following normalization to U6. MiRNAs showing more than 2-fold increase are highlighted in the red box (above) and those exhibiting more than 2-fold decrease in the black box (below). miR-15 which did not show a change is also highlighted in green.

B. The expression of specific miRNA identified in A were verified by carrying out independent transfections followed by qPCR in RCC4 cells transfected with either non-targeting siRNA or ANKHD1 siRNA. All experiments were performed three times and P values of less than 0.05 were considered significant using a student-t-test.
Figure 4: ANKHD1 binds miRNAs -29a, -196a and -205 via its single KH domain.

A. Endogenous RNA immunoprecipitations (RIP) for miR-29a-3p, -196a and 205 were performed using anti-ANKHD1 or IgG control antibodies, results are presented as fold enrichment over IgG. All experiments were performed three times and P values of less than
0.05 were considered significant using a student-t-test. B. A full-length flagged-tagged ANKHD1 construct (FL-WT) was used to generate a truncation mutation, a flag-tagged version of ANKHD1 that lacks the ankyrin repeats (KH-WT, 16kD). The canonical GxxG loop was mutated to GDDG to produce a flagged-tagged full-length mutant construct (FL-Mut). C. Sequencing was carried out to confirm the mutation in the FL-Mut construct. D-E. RIP pull downs were performed in RCC4 cells following transfection of either FL-WT, or FL-mut or KH-WT constructs. qPCR analysis of either miR-29a-3p (D) or miR196a (E) revealed that these physically interacted the full-length protein as well as the KH only proteins but were unable to interact with the mutant construct (FL-mut). F. RCC4 were transfected either with an empty vector (EV) or ANKHD1 full length (FL-WT) or the full-length mutant (FL-Mut) and cells stained with pH3 and subjected to flow cytometry to measure mitosis. Experiment was performed three times and a P value of less than 0.05 was considered as significant using a one-way Anova.
Figure 5: ANKHD1 regulates proliferation via miR-29a-3p/miR-196a/miR-205

A. RCC4 cells were transfected with either Non-target (NT) or ANKHD1 siRNA (ANKHD1 si) and co-transfected with an miR-29a-3p inhibitor. The number of RCC4 cells undergoing mitosis was studied by flow cytometry measuring number of pH3 positive cells. B. Experiments
were repeated three times and a P-value of less than 0.05 was considered as significant using a one-way Anova. C. ANKHD1 was also silenced in 786.O cells using two independent siRNAs (C, top panel) and number of mitosis measured. Inhibition of miR-29a (left) or miR-196a (middle) or miR-205 (right) was studied (C). D. To study how ANKHD1 control proliferation of 786.O cells, ANKHD1 was silenced using two independent siRNA and the expression of CCDN1, VEGFA and KLF4 was measured by qPCR. In addition to test whether the modulation of the target mRNAs is via miR-29a, cells were contransfected with a miR-29a-3p inhibitor. Results are normalised against HPRT expression and are shown as fold change when compared to non-target (NT) siRNA transfected cells. Experiments were repeated three times and a P-value of less than 0.05 was considered as significant using a one-way Anova.
Ankyrin repeat and Single KH domain 1 (ANKHD1) drives renal cancer cell proliferation via binding to and altering a subset of miRNAs
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*J. Biol. Chem.* published online April 25, 2018

Access the most updated version of this article at doi: [10.1074/jbc.RA117.000975](http://dx.doi.org/10.1074/jbc.RA117.000975)

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