miR-28-5p promotes chromosomal instability in VHL-associated cancers by inhibiting Mad2 translation

Michael P. Hell¹, Claudio R. Thoma¹, Niklaus Fankhauser¹, Yann Christinat¹, Thomas C. Weber², and Wilhelm Krek¹,³

¹ Institute of Molecular Health Sciences, ETH Zurich, Schafmattstr. 22, 8093 Zurich, Switzerland

² Rodent Center HCl, ETH Zurich, Wolfgang-Pauli-Str. 10, 8093 Zurich, Switzerland

³ Corresponding author: Wilhelm Krek

Email: wilhelm.krek@biol.ethz.ch

Tel: +41 44 633 34 47

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Precis

By identifying a potential mediator of chromosomal instability in VHL-associated cancers, this study suggests a novel microRNA-based therapeutic strategy to target aneuploid cells in VHL-associated cancers.
Abstract

Chromosomal instability enables tumor development, enabled in part by aberrant expression of the mitotic checkpoint protein Mad2. Here we identify a novel regulatory mechanism for Mad2 expression involving miR-28-5p-mediated inhibition of Mad2 translation, and we demonstrate that this mechanism is triggered by inactivation of the tumor suppressor VHL, the most common event in clear cell renal cell carcinoma (ccRCC). In VHL-positive cancer cells, enhanced expression of miR-28-5p diminished Mad2 levels and promoted checkpoint weakness and chromosomal instability. Conversely, in checkpoint-deficient VHL-negative renal carcinoma cells, inhibition of miR-28-5p function restored Mad2 levels, mitotic checkpoint proficiency and chromosomal stability. Notably, chromosome missegregation errors and aneuploidy that were produced in a mouse model of acute renal injury (as a result of kidney-specific ablation of pVHL function) were reverted in vivo also by genetic inhibition of miR-28-5p. Finally, bioinformatic analyses in human ccRCC associated loss of VHL with increased miR-28-5p expression and chromosomal instability. Together, our results defined miR-28-5p as a critical regulator of Mad2 translation and mitotic checkpoint function. By identifying a potential mediator of chromosomal instability in VHL-associated cancers, our work also suggests a novel microRNA-based therapeutic strategy to target aneuploid cells in VHL-associated cancers.
Introduction

The molecular origins of numerical chromosomal instability, resulting in aneuploidy in human cancers, are diverse. Prominent among them are defects in the mitotic checkpoint (1), which normally functions to protect against chromosome missegregation (2-4). A key mediator of this control pathway is the mitotic checkpoint protein Mad2 (Mad2L1). Mad2 protein levels vary significantly in human cancers (5-8) and studies in mice have shown that either increasing or decreasing Mad2 levels cause abnormal mitotic checkpoint function, resulting in chromosomal instability and tumour progression (9, 10). Several cancer-initiating mutational events such as those affecting the tumour suppressor genes RB1, TP53 or VHL have been linked to altered expression of Mad2 and chromosomal instability (5, 11), implying that part of their normal tumour suppressor function is to provide tight control over the levels of Mad2 to preserve normal mitotic checkpoint function and chromosomal stability.

Mutation of the VHL tumour suppressor gene, a signature lesion in ccRCC (12), is associated with low Mad2 expression (5). In accordance with this, VHL-/- renal carcinoma cells display a weakened mitotic checkpoint that can be rescued by ectopic expression of Mad2 (5). These results indicate that diminished expression of Mad2 is causally linked to the development of chromosomal instability in VHL-associated cancers. The mechanism actuated by loss of VHL function is not known.

Previous work suggested that the VHL gene product, pVHL, influences neither Mad2 transcription nor protein stability (5). Therefore, we aimed at exploring alternative mechanisms of Mad2 regulation as a function of pVHL status. Our
work identifies a novel Mad2 regulatory mechanism that involves translational inhibition of Mad2 via miR-28-5p binding to specific target sequences in the Mad2 3’UTR. We show that dysregulation of this miRNA-dependent mechanism is evoked by the inactivation of pVHL. Further functional analyses in renal carcinoma cells and mouse models suggest a key role for the miR-28-5p – Mad2 axis in mitotic checkpoint and chromosomal stability control.

Materials and Methods

Cell culture. RPE-1, HCT116, and IMCD-3 cells were obtained from the American Type Culture Collection (ATCC), resuscitated and cultured for 3-5 passages prior to freezing. Cell bank-authenticated cells were used over 3-4 month. HeLa E1 cells were donated by Patrick Meraldi. All cell lines were tested for the absence of mycoplasma by extracellular DNA staining. E13.5 Vhlfl/fl MEFs were obtained using standard techniques (mice: Jackson Laboratories, stock number: 004081). H2B-GFP or -mCherry expressing HeLa and H2B-mCherry 786-O cells (authenticated by verification of absence of functional pVHL) were generated by infection with H2B expressing viruses (5). All cells were cultured according to the supplier’s protocol in media supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/mL) (Life Technologies). Primary MEFs media were supplemented with β-mercaptoethanol (200 μM). Where applicable, stable pools were generated by puromycin selection (2 μg/mL: RPE-1: 10 μg/mL).
Mouse models. Kidney-specific Vhl knockout mice were obtained as described previously (13). Mice were kept under standard pathogen free conditions in accordance with Swiss animal welfare regulations. Unilateral kidney was performed as detailed in (14). In brief, the left kidney pedicle of female mice (age: 6-9 weeks) was clamped for 30 min at 37.5°C. If applicable, LNA 28-5p or ctrl was injected (2.5 mg/kg, s. c. flank) 12h prior to surgery and on day 3. 5.5 days post-surgery, both kidneys were isolated and immediately snap-frozen in liquid nitrogen for RNA extraction or formalin-fixed overnight and embedded in paraffin.

Fluorescence microscopy. Cells grown on a coverslip were fixed for 5 min with pre-chilled (-20°C) methanol, antibody stained (where applicable), counterstained with DAPI (4',6-diamidino-2-phenylindole, 1 µg/mL), and mounted in mowiol. Metaphase spreads were prepared as in (15). Mouse kidney sections (10 µm) from FFPE samples were cut on a HM 355S microtome (Microm AG). The following additional antibodies were used: anti-phosphorylated (Ser10) histone 3 (p-H3, Cell Signaling, 9706S), anti α-tubulin (clone YL1/2. Sigma-Aldrich), anti thiazide-sensitive sodium chloride cotransporter (NCC, Millipore, AB3553), anti-glucose transporter 1 (abcam, ab14683). Secondary antibodies were obtained from LifeTechnologies. Fluorescence in-situ hybridization (FISH) was performed with a probe described in (16). Fluorescence microscopy was performed on a Zeiss Axioplan2. Laser capture microdissection was performed on kresyl violet stained tissue (6 µm) on a Zeiss 200M PALM. Mad2 expression was
quantified on immunostained kidney sections, where distal cells were labeled with DBA (Vector Laboratories, RL-1032). Quantification was performed using ImageJ (National Institutes of Health) after background subtraction. Mitotic slippage experiments were described in (5).

**Bioinformatical analyses.** Mad2 targeting miRNAs were predicted *in silico* using the targetscan algorithm (17). miR-28-5p binding sites on Mad2 3’UTR were analyzed with RNAhybrid (18). Chromosome copy numbers were determined using SNP microarray datasets (GSE19949: ccRCC Fig. 6A, GSE19612: p53 data Fig. 6C, GSE19949: Fig. 6D). *VHL* mutation status was carefully assessed in the GSE19949 dataset. PennCNV (19) was used to assess copy number variations (CNV) excluding gonosomes. Affymetrix Power Tools (v1.14.4) generated genotyping calls, from which Log R Ratio (LRR) values and B Allele Frequency (BAF) values for each locus were calculated. PennCNV was used to find chromosomal regions exhibiting non-diploid copy-numbers (minimum of 2 adjacent SNP loci), and post-processed to determine average copy numbers per chromosome. The average and variance in copy number (CN) per chromosome was calculated by defining a chromosome map subdivided in 1000bp blocks specified by the positions of the array probes. Blocks affected by a region of aberrant CN as predicted by PennCNV were assigned the corresponding PennCNV copy numbers, otherwise CN = 2. The average CN of all blocks over the chromosome length and its variance was determined. Normalized miR-28-5p expression levels from ccRCC and matched normal tissue was obtained from (20) and processed as relative ratio ccRCC vs. normal for each patient. The same ratio
was calculated for the top 10% expressed microRNAs shown as frequency distribution.

**Statistical and error analysis.** Unless stated otherwise, results were presented from three or more biological replicates. Data are plotted as mean ± standard deviation (s.d.) or standard error of the mean (s.e.m.). If necessary, Gaussian error propagation was applied. If normal distribution could be assumed, a two-sided paired or unpaired Student’s *t*-test, otherwise the Mann-Whitney U test was utilized. p values: * p < 0.05, ** p < 0.01, *** p < 0.001. For clarity, non-significant associations were omitted unless instructive. Differences in the lagging chromosomes frequency were assessed with the Chi-squared (\( \chi^2 \)) test and metaphase counting was assessed using the two-sided Mood test (21). Mitotic slippage experiments were assessed using the Log Rank test.

Additional methods available in Supplemental Information.

**Results**

**pVHL augments the translation initiation of Mad2 in a transcript-specific manner mediated by the 3' UTR of Mad2**

Non-transformed retinal pigment epithelial (RPE-1) cells were infected with a lentivirus delivering short hairpin (sh)RNA targeting pVHL (shVHL) or a non-silencing control (shCtrl) and the polysome association profiles of the Mad2
mRNA were analysed (Supplemental Fig.1A, B). While depletion of pVHL did not affect the global distribution of ribosome-bound mRNA species (Fig. 1A), it affected the Mad2 mRNA association profile as evidenced by a shift of Mad2 mRNA towards lower density fractions in pVHL knockdown cells as a result of fewer ribosomes associated per Mad2 mRNA (Fig. 1B). The polysome distribution of β-actin mRNAs was not affected by pVHL, confirming that the effect on Mad2 mRNAs is specific (Fig. 1C). A similar specific shift for Mad2 mRNAs was seen when mouse Vhl was eliminated genetically by means of expression of Cre-recombinase in mouse embryo fibroblasts (MEFs) derived from Vhl<sup>fl/fl</sup> mice (Supplemental Fig.1C-E). Thus, pVHL promotes association of Mad2 mRNAs with ribosomes enhancing their translation.

As the 3' untranslated regions (3'UTR) of mRNAs are known to contain key regulatory elements contributing to translational regulation, we investigated next whether the above-noted effects of pVHL on Mad2 are mediated via the Mad2 3'UTR. We transfected cells with a reporter plasmid containing human Mad2 3'UTR downstream of the firefly luciferase gene (FL) and renilla luciferase (RL) under an independent promoter for normalization (Fig. 1D) together with either control or VHL targeting siRNAs. The plasmid lacking the 3'UTR of Mad2 served as control (Fig. 1D). Depletion of pVHL caused a specific reduction in the FL/RL ratio only in the reporter plasmid bearing the Mad2 3'UTR in HeLa and HCT116 cells (Fig. 1E and Supplemental Fig.1F). Also a reporter containing the mouse Mad2 3'UTR was specifically repressed by shRNA-mediated depletion of pVHL in murine IMCD-3 kidney cells (Fig. 1F).
To assess whether the observed effect is due to defects in translation initiation mediated by Mad2 3’UTR, we cloned viral internal ribosome entry site (IRES) sequences upstream the Mad2 3’UTR luciferase reporter (Fig. 1G). IRES sequences from hepatitis C (HCV) or encephalomyocarditis (EMCV) virus allow to bypass components of the mammalian translation machinery (22) and would thereby make the reporter resistant to the negative effects of pVHL depletion. When tested, this was indeed the case (Fig. 1H). Thus, impaired pVHL function negatively affects translational initiation of Mad2 via its 3’UTR.

miR-28-5p is a potent Mad2-targeting miRNA

MicroRNAs (miRNAs) represent a class of non-coding regulatory RNAs that have been implicated in posttranscriptional control either via degradation or translational inhibition of target mRNAs (23, 24). Therefore, we asked whether the 3’UTR of Mad2 is a target of one or more miRNAs able to inhibit Mad2 expression. miRNAs predicted by the targetscan algorithm (17) were transfected in HeLa cells. Among them only miR-28-5p (hsa-miR-28-5p) robustly decreased Mad2 protein levels (Fig. 2A). This negative effect on Mad2 protein abundance was also observed in other human cancer cells and in mouse IMCD3 kidney cells upon overexpression of miR-28-5p, which is sequence conserved between both species (Fig. 2B). QT-PCR confirmed the miRNA overexpression in each setting (Supplemental Fig. 2A). Likewise, stable lentiviral delivery of the primary miR-28 in RPE-1 cells, an experimental system that provides a more moderate expression of miR-28-5p (Supplemental Fig. 2B), also led to a downregulation of Mad2 protein (Fig. 2B). Importantly, expression of miR-28-5p did not affect Mad2 mRNA...
levels (Fig. 2C), supporting the view that miR-28-5p acts most likely by inhibiting Mad2 translation. miR-28-5p mediates its effects on Mad2 through the 3′UTR, since it failed to suppress the production of a green-fluorescence protein (GFP)-Mad2 fusion protein lacking the 3′UTR of Mad2 (Fig. 2D). In the same cells, endogenous Mad2 was downregulated by miR-28-5p (Fig. 2D). Similar results were obtained in murine cells (Supplemental Fig.2C). Binding site prediction with the RNAhybrid (18) algorithm suggested one potential site for miR-28-5p in the human (Fig. 2E) and two sites in the murine (Supplemental Fig.2E) Mad2 3′UTR. These binding sites are functional as their mutation in the corresponding Mad2 3′UTR luciferase reporter plasmid caused resistance of the reporter to the repressive effects of miR-28-5p (Fig. 2F and Supplemental Fig.2D,F). miR-28-5p-mediated repression of Mad2 production was also specifically cancelled by introducing different mutations in the seed sequence of miR-28-5p (Fig. 2G). Since the sequence of one miRNA mutant derivative, miR-28-5p-mut1, is perfectly complementary to the mutant binding site of human Mad2 3′UTR (mut), we tested whether in this constellation the Mad2 3′UTR (mut) reporter would now become sensitive to miR-28-5p-mut1. As shown in Fig. 2H, miR-28-5p-mut1 suppressed Mad2 3′UTR (mut) but not the corresponding Mad2 3′UTR (wt) reporter. The observation that both mutants complement each other argues in favour of a direct rather than an indirect interaction between miR-28-5p and the Mad2 3′UTR. Finally, the presence of a HCV- or EMCV-derived IRES in the Mad2 3′UTR dual-luciferase reporter plasmid rendered, similar to pVHL knockdown, the reporter resistant to the negative effects of miR-28-5p (Fig. 2I). Together,
these results strongly argue that miR-28-5p acts through specific sequences within the Mad2 3'UTR to reduce Mad2 translation.

**Overexpression of miR-28-5p impairs the mitotic checkpoint leading to chromosomal instability**

Next we explored whether expression of miR-28-5p would impair the mitotic checkpoint and promote chromosomal instability in *VHL*-proficient cancer cells. To this end, we performed time-lapse imaging of HeLa cells expressing fluorescently marked histone 2B (H2B) to visualize chromosomes in the presence of the microtubule depolymerising drug nocodazole, a mitotic checkpoint activator. Transfection of miR-28-5p (Supplemental Fig. 3A) affected the ability of these cells to nocodazole-induced arrest in prometaphase compared to control transfected cells (Fig. 3A, B and Supplemental movie 1,2), suggesting that they have undergone mitotic slippage. Also, it enhanced the frequency of chromosome missegregation errors such as multiple and single lagging chromosomes, while the number of chromosome bridges, which are believed to occur through a mitotic checkpoint-independent mechanism, remained unaffected (Fig. 3C). Finally, the spread in chromosome number, an indicator of chromosomal instability and the ensuing aneuploidy, was likewise increased by miR-28-5p expression (Fig. 3D). Similar results with respect to missegregation errors and chromosome number were obtained in HCT-116, RPE-1, and IMCD3 cells (Supplemental Fig. 3B, C). Furthermore, the mitotic slippage and chromosome missegregation phenotypes associated with increased expression of miR-28-5p was rescued by co-expression of a GFP-Mad2
plasmid (25) lacking the 3' UTR (Fig. 3E, F and Supplemental Fig.3D). We conclude that expression of miR-28-5p provokes a weakened mitotic checkpoint and chromosomal instability.

Inhibition of miR-28-5p derepresses Mad2 expression and restores the mitotic checkpoint of checkpoint-impaired renal carcinoma cells

To abrogate endogenous miR-28-5p function, a miR-28-5p-targeting locked nucleic acid (LNA)-based inhibitor, referred to as 28-5p LNA, was transfected in multiple cell lines. This resulted in efficient reduction of miR-28-5p levels compared to control inhibitor LNA (ctrl LNA) as detected by QT-PCR (Fig. 4A). Inhibition of miR-28-5p expression by 28-5p LNA caused increased expression of endogenous Mad2 protein compared to cells treated with ctrl LNA (Fig. 4B). QT-PCR reactions revealed that miR-28-5p inhibition did not affect Mad2 mRNA levels (Supplemental Fig. 4A). Reporter assays suggest that this effect is mediated by the 3'UTR of Mad2 and can be rescued by mutation of the miR-28-5p binding site in the Mad2 3'UTR luciferase reporter in 786-O and HeLa cells (Fig. 4C, S4B).

VHL-deficient 786-O renal cell carcinoma cells display low levels of Mad2 and are prone to mitotic slippage in that they fail to efficiently arrest in mitosis in response to nocodazole (5), thus offering a suitable cell-based model to further decipher the miR-28-5p – Mad2 axis in mitotic checkpoint and chromosomal stability control. Strikingly, transfection of 28-5p LNA but not control LNA in 786-O cells expressing fluorescently-marked histone 2B (H2B) to visualize chromosomes, increased the ability of these cells to arrest in
mitosis in response to nocodazole, suggesting a mitotic checkpoint restoration (Fig. 4D, S4C). 28-5p LNA treatment also reduced the frequency of anaphases with lagging chromosome errors, while the number of chromosome bridges remained unaffected (Fig. 4E). Finally, chromosome spreads revealed that upon prolonged treatment (6 days) with 28-5p LNA, numerical aneuploidy was reduced compared to ctrl LNA-treated 786-O cells (Fig. 4F). Thus, miR-28-5p acts as an endogenous regulator of Mad2 protein abundance that, when inhibited, is able to halt and partially revert chromosomal instability in mitotic checkpoint deficient cells.

**pVHL depletion-induced chromosomal instability is mediated via miR-28-5p**

Next we determined whether miR-28-5p expression is dependent on VHL status. As shown in Fig. 5A, knockdown of pVHL in either VHL-positive mouse IMCD3 or human RPE-1 cells induced miR-28-5p expression. miR-28-5p was likewise induced when Vhl was genetically eliminated by expression of Cre-recombinase in Vhlfl/fl mouse embryonic fibroblasts (MEFs, Fig. 5A). In each of these cell systems, the inhibition of pVHL function also provoked a similar upregulation of miR-28-3p (Supplemental Fig. 5A) and of the primary miR-28 transcript (Supplemental Fig. 5B), implying that pVHL affects miR-28 expression at the transcriptional level. Immunoblotting revealed that pVHL levels were reduced where expected (Supplemental Fig. 5C). Conversely, reexpression of pVHL in the VHL-negative ccRCC cell line 786-O (Supplemental Fig. 5C), resulted in reduced miR-28-5p (Fig. 5A), miR-28-3p (Supplemental Fig. 5A), and primary miR-28 levels (Supplemental Fig. 5B).
In accordance with these observations, and arguing in favour of a link between pVHL and miR-28-5p, both human and mouse Mad2 3’UTR (wt), but not the corresponding miR-28-5p mutant luciferase reporters, were suppressed upon knockdown of pVHL (Fig. 5B and Supplemental Fig.5D, respectively). Furthermore, inhibition of miR-28-5p via LNA inhibitor relieved the suppressive effects of pVHL depletion on the Mad2 3’UTR reporter plasmid (Fig. 5C). miR-28-5p inhibition also rescues the effect of pVHL knockdown on endogenous Mad2 (Fig. 5D). QT-PCR confirmed the expected changes in miR-28-5p levels (Supplemental Fig.5E). Finally, 28-5p LNA treatment also normalized both the mitotic slippage as well as the missegregation phenotype that resulted from pVHL depletion in HeLa cells (Fig. 5E, F). Collectively, these results suggest that miR-28-5p expression is induced by depletion or loss of pVHL function and that miR-28-5p functions as a relevant effector mediating mitotic checkpoint weakening and chromosomal instability in VHL-deficient cells by targeting Mad2.

Delivery of LNA-based miR-28-5p inhibitors rescue Vhl-loss-induced chromosome missegregation in mouse kidney epithelial cells following ischemic injury

To assess the relevance of this pathway in vivo, we took advantage of a Cre/loxP-mediated kidney-specific Vhl knockout mouse model (referred to as VhlΔ/Δ) allowing deletion of Vhl in distal tubule and collecting duct cells (13, 26). Indeed, compared to kidneys isolated from Cre-negative littermates, miR-28-5p levels were increased in VhlΔ/Δ kidneys (Fig. 6A). A similar increase was observed for miR-28-3p and primary miR-28 (Supplemental Fig. 6A, B).
Laser capture microdissection revealed a specific increase of miR-28-5p in isolated distal, where also loss of Vhl was confirmed, while no changes were observed in proximal tubule cells (Fig. 6B, Supplemental Fig. 6C, D).

We have recently shown that in an ischemic injury/regeneration model, kidney epithelial cells of VhlΔ/Δ mice display high rates of aneuploidy (14). Hence, we employed this in vivo model to explore miR-28-5p function with respect to its requirement for the development of chromosomal instability upon Vhl ablation. Prior to and 2.5 days after unilateral kidney ischemic surgery, 28-5p or control LNA inhibitors were injected subcutaneously into VhlΔ/Δ mice and chromosome missegregation frequency was assessed in kidney tubule cells 5.5 days post surgery (Fig. 6C). Importantly, only treatment with 28-5p LNA inhibitors reduced the occurrence of lagging chromosomes in VhlΔ/Δ animals specifically in distal tubules (Supplemental Fig. 6E), while the mitotic error rate was constant in proximal tubules (Fig. 6D). This functional rescue was also partially reflected in copy number alterations of chromosome 7, at least in the time frame of 5.5 days (Supplemental Fig. 6F). As shown in Supplemental Fig. 6G, injection of LNA 28-5p, but not control, reduced miR-28-5p levels. Moreover, Mad2 staining intensity in distal tubules, which was markedly reduced upon Vhl knockout, was specifically restored upon LNA 28-5p treatment (Fig. 6E, Supplemental Fig. 6H). The derepression of Mad2 protein levels was also confirmed by Mad2 immunoblotting in whole kidney lysates (Fig. 6F). Taken together, these data indicate that Vhl inactivation induces upregulation of miR-28-5p in the murine kidney, which acts as critical downstream mediator of chromosome (mis)segregation.
**VHL deficiency is associated with increased miR-28-5p expression and chromosomal instability in human ccRCC**

Finally, we addressed the implications of the pVHL-miR-28-5p-Mad2 axis for human ccRCC. As chromosome copy number changes represent the hallmark feature of aneuploidy, we bioinformatically analysed published SNP microarray datasets from sporadic ccRCC with known *VHL* status and normal kidney cortex (27). This analysis revealed that *VHL*-negative ccRCC display a much wider distribution of chromosome copy numbers compared to normal kidney (Fig. 7A). Interestingly, a similarly broad distribution was seen in *VHL*-positive ccRCC (Fig. 7A). To assess whether *VHL* inactivation preferentially leads to numerical (nCIN) rather than structural chromosomal instability (sCIN), as our results would suggest, we re-analysed the above-noted dataset. The basis of this analysis was that in nCIN, the copy numbers of individual loci vary little over the entire chromosome length, but change significantly upon chromosomal rearrangements, such as chromosome arm breaks or translocations (Fig. 7B). A SNP dataset with respect to p53 status served as internal control, as p53 mutations are known to induce sCIN (28, 29). Intriguingly, this analysis revealed a striking difference between *VHL*-negative and *VHL*-positive ccRCCs in that the former were characterized by nCIN and the latter by sCIN (Fig. 7C).

In parallel, we extracted miR-28-5p expression levels of ccRCCs and normal kidneys from RNA Seq data that were reported previously (30) and stratified these according to tumour grade. Already at low tumour grades, miR-28-5p levels were increased, a phenomenon that was apparent also in higher grades (Fig. 7D). Analysis of an independent dataset of ccRCCs and normal
matched kidney tissue (20) also revealed a significant and specific increase of miR-28-5p in ccRCC tumour tissue (Supplemental Fig. 7A). Even more importantly, aneuploidy levels, expressed as the variance of chromosome copy numbers from SNP data (31), correlate linearly with miR-28-5p expression levels in ccRCC stratified by grade (Fig. 7D and Supplemental Fig. 7B). Taken together, these results suggest a direct link between increased expression of miR-28-5p and state of aneuploidy in the context of ccRCC.

Discussion

Existing evidence suggests that increased and decreased expression, but not mutations, of the mitotic checkpoint protein Mad2 is a common event in human cancer (5-8, 32). These Mad2 expression changes are believed to contribute to aneuploidy and tumour development (9, 10, 33). The results of this study reveal a novel mechanism of Mad2 abundance control involving translational inhibition of Mad2 by miR-28-5p via specific target sequences in the Mad2 3’UTR. Disruption of this regulatory mechanism occurs in the context of VHL tumour suppressor inactivation in renal carcinoma cells, where aberrantly upregulated miR-28-5p induces, via Mad2 downregulation, mitotic checkpoint weakness and chromosomal instability (Fig. 7E).

A particular important facet of miRNA function is the ability of individual miRNAs to provide subtle control over gene expression ensuring optimal target gene dosage (34). Thus, miR-28-5p-mediated regulation of Mad2 may normally be dedicated to fine-tune Mad2 levels to balance against stress-induced numerical chromosomal instability. This interpretation is in line with
recent findings that amounts of checkpoint proteins dictate the strength of the mitotic checkpoint (35-37). Accordingly, miR-28-5p may be part of a mechanisms that provides robustness to the process of mitosis assuring error-free chromosome segregation.

Out data suggest a direct link between VHL inactivation as a tumour-initiating event and dysregulation of miR-28-5p – Mad2 expression. The mechanism(s) mediating miR-28-5p induction is not known. However, the proto-oncogene zinc finger and BTB domain containing 7A (ZBTB7A, also known as LRF) has been shown to negatively regulate miR-28 expression (38) and to compete with the Sp1 transcription factor for target binding (39). Whether ZBTB7A/LRF acts downstream of pVHL to control of miR-28 expression remains to be investigated.

As pVHL has been proposed to function as a ‘gatekeeper’ for the renal epithelium (40), its inactivation would be expected to instigate a program of numerical chromosomal instability (nCIN) early on during tumour development as also the marked increase in miR-28-5p levels in low grade tumours and the SNP analysis would suggest. It is conceivable that reduced availability of Mad2 checkpoint protein would, in the face of inactivation of additional ccRCC tumor suppressors such as BAP1 and/or PBRM1 (41, 42), augment the development of nCIN.

As complete lack of Mad2 is incompatible with cell survival (43), for VHL-deficient tumour cells to achieve subtle perturbation of the mitotic checkpoint that is attuned with cell proliferation, disruption of a miRNA-dependent mechanisms appears to be pertinent. miR-28-5p levels are reported to be upregulated in a variety of cancers (44-46). The identification of Mad2 as a
functional miR-28-5p target raises the possibility that in at least some of these cancers chromosomal instability may result from low levels of Mad2. Hence, it is tempting to speculate that miR-28-5p may function as a promoter of chromosomal instability in multiple cancers.

As a mouse model recapitulating key aspects of human ccRCC is not existing, we resorting to a mouse kidney ischemic injury model to evaluate the importance of miR-28-5p for Vhl loss-of-function-induced chromosomal instability in vivo. The ability of LNA-based miR-28-5p inhibitors to reduce the frequency of chromosomal missegregation errors in renal epithelial cells lacking Vhl, provide in vivo evidence that miR-28-5p acts downstream of pVHL and that its upregulation upon Vhl deletion is, at least in part, responsible for the observed chromosomal instability phenotype and can, block the occurrence of chromosome missegregation errors in the short-term in a Vhl−/− background. Consistent with a potential contributory function of miR-28-5p to specific aspects of tumour development in vivo is the observation that in a mouse xenograft model, miR-28-overexpressing HCT116 cancer cells form smaller primary tumours but are more prone to develop metastases (47). As overexpression of miR-28-5p in HCT116 causes downregulation of Mad2 and chromosomal instability (Fig. 2B), the delay in primary tumour growth in this model may be a result of considerable aneuploidy.

In summary, our findings provide a molecular explanation how aneuploidy may be instigated upon inactivation of a key renal tumour suppressor and draws attention to miR-28-5p as a critical regulator of Mad2 protein translation and mediator of aneuploidy. Inhibitors targeting this miRNA pathway could
represent therefore novel agents for therapeutic intervention of VHL-associated cancers.

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References


Figure Legends

Figure 1: Depletion of pVHL negatively regulates translation initiation of Mad2 mRNA.
A, Representative total RNA profile of density gradient fractionation of cell lysates from RPE-1 cells upon shVHL knockdown (N = 3). B, Distribution of Mad2 and C, β-actin mRNA determined by QT-PCR in fractions from density gradients described in A; normalized to LysA (bacillus subtilis) spike-in control (area under curve: 100%, mean ± s.d.). D, Schematic of dual firefly luciferase (FL) and renilla luciferase (RL) vectors with or without the full-length Mad2 3’ untranslated region (3’UTR) fused downstream the FL coding region. E, pVHL knockdown in HeLa cells transfected with either Mad2 3’UTR-containing or empty (no 3’UTR) luciferase reporter plasmids (N = 5, mean ± s.e.m.). F, Murine Mad2 3’UTR luciferase reporter assay after shRNA-mediated Vhl knockdown in IMCD-3 cells (N = 4, mean ± s.e.m.). G, Schematic illustration of reporter vectors with EMCV or HCV internal ribosome entry sites (IRES) inserted upstream of the Mad2 3’UTR luciferase reporter. EMCV: Encephalomyocarditis virus, HCV: hepatitis C virus. H, Effect of pVHL knockdown in HeLa cells transfected with control or IRES-containing Mad2 3’UTR reporter plasmids described in G (N = 3, mean ± s.e.m.).

Figure 2: Mir-28-5p is a Mad2-targeting miRNA.
A, Western blot of HeLa cells transfected with miRNAs predicted to target Mad2 probed with antibodies against indicated proteins. B, Western blot of lysates from human (HCT116) and mouse (IMCD-3) cells transfected with
miR-28-5p and from RPE-1 cells stably expressing lentivirally delivered miR-28, probed with antibodies against indicated proteins. C, QT-PCR analysis of Mad2 mRNA levels following miR-28-5p or miR-28 expression in indicated cells corresponding to B (N = 3, mean ± s.d.). D, HeLa cells were sequentially transfected with Mad2 Δ3'UTR and miR-28-5p and processed for immunoblotting with indicated antibodies. E, miR-28-5p binding site on human Mad2 3'UTR. Bold letters indicate mutations introduced to inactivate the miR-28-5p seed binding site. F, Luciferase assays in HCT116 cells co-transfected with control or miR-28-5p in the presence of wild-type (wt) or miR-28-5p-binding site mutant (mut) Mad2 3'UTR or 3'UTR-devoid reporter (N = 3, mean ± s.d.). G, Western blot for Mad2 of HCT116 cells transfected with wild-type (wt) or seed mutated miR-28-5p. H, Luciferase assays in HeLa cells co-transfected with human wild-type (wt) or miR-28-5p-binding site mutant (mut) Mad2 3'UTR reporter combined with control, miR-28-5p(wt) and miR-28-5p(mut1). The latter contains a seed sequence complementary to the mutated binding site in Mad2 3'UTR (mut). (N = 4, mean ± s.d.). I, Effect of miR-28-5p transfection on IRES-driven Mad2 3'UTR luciferase reporter (HeLa cells, N = 4, mean ± s.d.).

Figure 3: miR-28-5p weakens the mitotic checkpoint via Mad2 and induces aneuploidy.

A, Time from nuclear envelope breakdown (NEBD) to anaphase onset in nocodazole-arrested HeLa H2B-GFP cells transfected with miR-28-5p or siCtrl followed by time-lapse microscopy (N = 175/216, 3 independent experiments in triplicate, Log Rank test). B, Representative still images from
movies of HeLa H2B-GFP cells (histone 2B as nuclear surrogate marker) transfected with miR-28-5p and siCtrl. Scale bar: 10 µm. See also suppl. movie 1/2. C, Representative images of chromosome missegregation errors (nuclei stained with DAPI, scale bar: 10 µm) and quantification of their frequency upon transient miR-28-5p overexpression in HeLa cells or stable miR-28 overexpression in RPE-1 cells [lagg.: lagging, chrom.: chromosome, mult.: multiple (n ≥ 2), N > 150 (HeLa) or 300 (RPE-1) divisions per condition, two categorical (lagging vs. rest) χ² test]. D, Counting of chromosome numbers in metaphase spreads prepared from RPE-1 cells infected with miR-28 or control lentiviruses [N = 99 (ctrl), 103 (miR-28), Mood’s test, whiskers indicate minimum/maximum]. E, NEBD to anaphase onset timing in nocodazole-arrested HeLa H2B-mCherry cells co-transfected with miR-28-5p or siCtrl together with GFP-Mad2 devoid of its 3’UTR followed by time-lapse microscopy (only GFP-positive cells were analysed in 3 independent experiments in triplicate; Log Rank test). F, Analysis of chromosome segregation errors in the same samples shown in E [N > 100 divisions each, two categorical (lagging vs. rest) X² test].

Figure 4: Inhibition of miR-28-5p increases Mad2 protein levels and rescues mitotic slippage and the aneuploidy phenotype.

A, Analysis of miR-28-5p levels by QT-PCR in the indicated cell lines treated with locked nucleic acid (LNA) inhibitor against miR-28-5p or control (N = 3, mean ± s.d.). B, Western blot of cell lines treated as in A probed with antibodies against indicated proteins. Numbers under each lane represent relative Mad2 band density normalized to Cdk2. C, Inhibition of miR-28-5p by
LNA in 786-O cells transfected with luciferase reporter containing wild-type (wt) or miR-28-5p binding site mutant human Mad2 3’UTR (N = 3, mean ± s.d.). D, Nuclear envelope breakdown (NEBD) to anaphase onset timing in nocodazole-arrested 786-O H2B-mCherry cells transfected with control or miR-28-5p inhibiting LNA, followed by time-lapse microscopy (NEBD: nuclear envelope breakdown, 3 independent experiments in triplicate, Log Rank test).

E, Quantification of chromosome missegregation frequency upon miR-28-5p inhibition in 786-O cells by control or miR-28-5p inhibitory LNAs [lagg.: lagging, chrom.: chromosome, mult.: multiple (n ≥ 2), N > 100 divisions, two categorical (lagging vs. rest) χ² test]. F, Chromosome counts of metaphase spreads from 786-O cells after control or miR-28-5p LNA treatment for 6 days. Cells were re-transfected after 3 days. [N = 106 (LNA ctrl), 94 (LNA 28-5p), Mood’s test, whiskers indicate minimum/maximum].

Figure 5: miR-28-5p is upregulated upon pVHL depletion and mediates pVHL’s effect on Mad2 and chromosomal instability.

A, Analysis of mature miR-28-5p levels by QT-PCR with a Taqman probe in indicated mouse and human cells after treatment with either control or VHL shRNAs, Cre-recombinase in the case of mouse embryonic fibroblasts (MEF) carrying floxed Vhl alleles, or reexpression of pVHL in VHL-/- 786-O clear cell renal cell carcinoma cells (N = 3, mean ± s.d.). B, Luciferase reporter assays in HeLa cells co-transfected with siRNA against VHL or control and either wild-type (wt) or miR-28-5p binding site-mutated (mut) hMad2 3’UTR reporter plasmid (N = 3, mean ± s.d.). C, hMad2 3’UTR luciferase assays in HeLa cells with combined VHL knockdown and locked nucleic acid (LNA) mediated miR-
28-5p inhibition (N = 3, mean ± s.d.). D, HeLa cells were co-transfected with siRNA against VHL and miR-28-5p LNA inhibitor or corresponding controls as indicated and processed for Western blotting for indicated proteins. E, Analysis of nuclear envelope breakdown (NEBD) to anaphase onset time in nocodazole-arrested HeLa H2B-GFP cells treated as in D (2 independent experiments in triplicates, Log Rank test). F, Analysis of chromosome missegregation frequency of cells treated as in D [N > 150 divisions, two categorical (lagging vs. rest) χ² test].

Figure 6: miR-28-5p inhibition rescues pVHL loss-induced chromosome missegregation in a kidney injury/regeneration mouse model

A, Mature miR-28-5p levels determined by Taqman QT-PCR in total kidney RNA from kidney-specific Vhl knockout (VhlΔΔ) or Cre-negative control littermate (Vhl+/+) mice (N = 4, mean ± s.d.). B, Assessment of miR-28-5p levels in distal and proximal tubules isolated from Cre-negative and VhlΔΔ kidneys by laser microdissection (n = 3 mice each, approx. 20 spots, cf. Supplemental Fig. 6C, D). C, Schematic description of ischemic injury surgery: the left kidney pedicle is clamped (1), becomes ischemic (2), and reperfused (3). 5.5 days post-surgery kidneys are harvested, displaying an increased number of mitotic cells. D, Quantification of chromosome missegregation frequency in mitotic cells in distal and proximal tubules (differentiated by stainings presented in Supplemental Fig. 6E) in sections from ischemically clamped Cre-negative and VhlΔΔ kidneys, as well as VhlΔΔ mice additionally treated with miR-28-5p or control LNA inhibitor [3-4 mice per genotype, N(proximal/distal) = 41/21 (Cre-), 36/35 (VhlΔΔ), 30/36 (VhlΔΔ + ctrl]
LNA), 46/41 (VhlΔ/Δ + 28-5p LNA), two categorical (lagging vs. rest) $\chi^2$ test]. E, Representative pictures of Mad2 staining on kidney sections from the indicated animals co-stained with the distal tubule marker Dolichos Biflorus agglutinin (DBA). (background substracted, scale bar: 10 µm). F, Western blot with antibodies against Mad2 and Cdk2 from total kidney lysate from VhlΔ/Δ mice treated with LNA ctrl or LNA 28-5p (n = 3 mice each).

Figure 7: Human ccRCCs display a high frequency of numerical aneuploidy and are characterised by increased levels of miR-28-5p. A, Analysis of chromosome copy numbers extracted from published SNP microarrays in normal kidney and ccRCC biopsies with or without at least one functional VHL allele (see Materials and Methods). B, Rational to discriminate between nCIN and sCIN. The intra-chromosome copy number variance is determined as the variance of the copy numbers of all individual loci across a given chromosome. It is low in nCIN and high in sCIN. C, Discrimination between nCIN and sCIN by analysis of intra-chromosome copy number variance described in B (dataset derived from A and glioblastoma samples with or without p53 mutation; mean CN variance ± 95% confidence interval). D, Comparison of miR-28-5p expression with aneuploidy levels stratified by tumour grade. Aneuploidy levels were expressed as variance in inter-chromosome copy number in an independent ccRCC dataset [N(miR-28-5p/copy number events): normal: 5/1606, grade 1+2: 11/506, grade 3: 6/330, grade 4: 11/66]. E, Model explaining a role of the miR-28-5p – Mad2 axis in mitotic control as a function of pVHL status.
Figure 1

A. 

B. 

C. 

D. 

E. 

F. 

G. 

H.
Figure 2

A. Western blot analysis of Mad2 and Cdk2 in cell lines treated with different miRNAs.

B. Western blotting showing the expression of Mad2 and Cdk2 in HCT116, IMCD-3, and RPE-1 cells treated with miR-28 or pLenti.

C. Bar graph showing the relative mRNA levels of Mad2 in HeLa, HCT116, IMCD-3, and RPE-1 cells treated with siCtrl or miR-28-5p.

D. Western blot showing the expression of GFP Mad2 and actin in cells treated with miR-28-5p.

E. Schematic representation of the 3’UTR of hMad2 and the miR-28-5p binding site.

F. Bar graph showing the relative luciferase signal in cells treated with siCtrl or miR-28-5p.

G. Western blot showing the expression of Mad2 and actin in cells treated with different mutations in the 3’UTR.

H. Bar graph showing the relative luciferase signal in cells treated with hMad2 3’UTR wild type or mutant.

I. Bar graph showing the relative luciferase signal in cells treated with different IRES inhibitors.
**Fig. 3**

**A**
-48h → transfec miR → 0h → add nocodazole → imaging → 14h

A cumulative frequency graph showing NEBD to anaphase transition [h] for transfected miR samples.

**B**
- miR-28-5p
- siCtrl

Images at various time points:
-0h10, 0h00, 0h15, 0h30, 1h15

**C**
- Normal
- chr. bridge
- 1 lagg. chr.
- mult. lagg. chr.

**D**

A box plot comparing chromosome number across conditions, with significant differences indicated by ** and ***.

**E**

A cumulative frequency graph for NEBD to anaphase transition [h], showing P < 0.001 to all conditions.

**F**

A cumulative frequency graph comparing % of divisions across conditions, with significance levels indicated by *, **, and ****.

Legend:
- chr. bridges
- 1 lagg. chr.
- mult. lagg. chr.

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Fig. 4

A. Mat. miR-28-5p levels (normalized to RNU19) for RPE-1, 786-O, and HCT116.

B. Western blots for Mad2, Cdk2, and Mad2/Cdk2 for RPE-1, 786-O, and HCT116.

C. Relative luciferase activity for empty, hMad2, 3’UTR wt, hMad2 3’UTR mut.

D. Cumulative frequency of NEBD to anaphase transition for ctrl LNA (N = 119) and 28-5p LNA (N = 85).

E. Cumulative percentage of divisions for 786-O with ctrl LNA and 28-5p LNA.

F. Box plot of chromosome number for LNA ctrl 6d and LNA 28-5p 6d.
A

**knockdown** Cre **overex.**

- +VHL
- -VHL

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<th>Cell Type</th>
<th>rel. miR-28-5p levels (norm. to RNU19/sno202)</th>
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<td>IMCD-3</td>
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<tr>
<td>RPE-1</td>
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<tr>
<td>pMEFs</td>
<td><strong>2.0</strong></td>
</tr>
<tr>
<td>Vhl^{fl/fl}786-O</td>
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B

- siCtrl
- siVHL

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<th>28-5p mut.</th>
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<td>100%</td>
<td><strong>150%</strong></td>
<td><strong>100%</strong></td>
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C

- siCtrl
- siVHL

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<thead>
<tr>
<th>LNA control</th>
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<tr>
<td>Mad2</td>
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<tr>
<td>LNA ctrl</td>
<td>150%</td>
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<td>hVHL</td>
<td><em>n.s.</em></td>
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<tr>
<td>Cdk2</td>
<td>50%</td>
</tr>
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</table>

D

- siVHL
- LNA 28-5p

- Mad2
- hVHL
- Cdk2

E

- 48h
- 0h
- 14h

- Transfect LNA
- Add nocodazole

F

- % of divisions, cumulative
- 0%
- 10%
- 20%

- siCtrl
- siVHL

- 28-5p LNA

- chr. bridges
- 1 lagging chr.
- mult. lagg. chr.
Figure 6

A. miR levels in mouse kidney (norm. sno202)

B. rel. miR-28-5p levels (norm. to sno202) [a.u.]

C. LNA injection

1. Clamp left kidney
2. Ischemia
3. Kidney reperfusion

D. % of tubule cells, cumulative

E. Micrographs of kidney sections

F. Western blot analysis for Mad2 and Cdk2

Legend:
- n.s.
- **
- ***
- mult. lagg. chr.
- 1 lagg. chr.
- chr. bridge

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A

B

Numerical chromosome instability (nCIN)

SNP1: CN = 3
SNP2: CN = 3
→ CN variance low

Structural chromosome instability (sCIN)

SNP1: CN = 3
SNP2: CN = 2
→ CN variance high

C

D

E

pVHL-proficient

pVHL-deficient
miR-28-5p promotes chromosomal instability in VHL-associated cancers by inhibiting Mad2 translation

Michael P Hell, Claudio R. Thoma, Niklaus Fankhauser, et al.

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