miR-28-5p Promotes Chromosomal Instability in VHL-Associated Cancers by Inhibiting Mad2 Translation

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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. Cancer Res; 74(9); 2432–43. ©2014 AACR.

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 tumor progression (9, 10). Several cancer-initiating mutational events such as those affecting the tumor 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 tumor 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 tumor 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′ untranslated regions (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, resuscitated and cultured for 3 to 5 passages before freezing. Cell bank-authenticated cells were used more than 3 to 4 months. HeLa E1 cells were donated by Patrick Meraldi. All cell lines were tested for the absence of mycoplasma by extracellular DNA staining. E13.5 Flvcr2−/− mouse embryo fibroblasts (MEF) 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 mmol/L l-glutamine, penicillin (100 U/mL), and streptomycin (100 µg/mL; Life Technologies). Primary MEFs were supplemented with β-mercaptoethanol (200 µmol/L). 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 ref. 14. In brief, the left kidney pedicle of female mice (age: 6–9 weeks) was clamped for 30 minutes at 37.5°C. If applicable, locked nucleic acid (LNA) 28-5p or ctrl was injected (2.5 mg/kg, s.c.) 12 hours before 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 minutes with prechilled (−20°C) methanol, antibody stained (where applicable), counterstained with 4',6-diamidino-2-phenylindole (DAPI; 1 µg/mL), and mounted in mowiol. Metaphase spreads were prepared as in ref. 15. Mouse kidney sections (10µm) from formalin-fixed paraffin-embedded samples were cut on a HM 3555 microtome (Microm AG). The following additional antibodies were used: anti-phosphorylated (Ser10) histone 3 (p-H3, Cell Signaling, 9706S), anti-β-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 Life Technologies. FISH was performed with a probe described in ref. 16. Fluorescence microscopy was performed on a Zeiss Axiosplan2. Laser capture microdissection was performed on kresyl violet stained tissue (6µm) 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 ref. 5.

**Bioinformatical analyses**

Mad2-targeting miRNAs were predicted in silico using the targetscan algorithm (17). miR-28-5p binding sites on Mad2 mRNA were analyzed (Supplementary Fig. S1A and S1B). Although depletion of pVHL did not affect the global distribution of ribosomes-bound mRNA species (Fig. 1A), it affected the Mad2 mRNA association profile as evidenced by a shift of Mad2 mRNA toward 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 MEF derived from Vhlfl/fl mice (Supplementary Fig. S1C and S1D). Thus, pVHL promotes association of Mad2 mRNAs with ribosomes enhancing their translation.

As the 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 (FL) gene 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 Supplementary Fig. S1F). 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 because of 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

miRNAs represent a class of noncoding 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 IMCD-3 kidney cells upon overexpression of miR-28-5p, which is
sequence conserved between both species (Fig. 2B). Quantitative PCR (qPCR) confirmed the miRNA overexpression in each setting (Supplementary Fig. S2A). 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 (Supplementary Fig. S2B), 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, because it failed to suppress the production of a 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 (Supplementary Fig. S2C). 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 (Supplementary Fig. S2E) 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 Supplementary
Fig. S2D and S2F). 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). Because 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 corre-
sponding Mad2 3′UTR (wt) reporter. The observation that both
mutants complement each other argues in favor 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 fluo-
rescently marked histone 2B (H2B) to visualize chromosomes
in the presence of the microtubule depolymerizing drug noco-
dazole, a mitotic checkpoint activator. Transfection of miR-
28-5p (Supplementary Fig. S3A) affected the ability of these cells to
nocodazole-induced arrest in prometaphase compared with
control transfected cells (Fig. 3A and B and Supplementary
movies S1 and S2), suggesting that they have undergone
mitotic slippage. Also, it enhanced the frequency of chromo-
some missegregation errors such as multiple and single lagging
chromosomes, whereas the number of chromosome bridges,
which are believed to occur through a mitotic checkpoint-
independent mechanism, remained unaffected (Fig. 3C). Final-
ly, the spread in chromosome number, an indicator of chro-
mosomal instability and the ensuing aneuploidy, was likewise
increased by miR-28-5p expression (Fig. 3D). Similar results
with respect to missegregation errors and chromosome num-
ber were obtained in HCT-116, RPE-1, and IMCD-3 cells
(Supplementary Fig. S3B and S3C). Furthermore, the mitotic
slippage and chromosome missegregation phenotypes associ-
ated with increased expression of miR-28-5p was rescued by
coeexpression of a GFP-Mad2 plasmid (25) lacking the 3′UTR
(Fig. 3E and F and Supplementary Fig. S3D). We conclude that
expression of miR-28-5p provokes a weakened mitotic check-
point and chromosomal instability.

Inhibition of miR-28-5p depresses 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 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 with control inhibitor
LNA (ctrl LNA) as detected by qPCR (Fig. 4A). Inhibition of
miR-28-5p expression by 28-5p LNA caused increased expres-
sion of endogenous Mad2 protein compared with cells treated
with ctrl LNA (Fig. 4B). qPCR reactions revealed that miR-28-5p
inhibition did not affect Mad2 mRNA levels (Supplementary
Fig. S4A). 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 and Supplementary Fig. 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 chro-
mosomal stability control. Strikingly, transfection of 28-5p LNA
but not control LNA in 786-O cells expressing fluoressently
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 and Supplementary Fig. S4C). 28-5p LNA treatment also
reduced the frequency of anaphases with lagging chromosome
errors, whereas 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 with 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 pVHL-positive mouse IMCD-3 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 Vhfl/fl MEFs (Fig. 5A). In
each of these cell systems, the inhibition of pVHL function also
provoked a similar upregulation of miR-28-3p (Supplementary
Fig. S5A) and of the primary miR-28 transcript (Supplementary
Fig. S5B), implying that pVHL affects miR-28 expression at the
transcriptional level. Immunoblotting revealed that pVHL
levels were reduced where expected (Supplementary Fig.
S5C). Conversely, re-expression of pVHL in the VHL-negative
cRCC cell line 786-O (Supplementary Fig. S5C), resulted in
reduced miR-28-5p (Fig. 5A), miR-28-3p (Supplementary Fig.
S5A), and primary miR-28 levels (Supplementary Fig. S5B).

In accordance with these observations, and arguing in
favor 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 Supplementary Fig.
S5D, 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). qPCR confirmed the expected changes in miR-28-5p and siCtrl followed by time-lapse microscopy (N = 175/216; three 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 Supplementary Movies S1 and S2. 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 test, whiskers indicate minimum/maximum]. E, nuclear envelope breakdown (NEBD) to anaphase onset timing in nocodazole-arrested HeLa H2B-mCherry cells cotransfected 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 analyzed in three 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) χ² test]. n.s., nonsignificant. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

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; three 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 Supplementary Movies S1 and S2. 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 test, whiskers indicate minimum/maximum]. E, nuclear envelope breakdown (NEBD) to anaphase onset timing in nocodazole-arrested HeLa H2B-mCherry cells cotransfected 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 analyzed in three 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) χ² test]. n.s., nonsignificant. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

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 Vhl<sup>−/−</sup>) allowing deletion of Vhl in distal tubule and collecting duct cells (13, 26). Indeed, compared with kidneys isolated from Cre-negative littersates, miR-28-5p levels were increased in Vhl<sup>−/−</sup> kidneys (Fig. 6A). A similar increase was observed for miR-28-5p and primary miR-28 (Supplementary Fig. S6A

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and S6B). Laser capture microdissection revealed a specific increase of miR-28-5p in isolated distal, where also loss of Vhl was confirmed, whereas no changes were observed in proximal tubule cells (Fig. 6B and Supplementary Fig. S6C and S6D).

We have recently shown that in an ischemic injury/regeneration model, kidney epithelial cells of Vhl<sup>D/D</sup> mice display high rates of aneuploidy (14). Hence, we used this in vivo model to explore miR-28-5p function with respect to its requirement for the development of chromosomal instability upon Vhl ablation. Before and 2.5 days after unilateral kidney ischemic surgery, 28-5p or control LNA inhibitors were injected subcutaneously into Vhl<sup>D/D</sup> animals specifically in distal tubules (Supplementary Fig. S6E), whereas 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 (Supplementary Fig. S6F). As shown in Supplementary Fig. S6G, 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 and Supplementary Fig. S6H). 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 analyzed 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 with 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 reanalyzed 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
tumor grade. Already at low tumor 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 tumor tissue (Supplementary Fig. S7A). 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 Supplementary Fig. S7B). 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).
Mad2 expression changes are believed to contribute to aneuploidy and tumor 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 tumor suppressor inactivation in renal carcinoma cells, where aberrantly upregulated miR-28-5p induces, via Mad2 down-regulation, 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 tumor-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 tumor development as also the marked increase in miR-28-5p levels in low grade tumors 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 pVHL-deficient tumor cells to achieve subtle perturbation of the mitotic checkpoint that is attuned with cell proliferation arrest of a miRNA-dependent mechanisms seems 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 tumor development in vivo is the observation that in a mouse xenograft model, miR-28–overexpressing HCT116 cancer cells form smaller primary tumors 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 tumor 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 tumor suppressor and mediator of aneuploidy. Inhibitors targeting this miRNA pathway could represent therefore novel agents for therapeutic intervention of VHL-associated cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M.P. Hell, C.R. Thoma, W. Krek
Development of methodology: M.P. Hell, T.C. Weber
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pVHL Controls Aneuploidy via miR-28-5p

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