miR-34c-3p ablation of PRKAR2B regulates PKA independent of cAMP in *Theileria*-transformed leukocytes

miR-34c and PKA kinase activity

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Abstract*:*

Theileria annulata is a unique intracellular parasite able to transform its bovine host leukocytes. Cellular transformation is parasite-dependent and reverses upon drug-induced parasite death. Previously, we identified 19 miRNAs, whose levels are significantly altered upon *T. annulata* infection of bovine leukocytes. Here, we focus on the role of miR-34c in leukocyte transformation induced by *T. annulata*. We demonstrate how infection-induced increase in miR-34c-3p levels led to repression of prkar2b leading to activation of PKA independent of changes in cAMP levels. miR-34c-3p provoked increase in PKA activity drives the dissemination of *T. annulata*-transformed macrophages. Additionally, miR-34c-3p negatively regulates prkar2b expression and PRKAR2B protein levels in human cancer cell lines and miR-34c levels inversely correlate with amounts of prkar2b mRNA in brown adipocytes. Our findings represent a novel epigenetic and cAMP-independent way of regulating PKA activity in a range of cell types associated with cancer, diabetes and parasite diseases.

Author summary:

Tropical theileriosis is caused by *Theileria annulata* infection of bovine leukocytes. We describe how infection by *T. annulata* induces changes in infected host leukocyte miR-34c-3p levels to regulate bovine PKA kinase activity by ablating expression of the regulatory subunit *prkar2b*. Infection-induced changes in miR-34c-3p levels provide a novel epigenetic mechanism for regulating host cell PKA activity independent of fluxes in cAMP to aggravate tumor dissemination that underpins the pathogenesis of tropical theileriois caused by *T. annulata*

Introduction

MicroRNAs are a class of small non-coding RNAs and are key regulators of several biological processes ranging from development and metabolism to apoptosis and signaling pathways (1, 2). Indeed, their profiles are altered in many human diseases and particularly in cancer (3, 4), making them a major focus of research. The up- or down-regulation of miRNAs can cause drastic changes to gene expression due to the fact that miRNAs have up to hundreds of potential mRNA targets (5). Changes to gene expression can lead to malignant transformation, especially if miRNA targets include genes that regulate cell homeostasis, proliferation, cell cycle progression, adhesion, or dissemination (6).

Theileria parasites are obligate intracellular protozoa of the phylum *Apicomplexa* that contains many medically important parasites the best-known being *Plasmodium* the causative agent of malaria. The goal for *Theileria* and *Plasmodium* parasites is to infect their invertebrate arthropod hosts (ticks and mosquitoes, respectively) for successful transmission and continuity of the life cycle with the sexual forms – thus ensuring exchanges of genetic material via genetic recombination. To ensure transmission to blood sucking vectors the parasite population is amplified in the mammalian host. Post-inoculation by the insect vector, sporozoites of both species invade MHC class I- and class II-positive cells (leukocytes for *Theileria* and hepatocytes for *Plasmodia*), but thereafter their life cycles differ, as the *Theileria* parasite population amplifies within infected leukocytes. Apicomplexan parasites are able to alter the global gene expression patterns of their respective host cells by interfering with signaling cascades to neutralize host defences so improving their capacity to infect, proliferate and disseminate (7). *Apicomplexa* manipulate host cell miRNomes to their own benefit (8-10). We have shown that miR-126-5p by directly targeting and suppressing JNK-Interacting-Protein JIP-2 liberates cytosolic JNK to translocate to the host cell nucleus and phosphorylate c-Jun that trans-activates AP-1-driven transcription of *mmp9* to promote tumour dissemination of *Theileria*-transformed macrophages (11). Moreover, miR-155 is also induced by infection to supress De-Etiolated Homolog 1 expression that diminishes c-Jun ubiquitination (12). An increase in c-Jun levels leads to an augmentation in *BIC* (pri-miR-155**)** transcripts that contain miR-155, explaining how a positive feedback-loop contributes to the growth and survival of *Theileria*-infected leukocytes

(12). Furthermore, exosomes and their miRNA cargo play an important role in the manipulation of the host cell phenotype and the pathobiology of *Theileria* infections (13, 14).

Mammalian PKA is a hetero-tetrameric enzyme composed of two catalytic subunits associated with two regulatory subunits. There are four different regulatory subunit genes (R1A, R1B, R2A, and R2B) that exhibit differential expression patterns and play different roles in cell differentiation and growth control (15). The catalytic subunit PKA-C is bound to an inhibitory regulatory subunit (PKA-R), but upon binding of cAMP to the regulatory subunits, catalytic subunits are released to act as a serine/threonine protein kinase in both the cytoplasm and nucleus to phosphorylate the target proteins. PKA-C substrates include transcription factors such as CREB and NFκB and other proteins involved in developmental processes (16).

The role of miRNAs in infection is well established (17-20) and the post-transcriptional control of gene expression by miRNAs is increasingly recognized as a central part of host/pathogen interactions (21, 22). For these reasons, we have determined the expression profiles of host microRNAs in virulent and attenuated *T. annulata*-transformed macrophages and infected (TBL20) compared to non-infected (BL20) B cells by miRNome profiling (11). Herein, we functionally characterize the role of miR-34c-3p to demonstrate that by targeting *prkar2b* coding for the R2B (PRKAR2B**)** subunit miR-34c-3p regulates mammalian PKA activity independently of cAMP **(**Cyclic Adenosine Monophosphate). In doing so miR-34c impacts on the dissemination potential of *Theileria*-transformed macrophages.

Results

miR-34c-3p is differentially expressed in *T. annulata*-infected leukocytes:

T. annulata-transformed virulent macrophages disseminate in infected animals causing a disease called tropical theileriosis. However, upon long-term *in vitro* passaging their capacity to disseminate becomes attenuated and they are used as live vaccines to control disease (23). We focused on miR-34c, as its expression is upregulated in *Theileria*-infected virulent macrophages, but is significantly down-regulated following attenuation of their dissemination capacity (Figure 1 left and right panel). We artificially increased miR-34c levels of attenuated macrophages by

transfecting them with a miR-34c mimic (Fig 1B, left panel). Transfection of the mimic had no influence on amounts of an irrelevant miRNA (Fig. 1B, right panel).

Changes in miR-34c-3p levels influence dissemination of *T. annulata*-transformed macrophages:

Virulent macrophages display a high capacity to traverse Matrigel (24), and as miR-34c levels are uppregulated in virulent macrophages its regulatory effect on the capacity of *T. annulata*-transformed macrophages to traverse Matrigel was examined. Transfection of attenuated macrophages with the miR-34c-3p mimic restored Matrigel traversal to levels equivalent of virulent macrophages (Figure 2). Thus demonstrating that infection-induced increase in miR-34c-3p levels contributes to aggressive dissemination of *T. annulata*-transformed macrophages and that a drop in miR-34c-3p levels underpins the reduced dissemination of attenuated macrophages.

***prkar2b* is a novel direct target gene of miR-34c:**

As changes in miR-34c expression can modulate the dissemination phenotype of *Theileria*-transformed macrophages, the mechanism by which this is achieved was determined. Using two publicly available miRNA target prediction algorithms {miRWalk (25) and TargetScan (26)} the *Bos taurus* genome was searched for potential miR-34c-3p target genes and *prkar2b* was identified as a potential target. We have previously shown that cAMP levels and PKA activity influence the dissemination potential of *Theileria*-transformed macrophages (27) and as *prkar2b is* one of four genes coding for regulatory subunits of PKA we determine if *prkar2b* is a bona fide miR-34c target. First, *prkar2b* mRNA levels were measured in virulent and attenuated macrophages and following transfection of attenuated macrophages with the miR-34c-3p mimic (Figure 3A, left panel). Consistent with it being a direct miR-34c target *prkar2b* transcripts are more abundant in attenuated macrophages that have lower miR-34c-3p levels (see Figure 1) and PRKAR2B protein levels decreased in attenuated macrophages transfected with the miR-34c-3p mimic (Fig. 3A, right panel). To confirm that *prkar2b* is a direct target gene its 3'-UTR harboring an identified miR-34c seed sequence was subcloned into the psiCHECK-2 (Promega, # C8021) and transfected into attenuated macrophages together with the miR-34c mimic and Renilla luciferase activity monitored (Fig. 3B, left panel). Due to attenuated macrophages having

lower levels of miR-34c-3p they display greater luciferase activity than virulent macrophages. Upon mimic treatment luciferase activity in attenuated macrophages dropped significantly confirming that *prkar2b* is a direct target of miR-34c-3p. In addition, we synthesized full-length *prkar2b* with the 4 predicted miR-34 seeds mutated (see Suppl. Figure S1) and as a control synthesized full-length wild type (WT) *prkar2b*. The two synthesized versions of *prkar2b* were cloned with GFP fused to the N-terminus of PRKAR2B. The level of *prkar2b* transcripts was normalized to *gfp* mRNA to compensate for any differences in transfection efficiency and expression of the 2 plasmids and this showed that ablating the 4 seed sites rendered *prkar2b* transcripts resistant to miR-34c dicing (Fig. 3B, right panel). Taken together, this indicates that *prkar2b* expression is regulated by variations in miR-34c levels and confirms that *prkar2b* mRNA possess bona fide miR-34c seed sequences.

We have previously established that dissemination of *T. annulata*-transformed macrophages is cAMP-PKA-dependent (27-29). In Fig1B we showed that augmenting miR-34c levels increased Matrigel traversal suggesting that ablation of PRKAR2B expression by miR-34c increases PKA activity and macrophage traversal. Virulent macrophages display higher PKA activity compared to attenuated macrophages and transfection of attenuated macrophages with the miR-34c mimic restored PKA activity to virulent levels (Fig. 4, left panel). Thus, by ablating PRKAR2B miR-34c increases both PKA activity and dissemination potential of *Theielria*-tranformed macrophages.

PKA is known to phosphorylate the transcription factor cAMP response element binding protein (CREB) (30, 31), so for further confirmation of the capacity of miR-34c to modulate PKA activity the transcription level of CREB was estimated by CRE-driven luciferase assays to show that miR-34c mimic treatment induces an increase in CRE-driven luciferase activity (Fig. 4, right panel). Taken together, this demonstrated that mammalian PKA kinase activity can be regulated independent of changes in cAMP levels via miR-34c-3p-mediated ablation of *prkar2b*.

Regulation of *prkar2b* by miR-34c is a common mechanism:

Aberrant PKA signaling is involved in various types of human disease including cancer and obesity (32, 33). Since miR-34c regulates PKA activity independent of cAMP levels in *Theileria*-transformed macrophages, we asked if this could be a mechanism common to other tumor cell types and diseases. We found that overexpression of miR-34c-3p decreased *prkar2b*

mRNA and corresponding protein levels in two human cancer cell lines (HCT116: human colon cancer cell line and HL-60: human leukemia cell line) and miR-34c-3p levels are higher in brown adipocytes (BAT) compared to white adipocytes (WAT; Figure 5). The ensembles argue that cAMP-independent regulation of PKA activity due to miR-34c targetting of PRKAR2B could be common to many different pathologies.

Discussion

MicroRNA functions have been shown to be possibly associated with neoblast biology, development, physiology, infection and immunity of parasites (34). Importantly, parasite infection can alter host miRNA expression that can favor both infection and parasite clearance. Here, we characterize a novel role of miR-34c in modulating the cancer-like phenotype of *Theileria*-infected bovine macrophages. miRNAs are known to target many different genes and miR-34c-3p is no exception, since we were able to identify its seed sequence in just over 100 bovine genes whose expression is down-regulated in virulent macrophages (35), where miR-34c-3p levels are high, and whose expression increases in attenuated macrophages, where miR-34c-3p levels are low (Table S1). Just how many of the 100 genes are actually targeted by miR-34c-3p is difficult to say, as is whether ablation of a gene other than *prkar2b* contributes to the phenotype we observe upon miR-34c-3p mimic. However, and importantly, we confirmed that *prkar2b* is a direct miR-34c-3p-target gene in *Theileria*-trasnformed macrophages. Also, we've shown that miR-34c-3p-induced loss of PRKAR2B enhanced PKA activity to induce CREB transactivation both of which are important contributors to *Theileria*-transformed macrophage dissemination. We have previously described how TGF-β secreted by *T. annulata*-transformed macrophages contributes to sustaining PKA activity in virulent macrophages via PGE2 engagement of EP4 to augment levels of cAMP (27). Now, we provide an infection-induced epigenetic mechanism for regulating mammalian PKA activity independent of fluxes in cAMP. *T. annulata* infection and transformation of bovine macrophages targets mammalian PKA activity in 3 different ways; 1) by raising cAMP levels (27); 2) by suppressing the endogenous inhibitor PKIG (27) and 3) by miR-34c-3p ablation of PRKAR2B subunit expression and this three-pronged "attack" underscores the key role PKA plays in dissemination of *T. annulata*-transformed macrophages.

In addition to the novel role miR-34c-3p plays in *Theileria*-transformed macrophages we provide preliminary evidence that it could target *prkar2b* in some human cancer cell lines and brown adipocytes. Many studies have shown that PRKAR2B is highly expressed in certain tumors and in white adipocytes. For instance, PRKAR2B is highly expressed in prostate cancer and involved in proliferation and metastasis (36). This suggests that miR-34c-3p mimics and antagonists could have therapeutic use in the treatment of not only parasite diseases like tropical theileriosis, but also some types of cancer and perhaps obesity. Resumed in Figure 6 a schematic representating how miR-34c -3p via ablation of of PRKAR2B regulates PKA kinase activity independent of fluxes in cAMP.

Materials and Methods

Cell culture: Cells used in this study are *T. annulata*-transformed Ode macrophages, where virulent macrophages used corresponds to passage 62 and attenuated macrophages to passage 364 (37). Non-infected BL20 and *T. annulata*-infected BL20 (TBL20) cells used have been previously characterized (38, 39). All cells were incubated at 37°C with 5% CO_2 in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% Fetal Bovine Serum (FBS), 2mM L-Glutamine, 100 U penicillin, 0.1mg/ml streptomycin, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 5% 2-mercapthoethanol for BL20 and TBL20.

RNA extractions: Total RNA of *Theileria*-infected leukocytes was isolated using the Direct-zol RNA Kit (Zymo Research # R2070) and total RNA designated for miRNA experiments was extracted using the mirVana miRNA isolation kit (Thermo Fisher) according to the manufacturer's instructions. The quality of extracted RNA was verified using a Bioanalyzer 2100 and quantification carried using Qubit (Invitrogen, catalogue number Q10210).

qRT-PCR for miRNAs: RNA extracted with the mirVana kit was used. cDNA was synthesized using the TaqMan microRNA RT kit (Applied Biosystems) following the manufacturer's instructions. A total of 10 ng of RNA was used in the reverse transcription reaction with miRNA-specific primers. The real-time reactions were performed in a 7500 HT Fast Real-time PCR system (Applied Biosystems). Data was analysed using the $2^{-\Delta\Delta CT}$. The cycle threshold (Ct)

values from the selected miRNA targets were subtracted from the Ct values of the endogenous small noncoding RNA control RNU6B (control miRNA assay; Applied Biosystems).

qRT-PCR for mRNA: Total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied biosystems, catalogue number 4368814) as follows: 1 μg of total RNA, 2 μL of RT buffer, 0.8 μL of 100mM dNTP mix, 2.0 μL of 10X random primers, 1 μL of MultiScribe reverse transcriptase and Nuclease-free water to a final volume of 20 μL. The reaction was incubated 10 min at 25°C, 2 h at 37°C then the enzyme inactivated at 85°C for 5 min. Real time PCR was performed in a 20 μL reaction containing cDNA template, 10 μL 2X Fast SYBR Green Master Mix and 500 nM of forward and reverse primers. The reaction was run on the 7500 HT Fast Real-Time PCR System (Applied Biosystems). GAPDH was used as a housekeeping gene and the results were analyzed by the $2^{-\Delta\Delta CT}$ method. The error bars represent the SEM of 3 biological replicates.

Western Blotting: Cells were harvested and extracted by 1x mammalian lysis buffer (Abcam # ab179835) supplemented with protease and phosphatase inhibitor cocktail (#78440, Thermo Fisher). Protein concentration was determined by the Bradford protein assay. Cell lysates were subjected to Western blot analysis using conventional SDS/PAGE and protein transfer to nitrocellulose filters (Protran, Whatman). The membrane was blocked by 5% non-fat milk-TBST (for anti-PRKAR2B and anti-GAPDH), or 3% non-fat milk-PBST (for anti-actin antibody) overnight at 4 ^{0}C. Antibodies used in immunoblotting were as follows: rabbit polyclonal antibody anti-GAPDH (Merck Millipore #ABS16), mouse monoclonal antibody anti-PRKAR2B (#610625, BD transduction laboratories) and goat polyclonal antibody anti-actin (Santa Cruz Biotechnologies I-19). After washing, proteins were visualized with ECL western blotting detection reagents (Thermo Scientific) with fusion instrument. The β-actin was used as a loading control throughout all experiments.

Fluorescent activated cell sorting (FACS) of GFP-PRKAR2B expressing cells: Wild type *prkar2b* subunit of bovine PKA and mutant *prkar2b* (harboring mutations that ablate miR-34c seed sites) were synthesized (GenScript) and cloned into pmaxGFP vector (LONZA). Virulent *T. annulata*-transformed macrophages were transfected with plasmids expressing wild type- or

mutant-PRKAR2B with GFP fused to the N-termini. Twenty-four hours post-transfection GFP expressing cells were FACS sorted by MoFlo Astrios (Beckman) at the cytometry and immuno-biology facility (CYBIO) of the Cochin Institute. FACS-sorted cells were transfected with mir-34c microRNA inhibitor or mimic and 24 h post transfection protein total RNA was prepared, as described above.

Matrigel Chamber assay: The dissemination capacity of Ode macrophages was assessed *in vitro* using Matrigel migration chambers, as described (40). The Culture Coat Medium BME (basement membrane extract) 96-well assay was performed according to Culturex instructions (catalog number 3482-096-K). After 24 h of incubation at 37°C, each well of the top chamber was washed once in buffer. The top chamber was placed back on the receiver plate. One hundred microliters of cell dissociation solution-Calcein AM was added to the bottom chamber of each well, and the mixtures were incubated at 37°C for 1 h with fluorescently labelled cells to dissociate the cells from the membrane before reading at 485-nm excitation and 520-nm emission wavelengths, using the same parameters as those used for the standard curve.

Transfection: Macrophages were transfected by electroporation using the Nucleofector system (Amaxa Biosystems). A, total of 5×10^5 cells was suspended in 100 µL of Nucleofector solution mix with 400pM of mimic of miR-34c-3p and subjected to electroporation using the cell line-specific program T-O17 for *Theileria*-infected macrophages. After transfection cells were suspended in fresh complete medium and incubated at 37°C with 5% CO_2 for 48 h.

CREB-luciferase assay: After transfection cells were suspended in fresh complete medium and incubated at 37°C, 5% CO_2 for 24 h and then lysed after 48 h post-transfection. Measurements of luciferase and β-galactosidase activities were performed using the Dual Light Assay system (Life Technologies) and luminometer Centro LB 960 (Berthold) according to the manufacturer's instructions.

Measurement of PKA activity: *Theileria*-infected macrophages were transfected with the specific mimic of miR-34c-3p. Samples were then collected, centrifuged at 1500rpm for 5 min and lysed in recommended lysis buffer [20mM MOPS, 50mM β-glycerolphosphate,

50mM sodium fluoride, 1mM sodium vanadate, 5mM EGTA, 2mM EDTA, 1% NP40, 1mM dithiothreitol (DTT), 1mM benzamidine, 1mM phenylmethane-sulphonylfluoride (PMSF) and 10µg/mL leupeptin and aprotinin. The total amount of protein in the supernatant was measured by a Bio-Rad protein assay, based on the method of Bradford, using BSA as a standard. PKA activity was measured using an ELISA kit (PKA kinase activity kit, Abcam 139435) according to the manufacturer's instructions.

Statistical Analysis: Data were analysed with the Student's two-tailed T-tests with at least N = 3 for each experiment. All values are expressed as mean+/-SEM. In each figure, the following symbols represent respective p-value ranges: *:P<0.05; **: p<0.005, ***: p < 0.001.

Acknowledgments:

This study was supported by a Competitive Research Grant from the Office for Sponsored Research (OSR-2015-CRG4-2610) at King Abdullah University of Science and Technology (KAUST) awarded to AP and GL. MH, FBR and ZR acknowledge KAUST for Postdoctoral fellowships. SM and TB were supported by AP baseline funding (BAS/1/1020-01-01) from KAUST. GL also acknowledges ANR-11-LABX-0024 and core support from INSERM and the CNRS.

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Figure legends

Figure 1: miR-34c-3p is differentially expressed in *T. annulata*-infected leukocytes A left. miR-34c-3p levels in uninfected B lymphocytes (BL20) and levels increase when BL20 are infected with *T. annulata* (TBL20). **A right.** Levels of miR-34c-3p are high in virulent

macrophages and decrease in attenuated macrophages. **B left panel**. miR-34c-3p levels in attenuated macrophages increase following their transfection with miR-34c-3p mimic. **B right panel**. Transfection of attenuated macrophages with the miR-34c-3p mimic specifically augments only miR-34c-3p levels, since amounts of miR-30f in attenuated macrophages are unaltered following transfection of the miR-34c-3p mimic. Data represented as mean ±SEM. *n*=3. ***$P < 0.001$ compared to V; ###$P < 0.001$ compared to A. V stands for virulent, A for attenuated, Am: attenuated transfected with miR-34c mimic.

Figure 2: miR-34c-3p levels impact on dissemination potential of *T. annulata*-transformed macrophages. The ability of virulent macrophages (V) to migrate in Matrigel is higher compared to attenuated macrophages (A). Overexpression of miR-34c-3p in attenuated macrophages (Am) restores Matrigel traversal to virulent macrophage (V) levels. Data represent mean ±SEM. ***$P < 0.001$ compared to V and ### $P<0.001$ compared to A.

Figure 3: *Prkar2b* is a direct miR-34c-3p-target gene. A. *Theileria*-transformed macrophages transfected with the miR-34c-3p mimic (Am) down regulate both *prkar2b* transcripts (**left panel**) and PRKAR2B protein levels (**right panel)**. For qPCR normalization relative expression of *prkar2b* was determined with regard to actin gene expression in both virulent macrophages and attenuated macrophages transfected or not with miR-34c mimic. **B. Left panel.** Reflecting greater *prkar2b* expression in attenuated macrophages *prkar2b*-luciferase activity is also higher in attenuated (A) than virulent macrophages (V). Luciferase activity decreases when attenuated macrophages are transfected with the miR-34c-3p mimic (Am). **Right panel**. mRNA level of *prkar2b* in virulent macrophages transfected with either WT- or mutant-PRKAR2B expression plasmids. Mutation of the 4 seed sites rendered *prkar2b* transcripts resistant to miR-34c dicing.

Figure 4: miR-34c-3p-induced drop in PRKAR2B upregulates PKA kinase activity of *T. annulata*-infected macrophages. Left panel. *T. annulata*-infected virulent macrophages (V) have higher PKA activity than attenuated (A) macrophages and overexpression of miR-34c-3p restores PKA activity in attenuated macrophages. **Right panel.** Overexpression of miR-34c-3p in attenuated macrophages increases CREB-transactivation. (Data represented as mean ±SEM. *n*=3. ***$P < 0.001$ compared to V; ###$P < 0.001$ compared to A.

Figure 5: miR-34c and *prkar2b* expression levels in a human cancer cell lines (HL60 & HCT116) and adipose tissues. A. Overexpression of miR-34c-3p ablates PRKAR2B expression in HL60. **B**. BAT has higher levels of miR-34c and lower levels of PRKAR2B compared to WAT. Data represent mean ±SEM. n=3. ***P < 0.001.

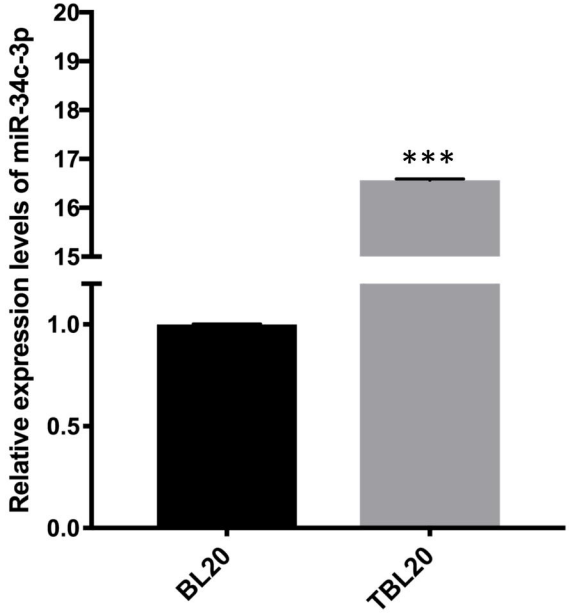
Figure 6: Model proposing how miR-34c-3p by targeting PRKAR2B alters PKA kinase activity independent of fluxes in cAMP. PKA is a heteromer composed of two catalytic subunits bound to two regulatoy subunits. Classical activation of PKA occurs when cAMP binds to regulatory subunits causing a conformational change in catalytic subunits from an inactive to an active kinase. miR-34c-3p can regulate PKA catalytic activity independently of cAMP by ablating *prkar2b* leading to reduced PRKAR2B expression and greater PKA catalytic activity towards target proteins in the nucleus and cytosol.

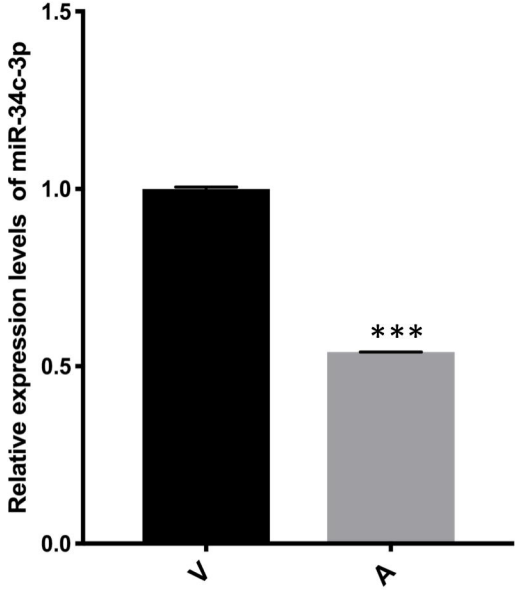
Suppoting information:

Table S1: List of genes harboring miR-34c-3p seed sequences whose transcription is upregulated in attenuated macrophages.

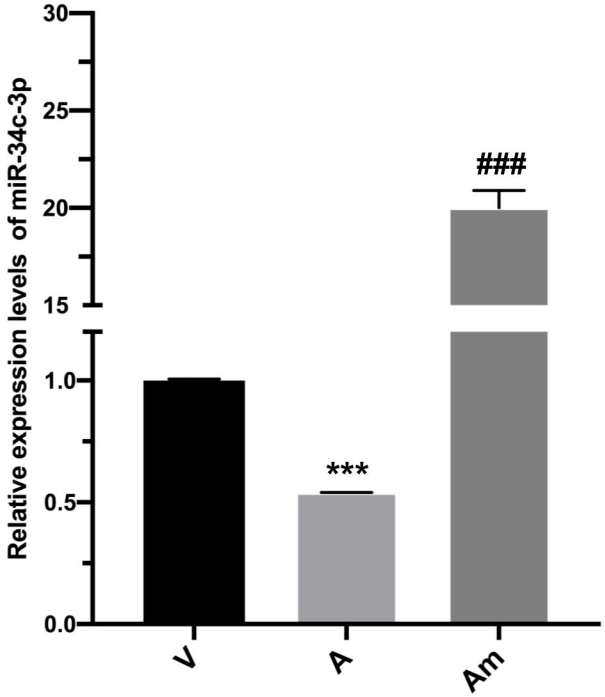
Figure S1: Sequences of wild type (WT) and mutant *prkar2b* with all 4 predicted miR-34 seeds mutated. Original seed sites are highlighted in yellow and mutated sites in green.

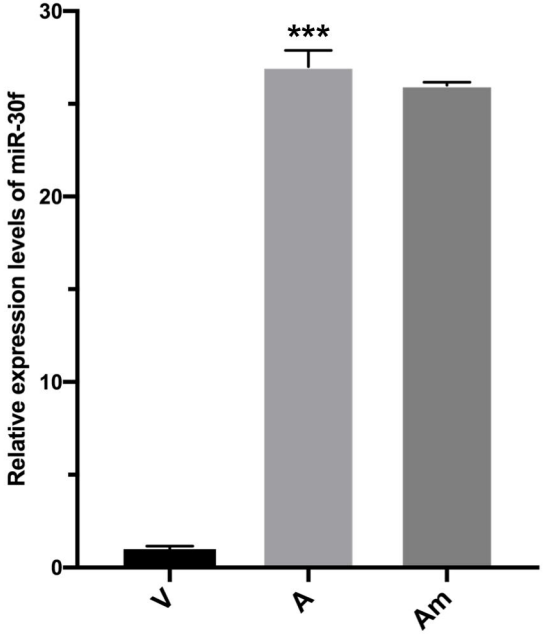
A

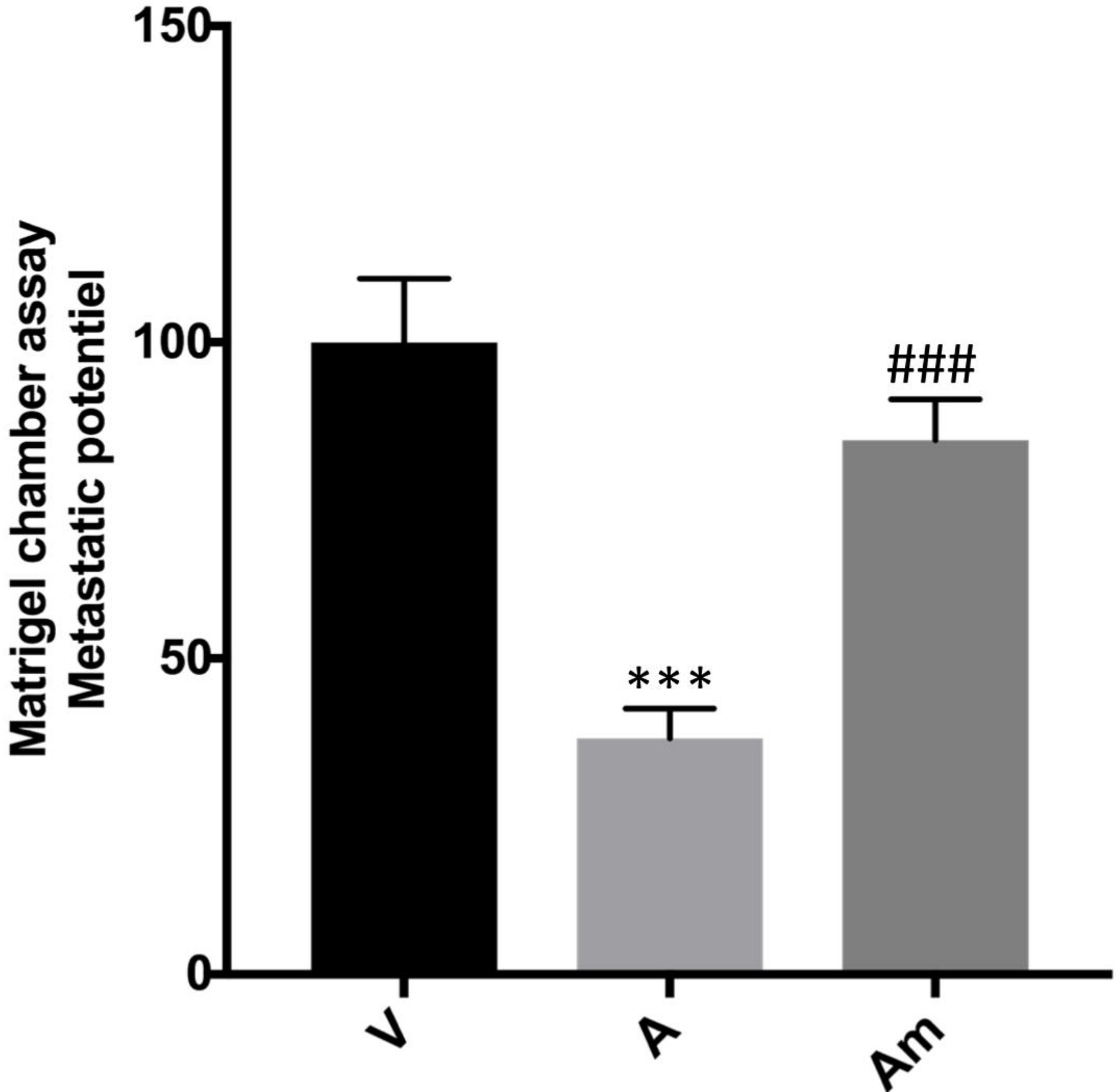

Relative expression levels of miR-34c-3p
20
19
18
17
16
15
1.0
0.5
0.0
***
BL20
TBL20


Relative expression levels of miR-34c-3p
1.5
1.0
0.5
0.0
***
V
A

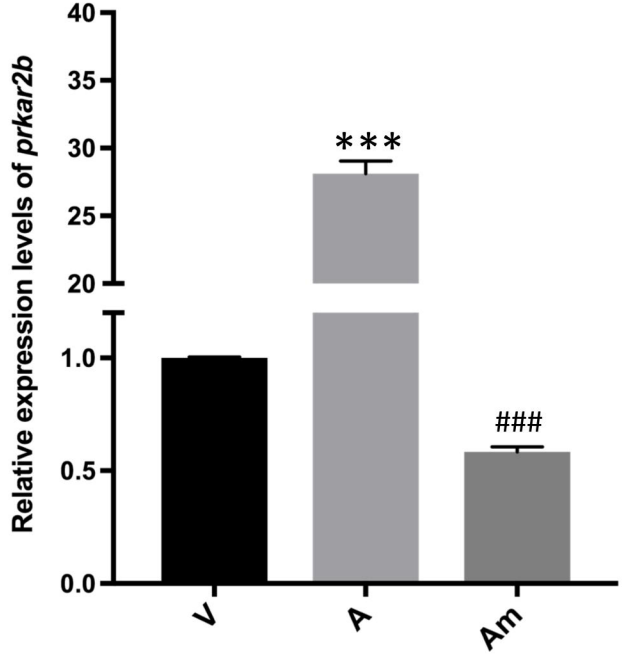
B

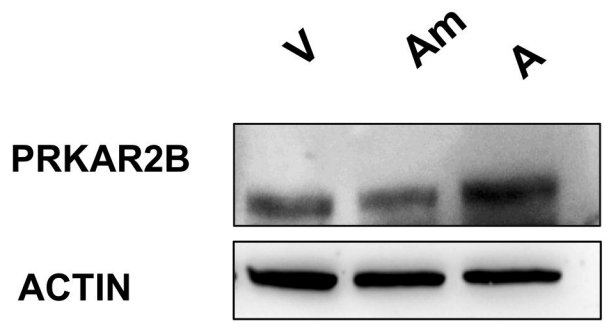

Relative expression levels of miR-34c-3p
30
25
20
15
1.0
0.5
0.0
***
###
V
A
Am


Relative expression levels of miR-30f
30
20
10
0
***
V
A
Am

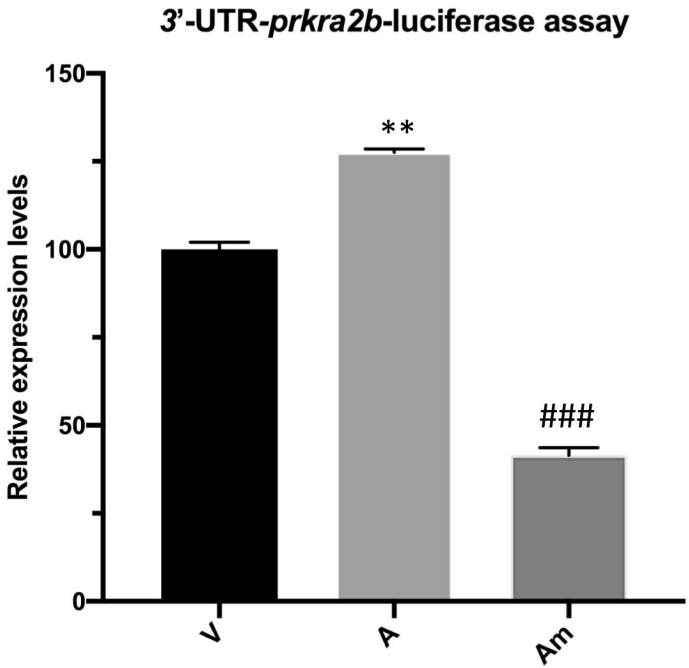

Matrigel chamber assay
Metastatic potentiel
150
100
50
0
***
###
V
A
Am

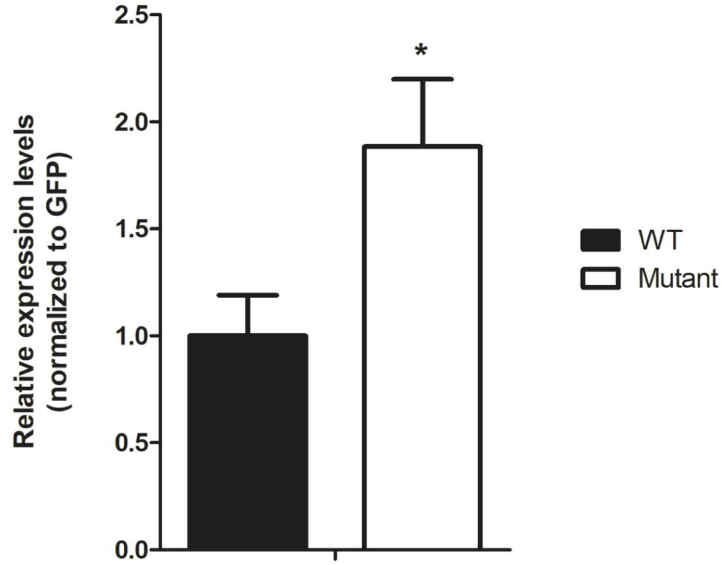
A

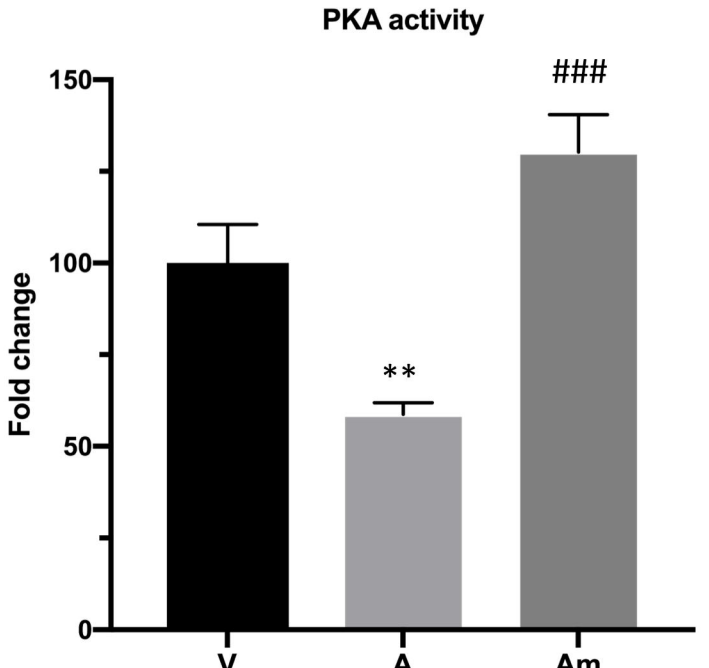

Relative expression levels of prkar2b
40
35
30
25
20
1.0
0.5
0.0
***
###
V
A
Am

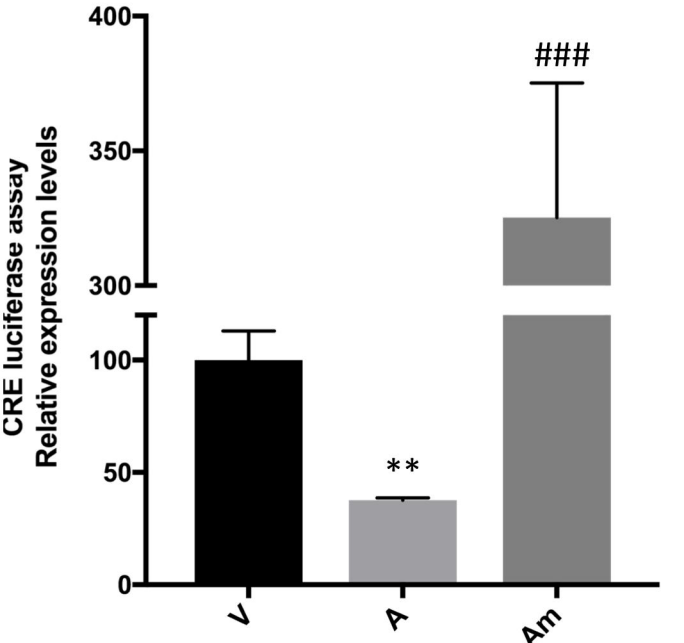

V
Am
A
PRKAR2B
ACTIN

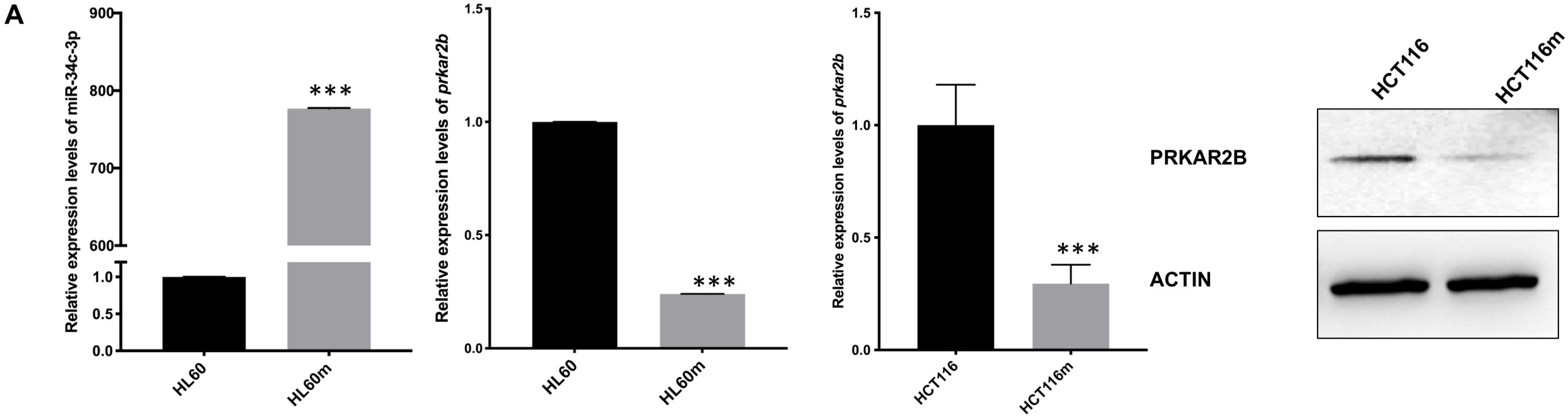
B

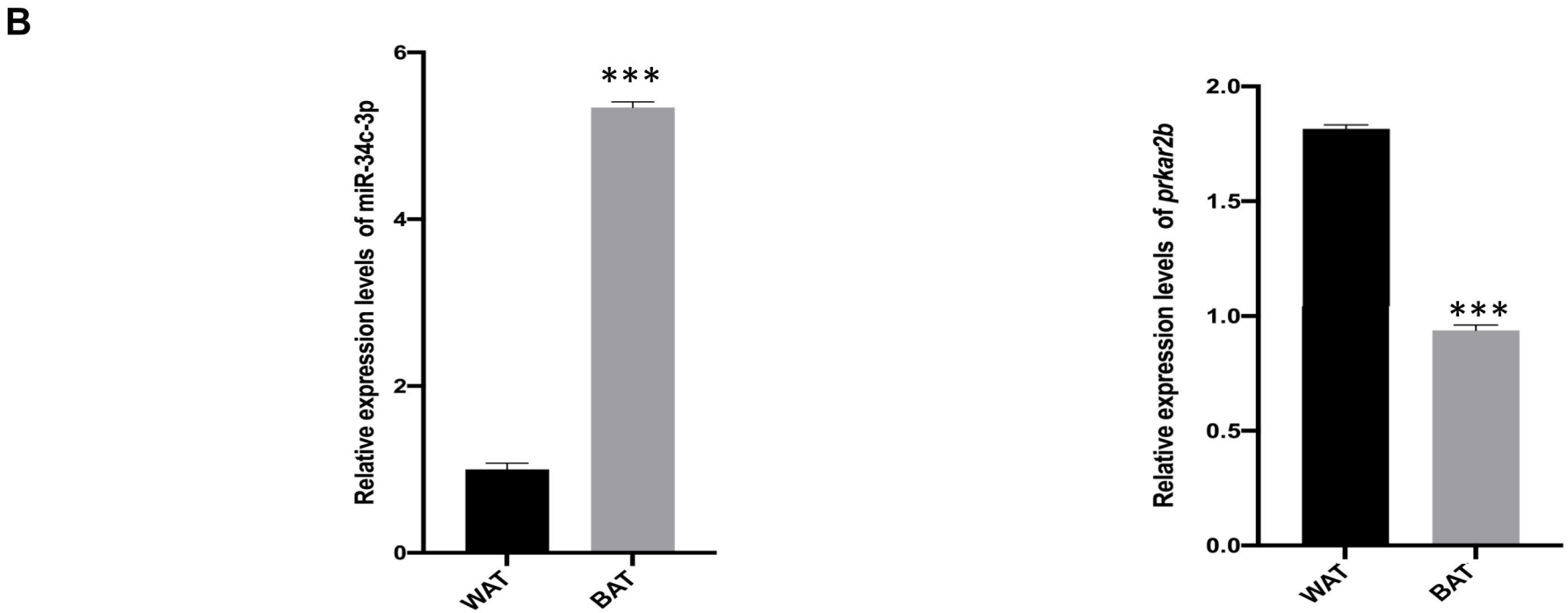

3'-UTR-prkra2b-luciferase assay
Relative expression levels
150
100
50
0
**
###
V
A
Am

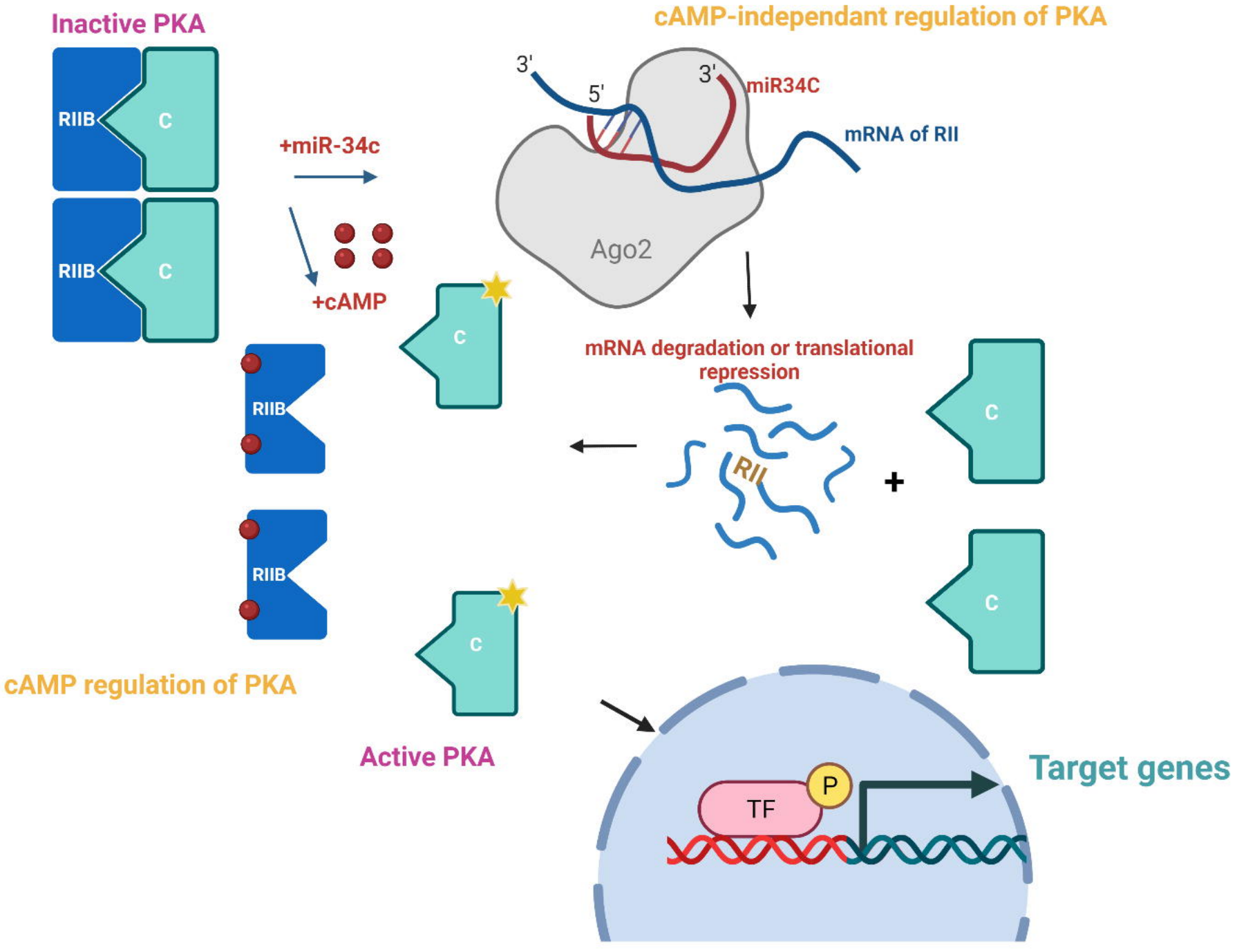

Relative expression levels
(normalized to GFP)
2.5
2.0
1.5
1.0
0.5
0.0
*
WT
Mutant


PKA activity
Fold change
150
100
50
0
**
###
V
A
Am


CRE luciferase assay
Relative expression levels
400
350
300
100
50
0
**
###
V
A
Am


A
Relative expression levels of miR-34c-3p
900
800
700
600
1.0
0.5
0.0
***
HL60
HL60m
Relative expression levels of prkar2b
1.5
1.0
0.5
0.0
***
HL60
HL60m
Relative expression levels of prkar2b
1.5
1.0
0.5
0.0
***
HCT116
HCT116m
HCT116
HCT116m
PRKAR2B
ACTIN


B
Relative expression levels of miR-34c-3p
6
4
2
0
***
WAT
BAT
Relative expression levels of prkar2b
2.0
1.5
1.0
0.5
0.0
***
WAT
BAT


Inactive PKA
RIIB
C
RIIB
C
+miR-34c
+cAMP
cAMP-independant regulation of PKA
3'
5'
3'
miR34C
mRNA of RII
Ago2
mRNA degradation or translational repression
RII
+
C
C
C
RIIB
RIIB
C
cAMP regulation of PKA
Active PKA
TF
P
Target genes