

## Absence of Dicer in monocytes and its regulation by HIV-1

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Running Title: Absence of Dicer in monocytes and its regulation by HIV-1

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### Abstract

MicroRNAs are a class of small RNA molecules that function to control gene expression and restrict viral replication in host cells. The production of miRNAs is believed to be dependent upon the Dicer enzyme. Available evidence suggests that in T lymphocytes, HIV-1 can both suppress and co-opt the host's miRNA pathway for its own benefit. In this study, we examined the state of miRNA production in monocytes and macrophages, as well as the consequences of viral infection upon the production of miRNA. Monocytes in general express low amounts of miRNA related proteins, and Dicer in particular could not be detected until after monocytes were differentiated into macrophages. In the case where HIV-1 was present prior to differentiation, the expression of Dicer was suppressed. MicroRNA chip results for RNA isolated from transfected and treated cells indicated that a drop in miRNA

production coincided with Dicer protein suppression in macrophages. We found that the expression of Dicer in monocytes is restricted by miR-106a, but HIV-1 suppressed Dicer expression via the viral gene Vpr. Additionally, analysis of miRNA expression in monocytes and macrophages revealed evidence that some miRNAs can be processed by both Dicer and PIWIL4. Results presented here have implications for both the pathology of viral infections in macrophages and the biogenesis of miRNAs. First, HIV-1 suppresses the expression and function of Dicer in macrophages via a previously unknown mechanism. Second, the presence of miRNAs in monocytes lacking Dicer indicates that some miRNAs can be generated by proteins other than Dicer.

### Background

The Human Immunodeficiency Virus 1 (HIV-1) infects multiple types of cells, including

CD4+ T-cells, macrophages, monocytes, dendritic cells, and microglial cells (1-5). It has also been reported that latently infected hematopoietic stem cells can be detected in HIV-1 positive patients (6,7). But despite the range of susceptible cell types, HIV-1 is typically discussed in the context of T-cell infection and the resulting depletion of CD4+ T-cells. Conversely, research into HIV-1 infection in the context of monocytes and macrophages remains underdeveloped. Monocytes and macrophages have been previously shown to harbor both productive and latent infections, serving as a part of the viral reservoir that could potentially be essential to chronic infection (8,9). Unlike T-cells, macrophages can resist the cytotoxic effects of HIV-1 infection and persist for several months while producing new infectious viral particles (10-12). In fact, it has been observed that latently infected macrophages are responsible for a large amount of viral resurgence after cessation of anti-retroviral therapies (13). Interestingly, we have previously observed that monocytes differ greatly from T-cells in their expression of miRNA related enzymes. Specifically, monocytes appeared to be deficient for the Dicer enzyme (14). This observation was intriguing because previous reports have indicated that HIV-1 can manipulate the host's RNA interference (RNAi) pathway in order to either promote viral latency or suppress the innate defenses against infection. Realizing that monocytes and monocyte-derived macrophages (MDMs) may be deficient for key enzymes, we sought to expand our investigation of HIV-1 and its regulation of RNAi to relevant cell types beyond T-cells.

The RNAi pathway is a regulatory and innate viral defense mechanism that is conserved in eukaryotes. RNAi uses short RNA oligonucleotides (~18-21nt) to restrict the expression of RNA transcripts in a sequence specific manner (15). Within humans, small RNAs are typically assigned to one of three classes. The first two classes, microRNAs (miRNAs) and short interfering RNAs (siRNAs), are believed to be generated by the

Dicer endonuclease. During their biogenesis, miRNAs and siRNAs are cleaved from either endogenous or foreign double-stranded RNA molecules, respectively. After being released into the cytoplasm, miRNAs and siRNAs hybridize to RNA transcripts and guide effector protein complexes to either degrade the targeted message or prevent its translation (16). The third class of small RNA molecules is known as PIWI-associated RNA (piRNA), which is believed to be generated by PIWI proteins and an unidentified nuclease. Typical piRNAs are between 26 and 33 nt in size (17). While initially thought to be restricted to germline cells, multiple reports have indicated that PIWI proteins and piRNAs are present in somatic cells (18-20). Unlike miRNA and siRNAs, piRNAs appear to initiate the formation of repressive chromatin in order to suppress retroelements and transposons in the genome (17,21).

It has been demonstrated that RNAi is capable of restricting HIV-1 replication by targeting viral transcripts with either miRNAs or siRNAs (14,19,22,23). One particularly convincing set of experiments demonstrated that the ablation of Dicer with siRNAs greatly increased viral replication in infected T-cells (24). Predictably, the virus would benefit from the suppression of RNAi and there is evidence to suggest that HIV-1 does inhibit Dicer activity in T-lymphocytes. For example, several reports have provided evidence that the viral Tat protein is capable of directly interacting with Dicer (25,26) and suppressing the ability of Dicer to generate miRNAs and siRNAs (24). Interestingly, there has been some past debate as to whether or not HIV-1 produces miRNAs of its own, so called viral miRNAs (27). Recent evidence supports the idea that HIV-1 encodes multiple conserved RNA stem-loop structures that can act as a source of viral miRNAs (28). The earliest direct evidence of viral miRNA was found by Northern blot analysis (14,29). More recent publications have verified that viral miRNAs are produced, using powerful techniques such as primer directed sequencing (30) and pyrosequencing (19). Of particular note is the HIV-1 TAR element, which is

produced during both productive and latent infections, can be processed into a viral miRNA. To date, the TAR miRNA has been demonstrated to have two known functions in the maintenance of viral latency. The host's miRNA machinery can use the TAR miRNA to guide repressive chromatin structure to the viral LTR (14,31). Additionally, the viral miRNA has the ability to downregulate multiple endogenous genes touching on pathways such as DNA repair, cytokine signaling pathways, and G-protein receptor signaling pathway. Functionally, the expression of the viral miRNA has been shown to suppress T-cell apoptosis in response to serum starvation, adding weight to the argument that the viral miRNA promotes the survival of latently infected host cells (30). During the course of our previous work, we observed that both primary and monocytes cell lines have lower levels of Dicer than T-cells. Consequently, we sought to establish if monocytes were also deficient for the other components of the miRNA machinery, and if viral infection affected the miRNA pathway.

In this report, we expand upon this initial observation to show that the Dicer protein cannot be detected in monocytes until forced to differentiate into MDMs. The other members of the classical miRNA pathway (Drosha, Ago1, and Ago2) were found to be expressed in monocytes, but at much reduced levels compared to T-cells. Furthermore, monocytes which are transfected with a HIV-1 plasmid show suppressed Dicer levels after differentiation, with a resulting drop in miRNA production. This HIV-1 specific suppression of Dicer expression was found to require the viral gene Vpr. Finally, we uncovered evidence that PIWIL4, a human homologue of the Drosophila PIWI gene, may contribute to miRNA generation. As a whole, these results indicate that monocytes and MDMs are very different from T-cells in their capacities to generate miRNA. Furthermore, the observation that HIV-1 can suppress Dicer levels in MDMs has important implications for future investigations into the topics of infection in macrophages and viral suppression of RNAi.

## Methods

### Cell culture

CEM, ACH2, 8E5, Jurkat, J1.1, 293T, U1 and U937 cell lines were obtained from the AIDS Reagent program. HCT-116 WT and HCT-116 Dicer<sup>-/-</sup> cell lines were a gift from Dr. B. Vogelstein and were generated as previously described (32). Suspension cell lines were maintained in RPMI-1640 with L-glutamine and Penicillin/Streptomycin with 10% FBS. Adherent cell lines were cultured in DMEM supplemented with L-glutamine and Penicillin/Streptomycin with 10% FBS. HCT-116 WT and Dicer<sup>-/-</sup> cells were cultured in McCoy's 5A Modified Media supplemented with L-glutamine and Penicillin/Streptomycin with 10% FBS. For experiments, cells were always harvested during log phase growth. For experiments, cells were always harvested during log phase growth. To induce maturation of monocytes, cells were stimulated once with 250 nM PMA and allowed to differentiate for 96 hours.

PMBCs were obtained from the blood of healthy donor (YW), and purified by centrifugation through a layer of Lymphocyte Isolation Medium. Cells were resuspended in serum-free RPMI and plated on culture dish for 1 hour at 37°C. Nonadherent lymphocytes were removed and the adherent monocytes were cultured in RPMI plus 10% heat inactivated FBS. Macrophages were further differentiated by incubating in 10 ng/ml M-CSF or PMA for 1 week with medium change every 2 days.

### Western Blots

Cell extracts were resolved by SDS-PAGE on 4–20% Tris-glycine gels (Invitrogen). Proteins were transferred to Immobilon membranes (Millipore) at 200 mA for 3 hours. Membranes were blocked with Dulbecco's phosphate-buffered saline (PBS) + 0.05% Tween-20 + 3% BSA. Primary antibody against specified antibodies was incubated with the membrane in PBS + 0.1% Tween-20 + 3% BSA overnight at 4°C. Membranes were washed three times with PBS + 0.1% Tween-20 and incubated with

HRP-conjugated secondary antibody for two hours at 4°C. HRP luminescence was elicited with SuperSignal West Dura Extended Duration Substrate (Pierce) and visualized on a Kodak 1D image station. Antibodies used for Western Blots were obtained from Abcam (Dicer, ab1460; Drosha, ab12286; Ago1, ab5070; Ago2, 57113), Santa Cruz ( $\beta$ -actin, sc-1615), and Abnova (PIWIL4, H00143689-B10P). Densitometry for Western blots was performed using the Carestream software package from Kodak. Levels of  $\beta$ -actin were used to normalize all densitometry data.

Multiple Western blots were performed for the various miRNA-related proteins using various cell lines. Semiquantitative data for protein expression levels were obtained by running multiple Western blots with increasing amounts of whole cell extract (WCE) until the protein (e.g. Dicer, Drosha) was observable. Expression was converted to percentages by comparing protein expression in all other cell lines to protein expression in CEM cells, with number rounded to the nearest integer. Proteins that were not observable even in 195  $\mu$ g of WCE were marked as having 0% expression. The term “nd” indicates that no data is present for a particular cell line.

### Electroporations and transfections

For electroporations, U937 cells were resuspended at 3 million cells in 250  $\mu$ l of RPMI. A total of 5  $\mu$ g of either pGFPmax (Amara) or pNL4-3 was then added to the cell suspension. Cells were pulsed a single time at 210 V, 800  $\mu$ F, and low resistance. Electroporated cells were then immediately transferred to RPMI-1640 with L-glutamine and Penicillin/Streptomycin with 10% FBS and plated in 6-well plates. Twenty-four hours after electroporation, cells were either treated with 250 nM PMA or an equal volume of vehicle and allowed to differentiate for 96 hours. The vehicle for PMA is 100% ethanol. Adherent cells were harvested with Trypsin-EDTA and cell scraping. For transfections, 293T cells were seeded in a 24-well culture plate at 40,000 cells/well. The following day the cells were transfected with 400 ng of the

appropriate empty psiCheck2 or cloned plasmid using Attractene (Qiagen) lipid reagent. For functional Dicer assay in monocytes and T-cells, the following shRNA Luciferase was used:

5'GGUGGCUCCCGCUGAAU  
UGGAGAAC; 3'UUCCACCGAGGGCGA  
CUUAACCUGAAG.

The si-PIWIL4 was SMARTpool siRNA D-004644-01 to 04; and the si-Dicer was SMARTpool siRNA D-003483-01 to 04 from Thermo scientific.

### Isolation and hybridization of miRNAs

MicroRNAs were isolated from cells using the mirVana miRNA Isolation kit (Ambion) according to the manufacturer's protocol. RNA from treated and untreated U937 cells was separated into two fractions using this protocol, a large RNA fraction and a “miRNA-enriched” fraction. A total of 250 ng of RNA from each miRNA-enriched fraction was hybridized on an Agilent Human microRNA V2 microarray. Hybridization, post-hybridization washing and scanning were performed using standard Agilent protocols (Agilent Technologies). The quality and consistency of individual hybridizations was confirmed by monitoring the following parameters in the Agilent output file: single channel total fluorescence signal is 5 times the additive error, signal-to-noise ratio greater than 5-fold, percent saturated features less than 1%, and percent coefficient of variation (%CV) for replicate probes less than 10%. MicroRNA annotation and identification was cross referenced back to various miRNA databases such as miRBase (<http://microrna.sanger.ac.uk/sequences/>). Data was normalized by quantile normalization and the GC-RMA procedure implemented in Partek Genomics Suite (Partek Incorporated) and GeneSpring GX (Agilent Technologies).

### Poly-A RT-PCR

For poly-A RT-PCR detection of miRNAs, 400 ng of RNA from the miRNA-enriched fraction was used to generate cDNA using the Quantimir kit (SBI) according to the manufacturer's protocol. Briefly, small RNA species are poly-adenylated and then RT

reactions are performed with a company-provided RT primer. For PCR, a universal reverse primer is provided by the manufacturer. Specific miRNA forward primers are identical in sequence to the miRNA of interest. Q-PCR performed using the DyNAmo HS SYBR Green qPCR Kit (NEB) in an ABI Prism 7100 thermal cycler. Data analysis was carried out using the SDS software package. For miRNAs that produced multiple bands, PCR products corresponding to the amplified miRNAs were run on a 3.5% agarose gel and quantified using the Kodak 1D software package

### RT-PCR and primers

For mRNA analysis of miRNA-related enzymes following PMA treatments, total RNA was isolated from cells using Trizol (Invitrogen) according to the manufacturer's protocol. For analysis of HIV-1 mRNA in electroporated cells, total RNA was isolated and separated into two fractions, a large and a small RNA fraction, using the mirVana miRNA Isolation kit (Ambion) according to the manufacturer's protocol. A total of 1 $\mu$ g of RNA from the large RNA fraction was treated with 0.25 mg/ml DNase I for one hour, followed by heat inactivation at 65°C for 15 minutes. A total of 1 $\mu$ g of total RNA was used to generate cDNA with the iScript cDNA Synthesis kit (Bio-Rad) using oligo-dT reverse primers.

### qPCR of RNAi related genes

U937 cells were harvested at days 0, 1 and 4 after PMA treatment. RNA was extracted by Trizol reagent (Invitrogen). One microgram of total RNA was reverse-transcribed using the Superscript III First-Strand Synthesis kit (Invitrogen). Following RT, 1 micro-liter of cDNA was used for qPCR. Quantitative PCR was performed using Brilliant II SybrGreen qPCR master mix (Stratagene) and primers for GAPDH

(GGAAGGTGAAGGTCGGAGTCAA, CCTTGACGGTGCCATGGAAT), Ago2 (TTCATCGTGGTGCAGAAGAG, CCCAGAGGTATGGCTTCCTT), Dicer (TCGCTGGCTGTAAAGTACGA, TTCAAGCAATTCTCGCACAG) and

Drosha (TGCAACTGGTAGCCACAGAG, ACACTGCTGAAGCTGGGATT). PCR was performed on a BioRad CFX384 Real-Time System. Expression of each transcript was normalized to GAPDH and displayed as fold change

### Cloning

The psiCheck2 vector was purchased from Promega. Vector was digested with XhoI (NEB) and NotI (NEB) for 1 hour at 37°C then heat killed at 65°C for 20 minutes. The vector was then treated with Antarctic Phosphatase (NEB) at 37°C for 30 minutes. The digested vector was purified by phenol/chloroform extraction followed by ethanol precipitation. Inserts corresponding to two regions of Dicer cDNA and three regions of the Drosha cDNA were generated via PCR amplification. PCR products were gel purified and then digested with XhoI and NotI. Digested inserts were gel purified to remove enzyme and digest fragments. Ligations were performed using DNA T4 DNA ligase (Promega) at a 1:3 molar ratio of vector to insert.

### Luciferase Assay

Luciferase assays were performed with Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's recommendations. Briefly, 293T cells were harvested 48 hours after transfection and 75  $\mu$ l aliquots from each transfection were transferred into a Costar 3610 96-Well Assay plate. Cells were lysed with an equal volume of Reagent #1 and incubated at 37°C for 10 minutes before measuring luminescence at 570 nm. An equal volume of Reagent #2 was then added and incubated at 37°C for 10 minutes before measuring luminescence at 460 nm. Luminescence was measured on a BioTEK Synergy HT Multi-Mode microplate reader.

### Binding site prediction

Predictions for miRNA binding sites were generated using the MiRanda Algorithm v1.0b distributed by the Computational Biology Center of Memorial Sloan-Kettering Cancer Center. The entire CDS for both

Dicer (NM\_177438) and Drosha (NM\_013235) were utilized to predict hybridization between miRNA and mRNA transcripts.

## Results

### Monocytes are deficient for key miRNA processing proteins.

We have previously observed that some of the components of the RNAi machinery may be either low or undetectable in monocytes and/or macrophages (14). In fact, upon close examination of the literature we were unable to clearly define if the components of the classic RNAi machinery were detectable in various HIV-1 susceptible cell types including monocytes, microglial cells, astrocytes, or CD34<sup>+</sup> hematopoietic stem cells. Indeed, the bulk of the existing literature describes the miRNA machinery in the context of epithelial cell lines (derived from cervical, lung, and breast cancers), endothelial vascular cell lines, and lymphocyte cell lines (33-38). In the current manuscript we decided to look at the expression of Dicer and other miRNA related protein in monocytes. The results of such an experiment are shown in Figure 1A, where Western Blot analysis of T-cells compared to monocytes showed no detectable levels of Dicer, Drosha, Ago1, and Ago2 (compare Lanes 1 and 2). Interestingly, when monocytes were treated with PMA or M-CSF, which induces a differentiation of these cells into macrophages, these RNAi components were detectable (lane 4 and 5). Treatment with PMA or M-CSF, but not vehicle, also resulted in visible morphological changes to U937 cells, which became adherent and formed pseudopodia as MDMs (data not shown). In the case of Ago1, a band appears in after vehicle treatment (Lane 3), but the size of the band is larger than the expected size for Ago1, which is observable in lymphocytes (Lane 1) and MDMs (Lanes 4 and 5). Nevertheless, there is a clear pattern present. Inducing the differentiation of monocytes results in the upregulation of miRNA related proteins.

Results in panel A used a low total amount of protein extract (50 µg) and therefore it may be possible that our Western Blot detection assay was not sensitive enough to detect these components. Therefore, we next performed another experiment with a greater amount of extract to detect whether the components of the RNAi machinery were present in these cells. Results in panel B show that at increasing concentrations of the extract (65 µg and 195 µg) all the components of the RNAi machinery were detectable (albeit at lower levels compared to T-cells) with the exception of the Dicer protein. In order to determine if these results were general to all monocytes or unique to U937 cells, we performed Western blots for the miRNA related proteins in multiple related cell lines. The results are compiled in Table 1, where there is a clear trend separating cell lines derived from the lymphoid lineage and cell lines derived from the myeloid lineage. Namely, myeloid cells appear to be generally deficient for the proteins related to miRNA biogenesis until terminally differentiated. Although these results are from cell lines, we have previously observed a similar deficiency with extracts from primary monocytes (14). Collectively these results indicate that monocytes in general express all of the classical miRNA related proteins at low levels, with the exception of Dicer.

With the observation that the protein levels for Dicer were undetectable by Western blot, we proceeded to perform RT-PCR on monocyte cells to investigate whether the expression of Dicer mRNA was dependent upon PMA stimulation. The results of this experiment (Figure 1C) show that mRNA for Dicer was detected in both CEM and untreated U937 cells (Lanes 1 and 2 respectively). Furthermore, the results in panel C show that mRNA for Dicer was expressed in monocytes at all time points (Lanes 3-6) and did not increase over time after PMA stimulation. Conversely, the results in Figure 1D clearly show that expression of the Dicer protein gradually increased over time after treatment with PMA (Lanes 3 through 6).

As differentiation of U937 cells using PMA results in increasing expression of Ago2, Dicer and Drosha we sought to determine if the levels of the mRNA transcripts for each of these genes changed in response to PMA treatment. Total RNA from U937 cells at day 0, 1 and 4 after treatment was reverse transcribed and cDNA used for qPCR. Absolute quantity of each transcript was normalized to GAPDH and is shown as fold change from Day 0 (Figure 1E). Dicer mRNA levels drop by greater than 50% from day 0 to day 4 following PMA treatment. Drosha and Ago2 levels fluctuate at one day post PMA treatment, but rise to 1.7 and 1.6 fold higher than baseline by day 4. As a whole, these results indicate that the Dicer mRNA is present in undifferentiated U937 cells and is likely repressed at the level of translation.

Finally to assess whether functional Dicer protein was available in these cells but simply not detectable with our current methods, we utilized a function transfection assay. Here we transfected HIV-LTR-Luc with Tat in presence of either shRNALuc or siLUC and scored for luciferase activity from these cells. Results of such an experiment are shown in Figure 1F, where transfection of siLuc, and not shRNA was able to downregulate luciferase protein levels in monocytes. Positive control experiments in T-cells showed downregulation of luciferase activity using shRNA-luc further indication a functional Dicer in these cells. Since repression of translation is often mediated by specific miRNA interactions with the mRNA. These results therefore suggest a hypothesis where monocytes might restrict the expression of Dicer via the miRNA pathway. This hypothesis is elaborated upon later in this manuscript.

#### **HIV-1 suppresses the expression of Dicer in MDMs.**

It is known that infected MDMs produce virus while infected monocytes produce relatively little virus (39-41). As our earlier results from monocytes and MDMs indicated that Dicer

expression correlated with differentiation, we hypothesized that viral infection might induce the expression of Dicer and the differentiation of monocytes in order to promote efficient viral replication. In order to test this hypothesis, we electroporated the wild type HIV-1 clone pNL4-3 into U937 monocytes, then treated infected monocytes with vehicle or PMA. As a negative control, the pGFPmax plasmid was also electroporated into U937 cells followed by treatment with either PMA or vehicle. U937 cells were then harvested and processed for both Western blots and RT-PCR. Results for this experiment can be seen in Figure 2A, where the cell extract was Western blotted for Dicer, Drosha, Ago2 and  $\beta$ -actin. In panel A, extract from uninfected and infected T-cell lines served as positive controls (Lanes 1 and 2, respectively) while extract from untreated U937 cells (Lane 3) served as a negative control. Cells transfected with the pGFPmax plasmid then treated with vehicle (Lane 4) or with PMA (Lane 5) showed that transfection with foreign DNA neither forced cells to differentiate, nor suppressed the effects of PMA treatment. However, cells transfected with the pNL4-3 plasmid then treated with vehicle (Lane 6) or PMA (Lane 7) showed interesting results. Contrary to our initial hypothesis, transfection of viral DNA did not promote the differentiation of monocytes to macrophages based on the lack of Dicer expression (compare Lanes 3 and 6) and lack of morphological changes (data not shown). Transfecting cells with pNL4-3 followed by treatment with vehicle neither induced the expression of the RNAi components nor induced cell adherence or pseudopodia formation. Furthermore, transfection with pNL4-3 suppressed the expression of Dicer even after PMA treatment (compare Lanes 5 and 7). In order to ensure that these results were not an artifact of our experimental design, this experiment was repeated using a different GFP vector and without any plasmid (data not shown). In each case, cells transfected with pNL4-3 and treated with PMA showed a suppression of Dicer levels when compared to the untransfected cells. Together, these results suggest that the

suppression of Dicer protein levels in MDMs is an HIV-1 specific phenomenon.

In order to confirm that HIV-1 mRNA was being produced in the transfected monocytes, RNA was isolated from CEM, ACH2 (an HIV-1 infected cell line), and transfected U937 cells using the mirVana RNA isolation kit. The isolated RNA was treated with DNase I and then used to perform RT-PCR for the viral Nef and Env genes, as well as the host GAPDH gene (Figure 2B). Uninfected CEM cells (Lane 1) showed no expression of HIV-1 mRNA while infected ACH2 cells (Lane 2) showed expression of both Nef and Env. HIV-1 message for both Nef and Env was observed in cells transfected with pNL4-3 (Lanes 5 and 6) whereas no viral mRNA was detected in untransfected cells (Lanes 3 and 4). Notably, there was a visible increase in Env message after PMA treatment (compare lanes 5 and 6) consistent with prior reports indicating that PMA treatment stimulates viral production (42,43). Finally, we utilized primary monocytes (donor YW) and asked whether the expression of Dicer increased once cells were differentiated into macrophages. Results in Figure 2C indicate that differentiated cell extracts (150 µg) showed abundance of Dicer (lane 2) and the levels were mostly diminished in the presence of pNL4-3 construct (Lane 4). Collectively these results indicate that increased Dicer levels in differentiated cell line or primary cells are regulated by HIV-1.

#### **HIV-1 suppresses miRNA production in MDMs.**

The lack of Dicer in undifferentiated monocytes and the suppression of Dicer in infected MDMs raise important questions about the state of miRNA biogenesis in both of these cell types. Results from animal models with conditional knockouts of Dicer have shown that a Dicer<sup>-/-</sup> genotype leads to developmental defects such as failure to differentiate, disruption of heterochromatin, and premature senescence, indicating that the production of miRNAs is important for cell survival and proliferation (44,45). Additionally, RNAi initiated by Dicer has

been implicated in the processes of restricting viral replication and promoting viral latency (30,31,46). The observed data raised two important and related questions: First, though Dicer could not be detected in undifferentiated monocytes, can any cellular miRNAs be detected in undifferentiated monocytes? Secondly, although HIV-1 transfection suppressed the expression of Dicer in MDMs, does this HIV-1 specific suppression result in a significant drop in miRNA levels?

To address both of these questions, we isolated small RNAs from transfected and untransfected monocytes and MDMs, then hybridized the RNA to an Agilent V2 Human miRNA chip capable of detecting 722 unique human miRNAs. Total RNA was isolated from four differentially treated groups; cells electroporated without plasmid plus vehicle (treatment 1) or plus PMA (treatment 2), and cells electroporated with pNL4-3 plus vehicle (treatment 3) or plus PMA (treatment 4). Isolated RNAs were column purified to capture small RNA species using the mirVana miRNA isolation kit, then end-labeled and hybridized on an Agilent Human miRNA V2 microarray. The microarray probes possess a secondary structure (resembling shepherd's crooks) that results in preferential binding for correctly sized miRNA only, and prevents binding by unprocessed hairpins and large RNA molecules. The results from this microarray indicated that miRNAs were indeed present in undifferentiated U937 monocytes, and that the expression of miRNA was altered by both PMA treatment and by transfection with the HIV-1 clone (Table 2). Furthermore, the results from this microarray were consistent with prior publications concerning MDMs and miRNA production. For instance, miR-155 is required for proper myelocyte development, and this miR was detected in both monocytes and MDMs (47). Similarly, the expression of miR-146b was shown to be upregulated by inflammatory cytokines (48) and a similar upregulation was observed in our PMA treated cells, as seen in Table 2. When comparing detectable miRNAs among the different treatments, we



observed that the expression of Dicer after PMA treatment significantly increased the abundance of several dozen miRNAs. The first column in Table 2 provides the name of each detected miRNA, followed by its fold increase in expression after the addition of PMA. The third column in Table 2 displays the fold change in miRNA expression between transfected monocytes (treatment 3) and transfected MDMs (treatment 4). From this table, it is clear that cells transfected with the pNL4-3 clone produce significantly fewer mature miRNAs than untransfected cells. The observed drop in miRNA expression also correlates with the observed suppression of Dicer protein levels shown in Figure 2. Taken as a whole, these results suggest that the expression of Dicer after PMA treatment resulted in an increase in miRNA production in MDMs, and that transfection with HIV-1 suppressed general miRNA biogenesis in infected MDMs.

Finally, it has previously been reported that overexpression of Tat can downregulate miRNA production in T-cells, but the overexpression of Tat alone did not alter protein levels of Dicer (26). Therefore, these experiments suggest that during the normal course of infection in monocytes and MDMs, HIV-1 suppresses RNAi by downregulating the expression of Dicer. To our knowledge, this is the first time that evidence has been presented indicating that HIV-1 has the potential to suppress Dicer protein levels in MDMs.

### **The viral protein Vpr is required for suppression of Dicer in MDMs.**

After observing that the HIV-1 virus is capable of suppressing Dicer protein levels, we were interested in determining if this observed suppression could be ascribed to a single viral gene. In order to carry out this investigation, we obtained several mutant strains of the pNL4-3 HIV-1 plasmid. These mutant strains were constructed as previously described by Sunamoto et al (49). Briefly, the mutant strains were generated by replacing the start codon of the individual viral ORFs with a stop codon. The mutant plasmids

which we have utilized here include a  $\Delta$ Rev,  $\Delta$ Nef,  $\Delta$ Vpr,  $\Delta$ Vpu, and  $\Delta$ Env mutation, referred to in this paper as “pNL4-3  $\Delta$ Rev”, “pNL4-3  $\Delta$ Nef”, etc. Since a  $\Delta$ Tat mutation would not allow efficient viral transcription, no  $\Delta$ Tat mutation was generated. Instead, a mutant possessing mutations for all ORFs except Tat was used so that the individual effects of Tat could be assessed. This last mutant plasmid can only produce the Tat and is referred to as “pNL4-3 Tat Only” in this paper.

Next we repeated our transfections of monocytes with these mutant constructs followed by PMA treatment. Four days after PMA treatment cells were harvested and whole cell extract was Western blotted for Dicer and  $\beta$ -actin. Western blot data was then quantified via densitometry. Dicer protein levels were normalized to  $\beta$ -actin levels (Figure 3A). Dicer protein levels are shown for untransfected and transfected MDMs in lanes 1 and 2. Comparatively, U937 cells that were electroporated with the pNL4-3 plasmid (Lane 2) experienced a five-fold reduction in Dicer protein levels compared to levels in transfected cells (Lane 1). Interestingly, cells transfected with the pNL4-3  $\Delta$ Vpr plasmid (Lane 6) showed a consistent inability to suppress Dicer protein levels, thereby indicating that the viral Vpr gene may be required for the suppression of Dicer.

While the results for cells transfected with pNL4-3  $\Delta$ Vpr show the most consistent evidence of mediating Dicer suppression, there was one other data point worthy of consideration. Cells transfected with the pNL4-3  $\Delta$ Nef plasmid (Lane 5) were unable to suppress Dicer levels. However, the results for the pNL4-3  $\Delta$ Nef plasmid showed a greater standard deviation than the results for pNL4-3  $\Delta$ Vpr plasmid. Nevertheless, this data may indicate a scenario in which both Vpr and/or Nef play a part in suppressing the host's expression of Dicer.

Finally we performed a time course experiment where cells were transfected with

various pNL4-3 wild types (WT) and mutant constructs, treated with PMA and processed for suppression of Dicer using western blots. Results in panel B indicate that Vpr mutant showed the most robust expression of Dicer at 48 hrs and Nef mutant virus showed Dicer expression after 96 hrs post PMA treatment. The effect of Vpr was further tested in U937 cells transfected with wild type Vpr where Dicer levels significantly decreased after 48 hrs of transfection in presence of PMA (panel C). Collectively, these results indicate that Vpr (and to some extent Nef) may be responsible for the observed suppression of Dicer in monocytes induced cells.

#### **Dicer is regulated by miR-106a in undifferentiated monocytes**

After data from our initial investigations showed that Dicer protein levels were absent in monocytes despite the presence of Dicer mRNA, we hypothesized that Dicer expression in undifferentiated monocytes was blocked at the translation stage. Since, miRNA inhibition of translation is a well known mechanism of translational inhibition, we examined our microarray data for any miRNAs which were both downregulated after PMA treatment and predicted to restrict Dicer mRNA translation. To accomplish this, we first consulted the lists of miRNA targets generated by the TargetScan and Pictar-Vert algorithms. Among those miRNAs which were downregulated more than 2-fold, only miR-18b was predicted by TargetScan to restrict Dicer expression.

The miR-18b gene is located on the X chromosome where it belongs to a polycistronic cluster of six miRNAs transcribed on the same mRNA. We refer to this miRNA cluster as the ChrX 17-92 cluster, as it possesses strong sequence similarity to the better characterized 17-92 cluster located on Chromosome 13. The members of the ChrX 17-92 cluster are depicted in Figure 4A, along with the fold change in expression following treatment of cells with PMA. Each member of the cluster was downregulated following PMA treatment, which is expected since all miRNAs in this cluster are derived from the

same mRNA transcript. However, we observed that the degree of downregulation was not equal for all cluster members, but was related to the position of each miRNA within the cluster. Most miRNAs are derived from RNA Polymerase II (Pol II) transcripts, but since this unusual pattern of downregulation would not be expected of a Pol II transcript, we explored the possibility that the ChrX 17-92 cluster may be transcribed by RNA Pol I or III. However, miR-106a and miR-18b were both found to be sensitive to low concentrations of  $\alpha$ -amanitin, indicating that this cluster is likely transcribed by RNA Pol II (data not shown). Therefore, the reason for the uneven downregulation of cluster members after PMA treatment is not immediately apparent and will require future research. Figure 4B shows the detected level of each miRNA after each treatment; 1) Untransfected cells, 2) Untransfected cells plus PMA, 3) pNL4-3 transfected cells, and 4) pNL4-3 transfected cells plus PMA. Transfection with pNL4-3 did not appear to affect levels of each miRNA (compare lane 1 to 3 or lane 2 to 4).

Since all of the miRNAs of the ChrX 17-92 cluster were downregulated by PMA treatments, all six members were further checked for binding to Dicer mRNA using the MiRanda v1.0b algorithm. The sequence for the Dicer mRNA was RefSeq NM\_177438, while the sequence for each miRNA was obtained from the Sanger Sequence Database ([www.mirbase.org](http://www.mirbase.org)). Using these sequences, the MiRanda algorithm provided three potential binding sites for miR-18b a single potential binding site for miR-106a (Figure 4C).

In order to verify these predictions, we PCR cloned the targeted regions of the Dicer mRNA into the 3' UTR of the Renilla Luciferase gene in the psiCheck2 plasmid. As a negative control, we cloned a region of the 5' UTR of the Drosha gene into the psiCheck2 reporter. Specific knockdown by a miRNA results in a drop in Renilla Luciferase activity. The plasmid constructs were transfected into 293T cells, then harvested and

assayed for Renilla Luciferase and Firefly Luciferase activity 72 hours later (Figure 4D). As a negative control, a PCR fragment from the 5'UTR region of the Droscha gene was inserted into the vector. The construct bearing the proposed target for miR-18b showed Renilla Luciferase activity comparable to the negative control, but the construct bearing the proposed binding site for miR-106a showed a significant drop in enzyme activity.

We then performed an siRNA experiment against miR-106a in undifferentiated primary monocytes (ZK) and observed a reversal of the endogenous Dicer levels in these cells after 72 hrs (Figure 4E). Although PMA treated cells (lane 3) at day 3 showed macrophage like phenotype, we observed no apparent change in structural shape of monocytes after simiR-106a treatment. Collectively, these results indicate that the expression of Dicer protein is likely restricted by miR-106a in undifferentiated monocytes.

#### **Evidence that PIWIL4 can play a part in the generation of miRNAs**

Lastly, since our initial results showed that Dicer is undetectable in undifferentiated monocytes, but small RNA species were still detected, we hypothesized that proteins other than Dicer may be involved in the production of mature miRNAs. As mentioned earlier, eukaryotic cells generate another set of small RNAs which are known as piRNAs. The generation of piRNAs is carried out by the members of the PIWI protein family in a Dicer independent fashion. PIWI proteins do not possess enzymatic activity, but instead protect short segments of double stranded RNA from degradation by an unknown ribonuclease (50). Although the expression of PIWI family members is often said to be restricted to reproductive tissues, several reports have presented evidence that PIWI proteins are expressed in somatic tissues as well (18-21). Based on these previous reports we focused on PIWIL4 since mRNA for this protein was reported to be ubiquitously expressed in human somatic tissues (18).

In order to confirm that PIWIL4 is expressed in the cell lines used in our previous experiments, we extracted protein from multiple cell lines and Western blotted for PIWIL4 and  $\beta$ -actin. The results shown in Figure 5A demonstrate that the full length PIWIL4 protein can indeed be detected in infected and uninfected T-lymphocytes (CEM, ACH2, 8E5, Jurkat, and J1.1 cells), monocytes (U937 and U1), and in the colorectal cancer cell line HCT-116. Uninfected cell lines (CEM, Jurkat, and U937) showed comparable protein levels to their infected clones (ACH2, 8E5, J1.1, and U1), indicating that infection with HIV-1 has no discernable effect on PIWIL4 levels. Furthermore, the absence of Dicer has no visible effect on PIWIL4 levels, as evidenced by the presence of PIWIL4 in both HCT-116 cells and the knockout HCT-116 Dicer<sup>-/-</sup> cell line.

As our previous results indicated that miRNA related proteins are induced by PMA, we were interested to know if PIWIL4 expression was similarly affected by PMA levels. The results of such an experiment are presented in Figure 5B. U937 cells were treated with PMA and harvested at 24 hour intervals. The protein extract was Western blotted for PIWIL4 and  $\beta$ -actin. Extract from untreated cells (Lane 1) showed that PIWIL4 is detectable prior to PMA treatment and protein levels were only slightly altered by PMA treatment over the course of four days (Lanes 2 through 5). These results suggest that unlike proteins in the miRNA pathway, the expression of the PIWIL4 protein was not dependent upon monocyte differentiation.

Our results confirmed that PIWIL4 is present in monocyte cells at time points when Dicer cannot be detected. In order to test our hypothesis that PIWIL4 may contribute to miRNA production in U937 monocytes, we performed poly-A RT-PCR with the Quantimir miRNA detection kit to look for multiple bands or a shift in the size of various miRNAs, thereby indicating that processing by PIWIL4 had occurred. The results from this analysis did not show obvious shifts in

size, but instead showed that mature miRNAs produced either single or paired bands on an agarose gel. Two miRNAs representative of our findings are shown in Figure 5C. Results for miR-18b (top panel) are representative for a miRNA which produces only single band on a gel. Interestingly, mature miRNA for miR-18b was detected in U937 cells both prior to and after PMA treatment (compare Lanes 1 and 3 to Lanes 2 and 4). However, many other miRNAs identified by poly-A RT-PCR in monocytes showed evidence of multiple bands. Among those miRNAs which produced a pair of bands, miR-572 showed the clearest evidence of processing by PIWIL4, as shown in the bottom half of Figure 5C. The miR-572 miRNA is derived from the 3' end of the pre-572 RNA hairpin and cleavage by Dicer would normally produce a miRNA roughly 19bp in length, corresponding to the smaller band visible in panel C. However, the larger band visible in panel C corresponds to a 32bp RNA molecule. Small RNA molecules in the range of 26 bp to 33 bp are typically classified as piRNAs. These results suggest that the pre-572 hairpin can be possibly processed by both Dicer and PIWIL4.

As the presence of multiple bands makes it difficult to perform qPCR analysis on the products of pre-572, we obtained densitometry data for these bands (Figure 5C, bottom). Consistent with the previous trend seen in Table 2, miRNA processing is suppressed in infected MDMs, as the intensity of the miRNA band is comparatively weaker in the infected cells than in untransfected cells (compare Lanes 1 to 3 and 2 to 4).

Finally, we performed a similar experiment in U937 cells with PMA, pNL4-3 and si-PIWIL4, but cells were undergoing apoptosis at much rapid rate (data not shown). However, we were able to transfect U937 cells with si-PIWIL4, with PMA and observed a dramatic decrease in the 32bp piRNA and a slight increase in the 19bp miRNA (Figure 5D). These results therefore provide validation for our earlier conclusions that 1) miRNAs are produced in both monocytes and MDMs using

proteins other than Dicer, and 2) that miRNA biogenesis is impaired in MDMs when transfected with an HIV-1 clone.

### Discussion

As indicated previously, there is an interesting interaction between HIV-1 and the host miRNA machinery. Current publications in the field indicate that HIV-1 encodes viral miRNAs of its own. These viral miRNAs, which are generated by Dicer, have been shown to promote viral latency in two distinct ways. Viral miRNA can protect the host cell from apoptosis (30) and also guide repressive chromatin remodeling proteins to the viral LTR (14,31). Conversely, HIV-1 is capable of suppressing RNAi in T-lymphocytes (24,26), and our results indicate that the same is true for monocytes and MDMs as well, though different mechanisms of suppression may be at work in the different cell types.

In this manuscript, we have focused on examining the miRNA machinery in monocytes and MDMs for several reasons. Of course, monocytes and MDMs are highly relevant in the course of HIV-1 infection, but more importantly, we had previously observed that monocytes appeared to be deficient for the Dicer enzyme (14). As Dicer is believed to be essential for both miRNA and siRNA production, it was our intention to better characterize the miRNA machinery in monocytes and MDMs both prior to and after transfection with viral DNA. Among our findings was the observation that monocytes did not appear to express any Dicer, and that transfection with the pNL4-3 plasmid unexpectedly resulted in the suppression of Dicer in MDMs. The suppression of Dicer proteins also coincided with a drop in miRNA production, confirming that viral transfection also leads to the suppression of miRNA and siRNA production in MDMs. Interestingly, it has been argued that MDMs do not typically support latent viral infections (51,52), and our results hint that MDMs may not be able to utilize the viral miRNA to silence viral transcription. This hypothesis might be tested in the future by over expressing Dicer in infected MDMs in order to see if the

percentage of latent infections increases as miRNA production increases. Surprisingly, when we used mutant variants of the pNL4-3 plasmid to investigate the mechanism underlying the suppression of Dicer levels, we found that the viral gene Vpr appeared necessary to maintain suppression. The presented data also suggested that the Nef gene may also be required to suppress Dicer levels, possibly indicating that perhaps both of these genes are simultaneously required to suppress Dicer. Interestingly, the pNL4-3 Tat only and pNL4-3 delta Rev plasmids also showed a very modest suppression of Dicer levels. These results could be interpreted to indicate that while the HIV-1 mRNA remains doubly-spliced, Tat is able to cause a modest suppression of Dicer in MDMs. One possible hypothesis arising here is that full length Tat can suppress Dicer levels, but short form of Tat cannot. Overall, the finding that Vpr was required to suppress Dicer expression in MDMs was unexpected. A review of the literature does not reveal any obvious connections between Vpr and the miRNA pathway, but it has been observed that while Vpr is dispensable for viral replication in T-cells, Vpr deficient viruses cannot efficiently replicate in macrophages (53,54). The underlying reasons for the requirement of Vpr in macrophages are not clearly understood, although it has been shown that Vpr is essential in maintaining the circularized extrachromosomal viral genomes that can be isolated from macrophages from infected patients (55,56). It may be that the suppression of RNAi is essential to preventing the silencing or degradation of circularized viral genomes in macrophages. However, more research will be required to fully investigate these hypotheses.

In addition to investigating how HIV-1 downregulates Dicer, we were also interested in understanding why the enzyme was undetectable in undifferentiated monocytes. The initial results indicated that Dicer expression was likely halted at the translational step, which hinted at the involvement of RNAi in a negative feedback loop. In fact, there is already one validated

case of such a feedback loop involving Dicer and miR-let-7, but our microarray results indicated no significant change in the miR-let-7 family after treatment with PMA, with the exception of an increase in the abundance of the non-guide strand products miR-let-7b\* and miR-let-7d\*. Instead, our results and target predictions indicated that miR-106a was likely responsible for restricting Dicer expression. However, these results led us to question whether or not a heavily restricted Dicer could be responsible for generating these miRNAs. As mentioned previously, an alternate, PIWI-dependent mechanism exists which can generate piRNAs from double stranded RNA. We hypothesized that in the absence of Dicer (or during its inhibition) precursor hairpins might accumulate and be processed by a member of the PIWIL4 family instead of Dicer. A prerequisite of this hypothesis is that a PIWI protein must be expressed in monocyte cells, which we were able to demonstrate for PIWIL4. Additionally, PIWIL4 was present at relatively stable levels regardless of PMA treatment or the presence of Dicer. Further analysis of miRNAs isolated from cells using poly-A RT PCR revealed that several miRNAs, as represented by miR-572, showed evidence of being processed by both Dicer and PIWIL4. One of the limitations of poly-A RT PCR is the requirement for a miRNA specific forward primer. However, if a hairpin precursor is processed differently by Dicer and PIWIL4, then the mature miRNA produced may have different, but overlapping sequences. Consequently, poly-A RT PCR may not detect miRNAs generated by PIWIL4 if the sequence of the mature product is shifted too much from the expected sequence of the Dicer product.

Overall, the findings presented in this paper hint at much larger questions with important implications for understanding HIV-1 pathogenesis. In the first set of experiments, we demonstrated that HIV-1 downregulates Dicer expression in MDMs, but note that the same finding has not been reported in T-lymphocytes. However, there is evidence that RNAi is suppressed in both cell types,

indicating that the virus may be taking advantage of differences between these two cell types in order to suppress RNAi by two independent mechanisms. While examining miRNA production in monocytes and macrophages, we obtained data indicating that miRNAs could be isolated from monocytes despite their apparent lack of Dicer.

Furthermore, we provided evidence that the PIWI proteins can become involved in the generation of miRNAs. The involvement of PIWI in miRNA generation raises interesting questions, such as whether PIWI products may substitute as miRNAs when Dicer is suppressed by the virus, or how PIWI products might aid in viral replication.

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### **COMPETING INTERESTS**

The author(s) declare that they have no competing interests

### **AUTHOR'S CONTRIBUTIONS**

WC and FK designed the experiments, and wrote the manuscript. RVD and ZK assisted in the design of experiments and performed western blots. LC and IG performed Western blots. KK aided in the preparation of the manuscript. SC and AN assisted with data analysis. TL and NL performed microarray hybridization and data analysis. FK oversaw the research and aided in the preparation of the manuscript.

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## Figure Legends

**Figure 1: Monocytes are deficient for key miRNA processing proteins.** **A)** U937 monocytes were treated with either 250 nM PMA or 50 ng/ml M-CSF for 96 hours to induce differentiation. For controls, cells were either untreated or treated with vehicle for 96 hours. Untreated CEM and ACH2 cells were harvested at log phase growth. Western blots show protein levels with 50  $\mu$ g of total protein. **B)** CEM and U937 cells were harvested during log phase growth. Lanes 1 and 2 were loaded with 65  $\mu$ g of protein and Lane 3 was loaded with 195  $\mu$ g of protein. **C)** U937 cells were treated with 250 nM of PMA and collected at 24 hour intervals. RT-PCR was performed to detect mRNA levels of Dicer, Drosha, Ago1, Ago2, and GAPDH. **D)** U937 cells were treated with 250 nM of PMA and collected at 24 hour intervals. A total of 65  $\mu$ g of whole cell extract was Western blotted for Dicer and  $\beta$ -actin. **E)** U937 cells were harvested at days 0, 1 and 4 after PMA treatment. RNA was extracted, retrotranscribed, and QPCR analysis was performed for GAPDH, Ago2, Dicer and Drosha. Expression of each transcript was normalized to GAPDH and displayed as fold change from day 0. **F)** Lack of functional Dicer in monocytes. CEM T-cells and U937 monocyte cells (five million) were transfected by electroporation with a combination of HIV-1 LTR-Luc (5  $\mu$ g), pcTat (3  $\mu$ g), siLuc (200 nM) or shRNALuc (500 nM). Cells (2 ml) were kept at 37 C for 48 hrs followed by Luciferase assay. Luciferase signal knockdown with shRNA requires both Dicer and Ago2 activity, while knockdown with siRNA only requires Ago2 activity.

**Figure 2: HIV-1 infection suppresses the expression of Dicer in U937 MDMs.** **A)** U937 cells were electroporated with either pGFPmax or with pNL4-3 HIV-1 vector. Cells were allowed to recover for 24 hours before treatment with either vehicle or 250 nM PMA for 96 hours. Cells were lysed and 65  $\mu$ g of whole cell extract was Western blotted for Dicer and  $\beta$ -actin. **B)** U937 cells were electroporated with pNL4-3 and allowed to recover for 24 hours before treatment with 250 nM PMA for 96 hours. CEM and ACH2 cells were harvested at log phase growth. Total RNA was extracted from the cells, treated with DNase I, and then used to perform RT-PCR for Nef, Env and GAPDH. **C)** PMBCs were obtained, purified, and cultured to isolate monocytes and macrophages. Monocytes were further differentiated into macrophages by incubating in media alone or PMA for 1 week with medium change every 2 days. Cells were then transfected with pNL4-3 (20  $\mu$ g) and plated for 3 days. Samples were collected and 150  $\mu$ g of extracts were western blotted for Dicer, Ago2 and  $\beta$ -actin.

**Figure 3: Vpr is required to suppress Dicer levels in U937 MDMs.** **A)** U937 cells were electroporated with no plasmid, the pNL4-3 HIV-1 plasmid, or a pNL4-3 mutant plasmid. Mutant plasmids possessed mutation to generate genotypes Tat Only,  $\Delta$ Nef,  $\Delta$ Nef,  $\Delta$ Vpr,  $\Delta$ Vpu, and  $\Delta$ Env. Cells were allowed to recover for 24 hours before treatment with 250 nM PMA for 96 hours. Cells were lysed and Western blotted for both Dicer and  $\beta$ -actin. Western blot results from three experiments were quantified by densitometry, where  $\beta$ -actin levels were used as an internal reference and data was normalized to Dicer levels in the untransfected cells. **B)** Similar to panel A, U937 cells were electroporated pNL4-3 HIV-1 Wild type and various mutant plasmids (10  $\mu$ g). Cells were allowed to recover for 24 hours before treatment with 250 nM PMA for 24, 48, 72, and 96 hrs. Cells were lysed and Western blotted for both Dicer and  $\beta$ -actin. **C)** Monocytes were transfected with 10  $\mu$ g CMV-Vpr (a VPr expression plasmid) and processed for western blot 48 hrs later using Dicer and  $\beta$ -actin antibodies.

**Figure 4: Dicer is repressed by miR-106a in undifferentiated monocytes.** **A)** A diagram of the six miRNAs (including miR-106a) belonging to the 17-92 miRNA cluster found on

chromosome X, as well as their drop in expression following PMA treatment. **B)** U937 cells were subjected to four different treatment prior to RNA isolation; 1) U937 cells + electroporation, 2) U937 cells + electroporation + PMA, 3) U937 cells + pNL4-3, and 4) U937 cells + pNL4-3 + PMA. Small RNA species were isolated from cells with the mirVana miRNA isolation kit and hybridized to an Agilent Human microRNA V2 microarray. Hybridization as detected by fluorescence is shown on a  $\log_{10}$  scale. The data shown is the average of two independent experiments. **C)** A diagram illustrating the location of the proposed binding sites for miR-18b and miR-106a on the Dicer mRNA. **D)** 293T cells were transfected in triplicate with psiCheck2 constructs bearing either a null target sequence, the miR-18b target sequence, or the miR-106a target sequence. Transfected cells were lysed 72 hours after transfection. Renilla Luciferase activity was normalized to Firefly Luciferase activity. **E)** Transfection of primary monocyte cells via electroporation with either siRNA against miR-106a or treatment with PMA. Cells were lysed 72 hours after electroporation and the lysates were Western blotted for Dicer protein.

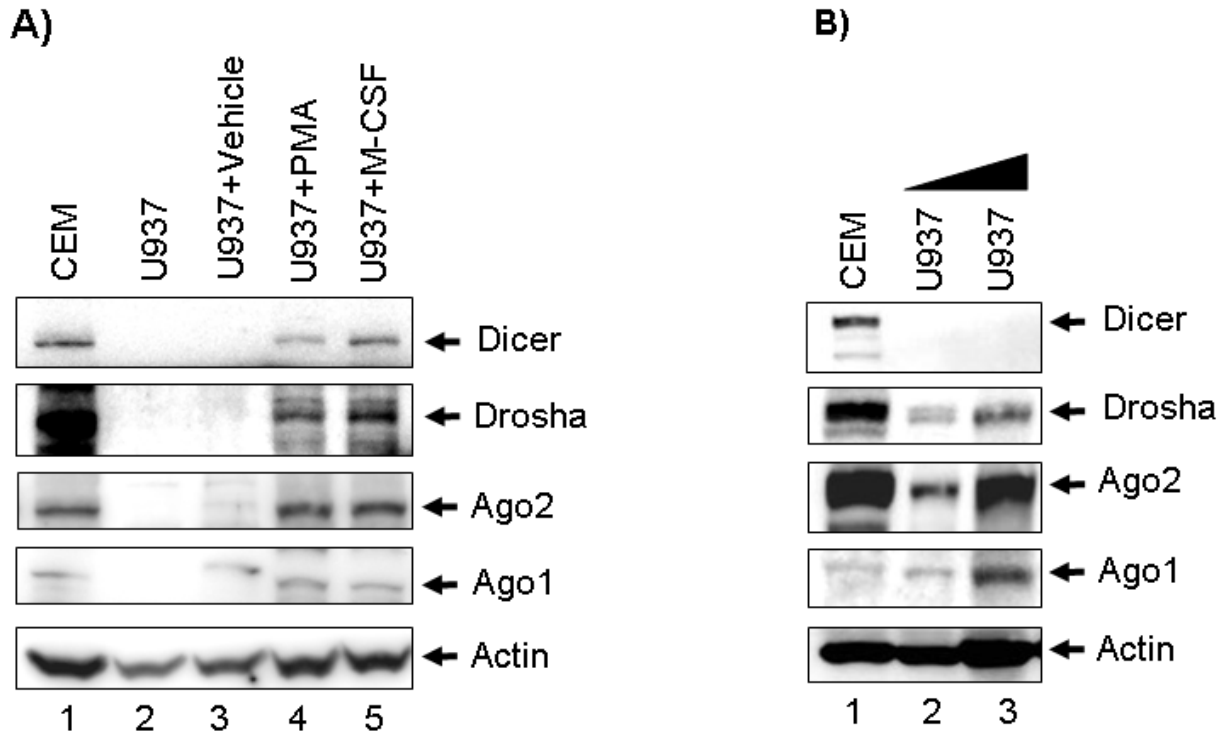
**Figure 5: PIWIL4 participates in the generation of some miRNAs.** **A)** Whole cell extract from CEM, ACH2, Jurkat, J1.1, U937, U1, HCT-116 WT and HCT-116 Dicer<sup>-/-</sup> cells during log phase growth. Whole cell extracts were Western blotted to detect the full length PIWIL4 protein and  $\beta$ -actin. **B)** U937 cells were treated with 250 nM PMA and harvested at 24 hour intervals. Cell extracts were Western blotted for PIWIL4 and  $\beta$ -actin. **C)** U937 cells were mock electroporated or electroporated with pNL4-3 and allowed to recover for 24 hours before treatment with either vehicle or 250 nM PMA for 96 hours. Small RNAs were extracted from U937 (Lane 1), U937 + PMA (Lane 2), U937 + pNL4-3, (Lane 3), U937 + pNL4-3 + PMA (Lane 4). *Top panel:* Poly-A RT-PCR for miR-18b shows a single band corresponding to a miRNA 20bp in size. *Bottom panel:* Poly-A RT-PCR for miR-572 shows two bands at corresponding to a miRNA 19 bp in size and a piRNA 32 bp in size. Densitometry showing the mean intensity for each of the individual bands present in panel C. **D)** Similar to panel C where U937 cells were treated with PMA and/or transfected with si-PIWIL4 (200 nM). Poly-A RT-PCR for miR-572 were performed and ran on a 3.5% agarose gel. Densitometry below shows the numbers for the corresponding miRNA 19 bp and piRNA 32 bp in size. Lane 3 is a mock control without any RNA added during the RT-PCR.

**Table 1: MicroRNA-Related Protein Expression in Various Cell Types (% present)**

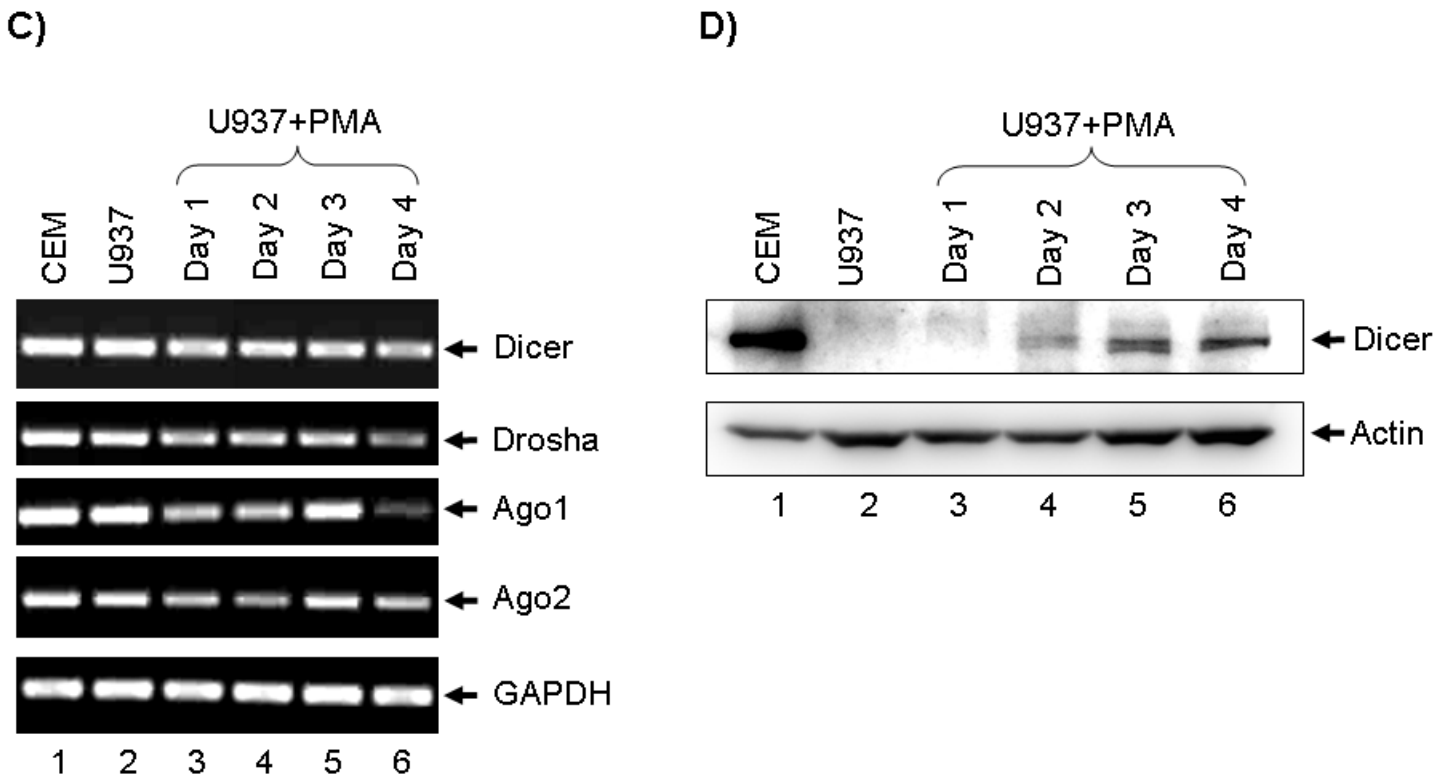
		Dicer	Drosha	Ago2	Ago1
Lymphoid Lineage	CEM	100	100	100	100
	ACH2	88	94	88	100
	Jurkat	94	88	88	100
	J1.1	94	88	88	100
Myeloid Lineage	HL-60	0	18	75	nd
	THP-1	0	18	75	nd
	U937	0	18	75	67
MDMs	U937 +PMA	25	33	94	100
	U937 +M-CSF	33	33	94	94

**Table 2: MicroRNA Levels Correlate to Dicer Protein Levels**

Name	Fold Change		Name	Fold Change	
	U937 vs. U937 +PMA	U937+pNL4-3 vs. U937+pNL4.3+PMA		U937 vs. U937 + PMA	U937+pNL4-3 vs. U937+pNL4.3+PMA
hsa-miR-146b-5p	471.9	30.7	hsa-miR-33b*	4.1	-2.1
hsa-miR-663	92.7	2.6	hsa-miR-1237	4.0	2.1
hsa-miR-572	31.4	1.1	hsa-miR-671-5p	3.9	-1.5
hsa-miR-134	26.4	1.2	hsa-miR-595	3.6	1.3
hsa-miR-765	25.6	-8.9	hsa-miR-940	3.6	1.4
hsa-miR-874	18.9	1.8	hsa-miR-29a	3.5	2.1
hsa-miR-513a-5p	18.5	3.8	hsa-miR-342-3p	3.5	2.3
hsa-miR-26a	16.9	1.8	hsa-miR-494	3.3	1.7
hsa-miR-1226*	16.0	1.8	hsa-miR-125a-3p	3.3	-1.1
hsa-miR-150*	13.9	6.7	hsa-miR-575	3.3	-2.0
hsa-miR-557	13.6	11.0	hsa-miR-768-3p	3.2	-1.6
hsa-miR-222	12.2	4.8	hsa-miR-1234	3.0	1.3
hsa-miR-630	11.9	-2.1	hsa-miR-34a	2.9	2.4
hsa-miR-520b	10.6	1.2	hsa-miR-23a	2.8	2.4
hsa-miR-483-5p	9.8	-2.6	hsa-miR-29b-1*	2.7	-1.7
hsa-miR-638	9.5	1.8	hsa-miR-365	2.7	3.1
hsa-miR-198	9.2	1.3	hsa-miR-132	2.7	1.4
hsa-miR-184	8.6	-6.5	hsa-miR-221	2.6	4.1
hsa-miR-345	8.1	1.2	hsa-miR-768-5p	2.6	2.4
hsa-miR-22	7.9	5.2	hsa-miR-181c	2.5	2.7
hsa-miR-29b	7.6	4.0	hsa-miR-371-5p	2.5	1.0
hsa-miR-513b	6.4	2.9	hsa-miR-877*	2.4	1.0
hsa-miR-1224-5p	6.3	1.3	hsa-let-7d*	2.4	-22.0
hsa-miR-520e	6.3	-2.2	hsa-miR-425*	2.3	-1.4
hsa-miR-135a*	6.1	1.2	hsa-miR-212	2.3	-1.2
hsa-miR-193a-5p	6.0	2.2	hsa-miR-32*	2.3	3.9
hsa-miR-1225-3p	5.6	1.9	hsa-miR-483-3p	2.2	-1.0
hsa-miR-188-5p	5.3	-1.0	hsa-let-7e	2.2	1.4
hsa-miR-1225-5p	4.9	2.8	hsa-miR-770-5p	2.2	-3.9
hsa-miR-877	4.9	3.5	hsa-miR-328	2.2	1.2
hsa-miR-623	4.8	-4.2	hsa-miR-1238	2.1	1.4
hsa-miR-602	4.6	2.4	hsa-miR-1224-3p	2.1	1.0



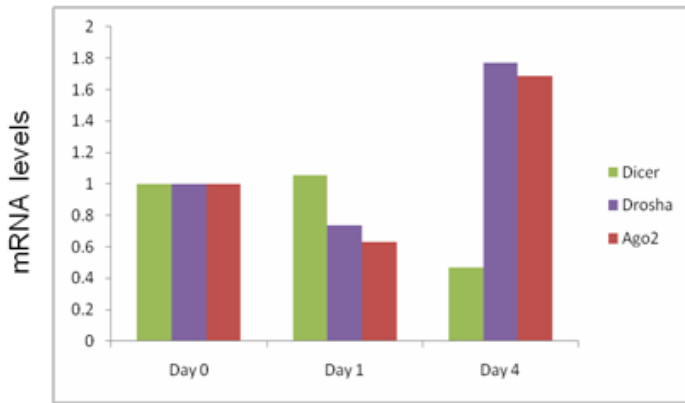
Coley et al. Fig. 1A,B



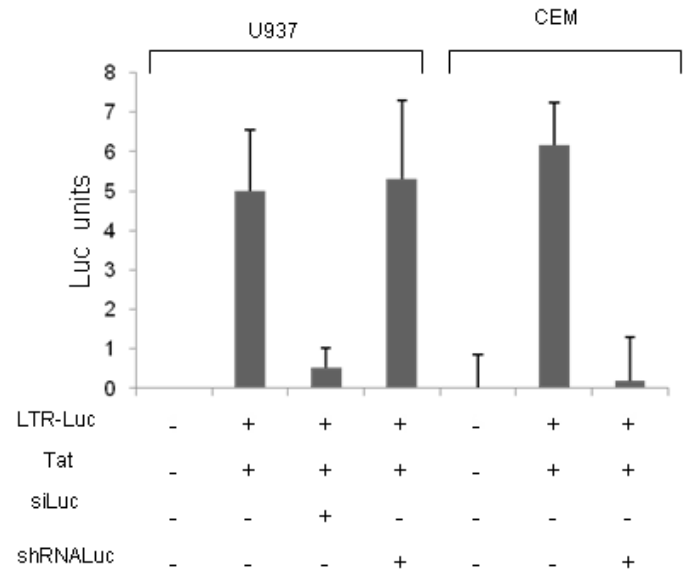
Coley et al. Fig. 1C,D



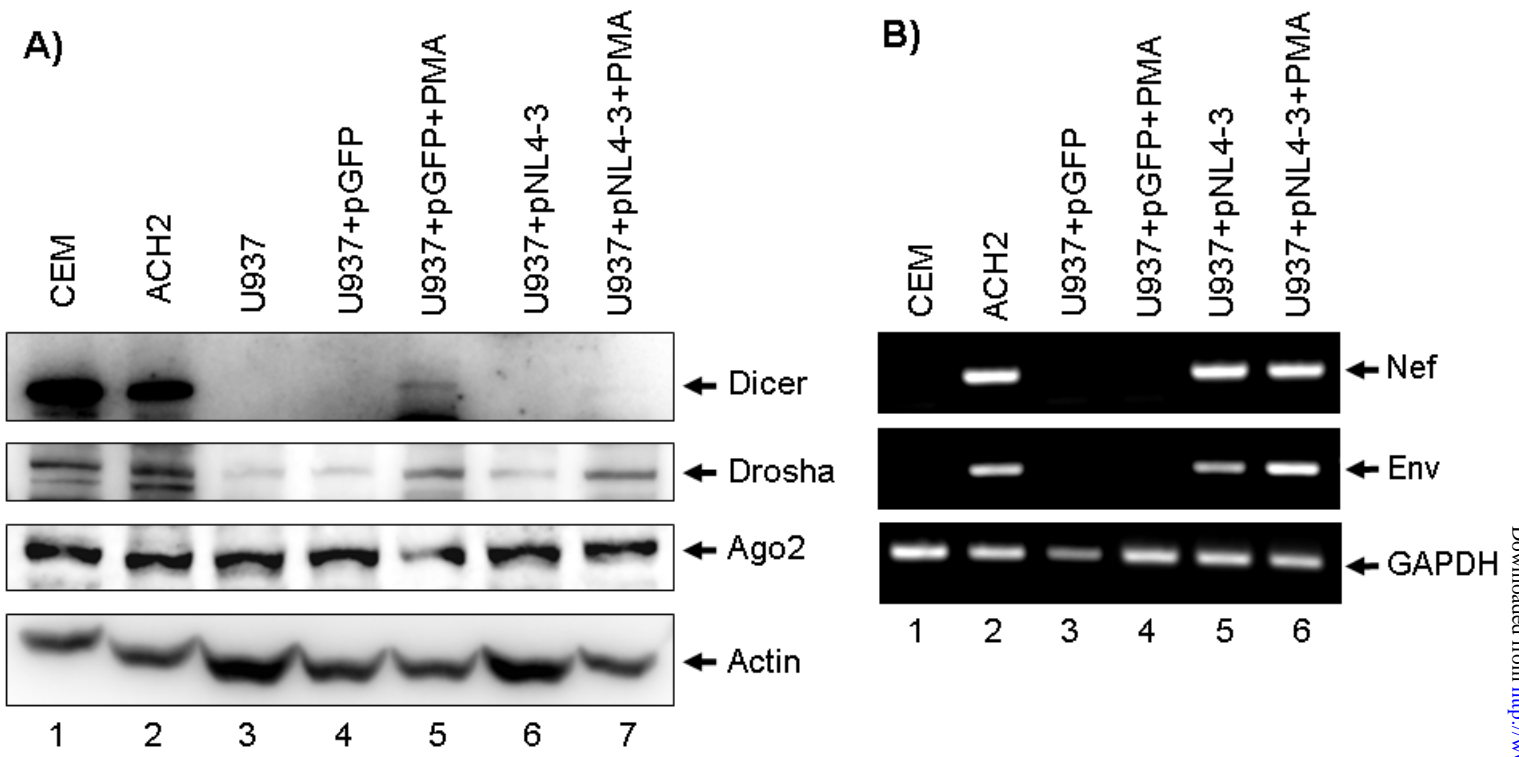
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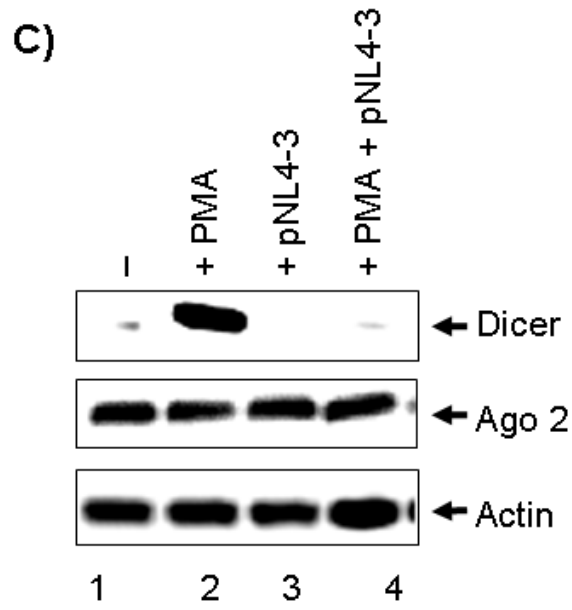
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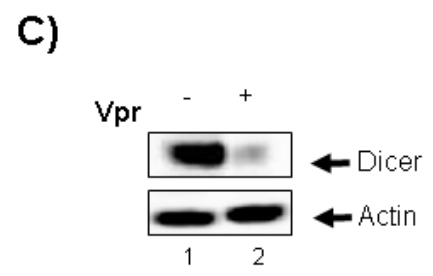
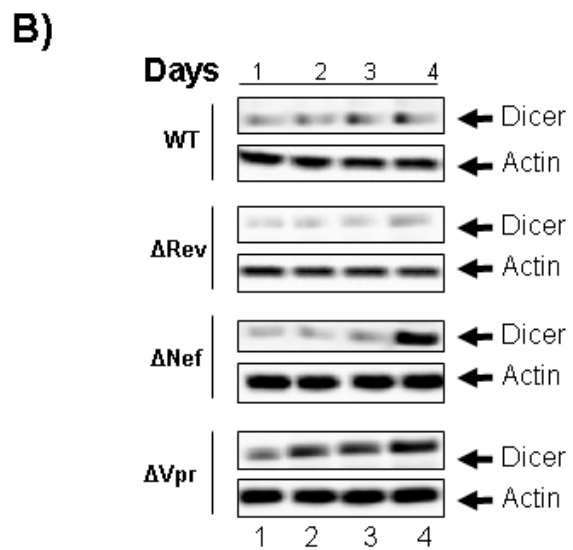
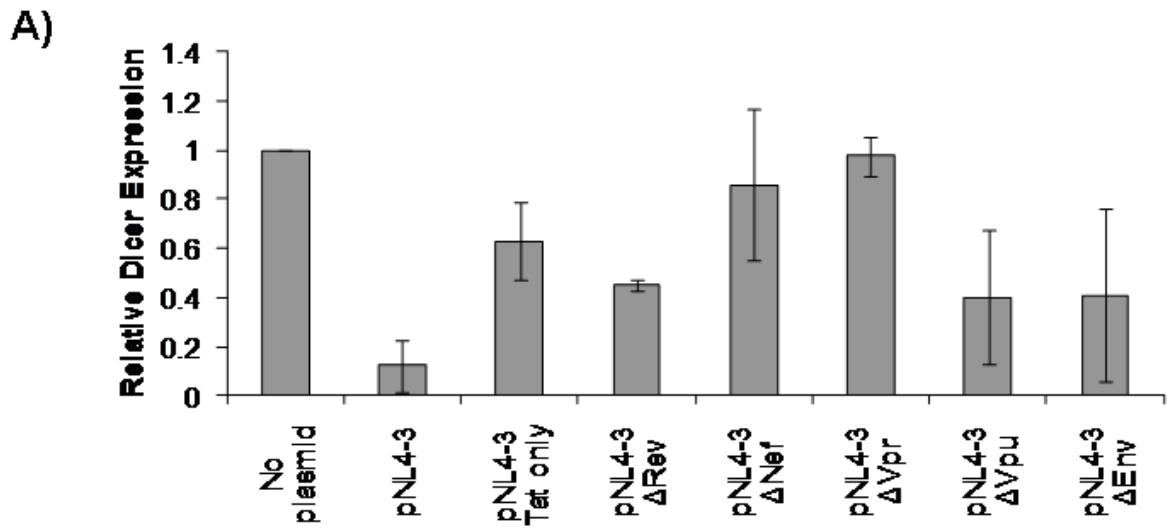
Coley et al. Fig. 1E, F



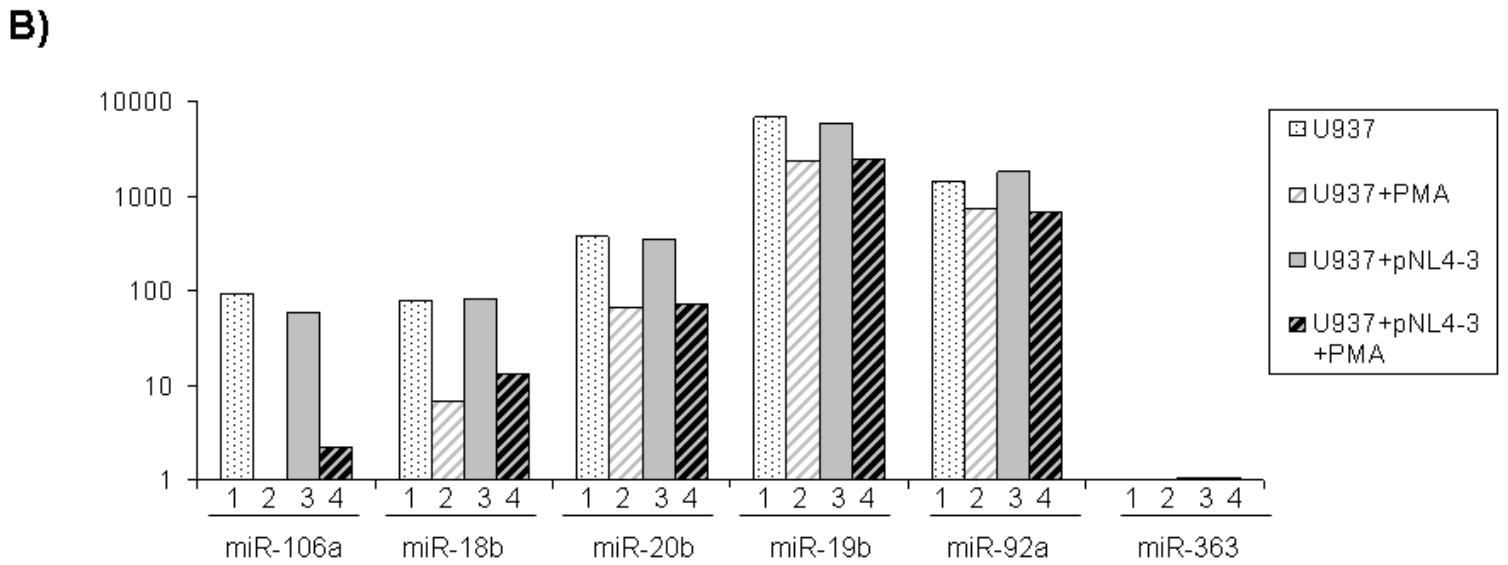
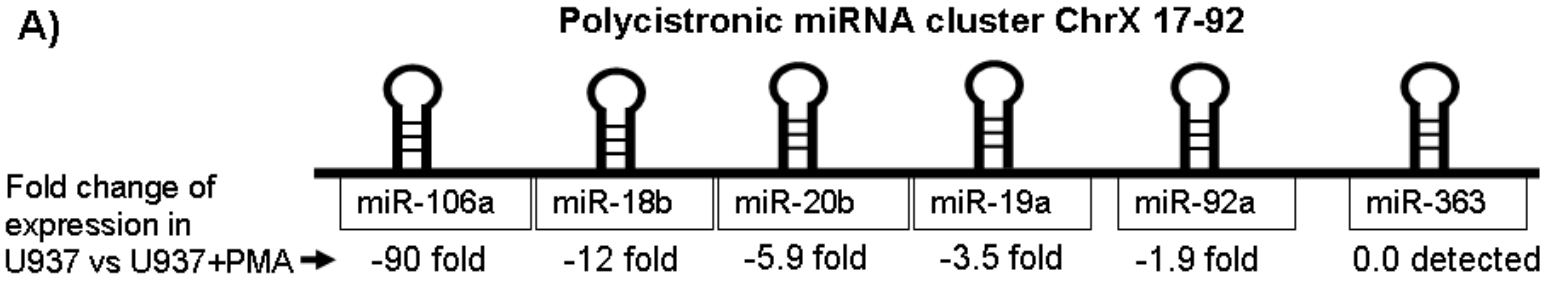
Coley et al. Fig.2A,B



Coley et al. Fig.2C

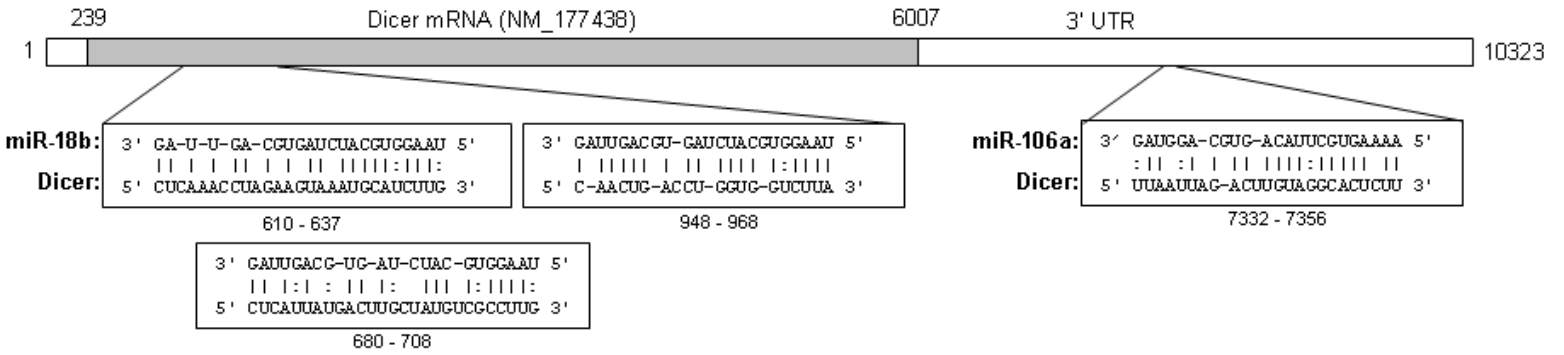


Coley et al. Fig.3A-C

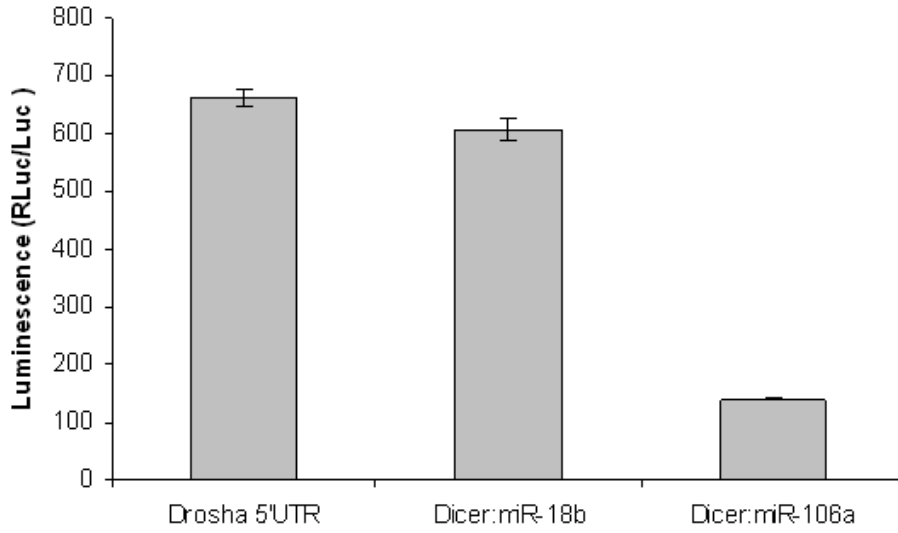


Coley et al. Fig.4A,B

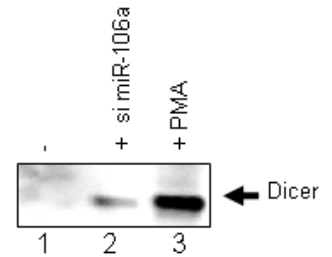
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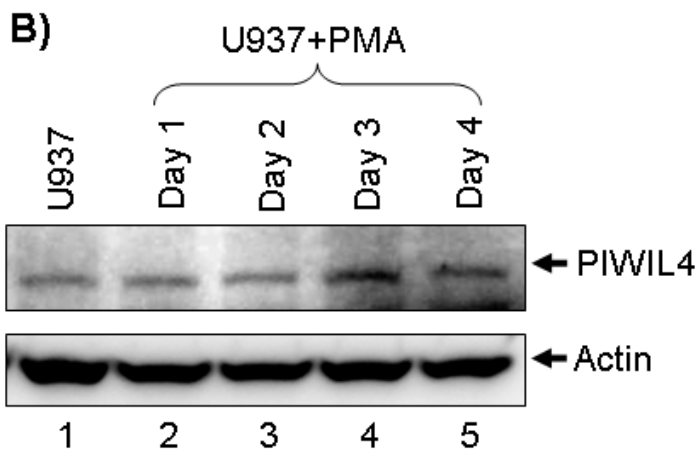
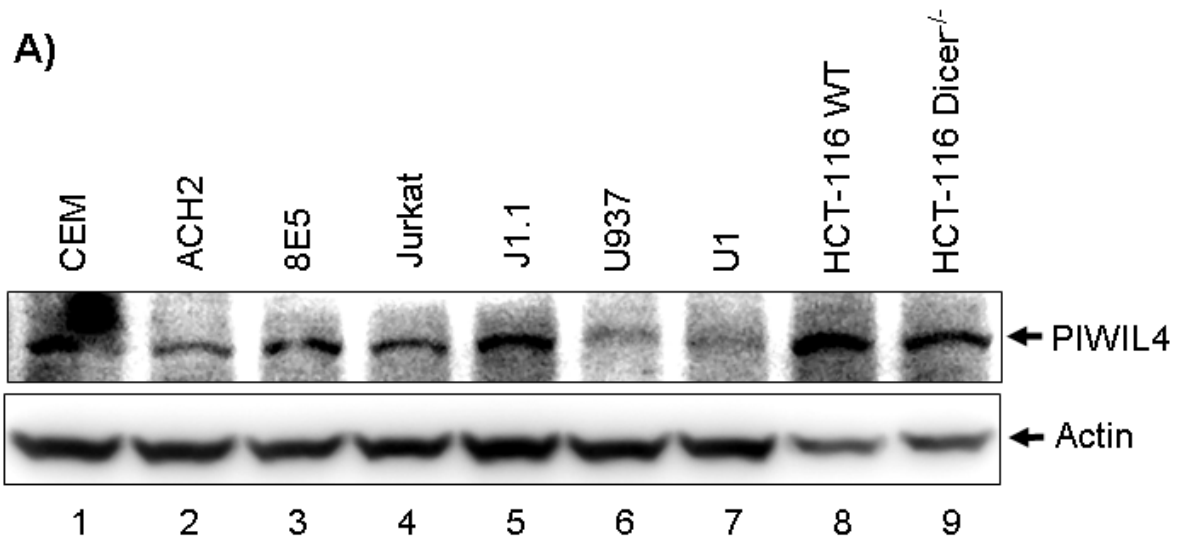
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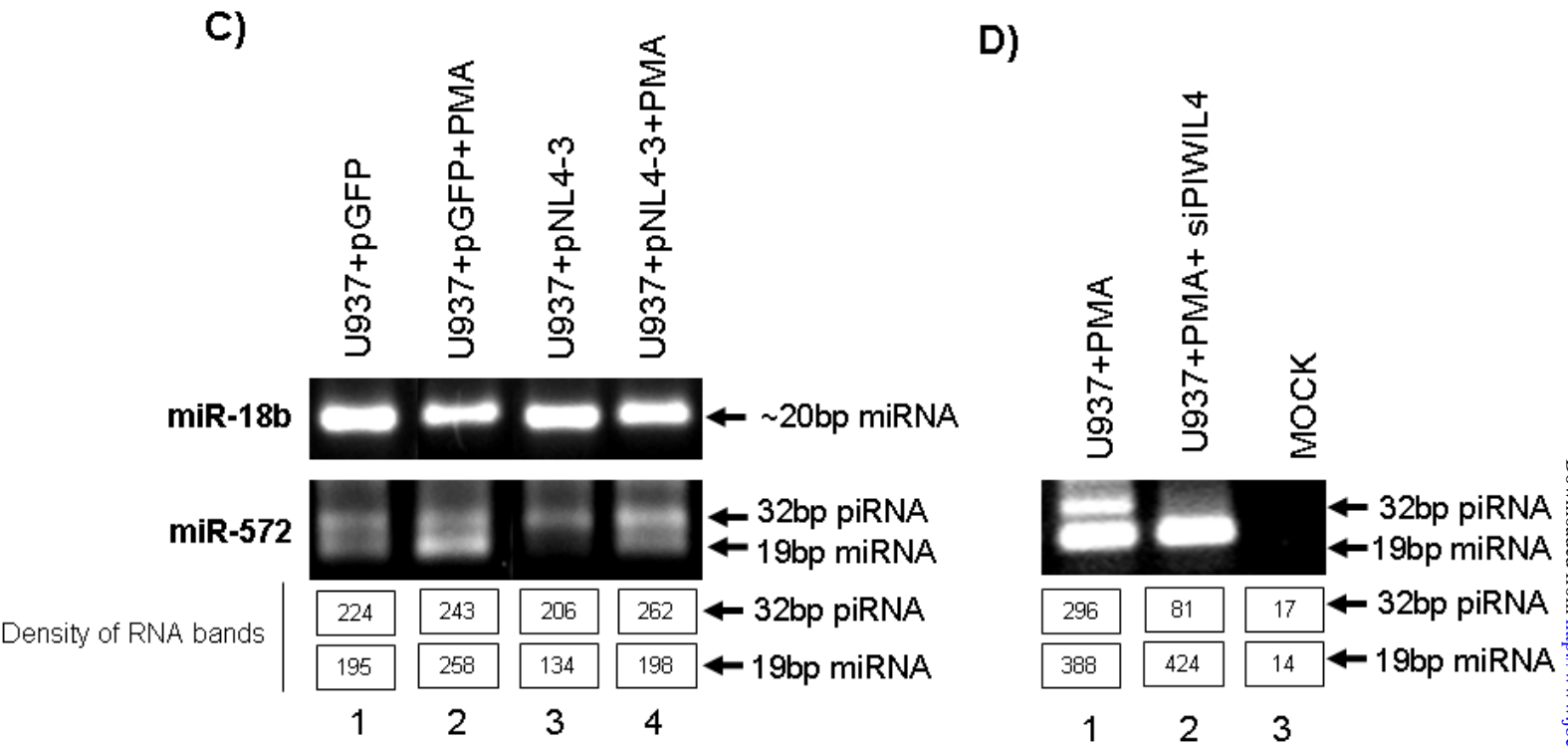
E)



Coley et al. Fig.4C-E



Coley et al. Fig.5A,B



Coley et al. Fig.5C,D



## **Absence of Dicer in monocytes and its regulation by HIV-1**

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