

GAM/ZFp/ZNF512B is central to a gene sensor circuitry involving cell-cycle regulators, TGF β effectors, Drosha and microRNAs with opposite oncogenic potentials

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ABSTRACT

MicroRNAs (miRNAs) are small regulatory RNAs targeting multiple effectors of cell homeostasis and development, whose malfunctions are associated with major pathologies such as cancer. Herein we show that GAM/ZFp/ZNF512B works within an intricate gene regulatory network involving cell-cycle regulators, TGF β effectors and oncogenic miRNAs of the *miR-17-92* cluster. Thus, GAM impairs the transcriptional activation of the *miR-17-92* promoter by c-Myc, downregulates *miR-17-92* miRNAs differentially, and limits the activation of genes responsive to TGF β canonical pathway. In contrast, TGF β decreases GAM transcripts levels while differentially upregulating *miR-17-92* miRNAs. In turn, *miR-17*, *miR-20a* and *miR-92a-1* target GAM transcripts, thus establishing a feedback autoregulatory loop. GAM transcripts are also targeted by miRNAs of the *let-7* family. GAM downregulates Drosha, the main effector of miRNA maturation in the nucleus, and interacts with it in a RNA-dependent manner. Finally, GAM modulates the levels of E2F1 and Ras, and increases apoptosis while reducing cell proliferation. We propose that GAM represents a new kind of vertebrate regulator aimed at balancing the opposite effects of regulators of cell homeostasis by increasing the robustness of gene circuitries

controlling cell proliferation, differentiation and development.

INTRODUCTION

MicroRNAs (miRNAs) have been implicated in the regulation of a number of fundamental processes, including muscle, cardiac, neural and lymphocyte development, or the regulation of both the innate and adaptive immune responses (1,2). MiRNAs originate from primary transcripts (pri-miRNAs) converted in the nucleus into precursor miRNAs (pre-miRNAs) by the RNase III Drosha, associated with DGCR8 to form the small microprocessor complex (3). Pre-miRNAs are then exported in the cytoplasm where the miRNA hairpin is cleaved by the RNase III Dicer within the RISC loading complex. The guide strand, which corresponds to the mature miRNA, is then incorporated into the RISC complex (3). MiRNAs and their transcriptional regulators usually form autoregulatory loops aimed at controlling their respective levels (4).

MiRNAs participate in gene regulatory networks whose molecular malfunctions are associated with major pathologies such as cancer (5–7). Thus, the *miR-17-92* cluster on chromosome 13, which appeared with the vertebrates, contains six miRNAs (*miR-17*, *-18a*, *-19a*, *-20a*, *-19b-1* and *-92a-1*). The *miR-17-92* cluster is amplified and overexpressed in B-cell lymphomas and solid tumors such as breast or small-cell lung cancers (5), where it may enhance oncogenesis by potentially targeting *E2F1*, *p21/CDKN1A* and *BCL2L1/BIM* (8). However, there is

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also some evidence suggesting that loss-of-function of *miR-17-92* miRNAs might be advantageous for cancer cells in certain settings. Indeed, loss-of-heterozygosity at the 13q31.3 locus has been observed in multiple tumor types, and a genome-wide analysis of copy number alterations in cancer revealed that the *miR-17-92* cluster was deleted in 16.5% of ovarian cancers, 21.9% of breast cancers and 20% of melanomas (9). The transcription of the *miR-17-92* cluster is controlled by c-Myc and E2F factors (10–13). Interestingly, *miR-17* and *miR-20a* target E2F1, while *miR-20a* also targets E2F2 and E2F3 (10,12). Two ortholog clusters are found on chromosomes 7 (*miR-106b-93-25*) and X (*miR-106a-18b-20b-19b-2-92a-2-363*), respectively. In contrast to *miR-17-92* miRNAs that appeared late in evolution, *let-7* miRNAs belong to a family of early developmental regulators well conserved during evolution (14). The *let-7* gene family expanded from one member in nematods to more than 10 in mammals. In *C. elegans*, *let-7* works as a master regulator of temporal patterning by controlling the transition from undifferentiated, proliferating stem cells to differentiated, quiescent cells (14). In human, *let-7* miRNAs are frequently downregulated in cancers like lung, colon or other solid tumors (5), and are therefore considered as tumor suppressor miRNAs in these types of cancers. In particular, *let-7* miRNAs target oncogenes of the *Ras* family (15) and *c-Myc*, and their expression in colon tumors results in reduced levels of both Ras and c-Myc (16).

GAM/ZFP/ZNF512B, a vertebrate-specific zinc-finger factor, was recently identified as a potential developmental regulator whose isoform shifts correlated with the main stages of skin and heart morphogenesis in chicken (17). In human, *GAM* transcripts are present in all tissues and cell types tested so far. Although *GAM* may possibly bind TC- and/or AG-rich DNA sequences (17), none of its target genes has been identified to date, and its functions remain elusive. Interestingly, *GAM* 3'-UTR (untranslated region) contains target sequences for ~150 miRNAs, several of whose, including *miR-17-92* and *let-7* miRNAs, have been implicated in the regulation of development and/or have been described either as oncomirs or tumor suppressor miRNAs. Given that miRNAs can potentially target tens to hundreds of different transcripts, we hypothesized that *GAM* and at least some of the miRNAs targeting its 3'-UTR may form regulatory gene circuitries aimed at regulating cell homeostasis through their combined effects on key regulators of cell proliferation. Here, we present the first evidence that *GAM* indeed may work as a molecular sensor potentially aimed at maintaining cell homeostasis by affecting the levels of regulators of cell homeostasis such as E2F1 and Ras, by modulating the levels of *miR-17-92* miRNAs, by regulating the levels of and interacting with Droscha, and by interfering with TGF β signaling.

MATERIALS AND METHODS

Cell culture and transfection

HEK-293, HepG2 and MCF7 were transfected using Lipofectamine 2000 (Invitrogen). K562 cells were

electroporated using the AMAXA kit. Whenever needed, cells were mock-treated or stimulated 34 h after transfection with TGF β (100 ng/ml, from R&D) for 14 h. Stealth oligonucleotides targeting *GAM* (*siGAM*), *Droscha* (*siDroscha*) and *c-Myc* (*siMyc*) were from Invitrogen.

Preparation of constructs

The human *GAM* cDNA was amplified by PCR from a mix of human cDNAs purchased from Clontech. The *miR-17-92* cluster promoter, the whole *miR-17-92* cluster sequence and the 3'-UTR of *GAM* were cloned by PCR from genomic DNA extracted from HEK-293 cells. *GAM* 3'-UTR was cloned downstream of the *luciferase* gene in the *XbaI* site of the *pGL3-Control* vector (Promega) or downstream of *GAM* encoding sequence in the *pCMV-HA* vector. The whole sequence of the *miR-17-92* cluster was inserted into the *pCMV* vector. The *c-Myc* expression vector was from Invitrogen. The sequences of the oligonucleotides used for cloning are available upon request.

Luciferase assays

HEK-293 cells plated in 12-well plates were transfected with 0.4 μ g of either the empty *pGL3-Control* vector or constructs containing miRNA target sites, the indicated pre-miRNAs (100 nM final, Ambion) and 20 ng of *Renilla* luciferase control vector (*pRL-TK* from Promega). Assays were performed 24 h after transfection using the Dual Luciferase Reporter Assay system (Promega). *Firefly* luciferase activity was normalized to *Renilla* luciferase activity. As a control, we used the pre-miR Precursor Molecule-Negative Control #1 (referred to here as control RNA) from Ambion. TGF β signaling in HepG2 cells was tested in sixplicates in 24-well plates using the Signal SMAD Reporter Assay Kit (Luciferase) from Super Array Bioscience Corporation. This kit is designed to monitor the activity of the canonical TGF β -induced signal transduction pathway, i.e. to give a measure of the rate of SMAD2 and/or SMAD3 nuclear transcriptional activity in conjunction with SMAD4 on a reporter construct containing a functional SMAD-binding site.

Western blots

Cells transfected and/or treated as needed were lysed 24 h or 48 h after transfection. Anti-p21/CDKN1A and α -tubulin antibodies were from Cell Signaling Technology. Anti-c-Myc and anti-Droscha antibodies were from Abcam. Anti-E2F1, anti-pan-Ras, anti-GAM/ZNF512B, anti-Dicer, anti-DGCR8 and anti β -Actin antibodies were from Santacruz.

Apoptosis and proliferation assays

Apoptosis and proliferation assays were conducted using the Mitochondrial Permeability Transition Detection Kit from Immunochemistry Technologies and the XTT Cell Proliferation Kit from Cayman, respectively, according to the manufacturer's instructions.

RNA and miRNA quantitative real-time PCR

RNAs extracted with TRIzol (Invitrogen) were subsequently subjected to DNase digestion (Turbo-DNase-Ambion). *GAM* and *Drosha* quantitative real-time PCRs (qRT-PCRs) were conducted after reverse transcription with the commercial ready-to-use primers/probe mix of Applied Biosystems. Values were normalized using *GAPDH* or β -*actin*. MiRNA qRT-PCR were performed using TaqMan MicroRNA Assays (Applied Biosystems). Values were normalized using *RNU-44*. Real-time PCRs were run in triplicates from three different cDNAs. As *miR-92a-1* and *-92a-2* sequences are 100% identical, they are collectively referred to as *miR-92a* in qRT-PCR experiments.

RNase-protection assays

Sense oligonucleotides used for the preparation of double-strand DNA templates for T7 RNA polymerase were as follows: *miR-17*: 5'-CAAAGTGCTTACAGTGCAGGTAG(T)₁₃; *miR-18a*: 5'-TAAGGTGCATCTAGTGCAGATAG(T)₁₃; *miR-20a*: 5'-TAAAGTGCTTATAGTGCAGGTAG(T)₁₃; RNAs containing *miR-92a1*: 5'-GTTTCTGTATGGTATTGCACTTGTCCGGCCTGTTGAGTTTG (the position of *miR-92a-1* is given by bolded underlined letters); *U6snRNA*: 5'-CGATACAGAGAAGATTAGCATGGCCCTGCGCAAGG. Antisense RNA probes were synthesized using the *mirVana* miRNA Probe Construction Kit from Ambion in the presence of α^{32} P-CTP (3000 Ci/mmol) and subsequently purified onto a denaturing TBE-Urea 15% polyacrylamide gel. The resulting antisense probes are 10-nt longer than the protected fragments. RNAs extracted with TRIzol (Invitrogen) or yeast tRNA were incubated overnight at 42°C in the presence of 8×10^4 cpm of the antisense RNA probes in the hybridization buffer provided by the *mirVana* miRNA Detection Kit from Ambion. RNase-digestion were done for 30 min at 37°C using a 1/50 dilution of the RNase A/RNase T1 solution provided by the same kit. Samples were subsequently separated on a denaturing TBE-Urea 15% polyacrylamide gel in the presence of radiolabeled Ambion Decade Markers (not shown on the figures due to the necessity to overexpose the gels to allow for the detection of the *miR-17-92* miRNAs). Gels were then subjected to autoradiography at -80°C in the presence of an amplifying screen.

Promoter activity studies

MCF7 cells were transfected with the empty *pGL3-Basic* (Promega) or a construct in *pGL3-Basic* containing different fragments of the *miR-17-92* cluster promoter inserted upstream of the *Luciferase* gene, together with the *pRL-TK* vector for internal control, along with either *pCMV-HA*, *pCMV-HA-GAM*, *pCMV-c-Myc*, or their combinations, or with a Control RNA or with either *siGAM* and/or *siMyc*. The *Firefly* luciferase activity was measured 48 h post-transfection and normalized to *Renilla* luciferase activity.

Protein interaction assays

HEK-293 cells were transfected with either an empty control vector or a construct expressing the tagged factor of interest. Cells were lysed 24 h after transfection in 1 ml of NP-40 lysis buffer supplemented with 0.5 mM ZnCl₂. Supernatants were incubated overnight with anti-HA (Covance) or anti-Flag (Sigma) antibody. HA- or Flag-expressing lysates were further incubated with protein A/G sepharose (Amersham). The bound proteins were subjected to three washes of 30 min each and then eluted by boiling for 5 min in the loading buffer. Whenever needed, RNase-digestion was conducted at 4°C for 30 min before addition of the anti-HA antibody using RNase A from Qiagen (200 µg/ml). Immunocomplexes were analyzed by SDS-PAGE electrophoresis followed by immunoblotting with anti-Drosha, anti-Flag or anti-HA.

Statistical analysis and quantification

Comparisons with the respective controls were done using the Student's *t*-test, and *P*-values are provided in the figure legends. Band quantification was done using the Adobe Photoshop software. The relative intensities of the bands of interest, preably normalized to either α -tubulin (western blots) or *U6snRNA* (RNase-protection assays), are given under each panel in percent of the respective control sample.

RESULTS

GAM transcripts are targeted by *miR-17-92* and *let-7* miRNAs

In silico analyzes using the TargetScan software (<http://www.targetscan.org>) showed that the 3'-UTR of human *GAM* contains conserved putative target sites for ~150 different miRNAs, including three sites for *miR-17-92* miRNAs (one for *miR-92a-1* and two for *miR-17/20a*) or their respective orthologs and three for *let-7* miRNAs (Figure 1A and Supplementary Tables S1 and S2). In order to validate these target sites, the 3'-UTR of human *GAM* was inserted in both orientations downstream of the *luciferase* gene in the *pGL3-Control* vector, providing us with sense (*Luc-1S*) and antisense (*Luc-1AS*) constructs collectively referred to as *Luc-1* on Figure 1A. Three shorter clones (*Luc-2*, *-3* and *-4*) were prepared similarly. Transfecting HEK-293 cells with *pre-miR-17*, *-20a* and *-92a-1* or their respective orthologs *pre-miR-106b*, *-93* and *-25* significantly decreased the expression of *Luc-1S* while remaining without effects on *Luc-1AS* (Figure 1B). Similar results were obtained with two *let-7* pre-miRNAs, namely *pre-let-7a* and *-let-7d* (Figure 1B). In contrast, *miR-155* remained without effects, in accordance with the fact that *GAM* 3'-UTR contains no *miR-155* target site. The expression of *Luc-2S* was also reduced by either *pre-miR-92a-1* or *-25*, but neither by *pre-miR-17* nor *-miR-20a*, due to the lack of their common target sites (Figure 1C). *Luc-2S* was also targeted by *let-7d* (Figure 1D). Furthermore, *miR-17*, *-20a*, *-106b* and *-93* targeted *Luc-3S* and/or *Luc-4S*, the latter being also

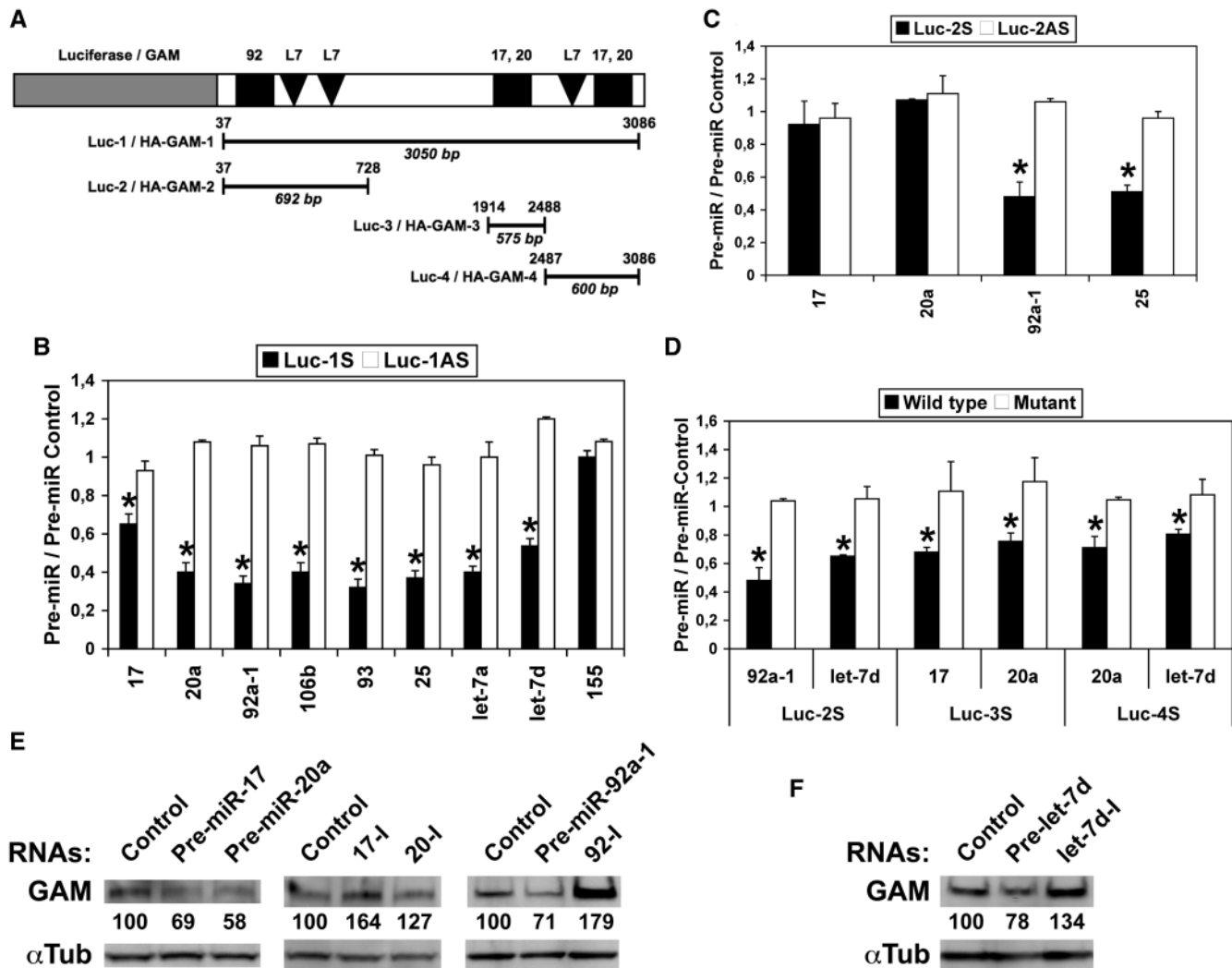


Figure 1. *GAM* transcripts are direct targets of *miR-17-92* and *let-7* miRNAs. (A) Schematic representation of *GAM* 3'-UTR constructs in *pGL3-Control* (*Luc-1*, -2, -3 and -4) and *pCMV-HA* (*HA-GAM-1*, -2, -3 and -4). Conserved putative target sites for *miR-17/20a* (17,20), *miR-92a-1* (92) and *let-7* (L7) miRNAs are indicated. The positions of the 5'- and 3'-limits of the inserts with respect to *GAM* 3'-UTR are indicated over the lines, and their respective lengths are given in bp below the lines. (B–D) Luciferase assays were done with *Luc-1* to *Luc-4* as indicated, both in sense (S) and antisense (AS) orientation. Mutations in constructs used in D are given in Supplementary Tables S1 (*miR-17-92* miRNA target sites) and S2 (*let-7* miRNA target sites). Bars show the ratios of the *Firefly* luciferase (normalized to *Renilla* luciferase) activity measured following transfection with the indicated pre-miRNAs to that obtained following transfection with the pre-miR Control for the same construct. Values represent the mean \pm SD ($n = 4$). (B and C) $*P < 0.001$, significantly different from pre-miR Control. (D) $*P < 0.005$, significantly different from wild type construct. (E and F) Endogenous *GAM* levels in HEK-293 cells transfected with either a pre-miR-Control, the indicated pre-miRNAs or antisense inhibitory RNAs to *miR-17*, *miR-20a* or *miR-92a* (17-I, 20-I and 92-I, respectively) were determined by western blotting.

targeted by *let-7d* (Supplementary Figure S1A and B). Finally, mutating the different target sites (Supplementary Tables S1 and S2) abolished the effects of the different pre-miRNAs (Figure 1D).

To determine if the targeting of *GAM* 3'-UTR by the above miRNAs would also reduce *GAM* protein accumulation, a human *GAM* cDNA encoding the same protein as GenBank [AB033022](#) sequence except for a conservative proline to leucine change was inserted upstream of the different parts of *GAM* 3'-UTR in the *pCMV-HA* expression vector (Figure 1A). As expected, the levels of HA-GAM-1 and HA-GAM-2 were reduced by either *pre-miR-17*, -20a, -92a-1 or their respective orthologs (Supplementary Figure S2A), and the

downregulation of HA-GAM-4 by *pre-miR-20a* was impaired by mutating the corresponding *miR-17/20a* target site (Supplementary Figure S2B). Finally, an anti-GAM antibody allowed us to definitely ascertain the downregulating effects of *pre-miR-17*, -20a, -92a-1 and -*let-7d* (Figure 1E and F, respectively). In contrast, as one would expect, transfecting HEK-293 cells with either antisense *miR-17*, -20a, -92a or *let-7d* inhibitory RNAs increased *GAM* levels (Figure 1E and F), which shows that both *miR-17-92* and *let-7* miRNAs regulate *GAM* expression *in vivo*. Collectively, these results give strong evidence that these two families of miRNAs should actually target the 3'-UTR of *GAM* transcripts in cell.

GAM regulates E2F1 and Ras levels as well as cell homeostasis

As *E2F1* was previously shown to be targeted by both *miR-17* and *miR-20a* (10,12), the blots from the middle and right panels of Supplementary Figure S2A were reprobed with an anti-E2F1 antibody. Expectedly, *pre-miR-20a* and *-106b* downregulated E2F1 and HA-GAM-1. Given that the two corresponding transcripts contain target sites for *miR-17/20a*, this provides good evidence that the effects of *pre-miR-17-92* miRNAs on HA-GAM levels were due to the production of fully functional miRNAs (Supplementary Figure S2A). In contrast, while downregulating HA-GAM-1 and HA-GAM-2, *pre-miR-92a-1* and its ortholog *pre-miR-25* increased E2F1 levels, suggesting that they may normally work to limit certain effects of *miR-17* and *miR-20a*, specially those related to the regulation of *E2F1* expression.

Given the opposite effects of *miR-17*, *-20a* and *-92a-1* on E2F1 levels despite their common downregulating effects on endogenous GAM (Supplementary Figure S2A and Figure 1E, respectively), we then checked if increasing GAM levels may have any consequences on E2F1 accumulation. Overexpressing *HA-GAM-2* reduced E2F1 level by ~40% (Figure 2A). In contrast, siRNAs directed against *GAM* transcripts (hereafter referred to as *siGAM*), which significantly decreased both *GAM* transcript and GAM protein levels (Supplementary Figure S3A–D), increased E2F1 levels by ~50% (Figure 5D). This suggests that GAM may somehow interfere with the effects of E2F1 on cell proliferation. To determine whether GAM may also affect other regulators of cell homeostasis, the blot of Figure 2A was reprobed with a pan-Ras antibody. Namely, Ras proteins function as intracellular switches in signal transduction cascades and, due to their ability to modulate transcription, they control cell growth and proliferation as well as other aspects of cell biology including senescence/cell-cycle arrest, differentiation and survival (18). Furthermore, like *GAM* transcripts, *Ras* transcripts represent validated targets of *let-7* miRNAs (15). In sharp contrast with E2F1, overexpressing *HA-GAM-2* increased Ras level ~9-fold (Figure 2A), while *siGAM* showed opposite effects (Supplementary Figure S4). Of note, while *HA-GAM-4* and *Ras* 3'-UTRs do not contain any *miR-92* target site, cotransfecting HEK-293 cells with *pre-miR-92a-1* and *HA-GAM-4* reduced the upregulation of Ras by GAM by ~50% (Figure 2B). This prompted us to look for the effects of *miR-92a-1* on *Ras* expression. As shown in Figure 2C, overexpressing *miR-92a-1* slightly decreased Ras levels. In contrast, transfecting HEK-293 cells with a *miR-92a* antisense inhibitory RNA increased Ras accumulation. Thus, *miR-92a-1* can downregulate Ras by targeting either endogenous *GAM* transcripts or other transcripts encoding positive regulators of *Ras*.

Finally, as mentioned earlier, *miR-17-92* and *let-7* miRNAs are respectively considered as oncogenes and tumor suppressor genes, due to their opposite effects on cell proliferation, apoptosis and more generally on cell homeostasis. *GAM* transcripts being a target of these two functionally opposite groups of miRNAs, and

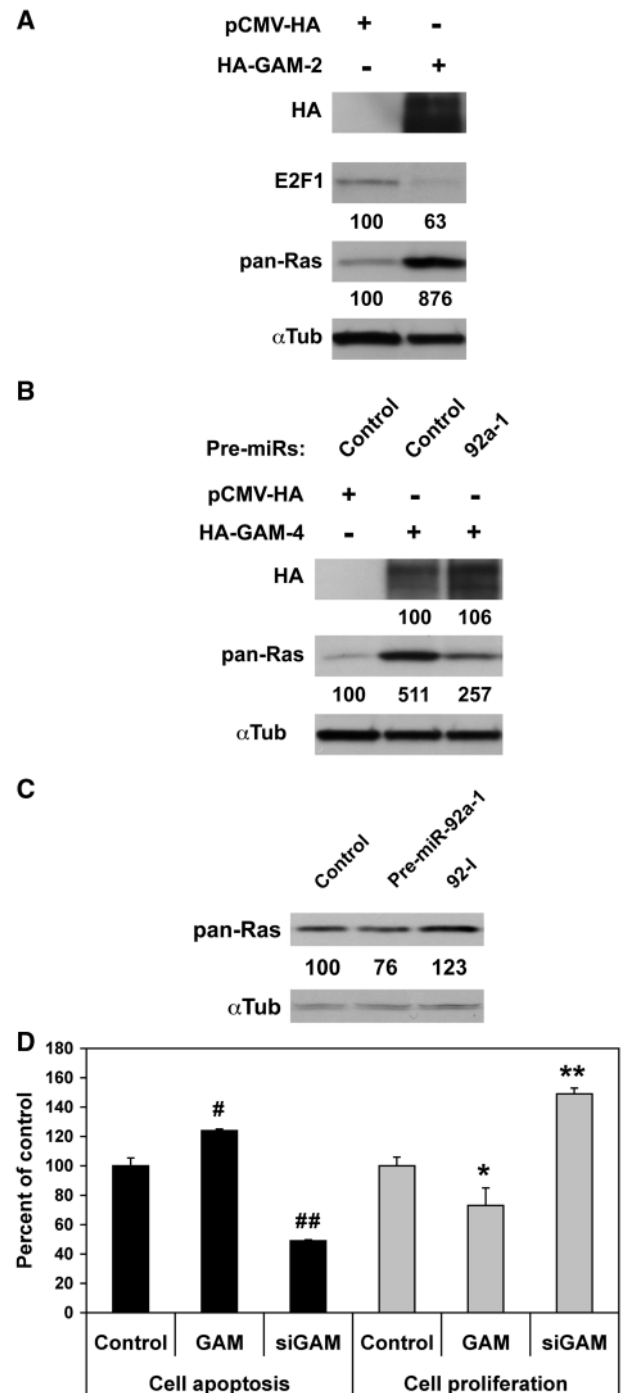


Figure 2. GAM modulates E2F1 and Ras levels, increases cell apoptosis and reduces cell proliferation. (A) HEK-293 cells were transfected with either *pCMV-HA* or *HA-GAM-2*. GAM effects on E2F1 and Ras were analyzed by western blotting. (B) HEK-293 cells were transfected with either *pCMV-HA* or *HA-GAM-4* along with a control RNA or *pre-miR-92a-1*. GAM effects on Ras were analyzed by western blotting. (C) Ras levels in HEK-293 cells transfected with either a pre-miR-control, *pre-miR-92a-1* or a *miR-92a* antisense inhibitory RNA (92-1) were determined by western blotting. (D) HEK-293 cells transfected as indicated were assayed 24 h later for apoptosis or proliferation. Values represent the mean \pm SD ($n = 3$). # and ##, Significantly different from apoptosis control, # $P = 0.063$, ## $P < 0.005$. * and **, Significantly different from proliferation control, * $P < 0.02$, ** $P < 0.005$.

GAM modulating E2F1 and Ras levels, we hypothesized that it might act as a molecular sensor aimed at maintaining cell homeostasis. Indeed, overexpressing *GAM* increased cell apoptosis while reducing cell proliferation, with *siGAM* having opposite effects (Figure 2D).

GAM decreases the expression of *miR-17-92* miRNAs differentially

Aimed at regulating the expression of their target genes, miRNAs tend to build feedback regulatory loops with their regulators (4). Thus, as *GAM* transcripts were targeted by *miR-17-92* miRNAs, and given that *GAM* and *miR-92a-1* had opposite effects on both *E2F1* and *Ras* expression (Supplementary Figure S2A and Figure 2A–C, respectively), we therefore hypothesized that *GAM* in turn may regulate *miR-17-92* miRNA

levels. Indeed, overexpressing *GAM* in HEK-293 (Figure 3A) or in K562 lymphoid cells (Supplementary Figure S5) decreased the levels of *miR-17-92* miRNAs. In contrast, the levels of these miRNAs increased following the transfection of HEK-293 (Figure 3B), HepG2 (Figure 6B) or MCF-7 breast cancer cells (Supplementary Figure S6) with *siGAM*. Finally, *GAM* overexpression also decreased the levels of *miR-17*, *-20a* and *-92a* in HEK-293 cells transfected with *Clu-81*, a 3837-bp expression construct containing the whole *miR-17-92* cluster (Figure 3C). This indicates that *GAM* generally works to impair any surge of *miR-17-92* miRNA levels. It also suggests that both the upregulation of *Ras* and the downregulation of *E2F1* by *GAM* (see hereabove) might be due at least in part to *GAM* downregulating effects on *miR-92a-1* levels.

Of note, and although qRT-PCR assays cannot discriminate between *miR-92a-1* and *miR-92a-2*, the relative

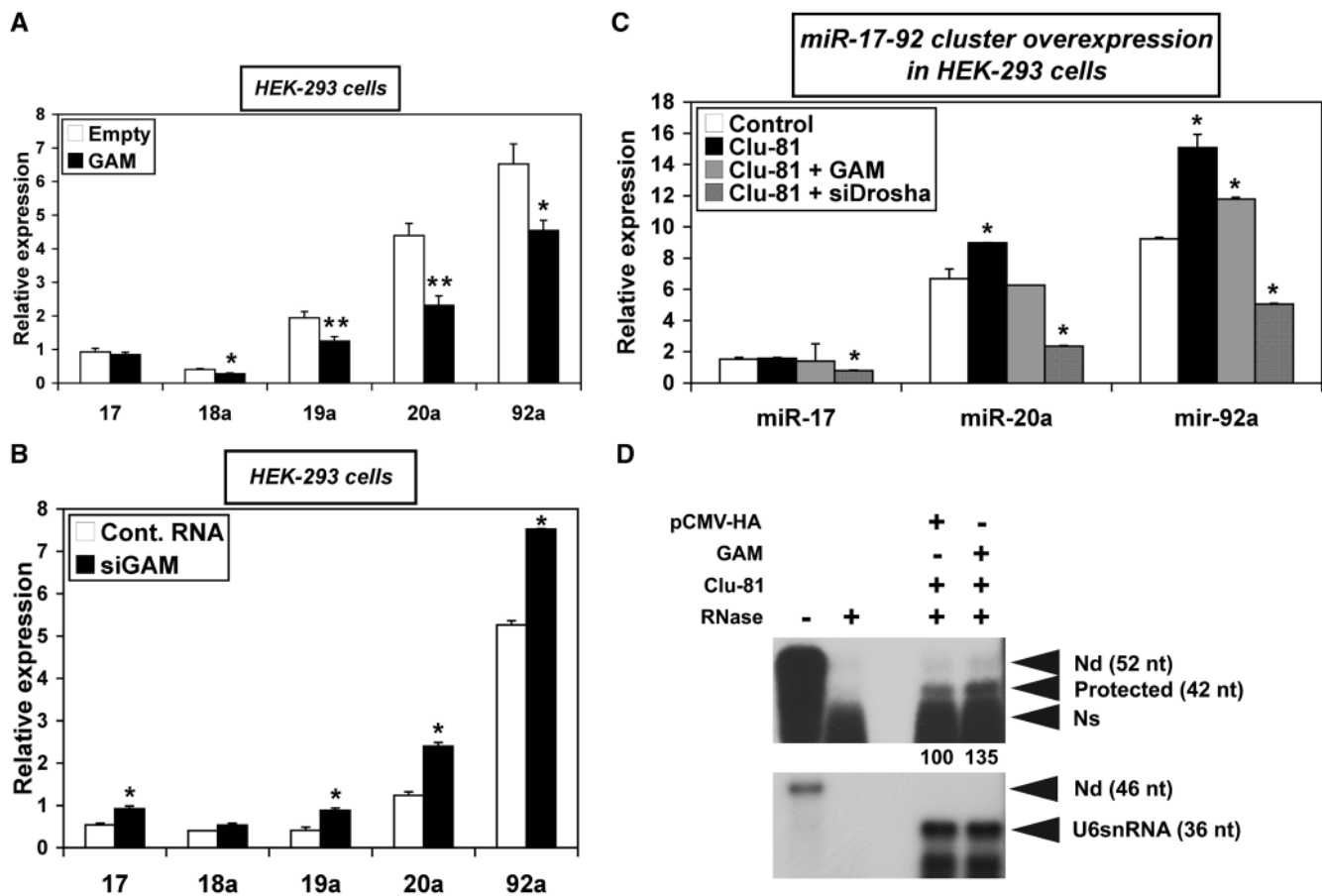


Figure 3. GAM downregulates *miR-17-92* miRNAs. (A and B). The relative levels of *miR-17-92* miRNAs in HEK-293 cells transfected with either the empty *pCMV-HA* vector or *pCMV-HA-GAM* (A), or with either a control RNA or *siGAM* (B), were determined by qRT-PCR. Values represent the mean \pm SD ($n = 3$). (A) * and **, Significantly different from empty control vector, * $P < 0.004$, ** $P < 0.0004$. (B) * $P < 0.005$, significantly different from control RNA. (C) HEK-293 cells were transfected with either a control *pCMV-HA/Control* RNA mix or a construct in the *pCMV* vector expressing the whole *miR-17-92* cluster (*Clu-81*) in combination with either a *pCMV-HA/Control* RNA mix, a *pCMV-HA-GAM/Control* RNA mix, or a *pCMV-HA/siDrosha* mix. *Clu-81* contains a 3837-bp insert bearing the whole *miR-17-92* cluster sequence (787 bp) plus 216-bp upstream of the first nucleotide of *pre-miR-17* and 2834-bp downstream of the last nucleotide of *pre-miR-92a-1*. The relative levels of the indicated miRNAs were determined by qRT-PCR. Values represent the mean \pm SD ($n = 3$). * $P < 0.01$, significantly different from control mix. (D) HEK-293 cells were transfected with *Clu-81* along with either the empty *pCMV-HA* vector or *pCMV-HA-GAM*. The relative levels of RNAs containing unprocessed *miR-92a-1* (42-nt long protected band) were determined by RNase-protection assays using 2 μ g total RNA per assay. The efficiency of RNase digestion was assessed in parallel following the incubation of each probe with the same amount of yeast tRNAs (two left lanes). ‘RNase’ controls correspond to a 15-fold dilution of the undigested sample. The relative intensities of the protected fragments, calculated using the *U6snRNA* as an internal control, are given in percent of the *pCMV-HA* control. Nd, non-digested, Ns, non-specific.

levels of expression of *miR-17-92* miRNAs located 3' of the cluster were generally higher than those with a more 5' position (Figures 3A and B, 6A and B and Supplementary Figures S5 and 6). These results indicate that *miR-17-92* miRNAs are processed with a differential efficiency from transcripts produced from the *miR-17-92* cluster host gene, i.e. *MIRHG1* or *C13orf25*. This was further confirmed by the fact that transfecting HEK-293 cells with *Clu-81* also lead to the differential expression of *miR-17*, *-20a* and *-92a* (Figure 3C), which suggests that HEK-293 cells may contain limiting amounts of unidentified factors required for the processing of each of the *miR-17-92* miRNAs.

Furthermore, in the four cell lines under study, *GAM* and/or *siGAM* affected the levels of the tested *miR-17-92* miRNAs differentially (Figures 3A–C, 6A and B and Supplementary Figures S5 and 6), suggesting that *GAM* may somehow affect their processing. Unfortunately, nothing is known about the processing of *miR-17-92* miRNAs beyond the fact that the production of *miR-18a* requires the presence of hnRNP A1 (19,20). It is also not known whether transcripts starting at either the TSS1 (transcription start site 1, i.e. the TSS of the *C13orf25* host gene) (13), the TSS2 (21), or both TSSs may function as *miR-17-92* primary RNAs, nor if any of these transcripts allows the simultaneous production of the six mature *miR-17-92* miRNAs, or not. Gaining definitive answers to these questions will obviously require a number of experiments and is well beyond the scope of this article. Nevertheless, to gain further evidence of *GAM* effects on *miR-17-92* miRNAs processing, we used RNase-protection assays to follow the levels of RNAs containing unprocessed *miR-92a-1* in HEK-293 cells transfected with *Clu-81* along with either an empty *pCMV-HA* control vector or a construct overexpressing *GAM*. Indeed, *GAM* overexpression increased the relative levels of RNAs containing unprocessed *miR-92a-1* by ~35% (Figure 3D). Given the facts that *GAM* is located in the nucleus [(17) and Tili *et al.*, unpublished results] and that *GAM* overexpression did not change the levels of Dicer, which further processes the pre-miRNAs in the cytoplasm (Supplementary Figure S7), the result of Figure 3D gives an independent indication that *GAM* may somehow decrease, directly or indirectly, the processing of *miR-17-92* primary transcripts in the nucleus.

GAM impairs the activation of the *miR-17-92* cluster by c-Myc

The expression of miRNAs can be regulated both transcriptionally and post-transcriptionally. The above results suggested that *GAM* downregulating effects on the expression of *miR-17-92* miRNAs come at least in part from post-transcriptional effects. To determine if the downregulation of *miR-17-92* miRNAs by *GAM* may also arise from effects on the *miR-17-92* cluster expression, we used five promoter constructs containing *miR-17-92* TSS2 (21) (Figure 4A). Four of them, but *Clu-5*, contain the Myc-binding site previously shown to be bound by c-Myc to transactivate the *miR-17-92* cluster (10). *Clu-1*, *Clu-2* and *Clu-3* were generated by fusing *Clu-4* to more

upstream sequences containing the TSS1 (13). These constructs were tested in MCF7 cells, where both *miR-17-92* miRNAs (22) and *c-Myc* (23) are well expressed. A high level of luciferase activity was produced from *Clu-1* and *Clu-2*, but the lack of a 123-bp fragment containing *E2F*-binding sites 1 and 2 (12) in *Clu-3* decreased the luciferase activity by ~75% (Figure 4B). This indicates that this 123-bp fragment is required for a robust expression of the cluster, in agreement with the fact that *E2F* factors have been shown to bind *E2F*-binding sites 1–4 and activate the transcription from the TSS1 (12,13). The activity of *Clu-4* was still lower, and that of *Clu-5* similar to the empty control vector. Overexpressing *GAM* decreased four times the activation of *Clu-1* and *Clu-2*, while remaining without significant effects on *Clu-3* (Figure 4B). In contrast, it increased the expression of *Clu-4* and *Clu-5*, which was probably due to off-target effects allowing the activation of the TSS2 through modifications of the levels of some of the transcriptional activators and repressors whose target sequences are known to be present in the *pGL3-Basic* vector. Regardless, these results suggest that *GAM* can decrease the transcriptional activation of the *C13orf25* host gene from the TSS1.

In agreement with previously published results (10), overexpressing *c-Myc* lead to a further activation of *Clu-1* to *Clu-4* (Figure 4C). The highest levels of expression were again obtained with *Clu-1* and *Clu-2*, indicating that transcription factors other than c-Myc, whose target sequences should be present both within and upstream the 123-bp fragment missing in *Clu-3*, were working along c-Myc to activate the expression of the reporter constructs. In contrast, siRNAs directed against *c-Myc* transcripts (*siMyc*) reduced the levels of expression of *Clu-1* and *Clu-2* to that of the empty *pGL3-Basic* vector, showing that the presence of c-Myc activity is mandatory for the activation of the *miR-17-92* cluster promoter from the TSS1, at least in the conditions of the experiment (Figure 4D). Coexpressing *GAM* along with *c-Myc* decreased the activating effects of c-Myc on *Clu-1* and *Clu-2* (Figure 4C). Of note, neither *GAM* overexpression nor *siGAM* affected c-Myc protein levels (Supplementary Figure S8), indicating that *GAM* activity interferes, directly or indirectly, with the transcriptional activation of the *miR-17-92* cluster by c-Myc. However, *GAM* overexpression did not reduce the activating effects of c-Myc on *Clu-3*, *Clu-4* and *Clu-5* (Figure 4C), suggesting that *GAM* effects might not be directed toward c-Myc itself but rather toward some of the unidentified transcriptional activators acting on the 123-bp fragment deleted in *Clu-3* and/or on sequences located upstream of *Clu-2*. To determine if *GAM* repressing effects were dependent on *E2F* sites 1 and 2, we then used constructs derived from *Clu-2* whose either *E2F* site 2 (*Clu-2-ME2*) or both *E2F* sites 1 and 2 (*Clu-2-ME1,2*) had been mutated (Supplementary Figure S9). These mutations did not change the levels of expression of *Clu-2*, indicating that the effects of *E2F* factors on this construct rather take place through *E2F* sites 3 and/or 4. Of note, these mutations also did not impair *GAM* repressing activity, indicating that *GAM* opposes the activating effects of some factors working on this 123-bp fragment either

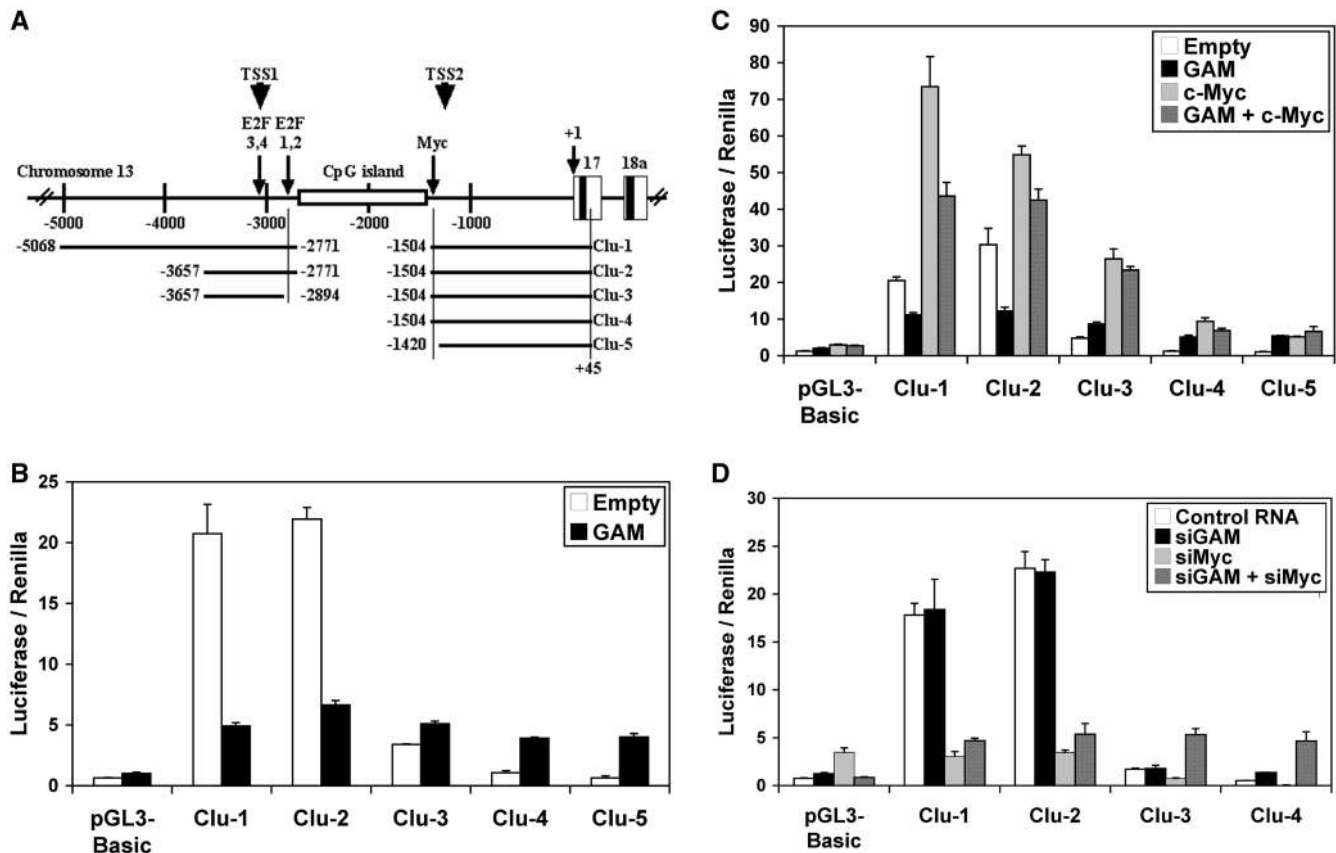


Figure 4. GAM impairs the activation of the *miR-17-92* promoter. (A) Schematic representation of the clones in *pGL3-Basic* containing different parts of the genomic region upstream of the *miR-17-92* cluster. The first nucleotide of *pre-miR-17* was arbitrarily used as nucleotide +1 for convenience. The ends of the DNA fragments used to generate the *Clu-1* to *Clu-5* constructs are indicated. The positions of one *c-Myc*-binding site (10) and of *E2F*-binding sites 1, 2 and 3, 4 (12) are indicated by an arrow. The approximate locations of *TSS1* (transcription start site 1) (13) and *TSS2* (21) are indicated by arrowheads. (B and C) Effects of GAM on the *miR-17-92* promoter either alone (B) or in conjunction with *c-Myc* (C). MCF7 cells were transfected with the empty *pGL3-Basic* vector or the *Clu-1* to *Clu-5* constructs as indicated along with the empty *pCMV-HA* vector or a construct expressing *GAM* and/or *c-Myc*. The *Firefly* luciferase activity was measured 48 h after transfection and then normalized to the *Renilla* luciferase activity. Values represent the mean \pm SD ($n = 4$). (D) Effects of siRNAs directed against *GAM* (*siGAM*) or against *c-Myc* transcripts (*siMyc*) on the *miR-17-92* promoter expression either alone or in conjunction. MCF7 cells were transfected with the empty *pGL3-Basic* vector or the *Clu-1* to *Clu-4* constructs as indicated along with a control RNA, *siGAM* and/or *siMyc*. The *Firefly* luciferase activity was measured 48 h after transfection and then normalized to the *Renilla* luciferase activity. Values represent the mean \pm SD ($n = 4$).

beside or along *E2F* factors. Finally, the lack of effects of *siGAM* showed that endogenous *GAM* activity is normally too low to impair the activation of the *miR-17-92* promoter (Figure 4D). Collectively, these results provide good evidence that *c-Myc* is critical for the *miR-17-92* cluster activation, and that *GAM* impairs the *miR-17-92* promoter activation by reducing, either directly or indirectly, the positive transcriptional effects of *c-Myc* and those of unidentified factors acting on the 123-bp fragment lacking in *Clu-3* construct or on sequences located upstream of *Clu-2*, at least in the conditions of the experiment. Similar experiments showed that *GAM* also impairs the activation of the *miR-17-92* cluster in HEK-293 and HepG2 cells (data not shown).

Of note, the activation of *Clu-4* and *Clu-5* following *GAM* overexpression (Figure 4B), of *Clu-5* following *c-Myc* and/or *GAM* transfection (Figure 4C), of the empty *pGL3-Basic* vector following *siMyc* transfection (Figure 4D), as well as of *Clu-3* and *Clu-4* following transfection with both *siGAM* and *siMyc* (Figure 4D)

most probably arose from off-target effects, known to occur with the *pGL3-Basic* vector.

GAM and Drosha modulate each other's levels

The differential effects of *GAM* on *miR-17-92* miRNA levels (Figures 3A–C, 6A and B and Supplementary Figures S5 and S6) suggested that *GAM* may somehow affect the processing of *miR-17-92* primary RNAs. This could possibly be done either by modulating, directly or indirectly, the levels of *Drosha* or those of factors required for the differential processing of *miR-17-92* miRNAs, or by interfering, directly or indirectly, with *Drosha* activity. Interestingly, *GAM* overexpression in HEK-293 cells decreased the levels of *Drosha* transcripts (Figure 5A) and, as shown by reprobating the blots from Figure 2A with an anti-*Drosha* antibody, of *Drosha* protein (Figure 5B). Accordingly, *siGAM* lead to an increase of both *Drosha* transcripts and *Drosha* protein levels (Figure 5C and D). Of note, *GAM* overexpression did

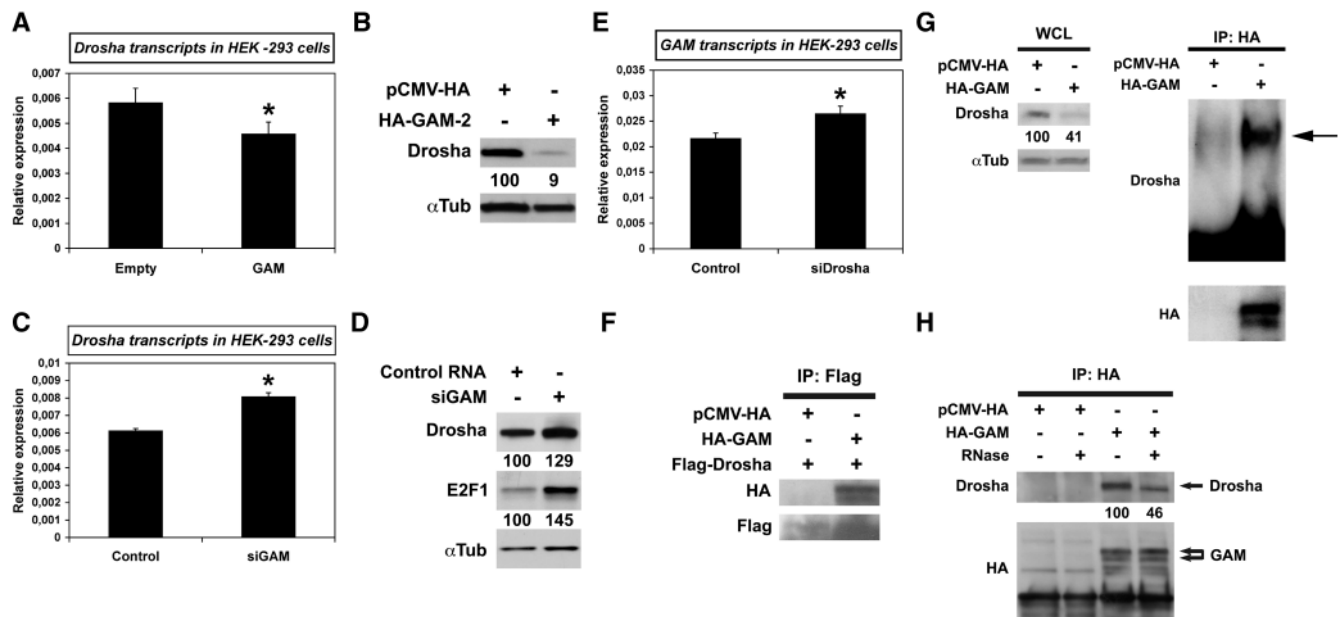


Figure 5. GAM downregulates Drosha, a critical effector of miRNA maturation, and GAM-Drosha interaction is RNA-dependent. (A and C) The relative levels of *Drosha* transcripts in HEK-293 transfected with either the empty *pCMV-HA* vector or *HA-GAM* (A), or with either a Control RNA or *siGAM* (C), were determined by qRT-PCR. Values represent the mean \pm SD ($n = 3$). * $P < 0.05$, significantly different from control RNA. (B and D) Drosha levels in extracts prepared from HEK-293 cells transfected with either the empty *pCMV-HA* vector or *HA-GAM-2* (B), or with either a Control RNA or *siGAM* (D), were analyzed by western blotting. The blots in B were from the same experiment as the blots of Figure 2A. (E) The relative levels of *GAM* transcripts in HEK-293 transfected with either the Control RNA or siRNAs directed against *Drosha* transcripts (*siDrosha*) were determined by qRT-PCR. Values represent the mean \pm SD ($n = 3$). * $P < 0.001$, significantly different from Control RNA. (F–H) HEK-293 cells were transfected with the empty *pCMV-HA* vector or *pCMV-HA-GAM*. In (F), cells were cotransfected with a *Flag-Drosha* construct. In (H), extracts were submitted to RNase digestion before immunoprecipitation as indicated. Immunoprecipitations were conducted using anti-Flag (F) or anti-HA antibody (G and H). Immunocomplexes were analyzed by western blotting with the indicated antibodies. In (G), the left blot (WCL) shows the levels of endogenous Drosha in whole cell lysates of HEK-293 cells transfected as indicated, before immunoprecipitation (these blots were prepared using 1/25th of the whole lysate subsequently used for immunoprecipitation).

not affect the level of DGCR8, the general partner of Drosha (Supplementary Figure S7). On the other hand, transfecting the cells with siRNAs directed against *Drosha* transcripts (*siDrosha*), which decreased *Drosha* transcripts and Drosha protein levels by $\sim 60\%$ (Supplementary Figure S3E and F), showed to increase *GAM* transcripts levels (Figure 5E), most probably by decreasing the levels of several of the miRNAs potentially targeting *GAM* transcripts. Thus, *GAM* and *Drosha* work within the same gene regulatory network and control, directly or indirectly, each other's expression.

The fact that GAM downregulates *Drosha* suggested that GAM may decrease the levels of most, if not all, miRNAs by decreasing the processing of their primary transcripts by Drosha. However, *GAM* overexpression in HepG2 did not decrease the levels of either *let-7d*, *let-7e* or *let-7f* and *siGAM* in contrast slightly but significantly downregulated *let-7d* (Figure 6E and F). This indicates that the repressing effects of GAM are not global but rather miRNA-specific. Accordingly, qRT-PCRs from RNAs extracted of SW480 colon cells as well as microarrays from RNAs prepared from THP-1 monocytic cells showed that GAM can upregulate or downregulate some miRNAs while leaving the levels of the others unchanged (Tili *et al.*, unpublished results). Thus, GAM-specific effects on miRNA expression should require the presence of specific trans-regulators of miRNA

processing. Finally, as *siGAM* did not increase Drosha levels in HEK-293 cells by $>30\%$ (Figure 5D), and given that Drosha is rather an abundant protein in these cells, as shown by proteomic analyses (Tili *et al.*, unpublished results), the differential downregulation of *miR-17-92* miRNAs by endogenous GAM cannot solely come from its capability to downregulate Drosha, and should also arise from GAM effects on at least some of those unidentified factors regulating the differential processing of *miR-17-92* primary RNAs by Drosha.

GAM interacts with Drosha in a RNA-dependent manner

As GAM affected both the transcription and the processing of *miR-17-92* miRNAs, and given that these two processes are generally coupled, we hypothesized that in addition to modulating the expression of *Drosha*, GAM may also possibly interfere with the processing of pri-miRNA by Drosha. Furthermore, a massive yeast two-hybrid screen suggested that GAM might directly interact with Drosha (24). Indeed, a Flag-tagged full-size Drosha allowed us to immunoprecipitate HA-tagged full-size GAM in HEK-293 cells using an anti-Flag antibody (Figure 5F). Accordingly, we were able to immunoprecipitate endogenous Drosha along with HA-GAM, confirming that GAM and Drosha may actually interact *in vivo* (Figure 5G). To gain further insights into the possible consequences of GAM-Drosha

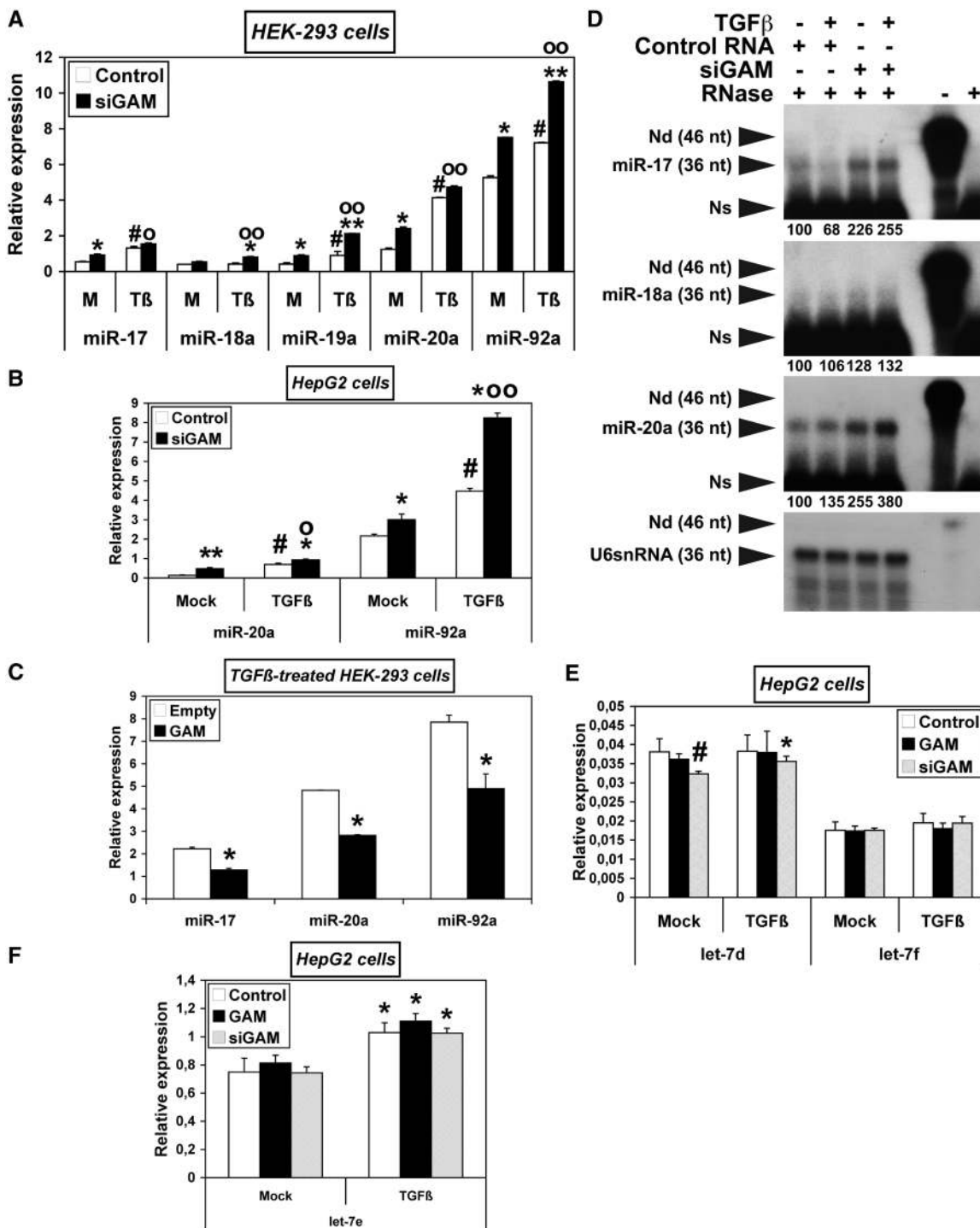


Figure 6. GAM opposes the upregulation of *miR-17-92* miRNAs by TGF β effectors. (A–C) HEK-293 (A and C) or HepG2 cells (B) were transfected with either a control RNA or *siGAM* (A and B), or with the empty *pCMV-HA* vector or *pCMV-HA-GAM* (C). After 34 h, they were either mock-treated (A and B) or treated with TGF β (A–C) as indicated. The relative levels of the indicated miRNAs were determined by qRT-PCR. Values represent the mean \pm SD ($n = 3$). The results obtained with mock-treated samples (M) in (A) are the same as those in Figure 3B. They are presented again along with the results obtained with TGF β -treated samples (T β) for easier comparison, as they were drawn from the same experiment. Panel A: * and **, *siGAM* significantly different from Control RNA for each treatment, *, $P < 0.005$, **, $P < 0.0005$; #, TGF β -treated Control RNA significantly different from mock-treated Control RNA, $P < 0.0002$; o and oo, TGF β -treated *siGAM* significantly different from mock-treated *siGAM*, o, $P < 0.003$, oo, $P < 0.0004$. Panel B: * and **, *siGAM* significantly different from Control RNA for each treatment, *, $P < 0.05$, **, $P < 0.002$; #, TGF β -treated Control RNA significantly different from mock-treated Control RNA, $P < 0.02$; o and oo, TGF β -treated *siGAM* significantly different from mock-treated *siGAM*, o, $P < 0.02$, oo, $P < 0.01$. Panel C: *, Significantly different from Empty control vector, $P < 0.05$. (D) The relative levels of *miR-17*, *miR-18a* and *miR-20a* were determined by RNase-protection assays. Total RNAs extracted from HepG2 cells ($10 \mu\text{g}$ per assay) treated and transfected as indicated (four left lanes) were hybridized with a radiolabelled RNA antisense probe complementary to the indicated miRNAs. The lengths of the antisense *miR-17*, *miR-18a* and *miR-20a* probes were made equal to that of the *U6snRNA* antisense probe by addition of a 3'-poly(A)-tail. Namely, the RNases used in the assay (RNase 1 and RNase T1) are not able to cut phosphodiester bonds following a non-paired A nt. The relative intensities of the protected fragments, given under each panel in percent of the

continued

interaction, we then pre-treated the cellular extracts with RNase A before immunoprecipitation. This reduced the amount of endogenous Drosha interacting with GAM by ~50% (Figure 5H), indicating that Drosha-GAM interactions take place when Drosha is engaged in pri-miRNA processing. However, transfecting HEK-293 cells with *Clu-81* did not allow us to precipitate Drosha more efficiently than in presence of an empty control vector (results not shown), suggesting that GAM differential effects on *miR-17-92* miRNAs processing require the presence of other factors present in limiting amount, most probably the very same factors responsible for the differential processing of *miR-17-92* miRNAs in untransfected cells (see hereabove). This would also explain why GAM was never reported to be part of the microprocessor complex. Finally, whether GAM competes with some of these *miR-17-92* processing factors for binding to Drosha and/or downregulates factors needed for the processing of miRNAs such as *miR-20a* or *miR-92a-1* remains to be determined.

GAM opposes the upregulation of *miR-17-92* miRNAs by TGF β

Usually, TGF β maintains tissue homeostasis and prevents incipient tumor formation by regulating cellular proliferation, differentiation, survival and adhesion as well as the cellular microenvironment. However, pathological forms of TGF β signaling can promote tumor growth and invasion, evasion of immune surveillance, cancer cell dissemination and metastasis (25). Importantly, miRNAs have recently been shown to regulate the TGF β signaling pathway. For example, the expression of *miR-21* in breast cancers predicts for elevated TGF β 1 expression and a poor clinical prognosis (26), while that in gliomas results in the suppression of multiple components of the TGF β signaling system, including its ligands (i.e. TGF β s 1 and 3), its receptors (i.e. T β R-II and T β R-III), and its effector molecules (i.e. Smad3, Daxx, and PDCD4) (27,28). Interestingly, the two-hybrid screen referred to here above also showed that GAM may interact with Smad1 (an infrequent transducer of TGF β signaling), and that siRNAs directed against *GAM* transcripts impaired the upregulation of endogenous *PAI-1* (*Plasminogen Activator Inhibitor-1*) by TGF β (24). Therefore, as the misexpression of *miR-17-92* miRNAs has been associated with cancers (5,9), we then looked for the effects of TGF β 1 (hereafter referred to as TGF β) on *miR-17-92* miRNA expression, and checked if these effects may be affected by GAM levels.

TGF β increased the levels of *miR-17-92* miRNAs in HEK-293 (Figure 6A), HepG2 (Figure 6B) and MCF7 cells (Supplementary Figure S6). These levels generally

increased further when cells were transfected with *siGAM*, indicating that *GAM* opposes the upregulation of *miR-17-92* miRNAs by TGF β . This was confirmed by the fact that *GAM* overexpression reduced the levels of *miR-17*, *20a* and *-92a* in HEK-293 cells treated with TGF β (Figure 6C). Interestingly, the upregulation of *miR-17-92* miRNAs was not uniform, suggesting that TGF β , as well as *GAM*, may somehow affect the maturation of *miR-17-92* miRNAs. The differential effects of TGF β and *siGAM* on *miR-17-92* miRNA expression were further confirmed in HepG2 cells by RNase-protection assays using probes specific of *miR-17*, *-18a* and *-20a* (Figure 6D). Together, these results show that, in HEK-293, HepG2 and MCF7 cells, the differential upregulation of *miR-17-92* miRNAs by TGF β is differentially buffered by endogenous *GAM* activity. Of note, the upregulation of *miR-17-92* miRNAs by TGF β effectors was not due to its inhibition of the downregulation of *Drosha* by endogenous *GAM*: namely, the TGF β treatment did not significantly affect *Drosha* transcripts nor *Drosha* protein levels (Supplementary Figure S10). This was further confirmed by the fact that, while also upregulating *let-7e*, TGF β did not change the levels of *let-7d* or *let-7f* (Figure 6E and F). Thus, like those of *GAM*, TGF β effects are miRNA-specific, and TGF β effectors most probably impact the levels of the same unidentified regulators of *miR-17-92* miRNA processing (see hereabove).

Finally, *siGAM* also increased the levels of *miR-106b* and *-93* in MCF7 cells, with or without TGF β treatment (Supplementary Figure S11), pointing to possible *GAM* effects on the two *miR-17-92* ortholog clusters.

GAM, *miR-17-92a* miRNAs and TGF β effectors work within the same gene regulatory network

Beside reducing the translation of their target transcripts, miRNAs also often drive their degradation (29). Therefore, we then checked for possible effects of *miR-17-92* miRNAs on *GAM* transcript levels. In HepG2 cells, *pre-miR-17* downregulated *GAM* transcript levels by 40% (Figure 7A). Of note, while *pre-miR-20a* and *-92a-1* reduced the levels of *GAM* transcripts by ~80%, their respective orthologs *pre-miR-93* and *-25* did not, showing that miRNAs with comparable effects on mRNA translation may well work differently on mRNA stability (Figure 7A). In accordance with the fact that no *miR-18a* target site is found in *GAM* transcripts, *pre-miR-18a* did not affect *GAM* transcript levels. The targeting of *GAM* transcripts by *miR-17-92* miRNAs was further confirmed in HEK-293 cells overexpressing *Clu-81* (Figure 7C).

Figure 6. Continued

control sample (lane 1), were calculated using the *U6snRNA* as an internal control. The efficiency of RNase digestion was assessed in parallel following the incubation of each probe with 10 μ g yeast tRNAs (two rights lanes). ‘RNase’ controls correspond to a 15-fold dilution of the undigested samples. Exposure time at -80°C was 36 h for *miR-17*, *miR-18a* and *miR-20a* probes, and 2 h for *U6snRNA* probe. Nd, non-digested, Ns, non-specific. (E,F) HepG2 cells were transfected with either a *pCMV-HA/Control* RNA mix (Control), a *pCMV-HA-GAM/Control* RNA mix (*GAM*) or a *pCMV-HA/siGAM* mix (*siGAM*). 34 h later, they were either mock-treated or treated with TGF β . The relative levels of *let-7d* and *let-7f* (E) and of *let-7e* (F) were determined by qRT-PCR. Values represent the mean \pm SD (n = 3). Panel E: #, mock-treated *siGAM* significantly different from mock-treated Control, $P = 0.01$; *, TGF β -treated *siGAM* significantly different from mock-treated *siGAM*, $P = 0.03$. Panel F: *, Significantly different from the corresponding mock-treated sample, *, $P < 0.02$.

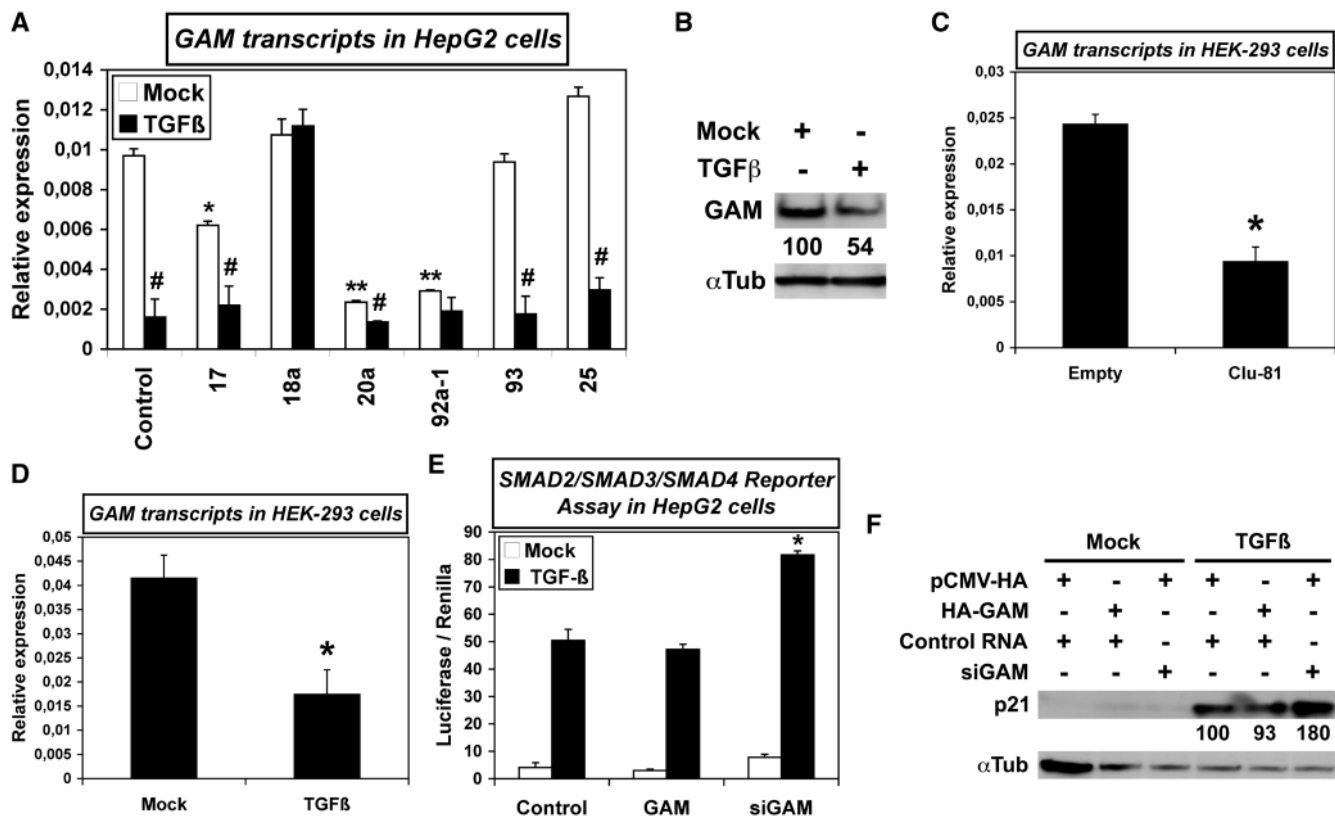


Figure 7. TGFβ and *miR-17-92* miRNAs downregulate *GAM* transcripts, while *GAM* impairs the canonical TGFβ signaling pathway. (A) The levels of *GAM* transcripts in HepG2 cells transfected with a pre-miR Control (Control) or the indicated pre-miRNAs, and either mock-treated or treated with TGFβ, were determined by qRT-PCR. Values represent the mean ± SD calculated from triplicate qRT-PCR reactions of the same cDNAs. #, TGFβ-treated sample significantly different from the corresponding mock-treated sample, $P < 0.003$. * and **, Pre-miR mock-treated significantly different from mock-treated pre-miR Control, * $P < 0.002$, ** $P < 0.001$. (B) *GAM* levels in HepG2 cells mock-treated or treated with TGFβ were analyzed by western blotting. (C,D) The relative levels of *GAM* transcripts in HEK-293 cells transfected with either the empty *pCMV* vector (Empty) or *Clu-81* (C), or either mock-treated or treated with TGFβ (D) were determined by qRT-PCR. Values represent the mean ± SD calculated from three independent reverse transcription reactions, each analyzed by qRT-PCR in triplicates. Panel C: *, Significantly different from Empty control vector, $P < 0.001$. Panel D: *, Significantly different from mock-treatment, $P < 0.001$. (E) After cotransfection with the *SMAD2/SMAD3/SMAD4* luciferase reporter plasmid along with either a *pCMV-HA/Control RNA* mix, a *pCMV-HA-GAM/Control RNA* mix or a *pCMV-HA/siGAM* mix, HepG2 cells were mock-treated or treated with TGFβ. The *Firefly* luciferase activity was measured 48 h after transfection and then normalized to the *Renilla* luciferase activity. Values represent the mean ± SD ($n = 6$). *, Significantly different from Control, $P < 0.005$. (F) The effects of *GAM* or *siGAM* on *p21/CDKN1A* expression in HepG2 cells mock-treated or treated with TGFβ were analyzed by western blotting.

We then checked if TGFβ may modulate the effects of *miR-17-92* miRNAs on *GAM* expression. TGFβ alone reduced *GAM* transcript as well as *GAM* protein levels (Figure 7A and B). A similar downregulation of *GAM* transcript level by TGFβ was also observed in HEK-293 (Figure 7D) and in MCF7 cells (Supplementary Figure S12). Of note, transfecting HepG2 cells with either *pre-miR-17*, *-20a*, *-92a-1*, *-93* or *-25* did not affect the downregulation of *GAM* transcripts by TGFβ (Figure 7A). This was probably due to the capability of TGFβ to upregulate endogenous *miR-17-92* miRNAs, and possibly also other miRNAs targeting *GAM* transcripts. However, we do not rule out the possibility that TGFβ signaling may also trigger miRNA-independent repression of *GAM*. Surprisingly, *miR-18a* impaired the downregulation of *GAM* by TGFβ (Figure 7A), possibly by targeting *SMAD2*, a transducer of TGFβ signaling whose 3'-UTR contains a *miR-18* target site, and/or some other repressors of *GAM*. Overall, these results suggest that the downregulation of *GAM* by the effectors

of TGFβ signaling is at least in part dependent of *miR-17-92* miRNA expression, and conversely that the upregulation of *miR-17-92* miRNAs by TGFβ might arise, at least in part, from its repressing effects on *GAM* expression. Of note, the conjugate effects of *GAM* and TGFβ on miRNA expression also appear to be miRNA-specific. Namely, TGFβ unexpectedly impaired the downregulation of *let-7d* by *siGAM* (Figure 6E), suggesting that *GAM* and TGFβ effectors, while having opposite effects on *miR-17-92* miRNA expression, may work together to keep *let-7d* levels above a certain threshold, in accordance with the fact that *GAM* and TGFβ were previously shown to collaborate and upregulate endogenous *PAI-1* (24).

Given the above interactions between *GAM* and TGFβ signaling, we further looked whether *GAM* may have broader effects on TGFβ canonical signaling pathway. In this pathway, the active TGFβ dimer signals by bringing together two pairs of receptor serine/threonine kinases known as the type I and type II receptors,

respectively. On binding TGF β , the type II receptors phosphorylate and activate the type I receptors that then propagate the signal by phosphorylating SMAD transcription factors, namely SMAD2 and SMAD3. Once activated, SMAD2 and SMAD3 form a complex with SMAD4, a binding partner common to all regulatory SMADs (30), and then shuttle to the nucleus. While *GAM* overexpression in HepG2 cells transfected with a luciferase reporter construct containing SMAD2-4 responsive elements remained without significant effects, targeting endogenous *GAM* transcripts with *siGAM* increased the luciferase activity by ~60% (Figure 7E). Accordingly, while *GAM* overexpression did not significantly impair the well established upregulation by TGF β of the cell-cycle regulator p21/CDKN1A, *siGAM* lead to a 80% increase of p21 level (Figure 7F). Thus, one possible way for GAM to reduce the upregulation of *miR-17-92* miRNAs by TGF β may be to impair SMAD activity. Furthermore, these results suggest that another important role for GAM may be to lower the plateau of activation reached by TGF β responsive genes under TGF β signaling.

Together, and taking also into account that *miR-20a* has been shown to target TGF β type II receptors (22), our results give strong evidence that GAM, *miR-17-92* miRNAs and the effectors of the canonical TGF β signaling pathway work within the same gene regulatory network.

DISCUSSION

Our study provides the very first data concerning the role of GAM, a previously uncharacterized factor, in human cells, in addition to shedding a new light on the regulation and functions of *miR-17-92* miRNAs. Altogether, our results show that GAM is central to a gene regulatory network, illustrated on the working model of Figure 8, through which: (i) *miR-17*, *-20a*, *-92a-1* and *let-7* miRNAs reduce *GAM* expression by targeting *GAM* transcripts; (ii) GAM downregulates *miR-17-92* miRNAs both

by impairing the transcriptional activation of the *miR-17-92* cluster by c-Myc and by differentially affecting the maturation of *miR-17-92* miRNAs; (iii) GAM also impairs the upregulation of *miR-17-92* miRNAs by TGF β effectors, possibly by decreasing the transcriptional activity of SMADs following the activation of the TGF β canonical pathway; (iv) GAM increases cell apoptosis while reducing cell proliferation, and modulates, directly or indirectly, the levels of E2F1 and Ras; and (v) GAM decreases the levels of and interacts in a RNase-dependent manner with Drosha, the main effector of miRNA maturation in the nucleus.

Using synthetic pre-miRNAs as well as antisense inhibitory RNAs for luciferase assays, qRT-PCRs and western blotting analyses, we provided strong evidence that endogenous *miR-17-92* and *let-7* miRNAs target *GAM* transcripts. Importantly, our experiments also showed that, while *miR-17*, *miR-20a* and *miR-92a-1* work together to downregulate *GAM* transcripts, *miR-92a-1* upregulates E2F1 in contrast to *miR-17* and *miR-20a*. As *miR-92a-1* is the only miRNA of the cluster with orthologs in non-vertebrates, it is possible that, following gene duplications in vertebrate lineage, evolution has kept the different *miR-17-92* miRNAs in cluster to establish a yet poorly understood mechanism of differential maturation, thus allowing for the fine tuning of their effects on their hundreds of respective target genes.

Our promoter studies confirmed the previously established activating effects of c-Myc on the *miR-17-92* cluster (10), and showed that GAM limits, directly or indirectly, c-Myc transcriptional activation of the *miR-17-92* cluster through TSS1 without affecting the levels of endogenous c-Myc, although the mechanistic bases of GAM/c-Myc interference remains to be elucidated. They revealed that the *miR-17-92* cluster constitutes a very complex transcriptional unit with regulatory sequences spanning over several thousands base pairs, from the site bound by c-Myc just upstream of the TSS2 (10) to an upstream 123-bp fragment containing *E2F1* sites 1 and 2 previously shown to allow the transcriptional activation of the cluster by E2F factors (12,13) to sequences located upstream of the TSS1 and the 5'-end of *Clu-2* (this article). In any case, it can be expected that the transcription of a cluster containing miRNAs targeting key regulators of cell proliferation would be regulated in a number of different ways. Of note, we have previously shown that chicken GAM interacts with the heterogenous nuclear ribonucleoprotein U (hnRNPU/SAF-A) (17), which is known to interact with different transcription factors, including the glucocorticoid receptor, a member of the superfamily of hormone nuclear receptors. Through this interaction, hnRNPU represses glucocorticoid-induced activation by sequestering the glucocorticoid receptors on the nuclear matrix (31). Further experiments would be required to determine whether interacting with hnRNPU might allow GAM to block the accession of c-Myc or other transcriptional regulators to the *miR-17-92* cluster promoter.

However, transcriptional regulation cannot explain why each of the *miR-17-92* miRNAs is processed differentially within a certain type of cells based on qRT-PCRs and RNase-protection assays. This form of differential

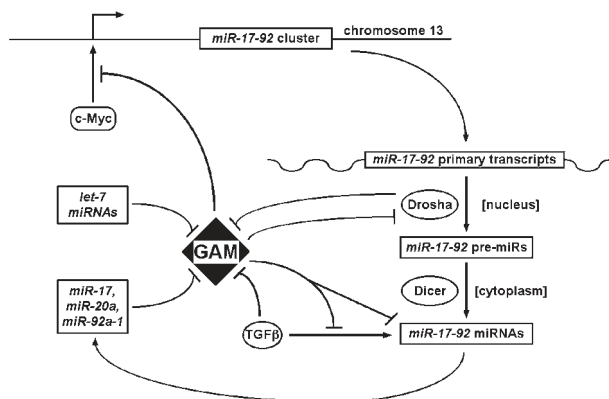


Figure 8. Working model depicting the intricate interaction loops between GAM, TGF β effectors, Drosha, *miR-17-92* and *let-7* miRNAs. For clarity, only the main interactions have been represented. Arrows indicate activating effects, and T-barred arrows inhibitory effects. The effects of c-Myc on the *miR-17-92* cluster were previously described (10). See text for more details.

expression of *miR-17-92* miRNAs seems similar to a certain degree in the different cell lines studied, which suggests that it may represent a more general phenomenon. Namely, it was previously shown that *miR-17-92* miRNA levels in mouse embryos are not uniform and change differentially according to the stage of development (32,33). Therefore, there is not much doubt that these miRNAs are processed with a differential efficiency from the *miR-17-92* primary transcripts. Interestingly, the relative levels of *miR-20a* and even more of *miR-92a* remained consistently higher than those of the miRNAs in a more 5'-position, whether cells were submitted to the different treatments, or not. One can hypothesize that the differential processing of *miR-17-92* miRNAs depends on their relative position in the cluster. Addressing this question will require to determine the levels of the different miRNAs after shuffling their respective pre-miRNA sequences within the expression constructs.

We further show that GAM and TGF β respectively downregulates and upregulates *miR-17-92* miRNAs in HEK-293, HepG2 and MCF7 cells, and that GAM also downregulates them in K562 lymphoid cells. In each case, the above effects were miRNA-specific. This suggests that GAM as well as TGF β effectors can modulate the levels of the factors which control the specific processing of each of *miR-17-92* miRNAs. To date, nothing is known about these regulators, except that hnRNPA1 is required to allow the specific processing of *miR-18a* (19,20). Nevertheless, the fact that *miR-18a* levels remained consistently low in the different cell lines, with or without treatment, indicate that these cells contain factors able to specifically bias the processing of *miR-17-92* primary RNAs toward the production of miRNAs such as *miR-17*, *miR-20a* and *miR-92a-1*. Furthermore, overexpressing the whole *miR-17-92* cluster using a high expression *pCMV* vector did not change *miR-17* expression, while increasing the levels of *miR-20a* and *miR-92a* by no more than about 30 and 60%, respectively. This suggests that the factors controlling the processing of *miR-17-92* primary transcripts by Drosha are present in limiting amounts, thus precluding the *in vitro* analysis of the consequences of GAM-Drosha interaction on the activity of a purified Microprocessor complex following *GAM* overexpression. Interestingly, GAM was previously shown to interact not only with hnRNPU/SAF-A, but also with hnRNP-M and Matrin 3, a component of the nuclear matrix (17). As hnRNPU and hnRNP-M4 have been found in the Microprocessor (34–36), and as hnRNPU has also been shown to interact with Matrin 3 (37), this raises the question of whether GAM might impair the access of Drosha to some of its target pri-miRNAs by relocating it in specific domains of the nuclear matrix.

Furthermore, while GAM downregulated both Drosha and *miR-17-92* miRNAs, TGF β signaling upregulated the same miRNAs without changing Drosha levels. Of note, the upregulation of *miR-17-92* miRNAs by TGF β may possibly help to understand how TGF β signaling can promote tumor growth and metastasis (25), given that *miR-17-92* miRNAs tend to increase cell proliferation and are usually considered as oncomiRs (5).

Furthermore, the effects of both GAM and TGF β effectors were miRNA-specific. For example, TGF β upregulated *let-7e* but did not change *let-7d* or *let-7f* levels in HepG2 cells. On the other hand, while overexpressing *GAM* or transfecting cells with *siGAM* did not affect the levels of *let-7e* or *let-7f* miRNAs, *siGAM* slightly but significantly decreased *let-7d* levels, an effect unexpectedly impaired by TGF β . Accordingly, qRT-PCR experiments in SW480 cells as well as miRNA microarrays in THP-1 monocytic cells showed that the levels of a number of miRNAs remain unchanged following transfection of these cells with either *siGAM* or a construct overexpressing *GAM*, while other miRNAs are either upregulated or downregulated (Tili *et al.*, unpublished results). Therefore, neither the downregulation of Drosha by GAM nor GAM-Drosha interaction leads to a general decrease of miRNA levels. Furthermore, neither GAM nor TGF β exert global effects on the activity of the miRNA processing machinery. In contrast, it is more likely that the miRNA-specific effects of both GAM and TGF β effectors may arise from their respective effects on the expression and/or the activity of factors controlling the processing of specific pri-miRNAs, especially that of *miR-17-92* primary transcripts. Of note, it has been previously shown that, depending on BMP or TGF β signaling, SMAD1, SMAD5 or SMAD3 can control Drosha-mediated miRNA maturation through their binding to DEAD box RNA helicase p68 (27). It would thus be very interesting to determine if SMAD2 and/or SMAD3 might similarly interfere with the processing of *miR-17-92* pri-miRNAs by Drosha under TGF β signaling, and, if this is the case, if GAM would be able to impair this interaction between SMAD2 or SMAD3 and Drosha. However, while we were able to confirm the previously established GAM-SMAD1 interaction (24), we did not find any GAM-SMAD2 or GAM-SMAD3 interaction (Tili *et al.*, unpublished results). Ultimately, elucidating how the differential processing of *miR-17-92* miRNAs is controlled and identifying their key target transcripts may be critical to understand the role of the *miR-17-92* cluster in tumor formation.

Importantly, in addition to opposing the upregulation of *miR-17-92* miRNAs by TGF β , GAM impaired the activation of TGF β responsive genes, while TGF β in turn downregulated GAM and *GAM* transcripts levels. This suggests that GAM may be a *bona fide* TGF β effector, potentially implicated in many aspects of TGF β signaling. It will thus be interesting to check whether GAM might differentially affect the cytostatic and the pro-metastatic activities of TGF β .

We also show that GAM modulates the levels of E2F1 and Ras at least in part through its repressing effects on endogenous *miR-92a-1*, while increasing cell apoptosis and reducing cell proliferation. The precise effects of GAM on such complex phenotypes will not be easy to determine. As GAM seems to regulate the levels of many miRNAs, and in particular miRNAs which potentially target hundreds of transcripts (e.g. \sim 1.000 for *miR-17* and *miR-20a*, and \sim 700 for *miR-92a-1*), GAM activity is likely to affect cell homeostasis in a number of different ways. Of note, it has also been shown that

TGF β 1 can overcome Ras mitogenic effects (38–40), and that Ras can counteract TGF β signaling by altering the expression of TGF β type II receptor (41). It would thus also be interesting to look whether GAM may play a role in these reciprocal interactions. Finally, the available public data show that *GAM* expression is higher in brain, kidney and lung tumors but lower in breast, colon, pancreas and prostate cancers, and that the expression of the *miR-17-92* cluster negatively correlates with that of *GAM* except in lung cells (<http://www.cgl.ucsf.edu/Research/genentech/gepis/index.html>). Given the above results, one can speculate that, while *GAM* has not yet been connected to a particular pathology, *GAM* misexpression may impair the proper balance between pro- and anti-proliferation factors, thus increasing the probability of tumor formation.

As a last remark, the emergence of the vertebrate lineage coincided with successive gene duplications, the apparition of new transcriptional regulators and new signaling molecules, and the acquisition of new functionalities such as a complex and more performing brain or adaptative immunity. It is thus probable that the new level of molecular complexity reached by vertebrates has been made possible by the development of robust genetic circuitries required to maintain cell homeostasis while allowing the organism to dynamically respond to an ever changing environment. Like *GAM*, TGF β as well as the *miR-17-92* cluster also appeared in vertebrates. It is therefore not surprising that they may be implicated in the control of cell homeostasis and interact with each other in so many different ways. As *GAM* transcripts are potentially targeted by ~150 miRNAs, *GAM* is likely to participate in feedback regulatory loops with many other miRNAs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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