Genome-wide Characterization of miR-34a Induced Changes in Protein and mRNA Expression by a Combined Pulsed SILAC and Microarray Analysis*§

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The gene encoding the miR-34a microRNA is a transcriptional target of the p53 tumor suppressor protein and subject to epigenetic inactivation in colorectal cancer and numerous other tumor types. Here, we combined pulsed SILAC (pSILAC) and microarray analyses to identify miR-34a-induced changes in protein and mRNA expression. pSILAC allowed to quantify the de novo protein synthesis of 1206 proteins after activation of a conditional miR-34a allele in a colorectal cancer cell line. ~19% of the detected proteins were differentially regulated, with 113 proteins being down- and 115 up-regulated. The proteins with a miR-34a seed-matching-sequence in the 3′-untranslated region (UTR) of the corresponding mRNA showed a clear bias toward translational repression. Proteins involved in DNA replication, e.g. the MCM proteins, and cell proliferation, were over-represented among directly down-regulated proteins lacking a miR-34a seed-match. The decrease in de novo protein synthesis of direct miR-34a targets correlated with reduced levels of the corresponding mRNA in most cases, indicating an interdependence of both types of regulation. In addition, 43 mRNAs encoding proteins not detected by pSILAC were down-regulated after miR-34a expression and contained miR-34a seed-matches. The direct regulation of selected miR-34a target-mRNAs was confirmed using reporter assays. Via down-regulation of the proteins encoded by these mRNAs miR-34a presumably inhibits glycolysis (LDHA), WNT-signaling (LEF1), invasion/migration (AXL) and lipid metabolism (ACSL1, ACSL4). Furthermore, miR-34a may activate p53 by inhibiting its acetylation (MTA2, HDAC1) and degradation (YY1). In summary, miR-34a presumably participates in multiple tumor suppressive pathways by directly and indirectly suppressing the expression of numerous, critical proteins. Molecular & Cellular Proteomics 10: 10.1074/mcp.M111.010462, 1–16, 2011.

The transcription factor encoded by the p53 tumor suppressor gene is activated by numerous cellular insults such as γ-irradiation or deregulated oncogene expression, which have the commonality of inducing DNA damage. Activated p53 regulates numerous genes, which mediate tumor suppressive processes as inhibition of cell-cycle progression and induction of apoptosis (1). Loss of p53 function is commonly observed during tumor development. Apart from regulating protein-coding genes p53 also controls microRNA (miRNA) encoding genes (2–3). miRNAs represent an abundant class of small ~21-nucleotide-long, noncoding RNAs involved in post-transcriptional control of gene expression. The influence of miRNAs on gene expression is predicted to be widespread, with more than 60% of human protein coding genes being subject to regulation by miRNAs (4). Among the p53-regulated miRNAs miR-34a seems to display the most pronounced induction by p53 (5–10). Ectopic expression of miR-34a induces apoptosis, senescence, cell cycle arrest and inhibits migration and invasion (2–3, 11). Therefore, miR-34a may be an important mediator of p53’s tumor-suppressive activities. Interestingly, miR-34a is silenced by CpG methylation in numerous types of tumors, among them colorectal cancer, and may therefore itself represent a tumor suppressor gene (10, 12). Several mRNAs have been shown to be direct miR-34a targets (11), which encode factors required for G1/S transition (c-MYC, E2F, CDK4, CDK6), anti-apoptotic proteins (Bcl2, SIRT1), but also proteins involved in invasion (c-MET). How-

The abbreviations used are: miRNA, microRNA; SILAC, stable isotope labeling with amino acids in cell culture; RIPA, radioimmunoprecipitation assay; HPLC, high performance liquid chromatography; ESI, electrospray ionization; MS/MS, tandem MS; TFA, trifluoroacetic acid; UTR, untranslated regions.

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ever, it is likely that miR-34a regulates additional, as yet unconfirmed targets, because bioinformatic predictions suggest that several hundred mRNAs contain matches to the miR-34a seed sequence.

MiRNAs regulate their targets via association of a 7 nucleotide stretch, the so-called seed-sequence, located in their 5'-portion with a complementary sequence in the 3' UTR of the target mRNA. Additional base pairing may occur via nucleotides in the middle and 3'-portion of the miRNA. Along with binding of the miRNA to target mRNAs the RISC/Ago2 complex is recruited to the mRNA. This complex mediates inhibition of translation initiation through interfering with elf4F-cap recognition and 40S small ribosomal subunit recruitment and/or by enhancing mRNA degradation through recruitment of the CCR4-NOT1 deadenylase complex. Because the relatively short seed-region is the primary determinant of target recognition, a single miRNA presumably regulates dozens or even hundreds of target mRNAs. However, because of the shortness of the seed-regions bioinformatic predictions of miRNA/mRNA interactions are not reliable for the identification of biologically relevant miRNA targets. Therefore, several attempts have been made to identify messenger RNAs that are subject to regulation by a specific miRNA using unbiased genome- or proteome-wide experimental approaches.

Because targeting of mRNAs through miRNAs often leads to degradation of the respective mRNA, microarray analysis of mRNA levels after ectopic expression of a miRNA can be used to identify miRNA targets. However, this approach is limited as it cannot detect miRNA targets that are solely regulated at the level of translational repression. Assuming that miRNAs in most cases only cause modest decreases in protein translation, the miRNA-mediated regulation of proteins with long half-lives may not be detected by measuring steady-state protein levels using standard proteomic quantification as SILAC (stable isotope labeling by aminos in cell culture) (17). This problem was solved by the introduction of pSILAC (pulsed SILAC), which facilitated the quantification of differences in protein translation rates caused by miRNAs (18).

Here we describe a global analysis of the effect of miR-34a expression on mRNA and protein expression using a combined microarray and pSILAC analysis. Our results indicate that miR-34a regulates numerous cellular pathways in addition to those described previously by modulating the expression of a large number of diverse proteins and mRNAs. Interestingly, almost all of the miR-34a-mediated regulations have the potential of contributing to tumor suppression.

**EXPERIMENTAL PROCEDURES**

**Generation of Cell Pools with Conditional miR-34a Expression**—The colorectal cancer cell line SW480 was kept in Dulbecco’s modified Eagle’s medium and 10% fetal calf serum (Invitrogen). SW480 cells were transfected with the episomal expression vector pRTS-miR-34a (9, 19) using Lipofectamine2000 (Invitrogen). Polyclonal cell pools were generated by selection with puromycin (2 μg/ml) for 10 days. For characterization of miR-34a targets, SW480 cells were transfected with the episomal pRTS-miR-34a expression vector. Polyclonal cell pools were generated by selection with puromycin (2 μg/ml) for 10 days. The percentage of RFP/GFP-positive cells was determined 48 h after addition of doxycycline at a final concentration of 100 ng/ml.

**Generation of Episomal Vectors for miR-34a Expression**—The pRTS vector is an improved version of the pRTS vector (Georg W. Bornkamm and Christian Berens, unpublished results). The pRTS expression vector was generated by replacing the KAR repressor domain containing Tet-trans-silencer of the pRTS (20) vector with an alternative trans-silencer containing a CtBP-recruiting PLDLS repression motif (21), and a relaxed effector specificity (22), together with an IRES-coupled puromycin-resistance gene. To generate the episomal pRTS-miR-34a vector, the pri-miR-34a cDNA sequence was excised with SfiI from pRTS-miR-34a and ligated into pRTS via the SfiI sites. The insert orientation and the miR-34a portion were verified by sequencing.

**GFP-Expression and Cell-cycle Analysis by Flow Cytometry**—Cells were seeded in six-well plates (2 × 10^5 cells/well) and cultured in the presence and absence of 100 ng/ml doxycycline, respectively. For flow cytometry, cells were trypsinized after 40 h for mRFP analysis and after 72 h for cell-cycle analysis. For the analysis of mRFP expression, cells were fixed in phosphate-buffered saline containing 3.5% (v/v) paraformaldehyde and 0.5% (v/v) Nonidet P-40 (Fluka) for 30 min on ice. Fixed cells were collected by centrifugation (200 × g, 5 min at 4 °C) and re-suspended in phosphate-buffered saline for FACS analysis.

For cell-cycle analysis cells were fixed with 70% (v/v) ethanol overnight at −20 °C, centrifuged by centrifugation (200 × g, 5 min at 4 °C) followed by a washing step in phosphate-buffered saline, and stained with 0.6 mg/ml propidium iodide (MP Biochemicals, Solon, OH) in the presence of 0.1% (w/v) Triton X-100 (Sigma) and 0.5 mg/ml RNaseA (Sigma). RNA digestion was carried out before FACS analysis at room temperature for 1 h. For DNA content analysis 10,000 cells and for detection of mRFP expression 25,000 cells per sample were analyzed with a FACS calibur device (BD Bioscience).

**Pulsed-SILAC Labeling**—An aliquot of 5 × 10^5 SW480 cells, harboring the pRTS-miR-34a vector, were seeded onto 10-cm dishes and grown in light DMEM (PAN Biotech) supplemented with light L-arginine (84 mg/l) and L-lysine (40 mg/l) and containing 10% dialyzed FBS (Hyclone, Thermo Scientific), 100 units/ml penicillin and 0.1 mg/ml streptomycin. Sixteen hours after pri-miR-34a induction with 100 ng/ml doxycycline cells were labeled by shifting them to heavy SILAC medium (84 mg/l 13C6-L-arginine and 40 mg/l 15N2-L-lysine and containing 20 ng/ml cytosine), and after 72 h for cell-cycle analysis. For the analysis of mRFP expression, cells were fixed in phosphate-buffered saline containing 3.5% (v/v) paraformaldehyde and 0.5% (v/v) Nonidet P-40 (Fluka) for 30 min on ice. Fixed cells were collected by centrifugation (200 × g, 5 min at 4 °C) and re-suspended in phosphate-buffered saline for FACS analysis.

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**Bis-Tris Gradient Gel Electrophoresis and Trypsin Digestion of Proteins**—Proteins were separated on a 4–12% NuPage Bis-Tris gradient gel (Invitrogen) according to the manufacturer’s instructions and stained with colloidal Coomassie Brilliant Blue G-250. Gel lanes were cut into 20 slices, which were immediately destained, washed, subjected to trypsin digestion, and prepared for LC/MS analysis as described previously (23).
flow reversed-phase capillary HPLC was carried out as follows: peptide mixtures were loaded onto one of two C18 µ-pre-columns (0.3 mm inner diameter × 5 mm, particle size 5 µm; PepMap, Dionex LC Packings) equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA), washed and preconcentrated for 5 min at a flow rate of 30 µl/min. The precolumn was then switched in line with a C18 RP nano LC column (75 µm inner diameter × 150 mm, particle size 5 µm; PepMap, Dionex LC Packings) and peptides were eluted using a binary solvent system consisting of 0.1% (v/v) formic acid (solvent A) and 0.1% (v/v) formic acid in 84% (v/v) acetonitrile (solvent B) with the following linear gradient: 5–40% solvent B in 150 min and 40–95% solvent B in 2 min. The column was then washed for 3 min with 95% solvent B and equilibrated with 5% solvent B (20 min) at a flow rate of 300 nl/minute. Precolumns were washed as follows: 5 min with 0.1% (v/v) TFA, 20 min with 0.1% (v/v) TFA in 50% (v/v) acetonitrile, and 10 min with 0.1% (v/v) TFA in 84% (v/v) acetonitrile before the column was re-equilibrated with 0.1% TFA.

The LTQ-Orbitrap XL instrument was equipped with a nano-electrospray ion source (Thermo Fisher Scientific) and distal coated SilicaTips (FS360–20-10-D, New Objective, Woburn, MA). The instrument was externally calibrated using standard compounds. To provide high mass accuracy, lock masses were routinely used for internal calibration. The general mass spectrometric parameters were as follows: spray voltage, 1.5 kV; capillary voltage, 4 V; capillary temperature, 200 °C; tube lens voltage, 100 V. For data-dependent MS/MS analyses, the software XCalibur 2.0 SR 2 (Thermo Fisher Scientific) was used. Full scan MS spectra (m/z 300 to 2000; resolution of 60,000, at m/z 400) were acquired in the orbitrap. Automatic gain control was set to 5 × 10^5 ions and a maximum fill time of 750 ms. After a brief survey scan, the six most intense multiply charged ions were selected for fragmentation by low energy collision-induced dissociation in the linear ion trap simultaneous with the completion of the MS scan in the orbitrap. The automatic gain control of the LTQ was set to 10,000 ions and a maximum fill time of 150 milliseconds. Fragmentation was carried out at a normalized collision energy of 35% with an activation q = 0.25 and an activation time of 30 milliseconds. The ion selection threshold was set to 5000. Fragmentation of previously selected precursor ions was dynamically excluded for the following 45 s.

**Mass Spectrometric Data Analysis**—Mass spectrometric data were processed using the software MaxQuant (version 1.0.13.13) (24). For peptide and protein identification, generated peak lists of MS/MS spectra were filtered to contain at most six peaks per 100 Da interval and correlated with the International Protein Index (25) human protein spectra. Peptide masses were filtered to contain at most six peaks per 100 Da interval. The local MaxQuant software package (26) was then switched in line with a C18 RP nano LC column (5 mm, particle size 5 µm; PepMap, Dionex LC Packings) and peptides were eluted using a binary solvent system consisting of 0.1% (v/v) formic acid (solvent A) and 0.1% (v/v) formic acid in 84% (v/v) acetonitrile (solvent B) with the following linear gradient: 5–40% solvent B in 150 min and 40–95% solvent B in 2 min. The column was then washed for 3 min with 95% solvent B and equilibrated with 5% solvent B (20 min) at a flow rate of 300 nl/minute. Precolumns were washed as follows: 5 min with 0.1% (v/v) TFA, 20 min with 0.1% (v/v) TFA in 50% (v/v) acetonitrile, and 10 min with 0.1% (v/v) TFA in 84% (v/v) acetonitrile before the column was re-equilibrated with 0.1% TFA.

Relative peptide and protein quantification by MaxQuant, based on two-dimensional centroid intensities of differentially labeled peptide species (see [24] for details), were performed automatically using the following settings: quantification was based on unique and “razor” peptides; “Re-quantify” and “Filter labeled amino acids” were enabled; low-scoring versions of identified peptides were excluded from quantification. Protein ratios reported by MaxQuant are the median of all peptide ratios assigned to a distinct protein or protein group. The variability (in %) is determined as standard deviation of the natural logarithms of all peptide ratios used to calculate the protein ratio multiplied by 100 (26). Systematic deviations such as mixing errors are corrected for by the MaxQuant algorithm by normalizing all protein ratios such that the median of all log-transformed ratios is zero (24). Raw mass spectrometric files, Mascot search results (dat/msm files), and MaxQuant output files are stored at the scientific file sharing network and data repository Tranche.

Data associated with this manuscript may be downloaded from the Tranche website www.ProteomeCommons.org using the hash: drmpxhWYi0VgjwrlcLC8v8BI25dXgU2bX1t/V1PJDJmdmvrRI3fexqMYF0GonyjWgLMG60DKG/n4eqd8H1bWEYwUAAAAAAAlw-- or will be provided on request.

**Microarray Analysis**—Expression of pri-miR-34a was induced in SW480 cells bearing the pRTS-miR-34a vector with 100 ng/µl doxycycline for 40 h. cDNA was generated from total RNA and amplified using the GeneChip WT cDNA Amplification Kit (Affymetrix, Santa Clara, CA). cDNA was labeled with the GeneChip WT terminal labeling kit and hybridized to Affymetrix Human Exon ST 1.0 arrays (Affymetrix) according to the manufacturer’s instructions. Arrays were hybridized, washed, stained, and scanned according to the manufacturer’s recommendations. Expression values were derived from CEL files using the Expression Console implemented by Affymetrix. The normalization method of choice was the robust-multichip average (RMA) provided by the software.

**Bioinformatic mir-34a Target Identification**—For bioinformatic identification of mRNAs containing miR-34a seed matches, we used a combined target prediction set generated by the TargetScan and Pictar algorithms (30–31). The data sets were obtained from http://www.targetscan.org and http://pictar.mdc-berlin.de. When using TargetScan, only phylogenetically conserved predictions were included.

**Cloning of 3’-UTRs**—The 3’-UTRs of the indicated target mRNAs containing putative miR-34a binding sites were PCR-amplified from oligo-dT-primed cDNA from SW480 cells with the Verso cDNA kit (Thermo Scientific). The 3’-UTRs were cloned into pGL3-control-MCS (32) and verified by sequencing. Seed matching sequences were mutated with the Quick Change Mutagenesis kit according to manufacturer’s instructions (Stratagene). Oligonucleotides used for cloning and mutagenesis are given in the supplementary Tables S5 and S6, respectively.

**Luciferase Assays**—H1299 cells were seeded in 12-well format at 1 × 10^4 cells/well, and transfected after 48 h with 100 ng of the indicated firefly luciferase reporter plasmid, 20 ng of Renilla reporter plasmid as a normalization control, and 25 nm of miR-34a pre-miRNA (Ambion, Austin; PM11030) or a negative control oligonucleotide (Ambion, neg. control #1). The miR-34as vector contains the exact complementary sequence of miR-34a and served as a positive control (32). Luciferase assays were carried out after 48 h with the Dual Luciferase Reporter assay system (Promega) according to manufacturer’s instructions. Fluorescence intensities were measured with a luminesimeter (Berthold) in 96-well format and analyzed with the simplicity software package (DLR).

**Western Blot Analysis**—SW480 cells harboring the pRTT-miR-34a vector were treated with 100 ng/µl doxycycline. SDS-PAGE and Western blotting were performed according to standard protocols.
Cells were lysed in RIPA buffer (50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 1% Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% sodium dodecylsulfate, complete mini protease inhibitors (Roche)). Lysates were sonicated and centrifuged at 16,060 g for 15 min at 4 °C. Per lane 40 μg of whole cell lysate was separated using 7.5% or 10% SDS-acrylamide gels and transferred on polyvinylidene difluoride membranes (Immobilon, Millipore). Antibodies used to detect the indicated proteins were: LDHA (Epitomics, 2091-1), LEF1 (Cell Signaling, C12A5), HDAC1 (Epitomics, 3426-1), MT2A (Cayman, 13778), and AXL (Cell Signaling, #4977).

Quantitative Real-Time PCR—RNA from SW480 cells was prepared with the RNaseasy Kit (Qiagen) according to manufacturer’s instructions. Oligo-dT-primed cDNA was prepared with the Verso cDNA kit (Thermo Scientific). Real-Time PCR was performed with a Light Cycler (Roche). A list of qPCR-primers is provided in supplemental Table S7.

RESULTS

Conditional miR-34a Expression—The colorectal cancer cell line SW480 was transfected with an episomal vector driving the expression of miR-34a pri-miRNA and mRFP from a bidirectional doxycycline (DOX)-inducible promoter. After selection of a hygromycin-resistant pool of cells, mRFP expression was determined by flow-cytometry (Fig. 1A). In the absence of DOX the vector containing cells displayed a minor increase in fluorescence. After treatment with DOX the fraction of positive cells increased to 73.8% with a more than 30 times higher mean fluorescence. Seventy-two hours after addition of DOX a threefold increase in the sub-G1 phase was detected, indicating the induction of apoptosis by ectopic miR-34a (Fig. 1B). This type of pro-apoptotic effect is known to result from ectopic expression of miR-34a (9). Ectopic expression of pri-miR-34a was verified by qPCR. We detected a ~300-fold increase of pri-miR-34a levels in DOX-treated cells after 72 h compared with the untreated control cells (Fig. 1C). Also the processed miR-34a displayed a pronounced increase (supplemental Fig. S1). Moreover, expression levels of the previously identified miR-34a targets CDK6 and c-MET (5, 7, 10) decreased by ~20% (CDK6) and ~40% (c-MET) after induction pri-miR-34a expression in these cells (Fig. 1D). As the ectopic expression of miR-34a resulted in the expected effects in SW480 cells, we employed these cells for pSILAC and microarray analyses.

Pulsed SILAC Analysis of miR-34a-Induced Changes in Protein Synthesis—To identify miR-34a targets, we performed pulsed SILAC (pSILAC) as outlined in Fig. 1E and described before (18, 33). In short, we induced ectopic pri-miR-34a expression for 40 h by addition of DOX. During the last 24 h the miR-34a expressing cells were pulsed with heavy (H) medium and the untreated control cells with medium-heavy (M) medium. Subsequently, protein samples were prepared and subjected to mass spectrometric analysis. These analyses were performed with biological triplicates. In total, 1818 proteins were identified, when detection of at least two matching peptides per protein was set as a requirement for unambiguous identification. Of these, 1206 proteins could be reliably quantified in at least two experiments and were therefore considered for identification and quantification of proteins and peptides see supplemental Table S1A and S1B. Remarkably, ectopic expression of miR-34a caused only modest changes in overall protein synthesis, with the majority of proteins having log2 (H/M) ratios between ~0.3 and 0.3 (Fig. 2A). Similar, overall moderate effects on global protein synthesis after ectopic miRNA expression have been reported previously (18). Among the 1206 proteins quantified in at least two experiments, 228 (~19%) were differentially regulated with log2 fold changes ≤−0.3 or ≥0.3 (+DOX versus −DOX). Among these the protein synthesis was down-regulated for 113 and up-regulated for 115 proteins (supplemental Table S1A).

Highly abundant ribosomal proteins, RNA binding proteins and metabolic enzymes were overrepresented among detected proteins, whereas proteins with generally low abundance, as transcription factors, were rarely detected (Fig. S2). Nonetheless, the fraction of bioinformatically predicted miR-34a targets among the proteins detected by pSILAC (41/1206 or approx. 3.4%) was in the range of miR-34a targets predicted based on the frequency of miR-34a seed-matching sequences present in mRNAs (623/17627 or approx. 3.5%, based on the number of annotated genes represented by the Affymetrix GeneChip Exon 1.0 ST array employed here), indicating that the quantitative proteomics approach does not per se cause a decrease in the fraction of detectable miRNA targets.

A cumulative distribution analysis of proteins with miR-34a seed-matching sequences in their respective 3′-UTRs revealed a reduced de novo protein synthesis when compared with proteins without miR-34a seed-matches (Fig. 2B). Moreover, the only miRNA binding site that was overrepresented in the 3′-UTRs of the down-regulated candidate proteins was that of miR-34a (supplemental Fig. S3). In order to determine whether the shift in protein synthesis as detected by pSILAC was dependent on the presence of predicted miR-34a binding sites in the 3′-UTRs, we performed the same analysis for proteins with predicted binding sites for the unrelated oncomiR-9 (34, 35) or the tumor suppressive miRNA let-7a (36) in their mRNAs. Both, miR-9 and let-7a have seed sequences that differ from the miR-34a sequence (Fig. 2C) and were not enriched in 3′-UTR of mRNAs targeted by miR-34a (Fig. 2D). Proteins that contain either miR-9 or let-7a binding sites in their 3′-UTRs did not show reduced protein synthesis after activation of miR-34a (Fig. 2E, F), nor was there a bias for the presence of either miR-9 or let-7a binding sites among the differentially translated proteins toward the down-regulated proteins, as detected for miR-34a (Fig. S4). These results indicate that even though the overall effects of miR-34a on global protein translation are moderate, miR-34a causes the specific translational repression of several proteins that is dependent on the presence of miR-34a binding sites in 3′-UTRs of their corresponding mRNAs.
Microarray Analysis of miR-34a Targets—Next, genome-wide mRNA expression was determined by microarray analysis 40 h after ectopic expression of pri-miR-34a in SW480 cells. Thereby, transcriptional down-regulation of 930 genes was detected, when mRNAs with a log2 fold change $\leq -0.3$ (+DOX versus −DOX) were considered (Fig. 3A, supplemental Table S2).

We analyzed whether the transcriptional changes could be ascribed specifically to expression of miR-34a. Compared with bioinformatically predicted targets of the unrelated miR-9 and let-7a miRNAs, which only showed minimal overlap with predicted miR-34a targets (Fig. 3B), 501 putative miR-34a target mRNAs with miR-34a seed-matching sequences in their 3'-UTRs were clearly biased toward lower expression levels when compared with mRNAs without miR-34a seed-matches (Figs. 3C, 3D, 3E). Therefore, the modest transcriptional down-regulation of a subset of mRNAs is specifically caused by expression of miR-34a and dependent on the presence of miR-34a seed-matching sequences in their 3'-UTR. Similar to the results obtained by pSILAC for proteins,
Identification of miR-34a Targets

**Fig. 2.** miR-34a expression results in specific changes in protein expression. A, Histogram of changes in protein expression after ectopic miR-34a expression for all 1206 proteins quantified in at least two out of three biological replicates. Translationally regulated genes with a log2 fold change $\leq -0.3$ or $\geq 0.3$ are highlighted as orange bars. Fold change denotes the ratio of peptide intensities of doxycycline-treated, heavy-labeled versus untreated, medium-heavy labeled cells. B, Cumulative distribution of miR-34a targets with $\geq 1$ seed-matching sequence in the 3'-UTR (red line) among proteins detected by pSILAC without the respective seed-matching sequence in the 3'-UTR of their mRNAs (black line). C, Sequences of the mature miR-34a, miR-9 and let-7a microRNAs. The seed sequences are highlighted in red, blue and green, respectively. D, Venn diagram of bioinformatically predicted targets of miR-34a, miR-9 and let-7a with $\geq 1$ seed-matching sequence in the 3'-UTR of the mRNAs corresponding to proteins identified by pSILAC. The total number of detected targets for each miRNA is given in brackets. E, F, Cumulative distributions of miR-9 and let-7a targets with $\geq 1$ seed-matching sequence in the 3'-UTR (blue or green lines) among proteins detected by pSILAC without the respective seed-matching sequence in the 3'-UTR of their mRNAs (black lines).
**Fig. 3. Identification of transcriptionally regulated miR-34a targets by microarray analysis.**

A. Histogram showing changes in mRNA levels after induction of ectopic pri-miR-34a expression for 40 h in SW480 cells by addition of DOX. Transcriptionally regulated genes with a log2 fold change $\leq -0.3$ or $\geq 0.3$ are highlighted as orange bars. Fold change denotes the ratio of gene expression values of DOX-treated versus untreated cells.

B. Venn diagram of the predicted target mRNAs of miR-34a, miR-9 and let-7a with $\geq 1$ seed-matching binding site in the 3'-UTR among all detected 17,326 mRNAs illustrate minimal overlap between the targets of these miRNAs. The total number of detected targets for each microRNA is given in brackets.

C, D, E. Cumulative distributions of miR-34a, miR-9 and let-7a targets with $\geq 1$ binding site in the 3'-UTR compared with mRNAs with no binding site.
the only seed-matching site that was over-represented among the down-regulated mRNAs was that corresponding to miR-34a (supplemental Fig. S5).

miR-34a Simultaneously Affects Protein Synthesis and mRNA Abundance—MiRNAs mediate the reduction of protein synthesis through inhibition of translation initiation and/or mRNA degradation. However, the relative contributions of each of these mechanisms to the down-regulation of the proteins encoded by the affected mRNAs and the order of events leading to reduced protein synthesis have not been fully resolved yet (37). To determine how miR-34a regulates expression of its target mRNAs, we compared changes in de novo protein synthesis with changes in mRNA abundance. The correlation between the changes in abundance of mRNAs and the encoded proteins was enhanced for mRNAs with miR-34a seed-matches, when compared with mRNAs lacking miR-34a seed-matches (Fig. 4A). Therefore, translational repression and mRNA abundance are presumably co-regulated for most miR-34a targets.

By combining the results generated by pSILAC and microarray analyses, we obtained a set of 39 predicted miR-34a targets that were amenable to quantification in both types of analysis (Fig. 4B). We found that the extent of translational regulation correlated with changes in abundance of the corresponding mRNA in most cases. For less pronounced regulations on the protein level exceptions to this pattern were detected, as for RRAS and SKIP.

Identification of miR-34a Targets—Next we determined which of the predicted miR-34a targets showed differential regulation either at the level of protein synthesis and/or at the level of mRNA expression as detected by pSILAC and microarray analysis, respectively. When considering proteins with a predicted miR-34a seed-matching site among the proteins detected by pSILAC, we found that 15 predicted targets were significantly down-regulated (log2 fold change <=-0.3), whereas only two predicted targets (STX17, PGM1) were translationally up-regulated with a log2 fold change >0.3 (Figs. 5A, 5B, supplemental Fig. S4A). Out of these 15 translationally down-regulated targets, eight were regulated both on the protein and mRNA level (Fig. 5A), whereas seven were mainly regulated at the level of de novo protein synthesis (with a log2 fold change <=-0.3 being the cutoff for the mRNA expression; Fig. 5B). These results indicate that the extent of regulation on the level of mRNA abundance of the miR-34a targets is high, but does not account for the effects on all target genes. At least some of the miR-34a targets, as NDRG1, ACSL1, and ABLIM1, seem to be only regulated at the level of de novo protein synthesis. In addition, we identified 43 mRNAs with miR-34a seed-matches that displayed decreased abundance, of which the corresponding proteins were not detected by pSILAC (Fig. 5C). We detected additional miR-34a targets by using the Miranda search algorithm (38), which requires less stringent seed-pairing for target prediction compared with TargetScan and Pictar (15) and thus resulted in an increased number of predicted miR-34a targets. Although the Miranda-generated set of predicted miR-34a targets contained a lower fraction of experimentally verified down-regulated genes both in the pSILAC and microarray analysis compared with a composite Target Scan/Pictar set (supplemental Fig. S6), we nevertheless could identify 132 additional, putative miR-34a targets that were down-regulated at the level of protein synthesis and/or mRNA abundance (supplemental Fig. S7, supplemental Table S4).

Pathways Affected by miR-34a Activation—A KEGG pathway analysis of the 113 proteins that were translationally down-regulated according to pSILAC revealed a striking enrichment for proteins that are involved in DNA replication, as well as a moderate enrichment for proteins involved in pyrimidine metabolism, and cell cycle regulation (Fig. 6A). Remarkably, we found almost all members of the DNA replication initiation complex (MCM2, MCM3, MCM4, MCM5, MCM6, and MCM7) to be translationally repressed. For the 115 translationally up-regulated proteins, we only detected minor enrichments of proteins involved in endocytosis as well as in several metabolic pathways (supplemental Table S3). Remarkably, some of the down-regulated proteins are common markers of cell proliferation (PCNA) or potential therapeutic targets in cancer therapy, such as RRM2 (39). The data indicate that one of the effects of miR-34a induction is an inhibition of DNA replication, either by regulation of the DNA replication machinery itself or by interference with the metabolic requirements of de novo DNA synthesis. A KEGG pathway analysis of the differentially regulated mRNAs showed that genes involved in cell cycle progression (p = 0.006) and DNA replication (p = 0.045) were slightly over-represented among the down-regulated mRNAs (log2 fold change <=-0.3, p < 0.05), further substantiating the influence of miR-34a on DNA replication initiation and cell cycle arrest (Fig. 6B). Among the up-regulated mRNAs, we found a significant increase in genes involved in the p53 signaling pathway (p = 0.011) and in apoptosis (p = 0.016; supplemental Table S3).

Confirmation of Direct Regulations by miR-34a—To validate whether the mRNAs and/or proteins down-regulated by miR-34a are direct targets of miR-34a, we performed dual reporter assays using reporter constructs containing the 3′-UTRs of representative mRNAs (Fig. 7A). Remarkably, all 12 tested reporter constructs were significantly repressed by ~20 to ~60% after cotransfection with a miR-34a mimic, whereas a control miRNA had no effect. To further validate the directness of the regulation by miR-34a, we mutated the miR-34a seed-matching sequences in six selected 3′-UTRs (Fig. 7B). Thereby, the repressive effect of miR-34a on the luciferase reporters of AXL, LEF1, MTA2, LDHA, and YY1 was reversed (Fig. 7C). In the case of TPDS2 mutation of the indicated seed-matching sequence only caused a partial resistance towards inhibition by miR-34a. This may be because of an additional weakly conserved miR-34a binding site in the 3′-UTR of TPDS2, which was not disrupted by mutation (Fig. 7B).
**Identification of miR-34a Targets**

**Fig. 4.** miR-34a-mediated changes in protein synthesis correlate with changes in mRNA abundance. **A,** Scatter plot correlating changes in protein synthesis as measured by pSILAC with changes in mRNA abundance as measured by microarray analysis. The Pearson’s correlation coefficient $R^2$ is shown for genes with predicted miR-34a seed-matching sequences compared with genes without miR-34a seed matches. **B,** Representation of 39 miR-34a targets detected both by pSILAC and microarray analysis containing miR-34a seed-matching sequences. The miR-34a targets are sorted on the basis of log2 fold changes in protein synthesis as measured by pSILAC and are represented as horizontal bars. In addition, the corresponding log2 fold changes in mRNA abundance for each miR-34a target as determined by microarray analysis are shown. The linear regression for changes in mRNA abundance is depicted as a black line. The regression coefficient $R^2$ is indicated.
YY1, which was not identified in our experimental screen, was analyzed on the basis of a bioinformatically predicted miR-34a binding site.

In addition, the expression of endogenous proteins encoded by miR-34a target genes was determined after ectopic pri-miR-34a expression (Fig. 7D). For all selected proteins a down-regulation was observed after ectopic miR-34a expression. We detected a reduction in protein levels between 24 and 72 h of miR-34a induction, albeit with different kinetics for every protein, which may be because of divergent protein half-lives or target mRNA abundance. AXL and LEF1 displayed an early reduction (i.e. after 24 h) of protein levels, whereas LDHA showed a detectable decrease only after 72 h. The histone deacetylase HDAC1 is a predicted miR-34a target that we found to be down-regulated by microarray analysis (supplemental Tables S2, S4). Although we could not unambiguously prove that HDAC1 is directly regulated by miR-34a using reporter assays, HDAC1 also displayed a robust decrease in protein levels after induction of miR-34a (Fig. 7D). At the mRNA level a decrease of all miR-34a target genes se-

Fig. 5. Identification of miR-34a targets by pSILAC and microarray analysis. A, Predicted miR-34a targets down-regulated translationally and on the mRNA level with log2 fold changes ≤−0.3. B, Predicted miR-34a targets down-regulated translationally with log2 fold changes ≤−0.3, but showing no or minor changes at the mRNA level (log2 fold changes >−0.3). C, miR-34a targets that were not detected by pSILAC, but showed down-regulation at the mRNA level with log2 fold changes ≤−0.3.
lected for analysis was detected 72 h after induction of ectopic pri-miR-34a expression (Fig. 7E).

DISCUSSION

The analyses presented here revealed numerous new direct miR-34a targets by proteomic and/or microarray analysis, which presumably represent mediators of the effects of miR-34a (summarized in Fig. 8). The directness of the regulations by miR-34a was confirmed by reporter gene assays for selected miR-34a target mRNAs. These exemplary analyses showed that the miR-34a-mediated repression of these proteins requires the presence of seed-matching sequences in the respective 3'-UTRs. On a proteome-wide scale ectopic expression of miR-34a caused moderate changes in protein translation. This is in accordance with a previous pSILAC study of miRNA-mediated changes in protein expression (18). However, the rather subtle changes in protein translation detected in this study were specific for miR-34a and in representative cases dependent on the presence on miR-34a seed-matching sequences in the 3'-UTR of the respective mRNAs. Similarly, we detected only minor, yet miR-34a-specific changes in mRNA abundances by microarray analysis.

Among the down-regulated proteins and mRNAs, we noted an overrepresentation of proteins involved in chromatin assembly (e.g. histones), DNA-replication initiation and cell-cycle regulation. Even though the down-regulation of these proteins, which do not contain predicted miR-34a binding sites in their 3'-UTRs, presumably is a secondary consequence of the direct miR-34a-mediated repression of cell cycle regulators (e.g. CDK4, CDK6, E2F, c-MYC), it reflects the inhibition of DNA replication and a cell cycle arrest as a major consequence of miR-34a activation.

Furthermore, we found that miR-34a targets exhibiting the most pronounced decrease in protein translation also tended to have the strongest decreases in mRNA levels. Even though the mechanistic connections of inhibition of protein transla-
Fig. 7. Experimental validation of miR-34a target regulation. A, Dual reporter assay in H1299 cells transfected with miR-34a mimics (pre-miR-34a) or control oligos and the indicated 3'-UTR-reporter constructs. Birc5/Survivin and EFNB1 have been identified as miR-34a targets based on the Miranda algorithm and their repression at the mRNA level (see supplemental Table S4). YY1 was analyzed on the basis of a bioinformatically predicted miR-34a binding site. Data are represented as mean ± S.D. (n = 3). B, Left, Schematic depiction of miR-34a seed-matching sequences and their targeted mutation in the 3'-UTRs of selected mRNAs. Black vertical bars: miR-34a seed-matching
Fig. 8. The p53-miR-34 pathway and its effects on multiple biological pathways. Schematic model of the regulation of and by the miR-34a miRNA. The indicated pathways are deduced from the known functions of the miR-34a targets identified in this and previous studies.

...tion and mRNA decay have not been fully elucidated, both act in concert on a large proportion of miR-34a target genes. In the following sections the putative biological consequences of the down-regulation of new, direct miR-34a targets are discussed.

The metabolic switch from oxidative phosphorylation to aerobic glycolysis, commonly referred to as the Warburg effect, is a hallmark of cancer cells (40). p53 inhibits glycolysis by at least two mechanisms (41), namely induction of TIGAR or SCO2 (42, 43) and thereby mediates tumor suppression. Here, we provide evidence that p53 may also inhibit glycolysis through activation of miR-34a, which directly represses lactate dehydrogenase (LDHA), a key enzyme required for aerobic glycolysis. LDHA converts pyruvate, the end product of aerobic glycolysis, to lactate and pharmacological or siRNA-mediated inhibition of LDHA prevents cancer cell proliferation (44).

The NAD-dependent deacetylase SIRT1, which also deacetylates p53, has been described as a miR-34a target (48). It has been proposed that p53, miR-34a and SIRT1 form a positive feed-back-loop, in which repression of SIRT1 leads to further activation of p53. Here we identified additional negative regulators of p53 as miR-34a targets: MTA2, HDAC1 and YY1. Therefore, it is tempting to speculate that miR-34a enhances p53 activity and tumor suppression by the combined down-regulation of these proteins. MTA2 and HDAC1 are components of the NURD complex, which mediates deacetylation and destabilization of the p53 protein (49). YY1, which modulates MDM2-mediated ubiquitination of p53 (50, 51), was directly repressed by miR-34a. While this manuscript was in preparation YY1 was also identified as a miR-34a target by others (52). HDAC1 is a histone deacetylase, which is required for cell cycle progression in normal and trans-
formed cells (53, 54). Interestingly, the p53 target gene p21 is repressed by HDAC1 (55, 56). Although HDAC1 may not to be a direct miR-34a target, we detected a robust decrease in both mRNA and protein levels of HDAC1 after expression of miR-34a. Thus, the inhibition of HDAC1 after induction of miR-34a may be one of several mechanisms by which miR-34a exerts its growth inhibitory effects.

AXL is a member of the TAM (Tyro-AXL-Mer) receptor tyrosine kinase (RTK) family. It was originally identified as a transforming gene in chronic myeloid leukemia (57, 58). AXL is involved in diverse aspects of tumor formation such as cell migration, proliferation and invasion (59, 60). Elevated expression of AXL has been associated with increased metastatic potential of breast cancer cells (61). Interestingly, another RTK, which confers metastatic potential, c-MET, is a direct target of miR-34a (62). Therefore, miR-34a might mediate suppression of metastasis by the combined down-regulation of several RTKs involved in invasion and metastasis.

Birc5/Survivin is an anti-apoptotic protein that is overexpressed in most human cancers because of activation by several oncogenic transcription factors, such as NFκB, STAT3, Notch and TCF4/β-catenin (63). The concomitant down-regulation of several anti-apoptotic proteins such as Survivin and Bcl2 (5) may therefore represent a mechanism that accounts for the tumor-suppressive function of miR-34a. Ephrin-B1 (EFNB1), which was identified as a miR-34a target in this study, is one of several ligands for the Ephrin-B1 receptor tyrosine kinase, which has been implicated in tissue architecture and organogenesis, as well as cancer progression (64).

Among the genes down-regulated either at the protein and/or mRNA level identified in this study that had a putative miR-34a binding site in their 3’-UTR, only CDK6, MYCN and Notch2 have previously been reported as direct miR-34a targets (7, 10, 32, 65). ACSL1 and AXL have been reported as direct miR-34a targets (66, 67) by others while this manuscript was in preparation.

Recently, the systemic treatment with miR-34a mimetics was shown to inhibit the growth of xenograft tumors in mice and the proliferation of prostate cancer stem cells (68–70). Therefore, ectopic miR-34a expression may serve as a tumor therapeutic means for human tumors in the future. A comprehensive knowledge of the targets and effects of miR-34a is of clinical relevance in order to evaluate the potential side effects of such therapies. The study presented here is an important step in the direction of obtaining a complete catalogue of miR-34a targets and will have to be extended to additional cellular systems and conditions. In the future, improvements in the sensitivity and dynamic range of proteomic and transcriptomic analysis may facilitate a more complete coverage of miRNA targets using this type of combined approach.

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