p53 downregulates Down syndrome-associated DYRK1A through miR-1246

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Several microRNAs mediate the functions of p53 family members. Here we characterize miR-1246 as a new target of this family. In response to DNA damage, p53 induces the expression of miR-1246 which, in turn, reduces the level of DYRK1A, a Down syndrome-associated protein kinase. Knockdown of p53 has the opposite effect. Overexpression of miR-1246 reduces DYRK1A levels and leads to the nuclear retention of NFATc1, a protein substrate of DYRK1A, and the induction of apoptosis, whereas a miR-1246-specific inhibitor prevented the nuclear import of NFATc1. Together, these results indicate that p53 inhibits DYRK1A expression through the induction of miR-1246.

Keywords: miR-1246; p53; DYRK1A; Down syndrome; NFATc1
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INTRODUCTION

The p53 tumour suppressor and its homologues, p63 and p73, exert their anti-tumour functions primarily by their transcriptional activity to induce the expression of protein-coding genes and microRNAs (miRNAs) responsible for apoptosis, cell growth arrest, differentiation and senescence (He *et al*, 2007; Raver-Shapira *et al*, 2007; Braun *et al*, 2008; Sachdeva *et al*, 2009; Sampath *et al*, 2009; Belyi *et al*, 2010; Su *et al*, 2010). However, their roles in animal development are distinct: p63 is more important for epithelial development (Mills *et al*, 1999); p73 is for the development of the central nervous and immune systems (Yang *et al*, 2000); and p53 is almost unnecessary for animal development (Donehower *et al*, 1992). In contrast to the many target genes identified for the p53 homologues (Stiewe, 2007), few miRNAs are known to be their targets (He *et al*, 2007), particularly specific to each of the homologues (Sampath *et al*, 2009; Su *et al*, 2010). In our initial attempt to identify possible miRNAs as targets for each homologue, we identified miR-1246 as a new

transcriptional target for the p53 family. This study shows a new p53 regulatory pathway, involving miR-1246, DYRK1A and NFAT, that is potentially important for tumorigenesis, suggesting that the p53 family might have a regulatory role in Down syndrome development.

RESULTS AND DISCUSSION

Identification of miR-1246 as a new p63 target

To identify p63-specific miRNA targets, we conducted an miRNA array analysis of RNA samples isolated from TAp63 γ or Δ N-p63 γ (also known as p40) expressed human lung carcinoma H1299 cells (Fig 1A), and found miR-1246 to be the most likely candidate target of TAp63 γ (Fig 1B; supplementary Fig S1 online). Interestingly, miR-1246 was only found in the human and ape genome, and its human gene is found on chromosome 2q31.1 (supplementary Fig S2A online). This result was confirmed by using quantitative reverse transcription (qRT)–PCR assays, as miR-1246 was markedly induced by TAp63 γ (Fig 1C), but not Δ N-p63 γ in H1299 cells (data not shown). Conversely, short-interfering RNA (siRNA) knockdown of endogenous p63 in human Hacat keratinocytes reduced the miR-1246 level (Fig 1D). Although TAp63 γ and Δ N-p63 α were knocked down by this siRNA (Fig 1D), the decrease of miR-1246 levels is probably due to the reduction of TAp63 γ , but not Δ N-p63 α , because overexpression of the latter failed to induce this miRNA (Fig 1A–C and data not shown). The genomic sequence encoding this miRNA has a highly conserved p53-responsive DNA element (p53RE) –194 bp upstream from the transcriptional initiation site (Fig 2A). To test if p63 binds to this p53RE sequence, we conducted a chromatin-associated immunoprecipitation (ChIP) assay after treating Hacat cells with etoposide using primers for the promoter or a p53RE-deficient DNA sequence. Endogenous TAp63 γ specifically bound to this miR-1246 promoter, but not to the control DNA sequence, as pulled down with 4A4 antibodies, but not nonspecific immunoglobulin G (Fig 1E). As shown in supplementary Fig S3B online, the ectopic p63 also bound to the miR-1246 promoter, but not to a control DNA location. Furthermore, we assessed luciferase expression driven by either a wild-type or a mutant-p53RE-motif-containing miR-1246 promoter (Fig 2D) in H1299 cells. TAp63 γ , but not Δ N-p63 γ , markedly induced luciferase activity when wild-type miR-1246 promoter was used (Fig 2E;

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Fig 1 | Identification of miR-1246 as a new p63 target. (A) Validation of p63 expression and activity in H1299 cells for microRNA (miRNA) array analyses in B. H1299 cells were collected for RNA and protein preparation 48 h after infection with adenoviruses encoding green fluorescent protein (GFP), GFP-p40, or GFP-TAp63 γ adenovirus (multiplicity of infection, 1000). Proteins (50 μ g per lane; the same for the following figures unless indicated) were analysed by SDS-polyacrylamide gel electrophoresis and detected by western blot analysis with the indicated antibodies. (B) Representative miRNA array data showing miR-1246 as the most likely candidate target for TAp63 γ . RNA samples prepared from panel A were subjected to miRNA microarray analysis. The complete data are shown in supplementary Fig S1 online. The level of miR-1246 was quantified on the basis of the fluorescence signal from the array and is presented in arbitrary units after it had been normalized against the signal from the GFP group (right panel). (C) Induction of mature miR-1246 by TAp63 γ . H1299 cells were collected 24 h after being transiently transfected with Flag-TAp63 γ for quantitative reverse transcription (qRT)-PCR analysis of mature miR-1246 levels and for western blot analysis with the indicated antibodies. (D) Decrease of miR-1246 by knocking down endogenous p63. Human Hacat cells were transfected with short-hairpin RNA (shRNA) specific to p63 and collected for western blot analysis with the indicated antibodies and qRT-PCR, as mentioned in C. (E) Endogenous p63 binds to miR-1246-promoter-containing wild-type p53RE, but not a control DNA sequence. Hacat cells were collected 12 h after treatment with etoposide for immunoprecipitation with the indicated antibodies, followed by PCR with primers for the miR-1246 promoter or negative control sequences. The sequences of these primers are listed in supplementary Table S1 online. Error bars represent standard deviation ($n = 3$). Ad, adenovirus; IgG, immunoglobulin G; IP, immunoprecipitation.

supplementary Fig S3A online). These results show that p63 induces the transcription of miR-1246.

miR-1246 is a target for the p53 family

To determine whether miR-1246 is a target for the p53 family, we overexpressed either p53 or p73 in H1299 cells and found that they can both induce the expression of this miRNA and p21 (Fig 2B), another p53 target (el-Deiry *et al*, 1993). In response to DNA damage caused by etoposide, miR-1246, similarly to p21, was induced in wild-type, but not mutant or null p53-containing human ovarian cancer cell lines (Fig 2C). This result was reproduced in other wild-type p53-containing human cancer cell lines (data not shown). In addition, knocking down p53 abrogated the induction of miR-1246 expression in response to DNA damage (supplementary Fig S4B online). Similarly, both p53

and TAp73 β induced the luciferase expression driven by the wild-type miR-1246 promoter (Fig 2E). Finally, p53 bound to the endogenous miR-1246 promoter, measured by ChIP assay (Fig 2F; supplementary Fig S4C online). These results indicate that miR-1246 is a new transcriptional target of the p53 family. Next, we focused on the functional effect of p53 on this miRNA.

miR-1246 targets DYRK1A on p53 activation

By using bioinformatic analysis using experimentally derived rules for miRNA target recognition (Bartel, 2009), we identified some target messenger RNAs (mRNAs) of miR-1246. One candidate mRNA with an ideal miR-1246-targeted 3'-untranslated region (3'UTR) sequence encodes DYRK1A (Fig 3A), a dual-specificity tyrosine (Y) phosphorylation-regulated kinase (Becker *et al*, 1998) that is highly expressed in patients with Down syndrome due to

Fig 2 | miR-1246 is a target for the p53 family. (A) Schematic representation of the p53RE sequence upstream from the miR-1246 gene. (B) Both p53 and Tap73β induce miR-1246 expression in cells. H1299 cells were transfected with the indicated plasmids and collected 36 h after transfection for analyses of miR-1246 levels by quantitative reverse transcription (qRT)-PCR and protein levels by western blot analysis with the indicated antibodies. MiR-1246 levels are presented as fold increase after normalization against glyceraldehyde-3-phosphate dehydrogenase mRNA. (C) DNA damage induces p53-dependent expression of miR-1246. Ovarian cancer cell lines SKOV3 (p53-null), A2780 (wild-type p53) and OV90 (mutant p53) were collected 18 h after treatment with 10 μM etoposide for western blot analysis with the indicated antibodies and qRT-PCR to detect miR-1246 levels. (D) Schematic of the pGL3 luciferase reporter constructs used. The plasmids either contain wild-type or mutant p53RE sequences of the miR-1246 promoter, as underlined. (E) Enhancement of miR-1246-promoter-driven luciferase activity by p53 family members. H1299 cells were co-transfected with the indicated plasmids and collected 48 h after transfection for assessment of luciferase activity, which was normalized against β-gal expression. (F) p53 binds to the endogenous p53RE-containing miR-1246 promoter. U2OS cells were treated with etoposide for 12 h to stimulate the endogenous p53 before chromatin-associated immunoprecipitation assays were conducted with D01 p53 antibodies and the primers listed in supplementary Table S1 online. Error bars represent standard deviation ($n = 3$). β-gal, β-galactosidase; Con, control; Eto, etoposide; IgG, immunoglobulin G; mRNA, messenger RNA; mut, mutant; p53RE, p53 responsive element; wt, wild type.

trisomy 21 (Kline *et al*, 2000). Overexpression of miR-1246, but not of control miRNA, in H1299 cells using the pSIR-miRNA vector reduced DYRK1A levels, as indicated by western blot analysis (Fig 3B). We then co-transfected p53-null human colon cancer HCT116 cells with the pSIF-miR-1246 expression vector and a luciferase reporter plasmid that carried either a wild-type or a mutant 3'UTR sequence derived from the DYRK1A mRNA (Fig 3C), and measured luciferase activity. MiR-1246, but no control miRNA, reduced the luciferase activity when only the wild-type 3'UTR sequence-luciferase reporter was used (Fig 3D). These results indicate that miR-1246 targets the 3'UTR sequence of the DYRK1A mRNA, reducing DYRK1A protein levels.

Consistent with the above results, siRNA knockdown of p53 in U2OS and HCT116 cells reduced the level of endogenous miR-1246, but increased the level of DYRK1A (Fig 3E; supplementary Fig S4A online). Etoposide induced p53 and miR-1246 (Figs 2C and 3F), but reduced DYRK1A levels (Fig 3F) and luciferase activity from a reporter plasmid with the wild-type, but not the mutant, miR-1246-targeted 3'UTR sequence of the DYRK1A mRNA (Fig 3G). The levels of p53 and miR-1246 were inversely proportional to those of DYRK1A in several human cancerous cells, lines tested (supplementary Fig S6 online), except MCF7 cells, in which p53 was less active due to highly expressed MDM2 and MDMX (Midgley & Lane, 1997). Next, we introduced either

Fig 3 | miR-1246 targets Down syndrome-associated DYRK1A in response to p53 activation. (A) Bioinformatic analysis shows the miR-1246-targeted 3'-untranslated region (3'UTR) sequence of DYRK1A mRNA. (B) Overexpression of miR-1246 decreases the level of DYRK1A protein in cells. H1299 cells were collected 36 h after transfection with the indicated plasmids for western blot analysis and quantitative reverse transcription (qRT)-PCR. (C) Schematic of the pMIR-DYRK1A luciferase reporter constructs used, which contain either a wild-type or a mutant miR-1246 target site derived from the DYRK1A mRNA. (D) Overexpression of miR-1246 specifically inhibits luciferase activity from the plasmid harbouring a wild-type, but not a mutant, targeting sequence. HCT116 $p53^{-/-}$ cells were co-transfected with the indicated plasmids and collected 48 h after transfection for luciferase assay. Luciferase activity was measured and normalized against β -gal expression. (E) Knockdown of p53 increases DYRK1A levels. U2OS or HCT116 cells were transfected with short-interfering RNA specific for p53 and collected for detection of miR-1246 using qRT-PCR and for DYRK1A and p53 protein levels by western blot analysis. Data for HCT116 are shown in supplementary Fig S4A online. The intensity of p53 and DYRK1A bands in western blot analysis were compared with that of β -actin and are presented in the middle graph of the panel. (F) Etoposide induced p53, but reduced DYRK1A levels in cells. U2OS cells were collected 18 h after treatment with 10 μ M etoposide. Whole-cell lysates were prepared for qRT-PCR and western blot analysis with the indicated antibodies. # indicates the left over signal of p53. (G) Etoposide reduces wild-type pmiR-DYRK1A luciferase activity. U2OS cells were co-transfected with the indicated plasmids and, 24 h after transfection, treated with dimethylsulphoxide (DMSO) or 10 μ M etoposide (Eto) for a further 18 h, and collected for luciferase assays; β -gal activity was used as an internal control. Error bars represent standard deviation ($n=3$). * P -value <0.01 . β -gal, β -galactosidase; mRNA, messenger RNA; MUT, mutant; WT, wild type.

an inhibitor specific to this miRNA or a control inhibitor into p53-containing human lung carcinoma A549 cells, and then treated the cells with etoposide followed by western blot analysis and qRT-PCR. Again, etoposide treatment induced p53 and reduced the level of DYRK1A (Fig 4A). By contrast, the miR-1246 inhibitor, but not its control, markedly increased the level of DYRK1A, regardless of the presence of etoposide (Fig 4A). This effect was specific to miR-1246 (right panel of Fig 4A), but not p21 (left panel of Fig 4A). These results indicate that DNA damage signals induce p53-dependent expression of miR-1246, which represses the expression of DYRK1A by targeting its 3'UTR RNA sequence.

miR-1246 inhibits DYRK1A activity

DYRK1A has been shown to phosphorylate NFATc1 (Arron *et al*, 2006; Gwack *et al*, 2006), a transcriptional factor that is essential for immune regulation and organ development (Shaw *et al*, 1988; Xanthoudakis *et al*, 1996) and linked to Down syndrome and cancer (Ryeom *et al*, 2008; Baek *et al*, 2009; Wu *et al*, 2010), and to suppress its functions by promoting its nuclear export (Arron *et al*, 2006; Gwack *et al*, 2006). MiR-199b, a transcriptional target of NFAT, was shown to target DYRK1A, thus forming a negative feedback loop between NFAT and DYRK1A (da Costa Martins *et al*, 2010). We aimed to determine whether miR-1246 affects this activity of DYRK1A by establishing a stable green fluorescent

Fig 4 | Silencing miR-1246 increases DYRK1A levels and activity. (A) Knockdown of miR-1246 by siRNA increases the DYRK1A protein level in A549 cells. A549 cells were transfected with the indicated oligos twice every second day. Six hours after the second transfection, cells were treated with 10 μ M etoposide or dimethylsulphoxide for 18 h and collected for western blot analysis (left panel) and qRT-PCR to detect miR-1246 levels after transfection with a miR-1246 inhibitor (right panel). (B) Overexpression of miR-1246 decreases DYRK1A protein levels in cells. Cells were transfected with miR-1246 mimic twice every second day and collected 24 h after the second transfection for western blot analysis. (C) Overexpression or knockdown of miR-1246 affects the nuclear import or export of NFATc1 through DYRK1A. U2OS cells stably expressing GFP-NFATc1 protein were transfected with miR-1246 mimic, its control mimic, the miR-1246 inhibitor or its negative control oligos. Transfected cells were treated with thapsigargin (TG) and cyclosporin A (CsA), as described in the Methods section. The quantification is shown in the right panel. (D) miR-1246 induces NFATc1 transcriptional activity. H1299 cells were co-transfected with the indicated pGL3-NFAT luciferase, and β -gal plasmids and collected 24 h after transfection for luciferase assay. Luciferase activity was normalized to β -gal and data are presented as mean $\pm$ standard error, $n = 3$. (E) miR-1246 induces apoptosis. A549 cells were double-transfected with miR-1246 or its control mimic. Cells were analysed by fluorescence-activated cell sorting 24 h after transfection. For FACS data, red indicates cell-cycle cells and green denotes sub-G1 apoptotic cells. The percentages of G1, G2, S, and sub-G1-phase cells are shown in the right panel. Error bars represent the results from two individual experiments. (F) Model of the DNA damage-p53-miR-1246-DYRK1A-NFATc1 pathway. Arrows on the right side of each term indicate the up or downregulation of a protein, miRNA or activity. β -gal, β -galactosidase; Con, control; Eto, etoposide; inhib, inhibitor; mim, mimic; siRNA, short-interfering RNA.

protein (GFP)-NFATc1 expressing U2OS cell line (Arron *et al*, 2006) and transfecting the cells with miR-1246 mimic, its control mimic, the miR-1246 inhibitor or its negative control inhibitor, as shown in Fig 4A. Cells were then treated with 1 μ M thapsigargin to deplete cellular Ca^{2+} by inhibiting the sarcoplasmic/endoplasmic reticulum ATPase and consequently activate the calmodulin-

dependent phosphatase calcineurin, which in turn dephosphorylates NFATc1 and promotes its nuclear import (Beals *et al*, 1997). After 60 min, cells were treated with 20 μ g/ml cyclosporin A (CsA), which inactivates calcineurin and thus reverses the phosphorylation status of NFATc1, endorsing its nuclear export (Nair *et al*, 1994). Transfected live cells were observed, their images were

taken under a fluorescence microscope and part of the cells was used for western blot analysis. Consistent with previous reports (Beals *et al*, 1997; Arron *et al*, 2006), GFP–NFATc1 showed a nuclear import followed by a nuclear export when cells were treated with thapsigargin followed by CsA (Fig 4C). However, introduction into the cells of miR-1246 mimics that reduced the level of endogenous DYRK1A (Fig 4B), but not its control mimic (Fig 4C), prevented the nuclear export of GFP–NFATc1 (Fig 4C). Conversely, overexpression of the miR-1246 inhibitor, but not its negative control (Fig 4C), blocked the nuclear import of NFATc1 by reducing miR-1246 and increasing DYRK1A levels (Fig 4A). In miR-1246 mimics and inhibitors, more than 80% of GFP–NFATc1, expressing cells showed nuclear retention or exclusion of NFATc1, as shown in Fig 4C. Additionally, overexpression of miR-1246 enhanced the NFATc1 transcriptional activity (Fig 4D) to produce interleukin-2 (supplementary Fig S5 online), whereas the miR-1246 inhibitor suppressed this activity (Fig 4D). Moreover, as DYRK1A was shown to suppress apoptosis (Laguna *et al*, 2008), overexpression of miR-1246 mimic, but not its control mimic, dramatically induced apoptosis, indicated by the increase of sub-G1 cells (Fig 4E). These results indicate that miR-1246 inactivates DYRK1A by repressing its expression.

In summary, we identify miR-1246 as a new transcriptional target for the p53 family (Figs 1,2). This miRNA downregulates the expression of DYRK1A by targeting its 3'UTR mRNA sequence, consequently activating NFAT1c and inducing apoptosis (Figs 3,4). Our study shows that p53 can repress the expression of DYRK1A through induction of miR-1246 and identifies a new downstream effector for this tumour suppressor (Fig 4F). Similarly, p63 and p73 also induced the expression of miR-1246 (Figs 1,2; supplementary Fig S3C online). Previous studies showed that DYRK1A can inactivate NFATc1 by phosphorylating it and excluding it from the nucleus (Arron *et al*, 2006; Gwack *et al*, 2006), whereas NFATc1 was recently shown to be required for the anti-cancer function of p53 in an animal model (Wu *et al*, 2010) and to be downregulated in skin cancers (Wu *et al*, 2010). Moreover, DYRK1A was shown to have anti-apoptotic activity (Laguna *et al*, 2008). Predictably, suppression of either NFATc1 activity or apoptosis by DYRK1A impairs p53 function. Hence, it is logical that p53 uses miR-1246 to deactivate DYRK1A (Fig 4F).

As DYRK1A is a key causative factor in the development of Down syndrome (Guimera *et al*, 1996; Song *et al*, 1996), our study not only identifies a possible new role of the p53 family in Down syndrome development through miR-1246, but also offers a native and useful molecule for the development of a therapeutic anti-Down-syndrome agent, which can be tested in a DYRK1A transgenic mouse model (Fernandez *et al*, 2007), as the miR-1246-targeted 3'UTR sequences derived from human and mouse DYRK1A mRNAs are identical (supplementary Fig S2B online).

METHODS

Cell lines. Unless indicated, all cells (H1299, A549, U2Os, HCT116) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 0.1 mg/ml streptomycin at 37 °C in 5% CO₂. SKOV3, A2780 and OV90 were obtained from Daniela E. Matei (IUSM) and cultured in DGEM (DMEM/M199 = 1:1), supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 0.1 mg/ml streptomycin at 37 °C in 5% CO₂. Human Hacat keratinocytes

were obtained from Dan F. Spandau (Indiana University School of Medicine).

ChIP–PCR. ChIP analysis was performed as described previously (Zeng *et al*, 2002) using p53 (DO-1) antibodies for exogenous p53 and Flag antibodies for overexpressed p63. Immunoprecipitated DNA fragments were analysed by semiquantitative and/or real-time PCR amplification using primers for miR-1246 and control genes. The primers are listed in supplementary Table S1 online.

Nuclear localization of NFATc1. U2OS cells, stably expressing GFP–NFATc1 (U2OS–NFATc1), were generated in the laboratory. Cells were transfected with miR-1246 mimic, inhibitor or negative control, as described as above. 36 hours after transfection, cells were treated with thapsigargin at a final concentration of 1 µM, images were taken under a Zeiss fluorescence microscope at the indicated times. 60 min after thapsigargin treatment, cells were washed twice with medium, followed by addition of CsA (final concentration 20 µg/ml) to the medium. Images of live cells were taken at the indicated time points. The result in Fig 4C was observed in more than 80% of GFP–NFATc1-expression cells.

See the supplementary information online for plasmids, adenovirus, antibodies, chemicals, RT–PCR, real-time qPCR, transient transfection, immunoprecipitation, western blot, knock-down of endogenous miRNAs and p53, flow cytometry and luciferase reporter assays.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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