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miR-24–mediated downregulation of H2AX suppresses DNA repair in terminally differentiated blood cells

Ashish Lal[1,4], Yunfeng Pan[2,4], Francisco Navarro[1,4], Derek M Dykxhoorn[1], Lisa Moreau[2], Eti Meire[3], Zvi Bentwich[3], Judy Lieberman[1] & Dipanjan Chowdhury[2]

Terminally differentiated cells have a reduced capacity to repair double-stranded breaks, but the molecular mechanism behind this downregulation is unclear. Here we find that miR-24 is upregulated during postmitotic differentiation of hematopoietic cell lines and regulates the histone variant H2AX, a protein that has a key role in the double-stranded break response. We show that the H2AX 3′ untranslated region contains conserved miR-24 binding sites that are indeed regulated by miR-24. During terminal differentiation, both H2AX mRNA and protein levels are substantially reduced by miR-24 upregulation in *in vitro* differentiated cells; similar diminished levels are found in primary human blood cells. miR-24–mediated suppression of H2AX renders cells hypersensitive to γ-irradiation and genotoxic drugs, a phenotype that is fully rescued by overexpression of miR-24–insensitive H2AX. Therefore, miR-24 upregulation in postreplicative cells reduces H2AX and makes them vulnerable to DNA damage.

Once a cell has terminally differentiated and no longer replicates its DNA, its need to repair DNA damage is reduced. Although ongoing DNA damage from oxidative metabolism and exogenous agents may be similar in dividing and nondividing cells, endogenous double-stranded breaks (DSBs) that occur during DNA replication and compromise genomic integrity are radically reduced or absent, and the danger of propagating damaged chromatin in progeny cells is minimized once a cell has stopped dividing. Nonetheless, cells that do not divide need to maintain the integrity of the genes they transcribe. For some long-lived and essentially irreplaceable cells, such as neurons, DNA repair may be more essential than for short-lived cells, such as terminally differentiated blood cells. Dividing cells handle the risk of creating DSBs during DNA replication by expressing and activating the homologous recombination repair machinery in a cell cycle–dependent fashion only during S phase. Moreover, during cell division, DNA-damage checkpoint proteins survey for unrepaired DNA damage to prevent cell-cycle progression at G1-S and G2-M. As a consequence of their reduced needs for DNA repair, nondividing cells have an attenuated DSB response[1].

The molecular mechanisms behind the downregulation of DNA repair in terminally differentiated cells are not well understood. In some cases, specific repair proteins are downregulated. For instance, Chek1, the orchestrator of cell-cycle arrest in response to replication-mediated DNA damage in proliferating cells, is not detected in terminally differentiated tissues[2]. Likewise, E2F1 and p53 expression are downregulated in terminally differentiated myotubes[3,4]. mRNA for Ku, the DNA binding proteins of the DNA-dependent protein kinase, which has a central role in DSB repair by nonhomologous end joining (NHEJ), decreases during differentiation of HL-60 cells into monocytes[5]. However, other repair pathways besides DSB repair, such as base excision repair (BER) and transcription-coupled repair, which repair lesions of equal importance in nondividing and dividing cells, may be undiminished after terminal differentiation.

MicroRNAs (miRNAs) are abundant small (~20–22 nt) non-coding RNAs that mediate sequence-specific post-transcriptional gene expression[6–8]. Bioinformatic studies predict that more than 30% of all human genes are targeted by miRNAs[9] and they affect a diverse array of biological processes, including development, differentiation, apoptosis and proliferation. Here we have investigated a connection between miRNAs and DNA repair. We found that expression of miR-24 and miRNAs that are clustered with it (members of the miR-23 and miR-27 families) are consistently upregulated during the terminal differentiation of two multipotent hematopoietic cell lines into multiple lineages.

The mRNA encoding histone variant H2AX, a key DSB repair protein, is a predicted target of miR-24. One of the earliest events in the DSB response is phosphorylation of H2AX at Ser139 by members of the phosphatidylinositol-3 kinase–like family of kinases[10]. Phosphorylated H2AX (termed γ-H2AX) participates in DNA repair, replication, recombination and regulation of the cell cycle[10]. The large domains of γ-H2AX generated at each DSB can be visualized by immunostaining as nuclear foci. γ-H2AX foci bind and retain an array of cell-cycle and DNA repair factors (cohesins, MDC1, Mre11, BRCA1, 53BP1) at the break site[11,12]. Notably, loss of a single H2AX allele compromises genomic integrity and enhances cancer susceptibility in mice[13,14]. This observation has both clinical and mechanistic

[1]Immune Disease Institute and Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, USA. [2]Dana-Farber Cancer Institute and Department of Radiation Oncology, Harvard Medical School, Boston, Massachusetts, USA. [3]Rosetta Genomics, Rehovot, Israel. [4]These authors contributed equally to this work. Correspondence should be addressed to J.L. (lieberman@idi.harvard.edu) or D.C. (dipanjan_chowdhury@dfci.harvard.edu).

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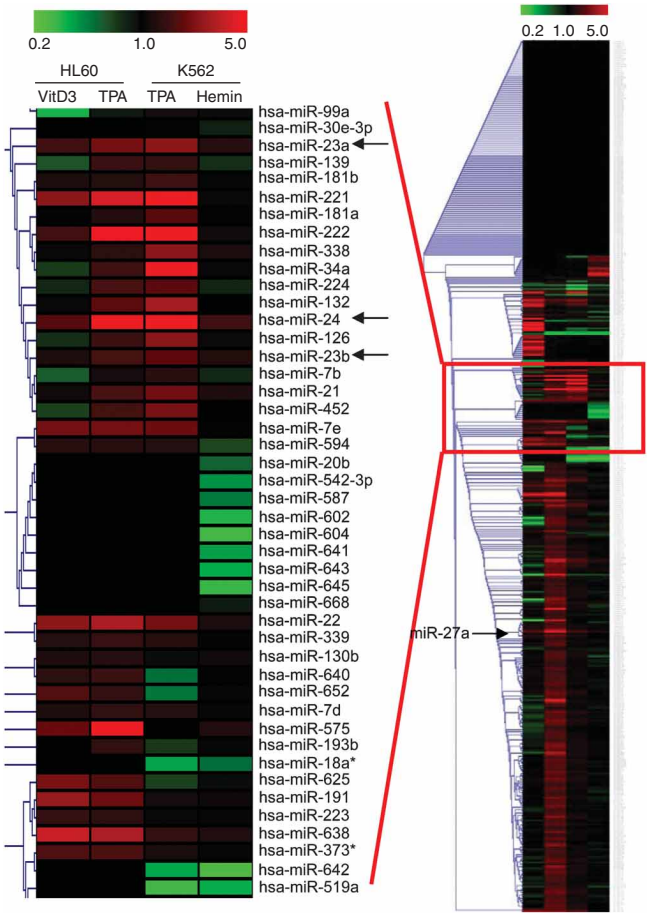

Figure 1 miR-24 is upregulated during hematopoietic cell differentiation into multiple lineages. Heat map for miRNA expression in HL60 and K562 cells differentiated into four different nondividing cell lineages, showing single-linkage hierarchical clustering, using Pearson squared as a distance metric. miRNA expression in each lane is analyzed relative to expression in control undifferentiated cells. The highlighted cluster shows miRNAs with similar expression profiles. Range is from ≤five-fold downregulation (green) to ≥five-fold upregulation (red). Arrows indicate miR-24 cluster miRNAs.

implications. The H2AX dosage effect may reflect its structural role in chromatin. H2AX constitutes ~15% of cellular H2A, and there are two H2A molecules per nucleosome. Thus, H2AX should be present, on average, in about one in three nucleosomes; this density is likely to be reduced in cells with less H2AX, resulting in disruption of H2AX function. Therefore, a subtle change in cellular H2AX, as might occur with miRNA targeting, may have an impact on DSB repair. Because of the crucial role of H2AX in DNA repair and the known consequences of haploinsufficiency, we focused on validating and studying the effect of miR-24 on H2AX in human cell lines.

RESULTS

miR-24 is upregulated in differentiated blood cells

To identify miRNAs regulating DNA repair during terminal hematopoietic cell differentiation, we analyzed miRNA expression by microarray in two human leukemia cell lines—K562 cells differentiated to megakaryocytes using 12-O-tetradecanoylphorbol-13-acetate (TPA) or to erythrocytes with hemin, and HL60 cells differentiated to macrophages using TPA or to monocytes using vitamin D3 (**Fig. 1**). Only a few miRNAs were consistently upregulated (by at least 40%) in all four systems of terminal differentiation: miR-22, miR-125a and members of the two miR-24 clusters—miR-24, miR-23a, miR-23b and miR-27a. miR-24 stood out as the most upregulated miRNA. The only member of the two miR-24 clusters that was not consistently upregulated was miR-27b, whose hybridization signal was substantially lower for all conditions than the other cluster members, suggesting that hybridization to that probe was inefficient. We therefore focused our study on miR-24, which we hypothesized might regulate terminal differentiation in nondividing cells across multiple cell lineages.

We verified the microarray results by quantitative reverse-transcription PCR (qRT-PCR). miR-24 was consistently upregulated during terminal differentiation of HL60 and K562 cells (**Fig. 2a,b**) and in differentiation of CD8 T cells, muscle cells and embryonic stem cells[15–17]. One of the biggest challenges in studying miRNAs is to identify target genes and correlate their downregulation with cellular properties. Computational algorithms have been developed to predict putative miRNA targets based on complementarity to the 3′ untranslated region (UTR) of the target message, particularly of miRNA nucleotides 2–8 (the 'seed' region)[18]. These tools (TargetScan, PicTar, rna22, miRanda) predict overlapping, but distinct, miR-24 target gene sets[18]. One strategy to counter this problem is to pursue targets predicted by multiple algorithms, and with a high prediction score. The DSB repair gene predicted by all algorithms with a high recognition score was *H2AFX*, encoding histone variant H2AX.

H2AX expression is downregulated during differentiation

H2AX mRNA and protein levels declined during K562 and HL60 cell differentiation (**Fig. 2c–e**). The H2AX transcript can be processed alternatively to a ~1.6-kb replication-independent transcript with a poly(A) tail or a ~0.6-kb transcript found only in dividing cells, which has a short 3′ UTR and lacks a poly(A) tail[19]. The shorter transcript, whose sequence is not annotated, might lack miR-24 recognition sites, because the H2AX transcript without the 3′ UTR is 505 bases long, leaving only about 100 bp for the 3′ UTR. This H2AX transcript containing a shorter 3′ UTR and expressed only in dividing cells could be an example of the recently described principle of preferential miRNA regulation of longer transcripts in nondividing cells[20].

By qRT-PCR, using primers from the H2AX coding region that measure both transcripts, we found a four-fold reduction in H2AX mRNA in TPA-treated K562 cells (**Supplementary Fig. 1a** online). Using primers specific for the longer transcript, H2AX mRNA declined by ~two-fold when K562 cells were differentiated by TPA to megakaryocytes or by hemin to erythrocytes, and when HL60 cells were differentiated by TPA to macrophages or by DMSO to granulocytes (**Fig. 2c,d**). The level of H2AX protein, measured after TPA induction, dropped by 14-fold in K562 cells and 4-fold in HL60 cells (**Fig. 2e**). The strong decrease in H2AX protein levels (relative to the modest decrease in H2AX mRNA level) during differentiation may be attributed to miR-24–mediated translational inhibition of the residual H2AX transcripts. We first detected increased miR-24 and reduced H2AX mRNA levels in TPA-differentiated K562 and HL60 cells 12 h after adding TPA, at which time the cells had stopped dividing (**Supplementary Fig. 1b–d**). The relatively high miR-24 and low H2AX mRNA and proteins levels in *in vitro* differentiated cells were comparable to levels in primary human peripheral blood monocytes and granulocytes (**Supplementary Fig. 2** online). The reduction in H2AX mRNA coincident with increased miR-24 in differentiated cell

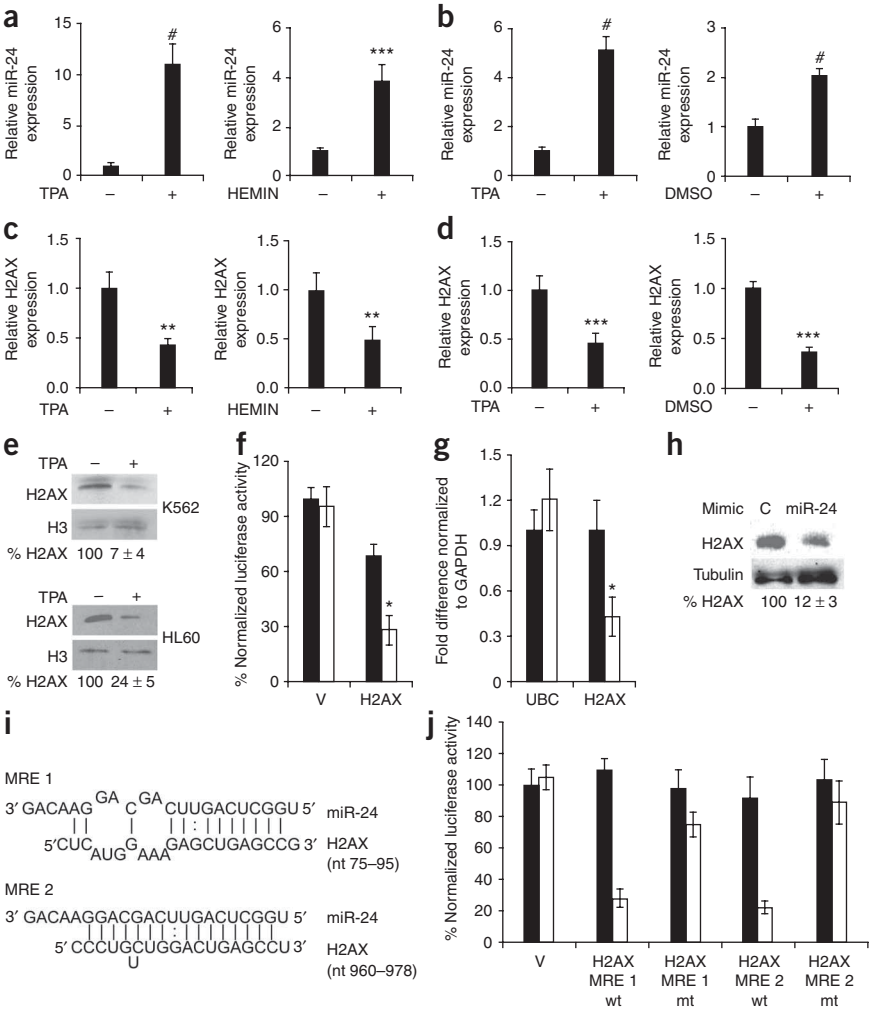

Figure 2 miR-24 downregulates H2AX expression during terminal differentiation. miR-24 levels, analyzed by qRT-PCR relative to U6, increase during differentiation of K562 cells (**a**) with TPA to megakaryocytes or hemin to erythrocytes (#, $P < 0.001$; ***, $P < 0.005$) and during differentiation of HL60 cells (**b**) with TPA to macrophages or DMSO to granulocytes (#, $P < 0.001$). Under the same differentiating conditions for K562 (**c**) and HL60 (**d**) cells, H2AX mRNA, normalized to GAPDH mRNA, is downregulated (**, $P < 0.01$, K562; ***, $P < 0.005$, HL60). (**e**) The H2AX protein level decreases after 2 d of TPA differentiation. Relative H2AX expression was quantified by densitometry using H3 as control. Lanes marked by '–' represent cells treated with vehicle alone. (**f**) miR-24 targets the 3′ UTR of H2AX mRNA in a luciferase reporter assay. HepG2 cells were transfected with control miRNA (black) or synthetic miR-24 (white) for 48 h and then with H2AX 3′ UTR-luciferase reporter (H2AX) or vector (V) for 24 h. Mean ± s.d., normalized to vector control, of three independent experiments is shown (*, $P < 0.001$). (**g**) miR-24 overexpression in HepG2 cells decreases H2AX mRNA, as analyzed by qRT-PCR normalized to GAPDH (white, miR-24; black, cel-miR-67) and protein (**h**) 48 h later. miR-24 overexpression does not alter UBC mRNA levels. (**i**) Schematic showing the predicted miR-24 binding sites (MRE) in the 3′ UTR of H2AX mRNA. (**j**) Suppression of the luciferase activity of a reporter gene containing in its 3′ UTR the two predicted miR-24 MRE, either wild-type (wt) or with mutated seed regions (mt). HepG2 cells were transfected with control miRNA (black) or miR-24 mimic (white) for 48 h and then with the indicated H2AX 3′ UTR-luciferase reporters or vector (V). Luciferase activity was assayed 24 h later. Mean ± s.d., normalized to vector control, of three independent experiments is shown. In all panels, mean ± s.d. is shown.

lines and primary blood cells could be due to miR-24 inhibition of H2AX mRNA expression and/or stability.

H2AX transcripts are targeted by miR-24

To verify that H2AX expression is regulated by miR-24, we tested the effect of miR-24 on luciferase expression from control or full-length H2AX 3′ UTR–containing reporter genes in hepatoma cell line HepG2. Luciferase activity was reduced more than two-fold by miR-24 expression (**Fig. 2f**). miR-24 overexpression in HepG2 cells also decreased the H2AX mRNA level by two-fold, whereas protein expression was reduced even more (approximately eight-fold) (**Fig. 2g**,**h**).

H2AX is a predicted miR-24 target by both TargetScan 4.2 and PicTar. Its 3′ UTR, which is 1,086 nucleotides long, encodes two evolutionarily conserved heptamer exact matches to the miR-24 seed, at positions 88–94 and 971–977, and each site has additional pairings to the 3′ region of miR-24 (**Fig. 2i**). PicTar predicts another conserved miRNA interaction (miR-328) with the H2AX 3′ UTR. To identify the miR-24 miRNA recognition elements (MRE) in the H2AX 3′ UTR, we inserted each of the predicted miR-24 MREs, as well as MREs with a mutated seed region, into the 3′ UTR of luciferase reporter genes. Luciferase activity was reduced approximately four-fold when either of the wild-type miR-24 MREs was inserted, but the mutated MREs (**Supplementary Table 2** online) had little effect (**Fig. 2j**). Therefore, miR-24 regulates H2AX expression by binding to the two sites predicted by TargetScan and Pictar. Although MRE2 would be found only in the longer H2AX transcript, MRE1 could potentially be present in both transcripts. The shorter transcript will need to be cloned to determine whether this is the case. Overexpressing miR-328, which is predicted (by PicTar) to target the 3′ UTR of H2AX, had no effect on luciferase activity or H2AX mRNA or protein levels, further underlining the specificity of the miR-24–H2AX interaction (**Supplementary Fig. 3a**,**b** online). Collectively, these results strongly suggest that miR-24 binds to the 3′ UTR of H2AX mRNA and downregulates its expression, probably by promoting both mRNA decay and inhibiting translation.

miR-24 affects DNA repair by regulating H2AX expression

To determine whether miR-24–mediated H2AX downregulation affects DSB repair, we first evaluated the most serious consequence of unrepaired DSBs, chromosomal instability, in K562 cells transfected with miR-24 or mock transfected. The transfection conditions were chosen to achieve a level of H2AX knockdown similar to what is observed during TPA differentiation (**Fig. 3a**). Metaphase spreads were prepared 24 h after low-dose γ-irradiation (**Supplementary Fig. 4** online). K562 cells overexpressing miR-24 had twice as many

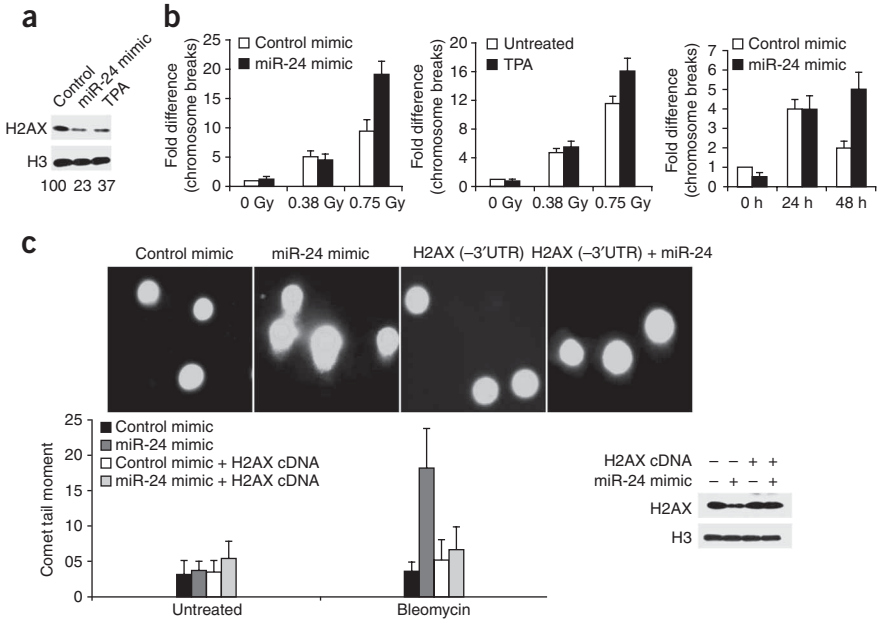

Figure 3 miR-24 expression impedes DSB repair and induces chromosomal instability of γ-irradiated K562 cells. (**a**) Transfection of miR-24 mimic into K562 cells reduces H2AX comparably to differentiation using TPA. H2AX was quantified relative to H3 protein by densitometry. (**b**) Chromosomal breaks were quantified 24 h after irradiation of K562 cells that were either undifferentiated or had been differentiated with TPA (middle) or transfected with miR-24 (left). Right, differences in chromosome breaks that were not present 24 h following exposure to 0.375 Gy of radiation were significantly different 48 h after irradiation in miR-24 versus mock-transfected cells. The mean ± s.d. of the number of chromosomal breaks and fragments per cell of three independent experiments normalized to the control is plotted. (**c**) Overexpressing miR-24 increases unrepaired DSBs, as measured by comet assay. K562 cells, transfected with expression plasmids encoding an miR-24 mimic and/or miR-24–insensitive H2AX were treated or not with bleomycin (0.5 μg ml^{-1}) for 12 h and analyzed by single-cell gel electrophoresis (comet assay) 12 h later. The H2AX protein level is compared to the H3 level in the immunoblot. H2AX levels, reduced by the miR-24 mimic, are rescued by the H2AX expression plasmid. Representative images from bleomycin-treated cells are shown above and the mean ± s.d. comet tail moment of three independent experiments for each condition below. Manipulating miR-24 or H2AX levels does not affect baseline DNA damage, but DNA damage after irradiation is significantly increased ($P < 0.001$) in miR-24 mimic–transfected cells, but only in the absence of H2AX rescue.

chromosomal breaks and fragments as control cells after exposure to 0.75 grays (Gy) of radiation ($P < 0.001$; **Fig. 3b**, left). Similarly, TPA-differentiated K562 cells were significantly more sensitive to 0.75 Gy than undifferentiated cells ($P < 0.003$; **Fig. 3b**, middle). Although there was not a significantly higher number of break 24 h after exposure to a lower dose of radiation (0.38 Gy), on the following day we saw more chromosomal instability at the same dose in miR-24–transfected cells (**Fig. 3b**, right). Undifferentiated and

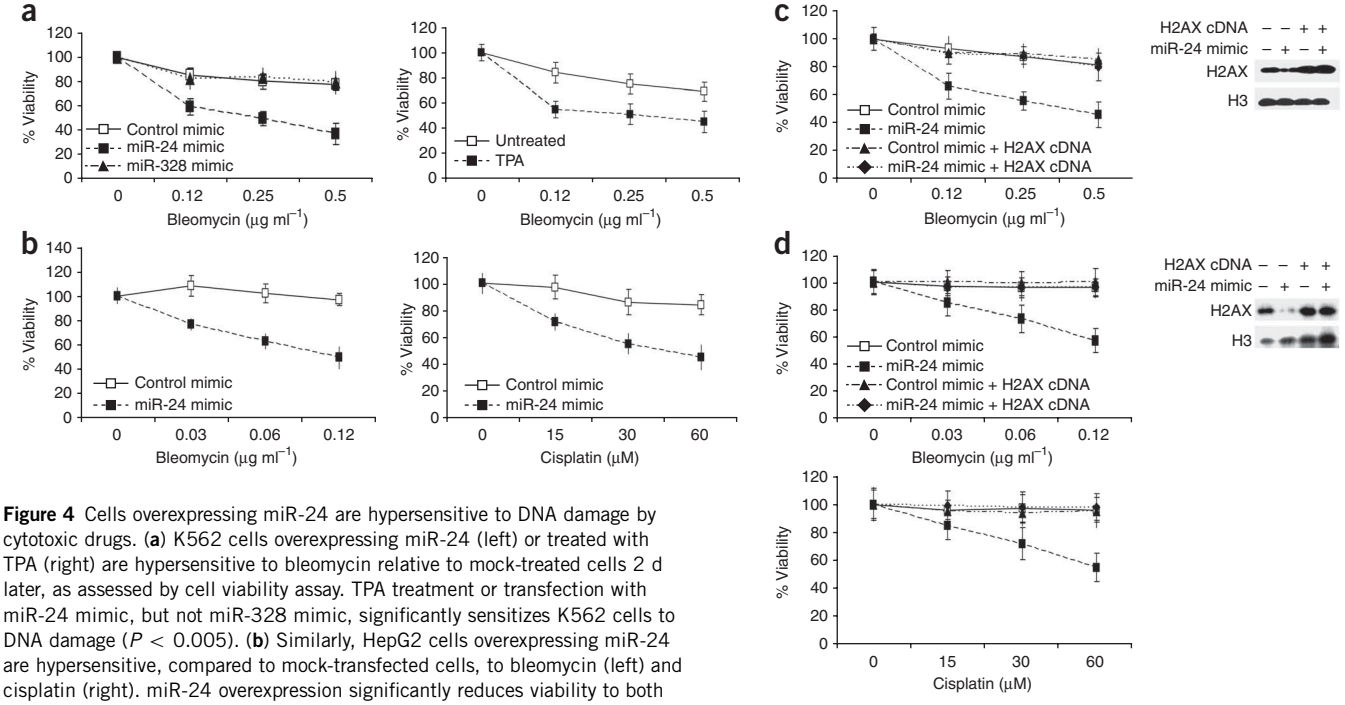

Figure 4 Cells overexpressing miR-24 are hypersensitive to DNA damage by cytotoxic drugs. (**a**) K562 cells overexpressing miR-24 (left) or treated with TPA (right) are hypersensitive to bleomycin relative to mock-treated cells 2 d later, as assessed by cell viability assay. TPA treatment or transfection with miR-24 mimic, but not miR-328 mimic, significantly sensitizes K562 cells to DNA damage ($P < 0.005$). (**b**) Similarly, HepG2 cells overexpressing miR-24 are hypersensitive, compared to mock-transfected cells, to bleomycin (left) and cisplatin (right). miR-24 overexpression significantly reduces viability to both genotoxic agents ($P < 0.004$). miR-24–mediated hypersensitivity of K562 (**c**) and HepG2 (**d**) cells is rescued by expression of miR-24–insensitive H2AX. Cells were mock transfected or transfected with miR-24 mimic or H2AX cDNA lacking the 3′ UTR or both. Cell viability was assayed 2 d after exposure to DNA damage and depicted relative to that of undamaged cells. Curves were generated from three independent experiments. Immunoblots demonstrate the miR-24–mediated decrease in H2AX protein and rescue by transfection with the H2AX cDNA (no 3′ UTR).

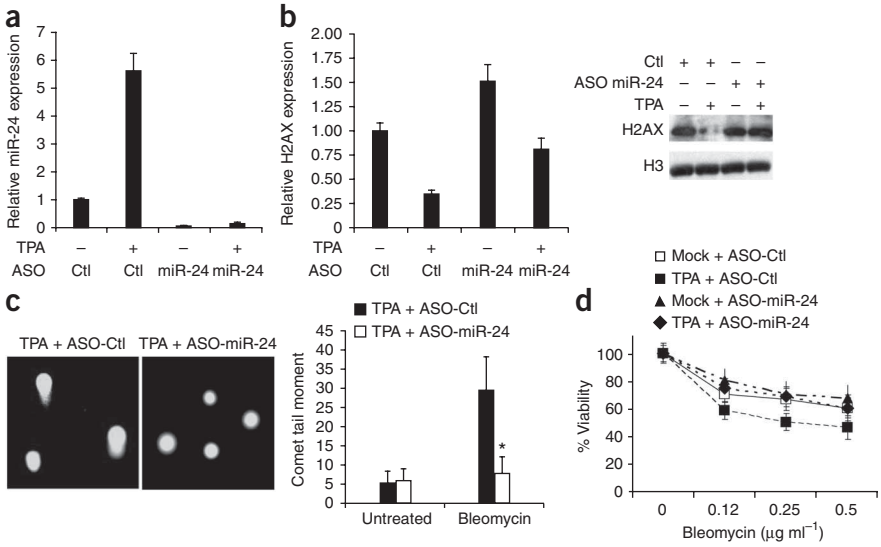

Figure 5 Antagonizing miR-24 enhances cell resistance to bleomycin. (**a**) miR-24 knockdown in K562 cells, treated or not with TPA, specifically decreases miR-24 levels, as assayed by qRT-PCR in cells transfected with miR-24 antisense oligonucleotides (ASOs) relative to control ASO (Ctl). miR-24 expression in ASO-treated cells, relative to U6 snRNA, is normalized to that in control ASO-treated cells. (**b**) miR-24 ASO enhances H2AX transcript (left) and protein levels (right) in K562 cells treated with TPA, but not in untreated K562 cells. (**c**) Transfection of miR-24 significantly enhances repair of bleomycin-induced DNA damage, as measured by comet assay, in TPA-treated K562 cells (*, $P < 0.001$). Representative images from bleomycin-treated cells are shown on the left and the mean ± s.d. comet tail moments of three independent experiments are shown on the right. (**d**) Relative to untreated cells, TPA treatment sensitizes K562 cells to bleomycin. Transfection of miR-24 ASO significantly blocks bleomycin-induced apoptosis of TPA-treated K562 cells ($P < 0.003$), but does not affect apoptosis of untreated K562 cells. Curves were generated from three independent experiments.

untransfected K562 cells, which have higher endogenous expression of miR-24 and four-fold less H2AX mRNA relative to HepG2 cells, also showed more chromosomal aberrations after irradiation than HepG2 cells (**Supplementary Fig. 5** online).

As another indicator of unrepaired DNA damage, we measured the persistence of DSBs by single-cell gel electrophoresis (comet assay) after low-dose bleomycin treatment (**Fig. 3c**). The comet moment quantifies the extent of unrepaired DNA damage. Although the basal comet moment was not significantly changed by miR-24 transfection, the frequency of comet tails was five-fold higher ($P < 0.001$) in miR-24 transfected cells, compared to control miRNA–transfected cells, after bleomycin treatment. To determine whether the effect of miR-24 on DSB repair was mediated via its effect on H2AX, we co-transfected K562 cells with miR-24 and a miR-24–insensitive H2AX expression plasmid without the H2AX 3′ UTR. The expression plasmid fully rescued the cells; cells overexpressing miR-24 and H2AX lacking the 3′ UTR showed no significant increase in the comet moment after bleomycin compared to cells transfected with the miRNA control and expression vector. This result strongly suggests that miR-24 regulates DSB repair by controlling H2AX.

Overexpression of miR-24 sensitizes cells to DNA damage

Because of impaired DNA-damage repair, H2AX deficiency also leads to increased cell death after exposure to genotoxic drugs. We compared cell viability of K562 cells overexpressing miR-24 or miR-328, and mock-transfected cells, after treatment with bleomycin (**Fig. 4a**, left). Consistent with the chromosomal breakage and comet assay analysis, cells overexpressing miR-24 were significantly hypersensitive to DNA damage, as were TPA-differentiated cells, relative to undifferentiated cells (**Fig. 4a**, right). miR-328 overexpression, which did not alter H2AX mRNA or protein levels (**Supplementary Fig. 2**), had no effect on bleomycin sensitivity. We further confirmed the effect of miR-24 on DNA-damage sensitivity by treating HepG2 cells transfected with a miR-24 mimic with bleomycin (**Fig. 4b**, left) and cisplatin (**Fig. 4b**, right). miR-24 significantly enhanced cytotoxicity caused by both drugs. The effect of miR-24 on survival was fully rescued by overexpressing miR-24–insensitive H2AX in both K562 (**Fig. 4c**) and HepG2 (**Fig. 4d**) cells. By contrast, overexpressing *CHEK1*, another miR-24 target gene involved in the response to DSBs (predicted by rna22), without its 3′ UTR did not rescue K562 cells (**Supplementary Fig. 6** online). Together these results suggest that H2AX is the key miR-24–regulated gene whose down-modulation inhibits the DNA-damage response in these terminally differentiated cells.

Suppressing miR-24 boosts DNA repair and reduces damage sensitivity

We next tested the effect of inhibiting miR-24 on sensitivity to genotoxic stress. When we transfected K562 cells with miR-24 antisense oligonucleotides, miR-24 expression was reduced, even during TPA differentiation (**Fig. 5a**). The reduction in miR-24, which correlated with enhanced H2AX mRNA and protein levels (**Fig. 5b**), had no effect on undifferentiated K562 cells, but significantly enhanced DNA repair (**Fig. 5c**) and reduced sensitivity to bleomycin in differentiated cells (**Fig. 5d**).

DISCUSSION

Why is there a mechanism to dampen DSB repair in terminally differentiated cells? One explanation is that most DSBs are generated during DNA replication and this mode of regulation allows differentiated cells to economize and conserve cellular resources under stress-free conditions. miRNA-mediated regulation is a particularly rapid and economic strategy to suppress gene expression in differentiated cells. In this case, by upregulating a single miRNA, miR-24, the terminally differentiated cell 'switches off' protein production of a whole set of genes, thereby affecting multiple genes. This could be more efficient than transcriptionally repressing each gene (especially if the locus is open and has recently been transcribed). Another possibility is that suppression of DSB repair in terminally differentiated cells sensitizes these cells to apoptosis. This may be preferred to error-prone repair via NHEJ (the primary mode of DSB repair in these cells), which would result in viable, but malfunctioning, cells. Although this solution makes sense for regenerating cells such as hematopoietic cells and myocytes, it might not be a good solution for long-lived terminally differentiated cells, such as neurons, with poor regenerative capacity. It will be worthwhile to determine whether miR-24 is upregulated during terminal differentiation of all cell types or only in lineages that are continuously renewing. It is noteworthy that at least one miR-24 cluster has been reported to be deleted in some poor-prognosis cases of chronic lymphocytic leukemia (CLL)[21], a disease in which key antiapoptotic genes are downregulated. Given

our findings here, inappropriate underexpression of miR-24 would be predicted to enhance DNA repair and thereby enhance resistance to cytotoxic cancer therapies.

This study focused on the effect of miR-24 on H2AX and DSB repair. Both H2AX mRNA and protein levels are reduced by miR-24 expression. miR-24 is likely to operate predominantly by inhibiting translation, as the effect on protein levels is greater than on mRNA. Mouse genetics has established that a 50% decrease in H2AX levels is sufficient to compromise genomic stability and induce tumorigenesis[13,14]. However, there are no reports regarding a physiologically equivalent scenario in human cells where inefficient DSB repair occurs as a result of reduced H2AX protein. This study not only establishes the novel connection between miR-24 and H2AX, but also demonstrates that terminally differentiated cells may represent a physiologically relevant setting in human cells where overexpression of miR-24 decreases H2AX levels, resulting in a diminished capacity to repair DSBs. Although current computational methods fail to detect additional DSB repair factors targeted by miR-24, other factors in the DSB response might be regulated by miR-24 expression. However, the observation that DSB repair was completely restored by overexpressing H2AX in differentiating cells, or cells overexpressing exogenous miR-24, suggests that the key target of miR-24 in DSB repair is H2AX. Furthermore, we investigated another miR-24 target involved in the cellular DSB response, CHEK1. Overexpressing miR-24–insensitive CHEK1 did not rescue the DNA repair phenotype induced by miR-24 (**Supplementary Fig. 5**). Future studies should explore whether these and other effects of miR-24 might have a role in sensitivity to DSBs and other forms of DNA damage.

METHODS

Cell culture and differentiation. HepG2 cells were grown in DMEM supplemented with 10% (v/v) FCS. HL60 and K562 cells were grown in RPMI-1640 supplemented with 10% (v/v) FCS. We treated K562 cells (0.5×10^6 cells per ml) with TPA (16 nM, 2 d) or Hemin (100 μM, 4 d) for differentiation into megakaryocytes or erythrocytes, respectively. To induce macrophage or granulocyte differentiation, we treated HL60 cells (0.5×10^6 cells per ml) with TPA (16 nM, 2 d) or DMSO (1.25%, 5 d), respectively. We isolated human polymorphonuclear neutrophils (PMN) from whole blood after removing mononuclear cells and platelets by Ficoll-Hypaque density gradient centrifugation. Erythrocytes were lysed by treatment with ice-cold isotonic lysis buffer (0.155 M NH_4Cl, pH 7.4). The remaining PMN cells were washed with Hanks' balanced salt solution and suspended in RPMI medium containing 10% (v/v) FCS. We isolated human macrophages from peripheral blood as described[22].

RNA isolation and quantitative reverse-transcription–polymerase chain reaction. Total RNA was isolated using Trizol (Invitrogen) and reverse transcribed using random hexamers and superscript II reverse transcriptase (Invitrogen). qRT-PCR was performed in triplicate samples using the SYBR Green master mix (Applied Biosystems) and the BioRad iCycler. Primers are provided in **Supplementary Table 1** online. Results were normalized to GAPDH. We carried out qPCR of miRNAs in triplicate using the TaqMan MicroRNA Assay from Applied Biosystems as per the manufacturer's instructions, normalized to U6 snRNA.

miRNA microrray. We performed miRNA microarrays as described[23].

miRNA mimic and antisense oligonucleotide transfection. We reverse transfected HepG2 cells (2.5×10^5 cells per well) with 30 nM miR-24 or control (cel-miR-67) mimics (Dharmacon) using NeoFx (Ambion) following the manufacturer's instructions. We transfected K562 cells with miR-24 or control mimics (100 nM) using Amaxa nucleofection following the manufacturer's protocol. K562 cells were treated with TPA (16 nM, 2 d) and were transfected with 100 nM miR-24 antisense oligonucleotides or antisense oligonucleotide negative control 1 (Ambion) using Lipofectamine 2000 (Invitrogen). After 36 h, we exposed these cells to the indicated concentrations of bleomycin and assessed cell viability 2 d later.

Luciferase assay. We reverse transfected HepG2 cells (2.5×10^5 cells per well) in triplicate with 30 nM miR-24 mimic, miR-328 mimic or control miRNA mimic. After 2 d, cells were transfected using Lipofectamine 2000 (Invitrogen) with psiCHECK2 (Promega) vector (0.5 μg per well) containing the 3′ UTR of H2AX cloned in the multiple cloning site of *Renilla* luciferase, or control. After 24 h we measured luciferase activities using the Dual Luciferase Assay System (Promega) and the TopCount NXT microplate reader (Perkin Elmer), as per the manufacturer's instructions. Data were normalized to Firefly luciferase. To test whether H2AX mRNA is directly regulated by miR-24, we cloned the two predicted MREs in the H2AX 3′ UTR into the multiple cloning site of psiCHECK2 and also the mutant versions that disrupted base-pairing between the binding sites and miR-24. HepG2 cells were co-transfected with these plasmids and miR-24 or control mimics for 48 h using Lipofectamine 2000, before we performed the luciferase assays as described above.

Immunoblot. We transfected K562 cells (1×10^6) with miR-24 mimics or control miRNA mimics (cel-miR-67) as described above. After 48 h we prepared whole-cell lysates using RIPA buffer (150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 50 mM Tris, pH 8.0). Protein samples were quantified using Bradford reagent (BioRad), resolved on 10% SDS-PAGE gels and analyzed by immunoblotting, probing with antibodies to histone H2AX (Upstate Biotech), CHEK1 (Cell Signaling), histone H3 (Cell Signaling) and tubulin (Sigma). All antibodies were used at a dilution of 1:1,000.

Chromosomal breakage analysis. We exposed K562 and HepG2 cultures in duplicate wells to the indicated doses of γ-irradiation and incubated at 37 °C for indicated times in 5% CO_2. Cells were harvested and processed for chromosomal analysis following standard methods[24]. We scored 50–75 Wright-stained metaphases for each condition for chromosomal aberrations.

Single-cell gel electrophoresis (comet) assay. We performed single cell comet assays as per manufacturer's instructions (Trevigen). Briefly, we transfected cells with siRNAs and, after 60 h, induced DSBs using camptothecin (2 μM, 1 h, 37 °C). Treated or untreated cells were collected, resuspended in ice cold PBS at 10^5 cells per ml, mixed with low-melt agarose (1:10 ratio) and spread on frosted glass slides. After the agarose solidified, the slides were successively placed in lysis and alkaline solutions (Trevigen). Slides were then subjected to electrophoresis (1 V per cm of distance between electrodes) for 10 min in 1× TBE buffer, and cells were fixed with 70% (v/v) ethanol and stained with SYBR Green. Nuclei were visualized using epifluorescent illumination on a Zeiss microscope and images analyzed with the National Institutes of Health ImageJ (http://rsbweb.nih.gov/ij/). DNA damage was quantified for 75 cells for each experimental condition by determining the tail moment, a function of both the tail length and intensity of DNA in the tail relative to the total DNA, using the software Comet Score (TriTek). Statistical analysis was done using the Student's *t*-test.

Cell viability assay. We seeded microRNA-transfected K562 or HepG2 cells (2×10^3 cells per 100 μl) into octuplicate microtiter wells, incubated them overnight and then treated them with the indicated reagents or medium for 48 h. Viability was measured using the CyQuant Cell Proliferation Assay Kit, as per manufacturer's instructions (Molecular Probes). Results were expressed as OD_{520} relative to that of untreated cells.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

Most of the experiments were performed collaboratively by A.L., Y.P. and F.N. Chromosome breakage analysis was done by L.M. Several constructs used in the

study were made by D.M.D. Z.B. and E.M. generated and analyzed the miRNA microarray data. J.L. and D.C. wrote the paper and conceived all the experiments with A.L.

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1. Nouspikel, T. & Hanawalt, P.C. DNA repair in terminally differentiated cells. *DNA Repair (Amst.)* **1**, 59–75 (2002).
2. Lukas, C. *et al.* DNA damage-activated kinase Chk2 is independent of proliferation or differentiation yet correlates with tissue biology. *Cancer Res.* **61**, 4990–4993 (2001).
3. Puri, P.L. & Sartorelli, V. Regulation of muscle regulatory factors by DNA-binding, interacting proteins, and post-transcriptional modifications. *J. Cell. Physiol.* **185**, 155–173 (2000).
4. Belloni, L. *et al.* DNp73α protects myogenic cells from apoptosis. *Oncogene* **25**, 3606–3612 (2006).
5. Yaneva, M. & Jhiang, S. Expression of the Ku protein during cell proliferation. *Biochim. Biophys. Acta* **1090**, 181–187 (1991).
6. Pillai, R.S., Bhattacharyya, S.N. & Filipowicz, W. Repression of protein synthesis by miRNAs: how many mechanisms? *Trends Cell Biol.* **17**, 118–126 (2007).
7. Ambros, V. The functions of animal microRNAs. *Nature* **431**, 350–355 (2004).
8. Bartel, D.P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297 (2004).
9. Sevignani, C., Calin, G.A., Siracusa, L.D. & Croce, C.M. Mammalian microRNAs: a small world for fine-tuning gene expression. *Mamm. Genome* **17**, 189–202 (2006).
10. Fernandez-Capetillo, O., Lee, A., Nussenzweig, M. & Nussenzweig, A. H2AX: the histone guardian of the genome. *DNA Repair (Amst.)* **3**, 959–967 (2004).
11. Petrini, J.H. & Stracker, T.H. The cellular response to DNA double-strand breaks: defining the sensors and mediators. *Trends Cell Biol.* **13**, 458–462 (2003).
12. Stucki, M. & Jackson, S.P. gammaH2AX and MDC1: anchoring the DNA-damage-response machinery to broken chromosomes. *DNA Repair (Amst.)* **5**, 534–543 (2006).
13. Bassing, C.H. *et al.* Histone H2AX: a dosage-dependent suppressor of oncogenic translocations and tumors. *Cell* **114**, 359–370 (2003).
14. Celeste, A. *et al.* H2AX haploinsufficiency modifies genomic stability and tumor susceptibility. *Cell* **114**, 371–383 (2003).
15. Tzur, G. *et al.* MicroRNA expression patterns and function in endodermal differentiation of human embryonic stem cells. *PLoS ONE* **3**, e3726 (2008).
16. Neilson, J.R., Zheng, G.X., Burge, C.B. & Sharp, P.A. Dynamic regulation of miRNA expression in ordered stages of cellular development. *Genes Dev.* **21**, 578–589 (2007).
17. Sun, Q. *et al.* Transforming growth factor-β–regulated miR-24 promotes skeletal muscle differentiation. *Nucleic Acids Res.* **36**, 2690–2699 (2008).
18. Bartel, D.P. MicroRNAs: target recognition and regulatory functions. *Cell* **136**, 215–233 (2009).
19. Mannironi, C., Bonner, W.M. & Hatch, C.L. H2A.X. a histone isoprotein with a conserved C-terminal sequence, is encoded by a novel mRNA with both DNA replication type and polyA 3′ processing signals. *Nucleic Acids Res.* **17**, 9113–9126 (1989).
20. Sandberg, R., Neilson, J.R., Sarma, A., Sharp, P.A. & Burge, C.B. Proliferating cells express mRNAs with shortened 3′ untranslated regions and fewer microRNA target sites. *Science* **320**, 1643–1647 (2008).
21. Calin, G.A. *et al.* MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. *Proc. Natl. Acad. Sci. USA* **101**, 11755–11760 (2004).
22. Song, E. *et al.* Sustained small interfering RNA-mediated human immunodeficiency virus type 1 inhibition in primary macrophages. *J. Virol.* **77**, 7174–7181 (2003).
23. Barad, O. *et al.* MicroRNA expression detected by oligonucleotide microarrays: system establishment and expression profiling in human tissues. *Genome Res.* **14**, 2486–2494 (2004).
24. Moorhead, P.S., Nowell, P.C., Mellman, W.J., Battips, D.M. & Hungerford, D.A. Chromosome preparations of leukocytes cultured from human peripheral blood. *Exp. Cell Res.* **20**, 613–616 (1960).