The kinase ABL phosphorylates the microprocessor subunit DGCR8 to stimulate primary microRNA processing in response to DNA damage

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The DNA damage response network stimulates microRNA (miRNA) biogenesis to coordinate repair, cell cycle checkpoints, and apoptosis. The multistep process of miRNA biogenesis involves the cleavage of primary miRNAs by the microprocessor complex composed of the ribonuclease Drosha and the RNA binding protein DGCR8. We found that the tyrosine kinase ABL phosphorylated DGCR8, a modification that was required for the induction of a subset of miRNAs after DNA damage. Focusing on the miR-34 family, ABL stimulated the production of miR-34c, but not miR-34a, through Drosha/DGCR8-dependent processing of primary miR-34c (pri-miR-34c). This miRNA-selective effect of ABL required the sequences flanking the precursor miR-34c (pre-miR-34c) stem-loop. In pri-miRNA processing, DGCR8 binds the pre-miR stem-loop and recruits Drosha to the miRNA. RNA cross-linking assays showed that DGCR8 and Drosha interacted with pri-miR-34c, but we found an inverse correlation between ABL-stimulated processing and DGCR8 association with pri-miR-34c. When coexpressed in HEK293T cells, ABL phosphorylated DGCR8 at Tyr267. Ectopic expression of a Y267F-DGCR8 mutant reduced the recruitment of Drosha to pri-miR-34c and prevented ABL or Drosha from stimulating the processing of pri-miR-34c. In mice engineered to express a nuclear import–defective mutant of ABL, miR-34c, but not miR-34a, expression was reduced in the kidney, and apoptosis of the renal epithelial cells was impaired in response to cisplatin. These results reveal a new pathway in the DNA damage response wherein ABL-dependent tyrosine phosphorylation of DGCR8 stimulates the processing of selective primary miRNAs.

INTRODUCTION

MicroRNAs (miRNAs) are short noncoding RNAs that inhibit gene expression by reducing the stability and the translatability of mRNA (1, 2). At least 60% of human mRNAs are targeted by miRNAs (3). The biogenesis of miRNAs involves transcription by RNA polymerase II to produce primary miRNA (pri-miRNA) with a local stem-loop that is cotranscriptionally recognized and cleaved by the DGCR8/Drosha microprocessor complex to produce the precursor miRNA (pre-miRNA) of ~70 nucleotides (nt) (4, 5). The pre-miRNA is further processed by Dicer (6, 7) and loaded onto the miRNA-induced silencing complex (5). The cotranscriptional cleavage of pri-miRNA to pre-miRNA is controlled by a variety of mechanisms (8, 9), including signal-induced Drosha interaction with pri-miRNA chromatin (10), RNA binding protein–assisted recruitment of DGCR8/Drosha to the pri-miRNA (11, 12), and regulation of Drosha/DGCR8 expression (13–16). Furthermore, a recent study has identified species-specific sequence motifs that are required for stem-loop recognition and processing (17).

In response to DNA damage, miRNA expression is altered to promote DNA repair, cell cycle checkpoints, and apoptosis (18, 19). Previous studies have uncovered several mechanisms for miRNA induction in DNA damage response (DDR), including transcription factor p53–dependent pri-miRNA transcription (20), p53- and RNA helicase–dependent pri-miRNA processing (10), and ataxia-telangiectasia mutated kinase–stimulated miRNA maturation (21). One of the most studied miRNA family is the p53-stimulated miR-34a, miR-34b, and miR-34c (20). Ectopic expression of miR-34a, miR-34b, or miR-34c causes growth arrest and apoptosis by decreasing the expression of pro- and antiapoptotic genes (20, 22, 23). In mice, knockout of the miR-34 family of genes did not disrupt p53-mediated responses to ionizing radiation (IR) (24), most likely because the miR-34 family shares redundant functions with the miR-449 family (25). The combined knockouts of the miR-34 and the miR-449 families caused developmental defects in mice (26). Although the miR-34 family of miRNAs may not be the essential downstream effectors of p53, they are excellent readouts for probing DDR-stimulated miRNA biogenesis. In mice, the basal and the IR-induced expression of miR-34a and miR-34c exhibit distinct tissue specificity (24), suggesting that these two related miRNAs are controlled by nonidentical mechanisms.

The ubiquitously expressed nonreceptor tyrosine kinase ABL contains three nuclear localization signals (NLS) and accumulates in the nucleus of DNA-damaged cells (27, 28). The nuclear substrates of ABL kinase include many regulators of transcription, such as the RNA polymerase II C-terminal repeat domain (29), the p53 family of transcription factors (30, 31), the E3 ubiquitin ligase MDM2 (32), its related MDMX (33), transcription factor YAP-1 (34), histone acetyltransferase Tip60 (35), and histone deacetylase-2 (36). Our laboratory has generated the Abl-μNLS allele in the mouse Abl1 gene by knock-in mutations of 11 amino acids to inactivate the three NLS (37). The Abl-μNLS mice are healthy and fertile, showing that the nuclear import of ABL is not essential to mouse development (37). However, cisplatin-induced apoptosis of renal proximal tubule epithelial cells (RPTCs) is defective in the Abl-μNLS mice, providing in vivo evidence for a role of nuclear ABL kinase in DNA damage–induced apoptosis (37). Because miRNAs play important roles in DDR-induced apoptosis (18), we investigated the role of ABL tyrosine kinase in DDR-induced miRNA expression.

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ABL kinase stimulates Drosha-dependent processing of pri-miR-34c from a minigene

To uncouple the effect of ABL on pri-miRNA transcription from that on processing, we tested its effect on miR-34c production from a minigene (Fig. 2A), in which the cytomegalovirus (CMV) promoter drives the transcription of 277 base pairs (bp) of exon sequence containing the pri-miR-34c stem-loop of 77 nt (predicted by miRbase, www.mirbase.org). transcription of the human pri-miR-34b/c gene locus, E1, upstream exon; EST, expressed sequence tag. (H) Expression of miR34b/c-E1 in cells treated as in (E) relative to vehicle treatment. Relative abundance values are means ± SD of at least three independent experiments. *P < 0.05, **P < 0.01, two-tailed t tests within cell line (C, D, F, and H); ***P < 0.001, two-way analysis of variance (ANOVA) test of interaction, with Bonferroni correction for the two main effect tests on the two cell lines (F).

RESULTS

ABL tyrosine kinase stimulates the production of miR-34c

To determine the effect of ABL kinase on DNA damage–induced miRNA expression, we measured the abundance of miRNAs by miRNA sequencing (miRNAseq) in human embryonic kidney (HEK) 293T cells after 24-hour treatments with vehicle, imatinib, doxorubicin, or a combined treatment of doxorubicin and imatinib. From 501 miRNAs sequenced with confidence across the four conditions, inspection of unsupervised hierarchical clustering found 93 miRNAs to be in a cluster induced by doxorubicin but not the imatinib + doxorubicin combination (Fig. 1A, boxed). Within this cluster, 22 miRNAs were significantly induced by doxorubicin (P < 0.05) (table S1), with miR-34c found as the top-ranked miRNA that was induced by doxorubicin but not by imatinib + doxorubicin (Fig. 1B). Previous studies have shown that doxorubicin-induced miR-34 family of gene expression is driven by kinase MK2 in HEK293T cells where p53 is inactivated (38, 39). This MK2-dependent induction of miR-34c was inhibited by imatinib; however, the related miR-34a was not found in the “doxorubicin but not imatinib + doxorubicin” cluster. In two p53-positive cell lines, HCT116 and MCF7, imatinib also dampened the doxorubicin induction of miR-34c (Fig. 1, C and D). In HCT116 cells, doxorubicin induction of miR-34b, miR-216a (2nd ranked in HEK293T cells) (Fig. 1B), and miR-335 (19th ranked) (table S1) was also inhibited by imatinib (fig. S1A). However, doxorubicin induction of miR-34a and three other miRNAs (miR-24-1, miR-27b, and miR-107) was not affected by imatinib (Fig. 1, C and D, and fig. S1B). To control for the off-target effects of imatinib, which also inhibits tyrosine kinases KIT, PDGFR, and ARG as well as nonkinase enzymes NQQ2 (40) and γ-secretase (41), we stably knocked down ABL (Fig. 1E) and showed that doxorubicin induction of miR-34c was reduced and the imatinib effect was abolished in ABL-knockdown HCT116 cells (Fig. 1F). We also expressed the mouse Abl (type IV) in the ABL-knockdown HCT116 cells and showed that the mouse Abl could restore the inhibitory effect of imatinib on miR-34c induction (fig. S1, E and F). These results established that imatinib inhibition of miR-34c production was ABL-dependent.

To determine whether ABL kinase is required for the doxorubicin induction of pri-miR-34c transcription, we measured the abundance of an upstream exon (E1) in this primary transcript (Fig. 1G). In HCT116 cells, doxorubicin induction of E1 expression was reduced by the knockdown of p53 but not by the knockdown of ABL (Fig. 1H), showing that this p53-dependent transcription of pri-miR-34c is not affected by ABL knockdown. However, imatinib could reduce the doxorubicin induction of E1, and this imatinib effect was similar in cells without or with ABL knockdown (Fig. 1I). This result suggested that the remaining ABL in the knockdown cells might contribute to the transcription of E1. Alternatively, imatinib might inhibit E1 transcription by targeting proteins other than ABL. Although ABL kinase could contribute to transcription, the result that ABL knockdown reduced the mature miR-34c, but not the pri-miR-34c, showed that ABL was also required for the processing of pri-miR-34c.
Abl kinase stimulates Drosha-dependent processing of pri-miR-34c from a minigene. (A) Diagrams of vector (Vec) and the minigenes (34a and 34c). The primer sets for the mature miRNAs and the uncult pri-miRNAs are shown. Numbers are genomic sequences (nt) flanking the pri-miRNA stem-loop. (B) Schematic of AblPPn depicting the activating mutations (42, 43). (C) Immunoblotting for ABL in HCT116 cells transfected with the indicated constructs. (D) Production of miR-34a and miR-34c in HCT116 cells transfected as indicated relative to vector-only-transfected cells. (E) Northern blots of total RNA from transfected HCT116 cells probed for miR-34a, miR-34c, and U6. (F) Expression of indicated mRNA in HCT116 relative to vector-only-transfected cells. (G and H) Production of miR-34c in 34c minigene–transfected 293T cells with the indicated cotransfections of AblPPn and Dicer–small interfering RNA (siRNA) (G) or with TN-Drosha or wild-type (WT) Drosha (H) relative to minigene-only–transfected cells. (I and J) Expression of pri-miR-34c in 34c minigene–transfected 293T cells with the indicated cotransfections of AblPPn, Drosha, or TN-Drosha (I) or treated with imatinib (J) relative to minigene-only–transfected cells. pTyr, phosphorysine. Data are means ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, two-tailed t-tests.

Flanking sequences promote Tyr phosphorylation of seven miRNAs predicted to be the targets of the miR-34 family members and found that doxorubicin treatment reduced the expression of all seven targets in HCT116 cells (Fig. S2A). Knockdown of ABL selectively interfered with this doxorubicin effect on two targets (SRSF2 and CCNE2), suggesting a more important role for miR-34b/c in targeting these miRNAs. Cotransfection of HCT116 cells with AblPPn and the 34c minigene also reduced the expression of these two and other mRNA targets of miR-34c (C41, CDK4, SRSF2, MET, E2F3, MYB, and CCNE2) (Fig. 2F and Fig. S2B), showing that AblPPn stimulated the maturation of a functional miR-34c from the 34c minigene. The AblPPn-stimulated miR-34c production was abolished by the knockdown of the Dicer ribonuclease (Fig. 2G) and by a transdominant catalytic mutant of Drosha (TN-Drosha) (44) (Fig. 2H). Expression of AblPPn also caused a reduction in pri-miR-34c, and this effect was abolished by TN-Drosha (Fig. 2I) or imatinib (Fig. 2J). These results showed that ABL kinase stimulated Drosha-dependent processing of pri-miR-34c derived from the 34c minigene.

Fig. 2. ABL kinase stimulates Drosha-dependent processing of pri-miR-34c from a minigene. (A) Diagrams of vector (Vec) and the minigenes (34a and 34c). The primer sets for the mature miRNAs and the uncult pri-miRNAs are shown. Numbers are genomic sequences (nt) flanking the pri-miRNA stem-loop. (B) Schematic of AblPPn depicting the activating mutations (42, 43). (C) Immunoblotting for ABL in HCT116 cells transfected with the indicated constructs. (D) Production of miR-34a and miR-34c in HCT116 cells transfected as indicated relative to vector-only-transfected cells. (E) Northern blots of total RNA from transfected HCT116 cells probed for miR-34a, miR-34c, and U6. (F) Expression of indicated mRNA in HCT116 relative to vector-only-transfected cells. (G and H) Production of miR-34c in 34c minigene–transfected 293T cells with the indicated cotransfections of AblPPn and Dicer–small interfering RNA (siRNA) (G) or with TN-Drosha or wild-type (WT) Drosha (H) relative to minigene-only–transfected cells. (I and J) Expression of pri-miR-34c in 34c minigene–transfected 293T cells with the indicated cotransfections of AblPPn, Drosha, or TN-Drosha (I) or treated with imatinib (J) relative to minigene-only–transfected cells. pTyr, phosphorysine. Data are means ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, two-tailed t-tests.

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Sequences flanking pre-miR-34c stem-loop determine the miRNA-selective effect of ABL Within the minigene sequences of pri-miR-34a and pri-miR-34c, the stem-loops are 53% identical, but the flanking sequences share only 27% identity (Fig. 3A and Fig. S3). In pri-miR-34a, the pre-miRNA hairpin that is conserved among the miR-34 family members is embedded in a larger stem-loop with extension beyond the predicted Drosha cleavage sites (Fig. 3A). To learn more about the minigene sequences that determine the miR-34c-selective effect of ABL, we swapped the flanking sequences to generate hybrid minigenes A34c (34a-flanking 34c-hairpin) and C34a (34c-flanking 34a-hairpin) (Fig. 3B). We also generated 34aS with a shortened stem-loop and C34aS, in which the shortened 34a stem-loop was embedded in 34c-flanking sequences (Fig. 3B). When co- transfected with AblPPn, a reduction in the relative abundance of pri-miRNAs was observed with miRNAs containing the 34c-flanking sequences, including 34c, 34aC, and 34aS (Fig. 3C). AblPPn did not stimulate the processing of pri-miR-34a or pri-miR-34aS, but it stimulated the processing of pri-miR-34c (Fig. 3C). These results suggested that the miR-34c stem-loop and flanking sequences could both mediate the effect of ABL. Coexpression with TN-Drosha (Fig. 3D) abolished the AblPPn-stimulated reduction in pri-miR-34c, pri-miR-34a, and pri-miR-34aS (Fig. 3E). These results showed that AblPPn stimulated the Drosha-dependent processing of pri-miRNAs that contained the flanking sequences in the 34c minigene.

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DGCR8 cross-linking to the 34c or the C34a pri-miRNA (Fig. 4A). Ectopic expression of Drosha also reduced the DGCR8-RNA interaction (Fig. 4A). However, expression of TN-Drosha did not reduce the DGCR8-RNA interaction, and furthermore, it prevented AbiPPn from reducing the cross-linking of DGCR8 to pri-miR-34c or pri-miR-C34a (Fig. 4A). These results showed an inverse correlation between processing and DGCR8 interaction with pri-miR-34c. Drosha or AbiPPn stimulated processing but reduced DGCR8-RNA cross-linking, whereas TN-Drosha inhibited processing but stabilized DGCR8-RNA interaction. This inverse correlation suggested that the pri-miR-34c flanking sequences caused the formation of a more stable DGCR8-RNA complex that was not efficiently processed by Drosha.

Phosphoproteomics studies have found that DGCR8 is phosphorylated at Tyr267 (www.phosphosite.org/homeAction.do) (47), but the biological significance of this phosphorylation was not investigated. The Tyr267 site is immediately N-terminal to the RNA binding heme domain (Rhed; amino acid 276 to 498), which a recent report suggests to also make contacts with the pre-miRNA stem-loop (48) (Fig. 4B). We found that cotransfection with AbiPPn increased Tyr267 phosphorylation of DGCR8, and that mutation of Tyr267 was sufficient to substantially reduce that phosphorylation (Fig. 4C). Coexpression of the wild-type DGCR8 did not interfere with AbiPPn-stimulated reduction in pri-miR-34c or increase in miR-34c (Fig. 4D); however, expression of Y267F-DGCR8 blocked these effects of AbiPPn (Fig. 4D). By RNA-qCLIP, we found that Y267F-DGCR8 also interacts with pri-miR-34c, but AbiPPn could not reduce this cross-linking (Fig. 4E). To determine how tyrosine phosphorylation of DGCR8 might affect the interaction of Drosha with pri-miR-34c, we did RNA-qCLIP with ectopically expressed Drosha. We detected a low amount of pri-miR-34c cross-linked to Drosha (Fig. 4F).

Coexpression of Drosha with AbiPPn, wild-type DGCR8, or Y267F-DGCR8 reduced the cross-linking of Drosha to pri-miR-34c (Fig. 4F). This reduction in Drosha cross-linking to pri-miR-34c could be due to processing of pri-miR-34c or lack of recruitment by DGCR8. To distinguish between these two possibilities, we measured the abundance of pri-miR-34c and found that coexpression with wild-type DGCR8 stimulated processing (loss of pri-miR-34c) but that coexpression with mutant Y267F-DGCR8 blocked Drosha-mediated processing (Fig. 4G). Thus, Y267F-DGCR8 has a dominant inhibitory effect on pri-miR-34c processing that is stimulated by AbiPPn or by Drosha. Because Y267F-DGCR8 inhibits Drosha cross-linking to and processing of pri-miR-34c, phosphorylation at Tyr267 of DGCR8 is likely to stimulate Drosha recruitment to pri-miR-34c.

**Nuclear import of ABL is required for miR-34c production in mouse tissues**

We have previously constructed the Abi-μNLS allele in which the three NLSs are inactivated by germ-line knock-in mutations in mice (Fig. 5A). Unlike the developmentally defective Abl knockout mouse (49), mice homozygous for the Abi-μNLS allele (the Abi−/− mice) are healthy and fertile, showing that nuclear entry of Abl is not required for mouse development (Fig. 5B). However, when treated with cisplatin to cause acute kidney injury, the apoptotic response of the RPTCs was found to be defective in the Abi−/− mice (37). We therefore examined the effect of the Abi-μNLS allele on miR-34c production in mouse tissues.

It was previously reported that IR induced the expression of the miR-34 family of genes in the mouse liver, thymus, and spleen, but not the kidney (24). Similarly, we found that cisplatin stimulated the production of miR-34a and miR-34c in the liver, thymus, spleen, but not the kidney of the Abi−/− mice (Fig. 5, C to F). This cisplatin-induced increase in miR-34a and miR-34c was not observed in the Abi+/− mice; thus, nuclear Abl is required for cisplatin to stimulate miR-34a and miR-34c production in the liver, thymus, and spleen (Fig. 5, D to F). We also measured the primary (upstream exon E1 and uncut pri-miRNA) and mature miR-34a and miR-34c in embryo fibroblasts from the Abi+/−:p53−/− and the Abi−/−:p53−/− mice (37). Even in the absence of p53, we found that doxorubicin stimulated the expression of E1-34a, pri-miR-34a, E1-34c, and pri-miR-34c in the Abi+/−, but not the Abi−/− fibroblasts, suggesting that nuclear Abl is required for pri-miR-34a and pri-miR-34c transcription in mouse embryo fibroblasts (fig. S4). Furthermore, the basal expressions of both the pri-miRNAs and the mature miR-34a and miR-34c were significantly reduced in the Abi−/−:p53−/− fibroblasts (fig. S4). These results showed that nuclear Abl

![Fig. 3. Sequences flanking pre-miR-34c stem-loop determine the miRNA-selective effect of ABL. (A) Schematics of pri-miR-34a and pri-miR-34c transcribed from the minigenes. Arrowheads denote the predicted Drosha cleavage sites. The pri-miR-34a contains upstream (light blue) and downstream (purple) extensions not in pri-miR-34c. Flanking sequences are indicated in orange (34a) or dark blue (34c). (B) Schematics of the hybrid minigenes with color-coding of sequence blocks shown in (A). Primers for measuring the uncut pri-miRNAs are shown as orange (34a-flanking) and blue (34c-flanking) arrowheads. (C) Expression of the indicated pri-miRNA in HCT116 cells cotransfected with the indicated minigenes and AbiPPn relative to vector cotransfected cells. (D) Immunoblotting of lysates from HCT116 cells cotransfected with AbiPPn and TN-Drosha as indicated. (E) Expression of the indicated pri-miRNA in HCT116 cells cotransfected with minigenes and the indicated plasmids relative to vector cotransfected cells. Data are means ± SD from three independent experiments. **P < 0.01, ***P < 0.001, two-tailed t tests.](http://stke.sciencemag.org/content/7/359/rc131)
Tyrosine phosphorylation of Tyr²⁶⁷ of DGCR8 stimulates pri-miR-34c processing

Our results show that ABL kinase stimulates pri-miR-34c processing through DGCR8 phosphorylation at Tyr²⁶⁷. Coexpression with activated AblPpn caused an increase in DGCR8 tyrosine phosphorylation that was diminished by mutation of Tyr²⁶⁷ to Phe²⁶⁷. The Y267F-DGCR8 can be cross-linked to pri-miR-34c, showing that this YF-mutation did not inhibit DGCR8-RNA interaction. Because Y267F-DGCR8 inhibited the AblPpn-stimulated reduction of pri-miR-34c, phosphorylation at Tyr²⁶⁷ was required for ABL to stimulate pri-miR-34c processing. We found that Y267F-DGCR8 also inhibited Drosha-stimulated processing of pri-miR-34c. Furthermore, Y267F-DGCR8 inhibited Drosha cross-linking to pri-miR-34c. Together, these results suggest that Tyr²⁶⁷ phosphorylation of DGCR8 may promote the recruitment of Drosha to pri-miR-34c to stimulate its processing.

A recent study showed that DGCR8 is phosphorylated by mitogen-activated protein kinase and a DGCR8 mutant with 23 phosphomimetic substitution (Mim23) promoted the expression of a cohort of progrowth miRNAs in HeLa cells (47). We found limited overlap between those miRNAs stimulated by the Mim23-DGCR8 and the miRNAs found in this study to be induced by doxorubicin, inhibited by imatinib, or induced by doxorubicin but not the imatinib + doxorubicin combination in HEK293T cells (fig. S5). Because Mim23 ratates 22 serine/threonine sites and 1 tyrosine site (Tyr²⁶⁷), the effect of Mim23-DGCR8 on miRNA expression may be skewed toward serine/threonine phosphorylations and did not reflect the effects of Tyr²⁶⁷ phosphorylation. Tyr²⁶⁷ is located immediately upstream to the Rhd domain that has RNA binding activity (48). It is conceivable that phosphorylation at Tyr²⁶⁷ may alter the Rhd-mediated interaction of DGCR8 with pri-miR-34c to promote Drosha recruitment and RNA cleavage. Alternatively, Tyr²⁶⁷ phosphorylation may release DGCR8 from the effect of an inhibitory factor that also interacts with pri-miR-34c to interfere with RNA processing.

Fig. 4. Flanking sequences promote and tyrosine phosphorylation reduces DGCR8 interaction with pri-miR-34c. (A) Percentage of input pri-miRNA cross-linked to endogenous DGCR8 in HCT116 cells transfected with the indicated minigenes and expression plasmids. Primers for quantitative reverse transcription polymerase chain reaction (qRT-PCR) of the pri-miRNAs are shown. (B) Domains of DGCR8. Rhed, RNA binding heme domain; WW, WW domain; dsRBD1 and dsRBD2, double stranded RNA binding domain 1 and 2. Phosphorylation site Tyr²⁶⁷ (Y267) is located between the NLS and the Rhed domain. (C) Immunoblotting of total lysates or anti-FLAG pull-down fractions from HCT116 cells transfected with the indicated plasmids. IP, immunoprecipitation. (D) Abundance of pri-miR-34c (left) and miR-34c (right) in cells cotransfected as indicated with the 34c minigene relative to minigene-only–transfected cells. (E) Percentage of input pri-miR-34c cross-linked to FLAG-epitope in HCT116 cells cotransfected with the 34c minigene and the indicated plasmids. (F) Percentage of input pri-miR-34c cross-linked to Drosha in HCT116 cells cotransfected with the indicated plasmids. (G) Immunoblotting (IB) (left) and pri-miR-34c expression (right) of HCT116 cells cotransfected with the 34c minigene and the indicated plasmids relative to minigene-only–transfected cells. Data are means ± SD of three independent experiments. *P < 0.05,**P < 0.01, ***P < 0.001, two-tailed t tests.

stimulated the transcription of pri-miR-34a and pri-miR-34c in fibroblasts, and this might explain the nuclear Abl requirement for DNA damage–induced production of both miR-34a and miR-34c in the mouse liver, thymus, and spleen. By contrast, in the kidney, the abundance of miR-34c was found to be comparable in the Abl+/+ and the Abl−/− mice; however, the miR-34a abundance may be skewed toward serine/threonine phosphorylations and did not reflect the effects of Tyr²⁶⁷ phosphorylation. Tyr²⁶⁷ is located immediately upstream to the Rhed domain that has RNA binding activity (48). It is conceivable that phosphorylation at Tyr²⁶⁷ may alter the Rhed-mediated interaction of DGCR8 with pri-miR-34c to promote Drosha recruitment and RNA cleavage. Alternatively, Tyr²⁶⁷ phosphorylation may release DGCR8 from the effect of an inhibitory factor that also interacts with pri-miR-34c to interfere with RNA processing.

DISCUSSION

Sequences flanking the pre-miR-34c stem-loop confer ABL dependence on processing

The Drosha/DGCR8 microprocessor must position itself at the pre-miRNA stem-loop for accurate processing (46, 50), and at least 40 nt of flanking sequences on each side of the stem-loop are required for efficient cleavage (51). A recent study identified three conserved sequence motifs that distinguish human pri-miRNAs for processing by Drosha/DGCR8 with two of those three motifs in the sequence flanking the stem-loop (17). These human-specific flanking sequence motifs can be found in the imatinib-insensitive and the imatinib-sensitive miRNAs and thus do not specify the dependency of processing on ABL. We did not identify conserved elements in the alignments of the sequences flanking the pre-miRNA stem-loops of the imatinib-sensitive miRNAs, suggesting that RNA secondary structure may also play a role in determining ABL dependency. We found that the 34c-flanking sequences can promote DGCR8 cross-linking to primary RNA containing either the pre-pri-miR-34c or the pre-pri-miR-34a stem-loops, and that this flanking sequence–stimulated DGCR8 cross-linking was inversely correlated with pri-miRNA processing. These findings suggest two possible ways for the pri-miR-34c flanking sequences to regulate processing: (i) the flanking sequences may directly contribute to the formation of a DGCR8-RNA complex that is not efficiently processed, or (ii) the flanking sequences may recruit another RNA binding protein to inhibit processing. An example of regulation of pri-miRNA processing by RNA binding proteins can be found with LIN28, which binds to the terminal loop of pri-let-7 to inhibit processing (52).
The natural text is as follows:

**Materials and Methods**

**Antibodies and plasmids**

Commercial antibodies used are for p53 (Santa Cruz Biotechnology), ABL 8E9 (PharMingen), Drosha (Abcam), DGC8R8 (Abcam), tyrosine phosphorylation (4G10, Millipore), and GAPDH (Millipore). The 34a and 34c minigenes were generated by ligating PCR products from the human mir-34a and mir-34c exons into Bam HI/Xba I–digested pKD1-miR-1225 (57). The vector control was generated by self-ligation of Pme I–digested 34c miRNA. The mutant minigenes A34c, C34a, 34aS, and 34aS were generated by three rounds of PCR. In the first round, the mutated upstream flanking region, mutated hairpin, and mutated downstream flanking region of each mutant minigene were PCR-amplified from 34a and 34c minigenes and gel-extracted. In the second round, the desired upstream flanking segment and the mutated hairpin were mixed as templates, PCR-ligated, and gel-extracted. In the third round, the second PCR product was mixed with the downstream flanking segment from the first PCR, ligated by PCR and gel-extracted. The product was then ligated back to the same restriction sites of the backbone plasmid as 34a and 34c. FLAG-Drosha, FLAG-DGCR8, and FLAG–TN-Drosha are gifts from V. N. Kim. The AblPPn plasmid was generated by two rounds of ligation: In the first ligation, Sbf I/Sal I digested fragment from CMV-Abl-PP (42) was ligated to PCR product of Sal I/µNES/Xba I fragment from Abl NES mutant plasmid (43) to generate an Sbf I/Xba I fragment containing NLS and µNES. In the second round, the Sbf I/Xba I fragment was further ligated into Sbf I/Xba I–digested CMV-Abl-PP-Nuc (AblPPn).

**Nuclear ABL stimulates miRNA expression and apoptosis**

Our finding that ABL kinase stimulates the production of a selective subset of miRNAs in DDR expands the biological functions of this capable signal transducer (53). Here, we identified miR-34c as an ABL-stimulated miRNA in human cell lines and mouse tissues. The miR-34 family of genes has been shown to stimulate apoptosis by targeting miRNAs that promote cell growth and survival (20, 22, 23). Because nuclear ABL kinase also stimulates apoptosis (37, 33), miR-34c may function as a downstream effector of nuclear ABL in the activation of apoptosis.

In mice, we have previously shown that Abl is not required for IR-induced apoptosis of neuroblasts or thymocytes (54). We have also found that nuclear Abl is not required for cisplatin to induce thymocyte apoptosis (37). Knockout of all three miR-34 family of genes in mice does not interfere with IR-induced thymocyte apoptosis either (24). Together, the mouse genetic results show that nuclear Abl stimulates miR-34a and miR-34c production in the mouse thymus, but this pathway is not essential to thymocyte apoptosis. However, we have shown that nuclear Abl kinase is required for cisplatin to induce apoptosis of the RPTCs (37). We also found that the abundance of miR-34c, but not miR-34a, was significantly reduced in the Abhrb kidney (Fig. 5C). These findings suggest that the proapoptotic function of the ABL–miR-34c pathway may be tissue-specific such that this pathway is important to the apoptotic response of RPTCs but not thymocytes. Besides miR-34c, the proapoptotic function of ABL kinase may involve the stimulation of others miRNAs, such as the imatinib-sensitive miR-216a and miR-335, which have also been shown to have proapoptotic functions (55, 56).

**miRNA sequencing**

Total RNA was isolated using TRizol (Life Technologies) and quality-controlled with the Bioanalyzer (Agilent). The miRNA libraries were prepared using the Illumina TruSeq Small RNA Sample Prep Kit and sequenced by the Illumina Genome Analyzer II. After barcode clipping, reads were aligned to the human genome (GRCh37/hg19) using the Burrows-Wheeler Aligner (http://bio-bwa.sourceforge.net/), and the miRNA read counts in each sample were normalized to the total read counts (58), and then normalized to the vehicle-treated sample, log2-transformed, median-centered, and hierarchically clustered using the Cluster and TreeView program (59). To rank the 93 miRNAs in the “induced by doxorubicin” but not “imatinib + doxorubicin” cluster, the P values for doxorubicin-induced increase in abundance were calculated using the statistics described in (60).

**RNA measurements**

Loop primers for reverse transcription and qPCR measurements of mature miRNAs are designed as described (61) and summarized in table S2, with other qPCR primers to measure pri-miRNAs and upstream exons. The authenticity of the loop primer–derived RT-PCR products was verified by hybridization and nucleotide sequencing (Fig. S1, C and D). Real-time PCRs were carried out using an ABI 7900HT fast real-time PCR system. For miRNAs, U6 was used as the reference gene. For endogenous pri-miRNAs and upstream exons, GAPDH was used as the reference gene.

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**Fig. 5. Nuclear import of ABL is required for miR-34c production in mouse tissues.** (A) Knock-in substitution mutations inactivate the three NLS in the mouse Ablµ/µNLS (µ) allele (27). (B) Expected and observed frequencies of Abl genotypes among 201 pups from breeding Abhrb with µNLS test and P value. (C to F) Abundance of miR-34a and miR-34c in mouse kidney (C), liver (D), thymus (E), and spleen (F) at 48 hours after intraperitoneal injection with vehicle (white bars) or cisplatin (gray bars) relative to vehicle-treated mice. Data are means ± SD of three mice. ***P < 0.001, two-way ANOVA test of interaction with Bonferroni corrections on the two main effect tests on the two mouse Abl genotypes in (C); *P < 0.05, ***P < 0.01, *P < 0.001, two-tailed t tests of the cisplatin effects on miRNA expression (D to F).
For pri-miRNAs from the transfected minigenes, the cotransfected GFP was used as the reference gene. Normalized \( \Delta C_T \) values (\( \Delta C_T \) experimental gene – reference gene) were from triplicate qPCR per biological sample. Relative abundance values were those of 2^\(-\Delta\Delta C_T\) by subtracting the \( \Delta C_T \) values of vehicle-treated or vector-transfected cells. Northern blotting of miRNA was performed using locked nucleic acid probes as described (62).

**Induction of DNA damage**

For imatinib and doxorubicin treatments, cells were grown to 70% confluent, pretreated with vehicle or 10 \( \mu \)M imatinib for 2 hours, and then treated with vehicle or doxorubicin (0.5 \( \mu \)g/ml (Sigma)) for additional 24 hours. For AblPPN- and minigene-transfected cells, imatinib treatment was for 12 hours at 10 \( \mu \)M. For cisplatin treatment, 8- to 10-week-old mice were given a single intraperitoneal injection of clinical-grade cisplatin at 20 mg/kg.

**Transfection**

Cells were transfected using GeneTrans II (Biomiga). For Dicer1 siRNA, cells were transfected with 25 nM of Silencer Select Negative Control No. 1 siRNA (Life Technologies) or Dicer1 siRNA SI00300006 (Qiagen) using RNAiFect (Qiagen) twice over 48 hours.

**Lentiviral infection**

Lentiviral particles were generated by transient transfection of 293T cells with a pLKO-shRNA (short hairpin RNA) expression plasmid targeting the human ABL (Sigma), and the packaging vectors expressing the HIV GAG/POL, REV, and the VSVG. The supernatants were collected at 12 hours at 10^6 pfu/ml.

**Whole-cell lysates and immunoprecipitation**

Radioimmunoprecipitation assay buffer [25 mM tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 10% glycerol, 1 mM phenylmethylsulfonfyl fluoride (PMSF), 1 \( \times \) protease inhibitor cocktail (Roche), 50 mM NaF, 10 mM sodium \( \beta \)-glycerophosphate, 10 mM NaPO_4, 10 mM NaVO_4] was used to lyse cells. To detect tyrosine phosphorylation of DGC8, cell lysate was immunoprecipitated with anti-FLAG beads and resolved by polyacrylamide gel electrophoresis (PAGE) and immunoblotting analysis.

**RNA cross-linking immunoprecipitation**

The RNA-qCLIP was performed exactly as described (63). Briefly, 10 million HCT116 cells were transfected with a minigene plasmid and then cross-linked at 48 hours after transfection. The sonicated lysate was treated with RQ1 deoxyribonuclease (DNase) (Promega) before incubation with antibodies. Random hexamers were used for the reverse transcription of the input or the immunoprecipitated RNA. After qPCRs, the \( C_T \) values from immunoglobulin G–precipitated samples were subtracted from the \( C_T \) values from antibody-precipitated samples. The subtracted \( C_T \) values were then normalized to the \( C_T \) values from the input RNA to calculate the percentage of input RNA recovered in each antibody-precipitated samples. Values are means and SE from three independent experiments, with triplicate qPCRs performed per sample.

**Biotin-labeled RNA pull-down of DGCR8**

The 34a, C34a, and 34c minigene plasmids were first linearized with Bgl II and gel-extracted. The RNA of the precursor minigenes was then generated by in vitro transcription with biotin-labeled uridine triphosphate (Roche) and T7 RNA polymerase (Promega) following the manufacturer’s instructions. The RNA products were treated with DNase (Sigma), purified with TRIzol (Life Technologies), and quantified. Nuclear extraction of HCT116 parental and ABL-knockdown cells was performed following Life Technologies’ online protocol. To perform the pull-down, 15 \( \mu \)l of NETN [20 mM tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA (pH 8.0), 0.1% NP-40, 10% glycerol, 1 mM diethiothreitol, 1 mM PMSF, 50 mM NaF, 10 mM \( \beta \)-glycerophosphate, 10 mM tetrasodium pyrophosphate, 10 mM sodium orthovanadate]–washed streptavidin beads (GE Healthcare) was mixed with 1 \( \mu \)g of either biotin-labeled RNA template and shaken at 4°C for 30 min. After three washes with NETN, the RNA-bound beads were mixed with 100 \( \mu \)g of HCT116 nuclear extract on a shaker at 4°C for 2 hours. After three more washes with NETN, SDS loading dye was added directly to the beads or 10 \( \mu \)g of the nuclear extract (input control) and boiled for 5 min. The supernatant was then resolved by PAGE and subjected to Western analysis with the indicated antibodies.

**Statistical analyses**

Statistical tests of qPCR results for miRNA gene expression were performed on the experimental \( \Delta C_T \) values (experimental gene – reference gene). Interaction contrasts from two-way ANOVA (Prism 5.0) were used to test for significance differences between genotypes in the effects of treatment with doxorubicin or cisplatin, that is, differences between the treatment effect comparing Abl\(^{+/−}\) versus Abl\(^{+/+}\) mouse tissues and fibroblasts or comparing parental versus ABL-knockdown HCT116 cells. The significance of the imatinib effect on doxorubicin induction of primary or mature miRNA expression within each cell line or genotype was determined by two-tailed t tests (Prism 5.0), as indicated by bars. A Bonferroni correction was used to adjust for multiple comparisons within each experiment.

**SUPPLEMENTARY MATERIALS**

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Fig. S1. Effects of ABL knockdown and imatinib on doxorubicin-induced miRNA expression.
Fig. S2. Expression of miRNAs predicted to be targets of the miR-3d family of miRNAs.
Fig. S3. Sequences flanking the pre-miRNA stem-loops did not affect DGCR8 binding in vitro.
Fig. S4. Reduced expression of pri-miR-34a and pri-miR-34c in Abl\(^{+/+}\) mouse embryonic fibroblasts.
Fig. S5. Limited overlaps between Mim23-DGCR8 stimulated progrowth miRNAs in HeLa cells and the miRNAs identified in this study.

Table S1. Ranking of 22 miRNAs in the “induced by doxorubicin but not by imatinib + doxorubicin” cluster by the significance of doxorubicin effect (from the miRNAseq data).
Table S2. Primers used in this study.

**REFERENCES AND NOTES**

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EnABLING DNA damage–induced miRNA production
DNA damage stimulates the production of microRNAs (miRNAs) that help coordinate DNA damage repair, cell cycle checkpoints, and apoptosis. Here, Tu et al. found that the tyrosine kinase ABL directly activated the production of a subset of DNA damage–induced miRNAs, particularly miR-34c. ABL phosphorylated the RNA binding protein DGCR8, which binds the enzyme Drosha to form the miRNA processing complex. Mutating the phosphorylation site in DGCR8 impaired the recruitment of Drosha to immature miR-34c and its subsequent processing in cultured cells, whereas expressing a nuclear import–defective mutant of ABL in mice reduced the abundance of miR-34c as well as apoptosis in kidney epithelial cells exposed to the DNA-damaging agent cisplatin. These results suggest that ABL coordinates aspects of the DNA damage response by promoting the production of some miRNAs.