Published in final edited form as:

Sci Signal. ; 8(383): ra64. doi:10.1126/scisignal.aaa4468.

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

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Abstract

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 comprised of the RNase 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-miRNA* stem-loop and recruits Drosha to the miRNA. RNA crosslinking 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 co-expressed 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

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Supplementary Materials

fig. S1. Effects of ABL-knockdown and imatinib on doxorubicin induced miRNA expression.

fig. S2. Expression of mRNAs predicted to be targets of the *miR-34*-family of genes.

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^{fl/fl}* mouse embryo fibroblasts.

fig. S5. Limited overlaps between Mim-23-DGCR8 stimulated pro-growth 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.

Author contributions: C.-C.T. designed and conducted the majority of experiments and prepared the manuscript. Y.Z., L.N., A.T. and P.S. conducted some of the experiments presented in the manuscript. P.S. and W.Y.T. reviewed and revised the manuscript. J.Y.J.W. conceived this study, designed the experiments and revised the manuscript.

Competing interests: The authors declare no competing interests.

Data and materials availability: The complete miRNAseq data set from this study has been deposited in National Center for Biotechnology Information's Gene Expression Omnibus and is accessible through GEO Series accession number GSE59028.

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 non-coding 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 microRNA (pri-miRNA) with a local stem-loop that is co-transcriptionally recognized and cleaved by the DGCR8/Drosha microprocessor complex to produce the precursor microRNA (pre-miRNA) of ~70 nt (4, 5). The pre-miRNA is further processed by Dicer (6, 7) and loaded onto the miRNA-induced silencing complex (miRISC) (5). The co-transcriptional 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 (ATM) 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-proliferation and anti-apoptotic 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 non-identical mechanisms.

The ubiquitously expressed non-receptor 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 repeated 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 lab

has generated the *Abl-μNLS* allele in the mouse *Abl1* gene by knockin mutations of 11 amino acids to inactivate the three NLS (27). 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 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.

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 miRNAseq in HEK293T 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 non-kinase enzymes NQO2 (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 expression was ABL-dependent.

To determine if 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 knockout 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. 1H). 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. While 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

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 CMV promoter drives the transcription of 277 bp of exon sequence containing the *pre-miR-34c* stem-loop of 77 nucleotides (nt) (predicted by miRbase <http://www.mirbase.org/index.shtml>) flanked by 100 nt each of upstream and downstream sequences. This minigene does not contain any of the *pre-miR-34b* stem-loop or surrounding sequences. For comparison, we also constructed a 34a-minigene with 310 bp of exon sequence containing the predicted stem-loop of 110 nt and flanking sequences (Fig. 2A). We expressed a constitutively activated nuclear ABL tyrosine kinase that contained two Pro mutations (P242E, P249E) to disrupt auto-inhibition (42) and a Leu mutation (L1109A) to inactivate the nuclear export signal (NES), called AblPPn (43) (Fig. 2, B and C). The amount of mature miR-34a increased in cells transfected with the 34a-minigene and co-transfection with AblPPn did not have a significant effect on the production of miR-34a (Fig. 2, D and E). Transfection with the 34c-minigene did not raise the abundance of miR-34c, but AblPPn co-transfection significantly stimulated miR-34c production from the minigene (Fig. 2D, E). We measured the expression of seven mRNAs 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*, *CCNE2*), suggesting a more important role for miR-34b/c in targeting these mRNAs. Co-transfection of HCT116 cells with AblPPn and the 34c-minigene also reduced the expression of these two and other mRNA targets of miR-34c (*CAV1*, *CDK4*, *SRSF2*, *MET*, *E2F3*, *MYB*, *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 RNase (Fig. 2G) and by a trans-dominant 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.

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, 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 minigenes containing the 34c-flanking sequences, including 34c, C34a, and C34aS (Fig. 3C). AblPPn did not stimulate the processing of *pri-miR-34a* or *pri-miR-34aS*, but it stimulated the processing of *pri-miR-A34c* (Fig. 3C). These results suggested that the miR-34c stem-loop and flanking sequences could both mediate the effect of ABL. Co-expression with TN-Drosha (Fig. 3D) abolished the AblPPn-stimulated reduction in *pri-miR-34c*, *pri-miR-C34a*, and *pri-miR-C34aS* (Fig. 3E). These results showed that AblPPn stimulated the Drosha-dependent processing of pri-miRNAs that contained the flanking sequences in the 34c-minigene.

Flanking sequences promote and Tyr phosphorylation reduces DGCR8 interaction with *pri-miR-34c*

DGCR8 has been shown to bind RNA without sequence-specificity (45), and its interaction with the pre-miRNA stem-loop is critical to Drosha recruitment and RNA processing (46). By RNA crosslinking immunoprecipitation (RNA-qCLIP), we could detect endogenous DGCR8 interaction with pri-miRNA transcribed from the 34c and the C34a minigenes but not that from the 34a minigene (Fig. 4A). This result suggested that the 34c-flanking sequences had a stabilizing effect on the DGCR8-RNA interaction. Expression of AblPPn caused a significant reduction of DGCR8 crosslinking 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 AblPPn from reducing the crosslinking 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 AblPPn stimulated processing but reduced DGCR8-RNA crosslinking; 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 (<http://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, aa 276–498), which a recent report suggests to also make contacts with the precursor miRNA stem-loop (48) (Fig. 4B). We found that co-transfection with AblPPn increased Tyr²⁶⁷ phosphorylation of DGCR8, and that mutation of Tyr²⁶⁷ was sufficient to substantially reduce that phosphorylation (Fig. 4C). Co-expression of the wild-type DGCR8 did not interfere with AblPPn-stimulated reduction in *pri-miR-34c* or increase in miR-34c (Fig. 4D); however, expression of Y267F-DGCR8 blocked these effects of AblPPn (Fig. 4D). By RNA-qCLIP, we found that Y267F-DGCR8 also interacts with *pri-miR-34c*, but AblPPn could not reduce this crosslinking (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). Co-expression of Drosha with AblPPn, wild-type DGCR8, or Y267F-DGCR8 reduced the crosslinking of Drosha to *pri-miR-34c* (Fig. 4F). This reduction in Drosha crosslinking 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 co-expression with wild-type DGCR8 stimulated processing (loss of *pri-miR-34c*) but that co-expression 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 AblPPn or by Drosha. Because Y267F-DGCR8 inhibits Drosha crosslinking 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 *Abl-μNLS* allele in which the three nuclear localization signals (NLS) are inactivated by germ-line knock-in mutations in mice (Fig. 5A). Unlike the developmentally defective *Abl*-knockout mice (49), mice homozygous for the *Abl-μNLS* allele (the *Abl^{μ/μ}* 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 renal proximal tubule epithelial cells (RPTC) was found to be defective in the *Abl^{μ/μ}* mice (37). We therefore examined the effect of the *Abl-μNLS* allele on miR-34c production in mouse tissues.

It was previously reported that ionizing radiation (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 *Abl^{+/+}* mice (Fig. 5, C to F). This cisplatin-induced increase in miR-34a and miR-34c was not observed in the *Abl^{μ/μ}* 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 the mature miR-34a and miR-34c in embryo fibroblasts from the *Abl^{+/+};p53^{-/-}* and the *Abl^{μ/μ};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 *Abl^{+/+}* but not the *Abl^{μ/μ}* fibroblasts suggesting that nuclear Abl is required *pri-miR-34a* and *pri-miR-34c* transcription in mouse embryo fibroblasts (fig. S4). Furthermore, the basal expressions of both the primary miRNAs and the mature miR-34a and miR-34c were significantly reduced in the *Abl^{μ/μ};p53^{-/-}* fibroblasts (fig. S4). These results showed that nuclear Abl 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-34a was found to be comparable in the *Abl^{+/+}* and the *Abl^{μ/μ}* mice; however, the miR-34c abundance was significantly reduced in the *Abl^{μ/μ}* mice (Fig. 5C). The miR-34c-selective effect of nuclear Abl is therefore observed in the mouse kidney, where nuclear Abl is also required for cisplatin to induce apoptosis (37).

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 the ABL-dependency. We found that the 34c-flanking sequences can promote DGCR8 crosslinking to primary RNA containing either the *pre-miR-34c* or the *pre-miR-34a* stem-loops, and that this flanking sequence-stimulated DGCR8 crosslinking 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).

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. Co-expression 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 crosslinking 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 (MAPK) and a DGCR8 mutant with 23 phosphomimetic substitution (Mim23) promoted the expression of a cohort of pro-growth 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 Mim-23 mutates 22 serine/threonine sites and 1 tyrosine site (Tyr²⁶⁷), the effect of Mim-23-DGCR8 on microRNA expression may be skewed towards 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.

Nuclear ABL stimulates microRNA expression and apoptosis

Our finding that ABL kinase stimulates the production of a selective subset of miRNAs in DNA damage response expands the biological functions of this capable signal transducer (53). In this study, 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 mRNAs that promote cell growth and survival (20, 22, 23). Because nuclear ABL kinase also stimulates apoptosis (37, 53), miR-34c may function as a downstream effector of nuclear ABL in the activation of apoptosis.

In the 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). Interestingly, 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 renal proximal tubule epithelial cells (RPTC) (37). We also found that the abundance of miR-34c, but not miR-34a, was significantly reduced in the *Abl^{fl/fl}* kidney (Fig. 5C). These findings suggest that the pro-apoptotic function of the ABL-miR-34c pathway may be tissue specific such that this pathway is important to the apoptotic response of RPTC but not thymocytes. Besides miR-34c, the pro-apoptotic 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 pro-apoptotic functions (55, 56).

Material and Methods

Antibodies and Plasmids

Commercial antibodies used are for p53 (Santa Cruz Biotechnology), ABL 8E9 (Pharmingen), Drosha (Abcam), DGCR8 (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-minigene. The mutant minigenes A34c, C34a, 34aS, and C34aS were generated by 3 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 generous gifts from V. N. Kim. The AblPPn plasmid was generated by two rounds of ligation: in the first ligation, *SbfI/SalI* digested fragment from CMV-Abl-PP (42) was ligated to PCR product of *SalI/μNES/XbaI* fragment from Abl NES mutant plasmid (43) to generate a *SbfI/XbaI* fragment containing NLS and μNES. In the second round, the *SbfI/XbaI* fragment was further ligated into *SbfI/XbaI* digested CMV-Abl-PP-Nuc (AblPPn). The integrity of all plasmids generated in this study was verified by sequencing. The cloning primer sequences are listed in table S2.

MiRNAseq

Total RNA was isolated using Trizol (Life Technologies) and quality-controlled with the BioAnalyzer (Agilent). The microRNA 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 microRNA reads were matched to the sequences of the mature human miRNAs in the mirBase (Release 17). Above 2 million unambiguous reads from each biological sample were mapped to a total of 501 annotated miRNAs. For unsupervised hierarchical clustering, 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 was 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 Supplementary 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. S1C, D). Real-time PCR reactions 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. For pri-miRNAs from the transfected minigenes, the co-transfected GFP was used as the reference gene. Normalized CT values (CT, experimental gene – reference gene) were from triplicate qPCR reactions per biological sample. Relative abundance values were those of 2^{-CT} by subtracting the CT values of vehicle-treated or vector-transfected cells. Northern blotting of miRNA was performed using locked nucleic acid (LNA) probes as described (62).

Induction of DNA damage

For imatinib and doxorubicin treatments, cells were grown to 70% confluent, pre-treated with vehicle or 10 μM imatinib for 2 h, and then treated with vehicle or 0.5 μg/ml doxorubicin (Sigma) for additional 24 hours. For AblPPn and minigene transfected cells, imatinib treatment was for 12 hours at 10 μM. For cisplatin treatment, 8–10 week-old mice were given a single intraperitoneal injection of clinical grade cisplatin at 20-mg/kg body weight.

Transfection

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

Lentiviral infection

Lentiviral particles were generated by transient transfection of 293FT cells with a pLKO-shRNA 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 48 hours after transfection and used to infect HCT116 cells in the presence of 8 µg/ml polybrene, followed by selection for stable infection by selection for resistance to puromycin (2.0 µg/ml).

Whole cell lysates and immunoprecipitation

RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 10% glycerol, 1mM PMSF, 1× protease inhibitors cocktail (Roche), 50 mM NaF, 10 mM Sodium β-glycerophosphate, 10 mM Na₄P₂O₇, 10 mM NaVO₄) was used to lyse cells. To detect tyrosine phosphorylation of DGCR8, cell lysate was immunoprecipitated with anti-FLAG beads, resolved by polyacrylamide gel electrophoresis and immunoblotting analysis.

RNA Crosslinking Immunoprecipitation (RNA-qCLIP)

The RNA-qCLIP was performed exactly as described (63). Briefly, 10 million HCT116 cells were transfected with a minigene plasmid and then crosslinked at 48 hours after transfection. The sonicated lysate was treated with RQ1 DNase (Promega) before incubation with antibodies. Random hexamers were used for the reverse transcription of the input or the immunoprecipitated RNA. Following qPCR reactions, the CT values from IgG-precipitated samples were subtracted from the CT values from antibody-precipitated samples. The subtracted CT values were then normalized to the CT values from the input RNA to calculate the percentage of input RNA recovered in each antibody-precipitated samples. Values are means and standard errors from 3 independent experiments, with triplicate qPCR reactions 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 UTP (Roche) and T7 RNA polymerase (Promega) following the instructions of the manufacturers. The RNA products were DNase (Sigma) treated, purified with Trizol (Life Tech), and quantified. Nuclear extraction of HCT116 parental and ABL knockdown cells was performed following Life Tech's online protocol. To perform the pull-down, 15 µl of NETN (20mM Tris HCl pH 8.0, 100 mM NaCl, 1 mM EDTA pH 8.0, 0.1% NP40, 10% glycerol, 1 mM DTT, 1 mM PMSF, 50 mM NaF, 10 mM β-glycerophosphate, 10 mM tetrasodium pyrophosphate, 10 mM sodium orthovanadate)-washed streptavidin beads (GE) were mixed with 1 µg of either biotin-labeled RNA template and shaken at 4°C for 30 min.

After 3 washes with NETN, the RNA-bound beads were mixed with 100 µg HCT116 nuclear extract on a shaker at 4°C for 2 hours. After 3 more washes with NETN, SDS loading dye was added directly to the beads or 10 µg 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 microRNA gene expression were performed on the experimental CT 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, i.e., differences between the treatment effect comparing *Abt*^{+/+} vs. *Abt*^{W^U} mouse tissues and fibroblasts or comparing parental vs. ABL-knockdown HCT116 cells. The significance of the imatinib effect on doxorubicin induction of primary or mature microRNA 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 Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. V. N. Kim for the Drosha-FLAG, the TN-Drosha-FLAG and the DGCR8-FLAG expression plasmids. We thank Dr. M. L. Hastings for providing the *miR-1225* minigene, the backbone of which was used to generate the 34a and the 34c minigenes. We thank Dr. K. Messer and Dr. M. Pu for consultation on statistical analyses. We thank J. Chen, D. Lau and A. Hwang for assisting with the experiments.

Funding: This research was supported by a grant (CA043054) from the National Cancer Institute (to J.Y.J.W.).

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Fig. 1. ABL tyrosine kinase stimulates the expression of *miR-34c*

(A) Unsupervised hierarchical clustering of miRNAseq results from 293T cells treated for 24 hours with V: vehicle, IM: imatinib (10 μ M), D: doxorubicin (0.5 μ g/ml), or I+D: a combination of imatinib and doxorubicin. 93 miRs were clustered into the “D but not IM +D” category. (B) Top five miRNAs from (A) increased by doxorubicin but not by the imatinib+doxorubicin combination. The *p*-values were calculated as described in the Methods. (C and D) Production of miR-34a and miR-34c in MCF7 (C) or HCT116 (D) cells at 24 hours after the indicated treatments with IM and/or D relative to vehicle treatment. (E)

Immunoblotting of total lysates from the indicated HCT116 cells at 24 hours of the indicated treatments. kd: knockdown. (F) Production of miR-34c in cells treated as in (E) relative to vehicle treatment. (G) Schematic of the human *miR-34b/c* gene locus. E1: upstream exon. (H) Expression of *miR34b/c-E1* in cells treated as in (E) relative to vehicle treatment. Relative abundance values are mean $\pm$ SD of at least 3 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 ANOVA test of interaction, with Bonferroni correction for the two main effect tests on the two cell lines (F).

Fig. 2. ABL kinase stimulates Drosha-dependent processing of *pri-miR-34c* from a minigene (A) Diagrams of vector (Vec), and the minigenes (34a, 34c). The primer sets for the mature miRNAs and the uncut *pri-miRNAs* are shown. Numbers are genomic sequences (nt) flanking the pre-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 co-transfections of AblPPn and Dicer-siRNA (**G**) or with trans-dominant Drosha (TN-Drosha) or wild-type 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 co-transfections of AblPPn, Drosha, or TN-Drosha (**I**) or treated with imatinib (IM) (**J**) relative to minigene-only transfected cells. pTyr: phospho-tyrosine. Data are mean $\pm$ SD of 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, two-tailed t tests.

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 co-transfected with the indicated minigenes and AbIPPN relative to vector co-transfected cells. **(D)** Immunoblotting of lysates from HCT116

cells co-transfected with AblPPn and TN-Drosha as indicated. (E) Expression of the indicated pri-miRNA in HCT116 cells co-transfected with minigenes and the indicated plasmids relative to vector co-transfected cells. Data are mean $\pm$ SD from 3 independent experiments. ** $P < 0.01$, *** $P < 0.001$, two-tailed t tests.

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Fig. 4. Flanking sequences promote and tyrosine phosphorylation reduces DGCR8 interaction with *pri-miR-34c*

(A) Percentage of input *pri-miRNA* crosslinked to endogenous DGCR8 in HCT116 cells transfected with the indicated minigenes and expression plasmids. Primers for qRT-PCR of the *pri-miRNAs* are shown. (B) Domains of DGCR8. NLS: nuclear localization signal, Rhed: RNA binding heme domain, WW: WW domain, dsRBD1 and dsRBD2: double strand 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. pTyr: phosphotyrosine. (D) Abundance of *pri-miR-34c* (left) and *miR-34c* (right) in cells co-transfected 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 co-transfected with the

34c minigene and the indicated plasmids. (F) Percentage of input *pri-miR-34c* crosslinked to Droscha in HCT116 cells co-transfected with the indicated plasmids. (G) Immunoblotting (left) and *pri-miR-34c* expression (right) of HCT116 cells co-transfected with the 34c minigene and the indicated plasmids relative to minigene-only transfected cells. Data are mean $\pm$ SD of 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, two-tailed t tests.

Fig. 5. Nuclear import of ABL is required for miR-34c production in mouse tissues
(A) Knock-in substitution mutations inactivate the three nuclear localization signals (NLS) in the mouse *Abl-μNLS* (μ) allele (27). **(B)** The expected and observed frequencies of *Abl*-genotypes among 201 pups from breeding *Abl*^{+/μ} mice with Chi-Square 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 h after intraperitoneal injection with vehicle (white bars) or cisplatin (grey bars) relative to vehicle-treated *Abl*^{+/+} tissues. Data are mean $\pm$ SD of 3 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 and *** P <0.001, two-tailed t tests of the cisplatin effects on microRNA expression (D to F).