UNIVERSITY OF CALIFORNIA, SAN DIEGO

Multipathway Regulation of DNA Damage Response by Nuclear ABL

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by

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The thesis of Louis Thien Toan Nguyen is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

I dedicate this work to everyone I have ever loved and cared for.

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I would first like to express my gratitude to Dr. Wang for being my teacher for these past few years and allowing me to work in her lab. Her guidance has led me to become a better student, scientist, and person.

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Figures 3, 4C, 5A, and 5B are reprints of the figures as they appear in: "The kinase ABL phosphorylates the microprocessor subunit DGCR8 to stimulate primary microRNA processing in response to DNA damage." Tu, Chi-Chiang, Yan Zhong, Louis Nguyen, Aaron Tsai, Priya Sridevi, Woan Yu Tarn, and Jean YJ Wang. *Science signaling* 8, no. 383 (2015): ra64. The thesis author was a co-author of this paper.

ABSTRACT OF THE THESIS

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Master of Science in Biology

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The ubiquitously expressed nonreceptor tyrosine kinase ABL becomes activated and accumulates in the nucleus when cells undergo DNA damage. Nuclear ABL can then stimulate apoptosis when the cell is unable to repair its DNA. Here we have discovered novel mechanisms in which nuclear ABL regulates cellular DNA damage response. We have identified that ABL can significantly enhance the production of the pro-apoptosis protein PUMA in a post-

transcriptional manner. We have also found that nuclear ABL is necessary for p21 activation following DNA damage in the absence of p53. We further expanded our studies into microRNAs, and have found that ABL can phosphorylate the Microprocessor protein DGCR8 in order to stimulate selective microRNA processing of pro-apoptotic microRNAs. Together, our results provide new insights on the role of ABL in DNA damage response.

I:

Introduction

Nuclear ABL Tyrosine Kinase

ABL is a ubiquitously expressed nonreceptor tyrosine kinase that can be activated by certain types of cell stress such as DNA damage (1). ABL is a 135kDa protein containing an N-terminal SH3, SH2, and kinase domain, as well as a C-terminal actin binding domain (2, 3). The ABL protein responds to cellular location cues through 3 nuclear localization sequences (NLS) as well as 1 nuclear export sequence (NES) (4). Being a nonreceptor kinase, ABL can be the substrate to many signals that each may activate different specific biological processes.

It has been shown that upon induction of DNA damage, the cell will shuttle ABL into the nucleus, causing it to accumulate (5). Activated nuclear ABL will then act on several pro-apoptotic pathways, including the p53 and p73 family of transcription factors (1, 6). Our laboratory has mutated the mouse ABL gene and generated a mutant ABL allele (ABL-μNLS) containing 3 inactive nuclear localization signals by knock-in mutation. Mice engineered with the ABL-μNLS allele are healthy and fertile, indicating that nuclear ABL is not essential to development (4). However, using a system consisting of cisplatin-induced DNA damage in renal proximal tubule epithelial cells, it was found that mice containing the ABL-μNLS allele are defective in apoptosis (7). Therefore it is evident that nuclear ABL is necessary for DNA-damage induced apoptosis.

DNA Damage Response

Genomic DNA in the cell is constantly receiving damage from a variety of sources. These sources can include DNA mismatches in replication, to environmental toxins that chemically react with DNA, to absorption of ionizing radiation (8). To ensure cell survival and replication, cells have evolved mechanisms to detect DNA damage and promotes its repair. In cases where DNA damage is too great, cells may proceed to undergo programmed cell death, known as apoptosis (9).

Upon DNA damage, there is an immediate activation of various kinases which serve as the primary signal transducers in the signaling cascade. These kinases will then phosphorylate key downstream effectors to amplify the DNA damage signal and activate various transcription factors such as p53 (9, 10). Transcription factors such as p53 will then serve to selectively amplify key genes. The result of this process is drastically increased levels of mRNAs and proteins related to cell cycle arrest, DNA repair, or apoptosis (9, 10, 11). However, recent studies have demonstrated that this drastic change of gene expression has also been linked to changes in microRNA (miRNA) expression. Studies have uncovered that certain miRNAs are stimulated following DNA damage (12, 13). One of the most studied miRNAs are those belonging to the miR-34 family. It was found that miR-34 expression causes cell cycle inhibition by decreasing the expression of pro-apoptotic genes. This expression also can promote apoptosis by decreasing the expression of anti-apoptotic genes (14, 15).

MicroRNAs and their biogenesis

MicroRNAs (miRNAs) are short non-coding RNAs of approximately 22 nucleotides in length that play a central role in post-transcriptional gene regulation (16). miRNAs are integrated into the RNA-induced silencing complex (RISC). The RISC regulates genes through complementary base pairing to messenger RNAs (mRNAs), reducing their stability and ability to be translated (17).

miRNA biogenesis begins with transcription by RNA polymerase II, generating a long primary miRNA (pri-miRNA) containing a local hairpin loop structure in which the mature miRNA transcript resides (18). This long pri-miRNA transcript is then processed by the Microprocessor complex, a complex consisting of the two proteins: Drosha and DiGeorge syndrome critical region 8 (DGCR8) (19). This processing consists of the cleavage of primary miRNA at specific sites to release the ~65nt hairpin loop structure, generating what is known as precursor miRNA (pre-miRNA) (20). The pre-miRNA is then exported out of the nucleus by the nuclear export protein Exportin-5 (21). In the cytoplasm, the pre-miRNA is further processed by the RNase enzyme Dicer that interacts with the hairpin and cleaves the loop structure to generate a mature miRNA duplex (22). A single strand of the miRNA duplex is then loaded onto the Argonaute protein to form the RNA-induced silencing complex (RISC) (17).

DGCR8 and the Microprocessor Complex

DGCR8 is an RNA binding protein that is primarily localized in the nucleus. DGCR8 is an 86 kDa protein which contains a nuclear localization signal, RNA binding heme domain, two double-stranded RNA binding domains, and a C-terminal tail region (23). In other organisms, DGCR8 is known as Pasha, or partner of Drosha. DGCR8 binds to Drosha, an RNase III enzyme, to form the Microprocessor complex (24).

The Microprocessor complex is a ~364 kDa heterotrimeric complex of one Drosha and two DGCR8 molecules. In this complex, the DGCR8 dimer interacts with the stem and apical elements of the pre-miRNA to stabilize and activate Drosha. Drosha serves as a "ruler" by measuring 11bp from the basal ssRNA-dsRNA junction and serves as the enzyme that cleaves the pri-miRNA to pre-miRNA (24).

It has been shown that p53 is able to interact with the Microprocessor through association with the RNA helicase p68. This interaction allows for the processing of pri-miRNA to pre-miRNA (25). Therefore, it is evident that miRNAs can be regulated through modulation of miRNA processing.

II:

Results

Nuclear ABL can regulate the protein level of p53 upregulated modulator of apoptosis, PUMA

To investigate the role played by ABL in DNA damage, Dr. Priya Sridevi, a research scientist in the Wang lab, was investigating the role of ABL nuclear localization on the apoptosis pathway. It was found that nuclear ABL was required for the sustained expression of p53 upregulated modulator of apoptosis (PUMA) in murine kidneys following treatment of the DNA damaging chemotherapy drug cisplatin. PUMA protein was induced in cells following 30 hours of treatment, but was unable to continue expression at 48 hours, even though there was PUMA mRNA. From this result, it was hypothesized that nuclear ABL could regulate the expression of PUMA post-transcriptionally.

In order to confirm that nuclear ABL could directly increase the protein level of PUMA, we carried out a transient co-transfection experiment in human embryonic kidney (HEK) 293T cells. We co-transfected in a PUMA-alpha (PUMA-α) expression plasmid, a mutated nuclear ABL kinase (AblppNES), and the anti-apoptosis protein Bcl2, a protein that would serve as antagonist to PUMA to prevent cells from dying. The mutated nuclear ABL kinase contains a P242E and P249E mutation to cause constitutive activation, and a L1109A mutation to inactivate nuclear export. We also transfected a mutated form of PUMA-α that lacked the BH3 domain (PUMA-dBH3), to determine whether PUMA protein expression could be studied in cells without the use of Bcl2 co-transfection,

which could lead to off target effects. The schematics for the mutant constructs are shown (figure 1A).

The cells were then subject to SDS-PAGE and western blotting. As shown in figure 1B, we found that PUMA-α protein can be expressed in the presence of Bcl2 at a minimal level. PUMA-dBH3 was also expressed at a minimal level in the presence of Bcl2. We found however, that the co-transfection of AblppNES, Bcl2, and either PUMA-α or PUMA-dBH3 increased their protein expression by over 20 fold over only PUMA and Bcl2. These results demonstrate that ABL kinase has a direct regulatory role over the protein level of the pro-apoptosis protein PUMA.

Nuclear ABL is required for the expression of p21 protein following DNA damage in p53-deficient cells

When cells undergo DNA damage, genes related to cell cycle arrest are induced to allow for DNA repair (9). In studying the role of nuclear ABL in programmed cell death, we decided to determine whether nuclear ABL could regulate a member of the p53 pathway responsible for cell cycle inhibition, p21.

We decided to do this study in murine embryonic fibroblasts 3T3 cells. We decided to use this cell line specifically as we have a mutant (3T3-μNLS) in which the 3 nuclear localization signals of ABL have been disrupted with knock-in mutations. We treated the cells with the chemotherapy drug doxorubicin to

induce DNA damage. We also decided to collect cells at specific time points to determine whether nuclear ABL regulation is time dependent.

The cells were then subject to SDS-PAGE and western blotting. As seen in figure 2A, in wild-type cells, we noticed that within 6 hours of treatment, the cell cycle inhibitor protein p21 has significantly increased expression, correlating to the cell responding to DNA damage. This expression is maintained into 24 hours of treatment, and decreases at 48 hours. We however discovered that in 3T3-μNLS cells, there is no induction of p21 whatsoever. We also checked p53 protein, and saw that there was no p53 protein found in either the 3T3 wild-type or 3T3-μNLS cells.

To determine whether nuclear ABL was inducing p21 expression in a transcriptional manner, we conducted quantitative reverse transcriptase PCR (qRT-PCR) on 3T3 wild-type and 3T3-μNLS cells treated with doxorubicin for 8 and 24 hours (figure 2B). We have found that doxorubicin treated 3T3 cells have 6 fold increase in p21 mRNA levels at 8 hours, and a 3 fold increase at 24 hours. In μNLS cells however, there is no increase in p21 mRNA, correlating to the western blotting results. Both wild-type and μNLS cells also do not have any p53 induction following DNA damage. We also checked two other members of the p53 DNA damage pathway, MDM2 and Noxa, and found that μNLS cells were also deficient in upregulating these proteins following DNA damage. From this result, we determined that nuclear ABL is required to induce p21 expression in a p53-null background.

ABL tyrosine kinase stimulates the production of miR-34c

Because miRNAs play an important role in DNA damage response, Dr. Eric Tu, a former post-doctoral investigator from our lab, conducted a miRNA sequencing experiment in human embryonic kidney (HEK) 293T cells. These cells were treated for 24 hours with vehicle, imatinib, doxorubicin, or a combined treatment consisting of both imatinib and doxorubicin. Imatinib would serve to limit any miRNAs that depend on ABL kinase for expression. Of the 501 miRNAs sequenced with confidence, 93 miRNAs were found to be induced by doxorubicin, but not imatinib + doxorubicin (figure 3A). 22 of the 93 miRNAs were found to be significantly induced, of which miR-34c was the most induced out of all miRNAs sequenced (figure 3B).

To validate this result, using HCT116 and MCF7 cell lines, qRT-PCR was used to measure the levels of miR-34c and its family member, miR-34a (figure 3C). It was found that doxorubicin induced the production of miR-34c by 10 fold in MCF7 and 30 fold in HCT116. miR-34a levels were also found to be increased in both cell lines. However, combined treatment with imatinib caused reduction in miR-34c levels in both cell lines, but not miR-34a.

Dr. Tu also created two “minigenes” that contained the primary step loop sequence of the miR-34a and miR-34c as well as 100nt of flanking sequence. A schematic of the “minigenes” are shown (figure 3D). A qRT-PCR was performed which showed that in the presence of AblppNES, there was significant increase of miR-34c, but not miR-34a (figure 3E). A transient co-transfection experiment

was then performed with a catalytically dead form of Drosha (TN-Drosha), AblppNES, and the miR-34c minigene. It was found that in the presence of TN-Drosha and AblppNES, miR-34c expression was stunted compared to only AblppNES (figure 3F). This result led to the conclusion that Abl was regulating miR-34c not by transcription, but by regulation of processing.

ABL regulates miRNA processing by phosphorylating DGCR8

To determine how ABL is regulating miRNA expression, we conducted an initial experiment to determine whether ABL could phosphorylate DGCR8. This was done by transient co-transfection of AblppNES and a FLAG-tagged DGCR8 (FLAG-DGCR8) in HEK293T cells. The cells were also treated with the ABL kinase inhibitor imatinib to determine whether phosphorylation of DGCR8 was specific to ABL. We then carried out an immunoprecipitation assay using a FLAG-specific antibody conjugated to magnetic beads on whole cell lysates.

The precipitated protein was then resolved on SDS-PAGE and subject to western blotting against an antibody specific for phosphorylated tyrosine. As seen in figure 4A, we determined that in the presence of AblppNES, FLAG-DGCR8 is significantly more phosphorylated. The addition of imatinib to inhibit AblppNES resulted in the disappearance of tyrosine phosphorylated DGCR8. This result demonstrates that ABL can regulate DGCR8 by phosphorylation.

We found that in various proteomics studies, DGCR8 is tyrosine phosphorylated on tyrosine 267. We conducted site-directed mutagenesis on the FLAG-DGCR8 construct, and created a FLAG-DGCR8 construct expressing a DGCR8 Y267F mutant (FLAG-DGCR8-Y267F). The mutation is shown in figure 4B. We again utilized transient co-transfection, and co-transfected AblppNES, FLAG-DGCR8, and FLAG-DGCR8-Y267F followed by immunoprecipitation by FLAG.

As seen in figure 4C, we determined that FLAG-DGCR8 phosphorylation was significantly reduced in the Y267F mutant, even in the presence of AblppNES. From this result, we have demonstrated that ABL may be regulating DGCR8 by phosphorylating DGCR8 on Y267.

To determine if phosphorylation of DGCR8 is stimulating miRNA processing, qRT-PCR was performed on HCT116 cell lysates by Dr. Tu. HCT116 cells were transfected with or without AblppNES and wild-type DGCR8 or DGCR8-Y267F. The miR-34c minigene was also co-transfected. The cells would then be subject to quantitative measurement of pri-miR-34c and miR-34c. As seen in figure 4D, in the presence of AblppNES and wild-type DGCR8, there was significantly less pri-miR-34c, but more miR-34c production than without AblppNES. With DGCR8-Y267F, there was no change in pri-miR-34c or miR-34c with or without the presence of AblppNES. These results indicate that phosphorylation of DGCR8 on Y267 increases the processing of miR-34c. Taken

together, we demonstrate that ABL is regulating selective miRNA processing by phosphorylating DGCR8 on Y267.

Nuclear ABL is required for the transcription of miR-34a and miR-34c in mouse embryonic fibroblasts

It has been demonstrated that the apoptotic functions of ABL kinase require nuclear localization of the protein. Therefore it was necessary to determine whether nuclear localization of ABL was required for its regulation of miRNA processing. We again used 3T3 cells that have the ABL μNLS mutant gene. We then treated these cells with doxorubicin to induce DNA damage. Following treatment, the cells were harvested and subject to qRT-PCR and western blotting. As seen in figure 5A, we determined that ABL is activated 9 hours post treatment by doxorubicin using a phosphorylated Y245 antibody that signifies ABL activation. ABL is increasingly activated at 24 hours, but decreases at 48, possibly due to cell death.

qRT-PCR was conducted on cells treated for 8 hours by Dr. Sridevi, and it was found that doxorubicin stimulated the expression of E1-34a, pri-miR-34a, E1-34c, and pri-miR-34c in the ABL wild-type, but not μNLS cells (figure 5B). This result suggests that nuclear ABL is required for the transcription of miR-34a and miR-34c in these cells.

DNA damage induced DGCR8 phosphorylation is not capable of being detected by standard immunoprecipitation and western blotting

From the results showing that AblppNES could tyrosine phosphorylate DGCR8, we sought out to determine whether a wild-type ABL would phosphorylate DGCR8 when cells undergo DNA damage. We decided on a transient co-transfection approach in HEK 293T cells in order to maximize the likelihood of capturing an event that could possibly be extremely transient or stoichiometrically minimal. We co-transfected a wild-type mouse ABL and FLAG-DGCR8 for 40 hours. We then treated cells with the potent DNA damaging agent cisplatin for 8 hours. We then performed FLAG immunoprecipitation and resolved the eluted proteins using SDS-PAGE and western blotting.

As seen in figure 6A, we see that FLAG-DGCR8 is phosphorylated at a basal level in cells. Co-transfection of the wild-type mouse ABL did not increase phosphorylation of DGCR8, indicating that DGCR8 is not phosphorylated by inactive ABL. However, the addition of wild-type ABL to cisplatin treated cells also did not show an increase in phosphorylation of DGCR8 as well. Treatment of the cells with imatinib did not decrease the level of DGCR8 phosphorylation. From these results, we have found that standard immunoprecipitation and western blotting techniques are insufficient in capturing the DNA damage induced phosphorylation of DGCR8 by ABL.

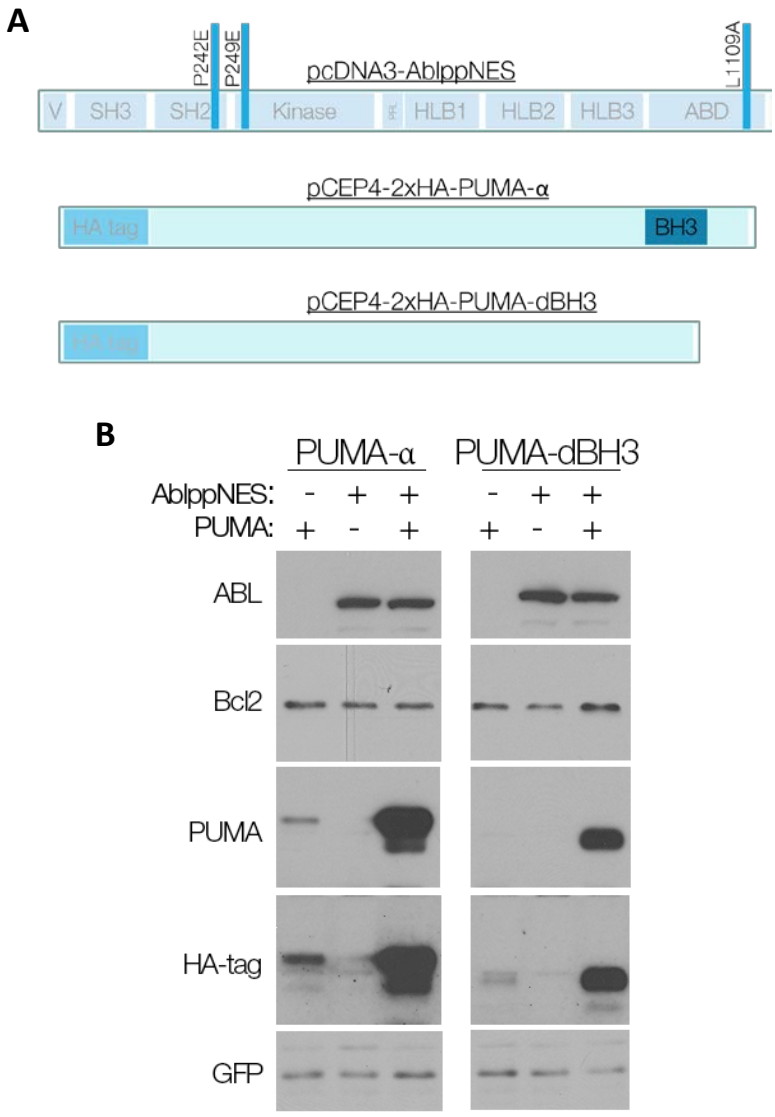

Figure 1. AblppNES can significantly enhance the protein level of the pro-apoptosis protein PUMA. (A) Diagram of the mutant constructs used in this transient co-transfection experiment. (B) Western blot from transient-co-transfection experiment probed with ABL, Bcl2, PUMA, HA-tag, and GFP specific antibodies. Results from both PUMA constructs, PUMA-α and PUMA-dBH3 are shown.

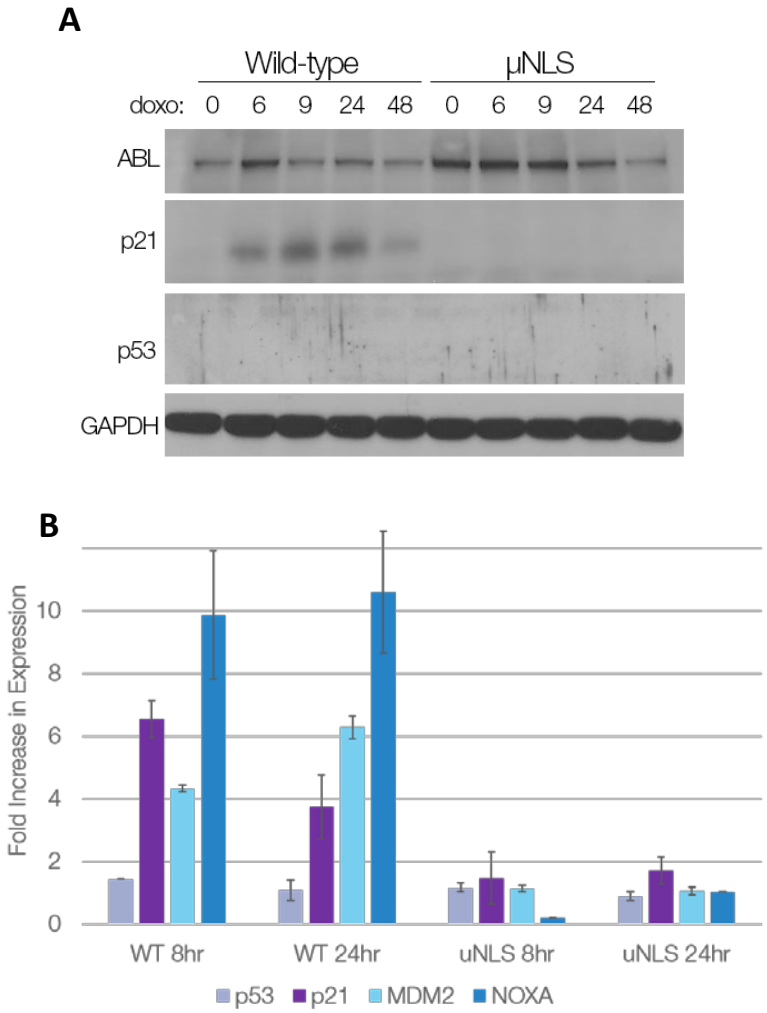

Figure 2. Nuclear ABL is required for p21 induction in the absence of p53. (A) Western blot corresponding to doxorubicin treatment of 3T3 wild-type and μNLS cells. Cells were treated with doxorubicin at a concentration of 0.2ug/ml for the indicated time shown. Western blots were probed with antibodies specific to ABL, p21, p53, and GAPDH. (B) qRT-PCR result for 3T3 wild-type and μNLS cells treated with 0.2ug/ml doxorubicin for 8 or 24 hours. Exon spanning primers specific to p53, p21, MDM2, and NOXA were used to measure mRNA levels. Relative increase in expression was calculated by 2^-(ddCT), and normalized to GAPDH mRNA levels.

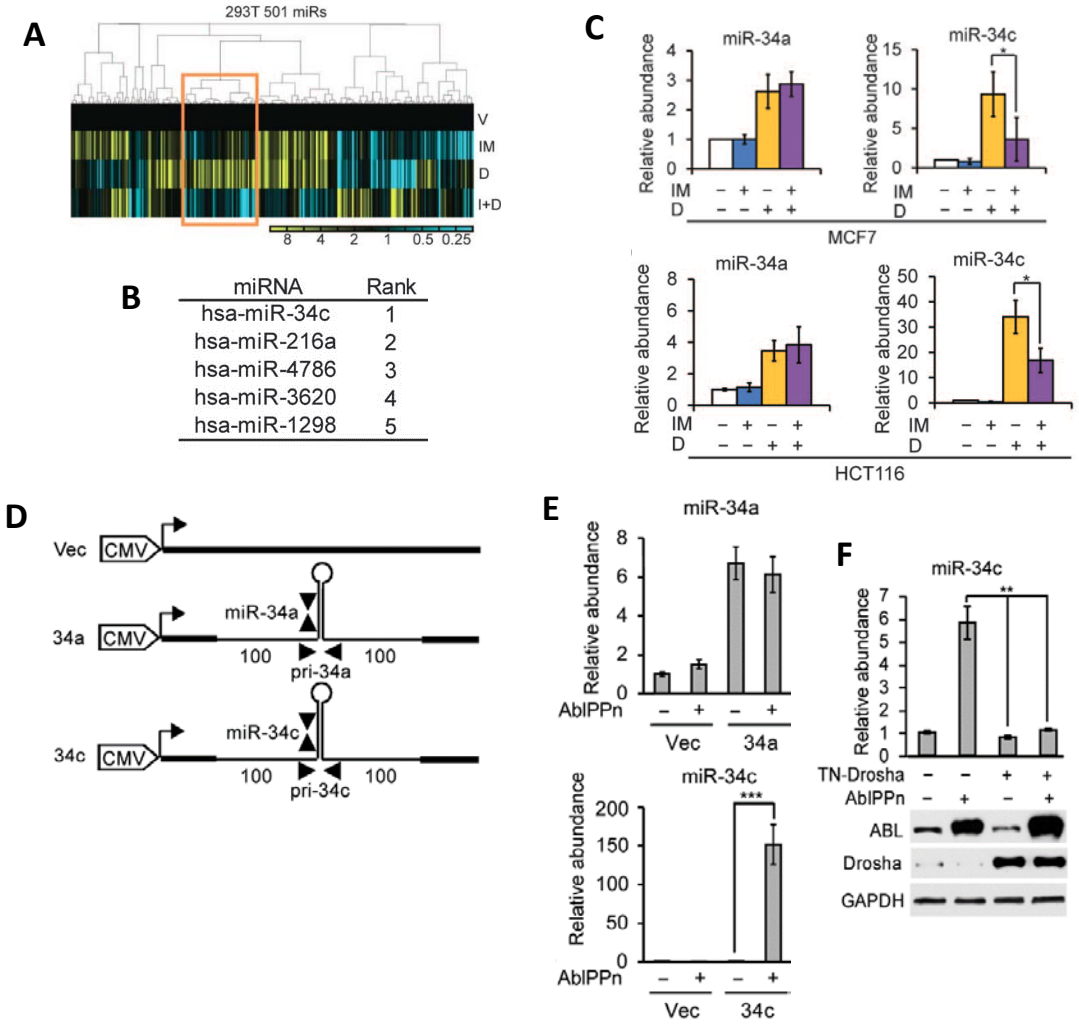

Figure 3. ABL can regulate miR-34c by regulating processing, but not miR-34a. (A) Representation of clustering in miRseq. 293T Cells were treated with vehicle, imatinib (10uM), doxorubicin (0.5ug/ml), or both. (B) Table showing the highest ranking miRNAs affected by the doxorubicin cluster, but not the doxorubicin+imatinib cluster. (C) qRT-PCR of miRNAs in MCF7 and HCT116 cells treated with doxorubicin or imatinib for 24 hours. (D) Schematic of minigenes created to express miR-34a or miR-34c. (E) qRT-PCR measurements of minigene mature miRNA levels in the presence of AblppNES. (F) Transient co-transfection of AblppNES with TN-Drosha and corresponding qRT-PCR to determine level of minigene transcribed mature miR-34c levels.

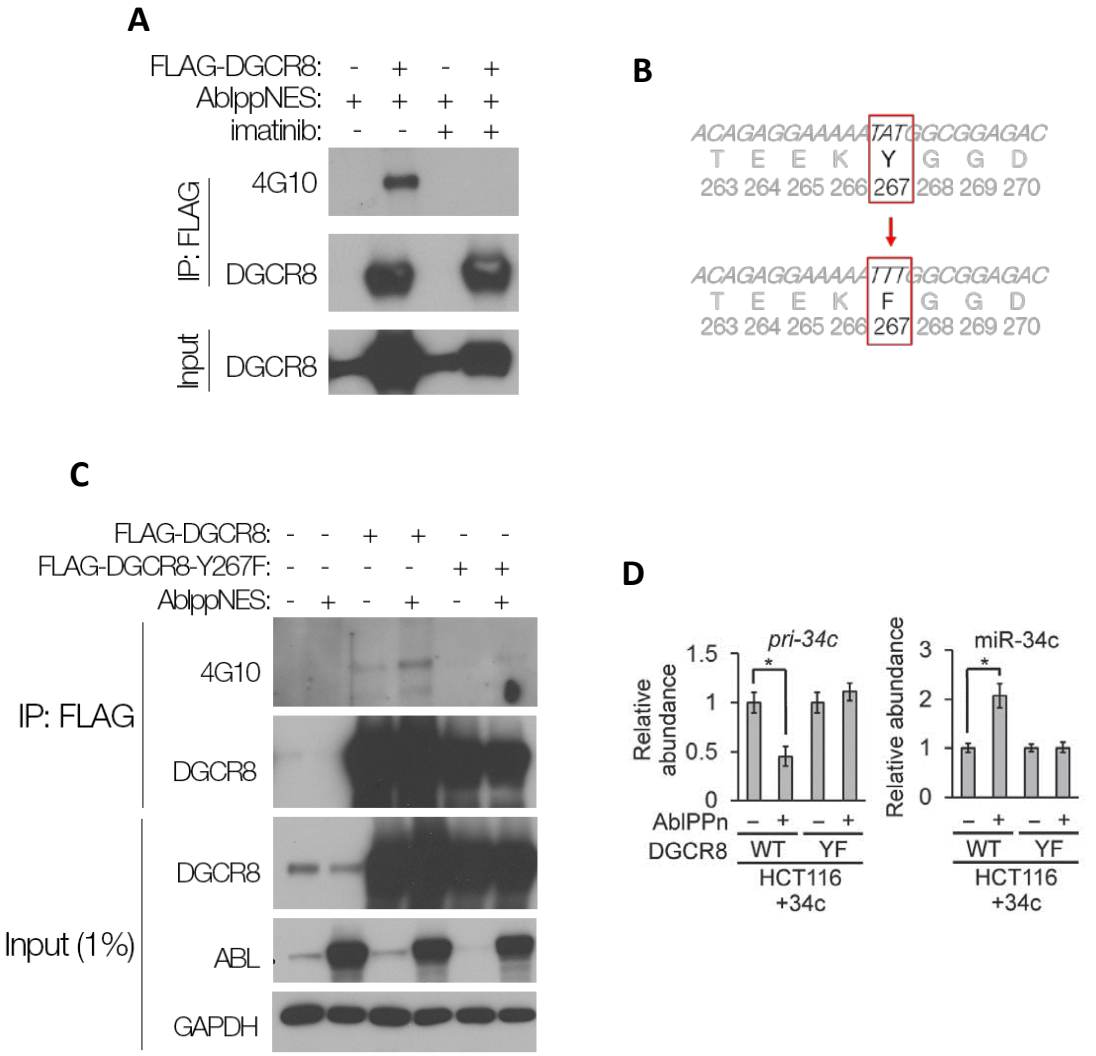

Figure 4. ABL phosphorylation of DGCR8 on Y267 is required for miR-34c processing. (A) FLAG-Immunoprecipitation of pY-DGCR8 in the presence of AblppNES. Cells were treated with imatinib for 2 hours prior to transfection. Western blots were probed with antibodies specific for phosphotyrosine (4G10) and DGCR8. (B) Schematic showing the site directed mutagenesis of DGCR8 Y267F. (C) Immunoprecipitation of pY-DGCR8 and pY-DGCR8-Y267F in the presence of AblppNES. Western blots were probed with antibodies specific for phosphotyrosine (4G10), DGCR8, ABL, and GAPDH. (D) qRT-PCR measuring pri-miR-34c and mature miR-34c levels from transient co-transfection of AblppNES, miR-34c minigene, and wild-type DGCR8 or DGCR8-Y267F.

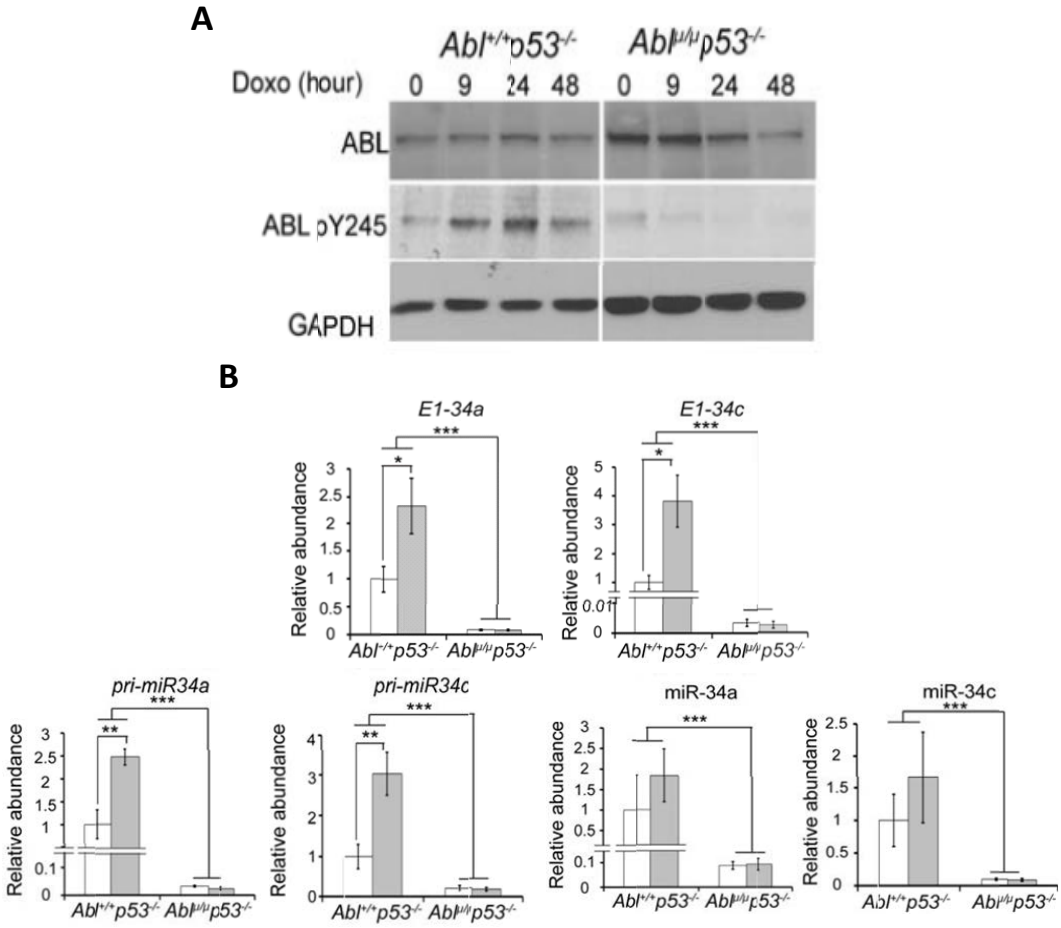

Figure 5. Nuclear ABL is required for miRNA transcription in 3T3 cells. (A) Western blot of 3T3 wild-type and μNLS cells treated with 0.2ug/ml doxorubicin for specified time. Blots were probed with antibodies specific to ABL, ABL-pY245, and GAPDH. (B) qRT-PCR measurements of upstream exon 1 (E1-34a, E1-34c), uncut primary miRNA (pri-miR-34a, pri-miR-34c), and mature miRNA (miR-34a, and miR-34c) in 3T3 wild-type and μNLS cells treated with 0.2ug/ml doxorubicin for 8 hours.

A

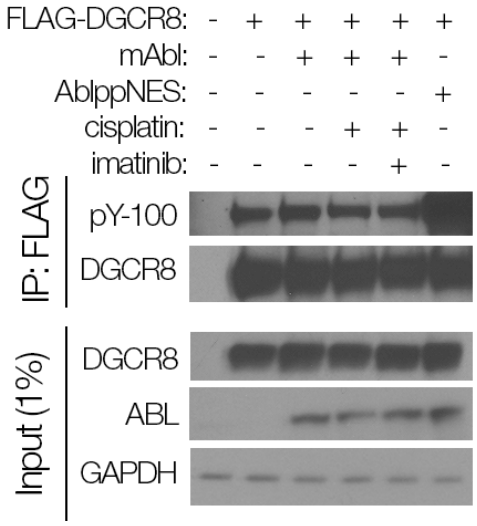

Figure 6. DNA damage induced increase in DGCR8 phosphorylation is not resolvable by standard immunoprecipitation and western blot. (A) FLAG-immunoprecipitation of transfected 293T cells pre-treated with imatinib (10uM) and then treated with cisplatin (20uM) for 8 hours. Western blots were probed with antibodies specific to phosphotyrosine (pY-100), DGCR8, ABL, and GAPDH.

III.

Discussion

The pro-apoptotic function of nuclear ABL kinase is well described in literature. However, much is still not known as to what are the downstream effectors regulated by nuclear ABL kinase as it is mediating DNA damage response. In this work we have demonstrated that presence of AblppNES causes a significant increase in expression of the pro-apoptosis protein PUMA, indicating that ABL can directly promote apoptosis by increasing apoptotic protein levels. We have also demonstrated that nuclear ABL is required for the expression of the cell cycle inhibitor p21 following DNA damage in the absence of p53. Finally, we have shown that ABL is capable of selectively regulating miRNA processing through phosphorylation of DGCR8, an essential subunit of the Microprocessor complex.

The investigation of nuclear ABL regulation of DNA damage response has identified that nuclear ABL is necessary for the sustained expression of the pro-apoptotic protein PUMA in post-transcriptional manner. Through transient co-transfection and western blotting, we have shown that PUMA can be expressed in cells in the presence of Bcl2. Presence of PUMA with AblppNES and Bcl2 however caused a 20 fold increase of PUMA protein levels, providing insight as to how ABL can mediate a pro-apoptotic response. Deletion of the BH3 domain also did not hamper the ABL effect on PUMA, demonstrating that ABL is not enhancing PUMA protein expression through inhibiting apoptosis. Although ABL has been shown to facilitate transcriptional activation of certain apoptotic pathways including p53, this new insight suggests that nuclear ABL can promote apoptosis in a p53-independent manner by directly facilitating increased

expression of downstream apoptosis proteins. It would be of interest to determine how exactly ABL is regulating PUMA, and whether this increased expression is also true for other apoptosis related proteins.

Our investigation into ABL mediated DNA damage response has led us to the result that nuclear ABL is required for regulation of p21 in the absence of p53. We demonstrate that wild-type 3T3 cells will induce p21 protein expression within 6 hours of DNA damage, signifying that the cells are attempting to prolong DNA repair. This p21 expression increases at 24 hours, but decreases at 48 hours, possibly due to the cells making the shift into apoptosis. In cells deficient of nuclear ABL, we have discovered that there is no p21 expression following DNA damage. At first, it was believed that because ABL is a regulator of the p53 pathway, p21 is being regulated by ABL through transcriptional regulation by p53. However, we found that these cells were deficient of p53 protein, suggesting that p21 expression in a p53 null background is entirely dependent on regulation by nuclear ABL. It is also known that the two p53 related proteins p63 and p73 are capable of transcriptionally regulating p21. However, it is also known that nuclear ABL regulates both p63 and p73. Therefore, this result may suggest that cells deficient of only p53 and nuclear ABL are completely incapable of cell cycle inhibition following DNA damage. The two possibilities from this result would be that DNA damaged cells would proceed to apoptosis or some other form of death more rapidly without cell cycle inhibition, or that cells have become more resistant to apoptosis from the lack of cell cycle inhibition. Further work will be necessary to elucidate which one of these possibilities is true.

It is described in literature that DNA damage response is a cell-wide phenomenon of which there are drastic changes in RNA and protein expression. Our investigation into the intrinsic mechanisms by which ABL facilitates DNA damage response also led to the hypothesis of whether ABL could affect miRNA response to DNA damage. Through miRseq and qRT-PCR validation, we have found that ABL kinase is necessary for the expression of miR-34c but not miR-34a. It was also found that ABL was regulating miR-34c through regulation of processing, not transcription. Through western blotting and qRT-PCR, we have found that an active nuclear ABL is required for transcription of miR-34a and miR-34c. Transient co-transfection of ABLppNES and DGCR8 followed by FLAG immunoprecipitation demonstrated that ABL significantly increases DGCR8 phosphorylation. Mutation of DGCR8 Y267 to F267 diminishes the level of DGCR8 phosphorylation in the presence of ABL indicating that this is the tyrosine that gets phosphorylated by ABL. Using qRT-PCR, we have found that DGCR8 tyrosine phosphorylation in the presence of ABLppNES reduces the amount of pri-34c by 2 fold, but increases the amount of the mature miR-34c by 2 fold, indicating promoted processing. The DGCR8-Y267F mutant does not affect the level of either pri-34c or miR-34c in the presence of ABL. These results pose the question as to how phosphorylation of DGCR8 enhances the processing of miRNAs. A possibility is that unphosphorylated DGCR8 is binding pri-miRNA, and is impeded from binding to Drosha. When DGCR8 becomes phosphorylated, it undergoes a conformational change in which allows it to bind to Drosha. This impediment may stem from the pri-miRNA sequence itself, or another unknown

RNA binding protein which blocks Drosha-DGCR8 binding until DGCR8 is phosphorylated. Continuing on this question, it would also be of interest to determine what other miRNAs require DGCR8 Y267 phosphorylation to be processed.

We also attempted to demonstrate increased phosphorylation of DGCR8 by ABL following DNA damage. However, we have found that standard immunoprecipitation and western blotting techniques are not sufficient to demonstrate this idea. We have found that following induction of DNA damage, we cannot demonstrate increased DGCR8 phosphorylation more than the basal amount present. It may be possible that DNA damage induced DGCR8 phosphorylation is an extremely transient phenomenon, of which would require a very intricate time-controlled experiment to show. However, we have seen that DGCR8 is phosphorylated at a basal level, of which is unaffected by treatment with imatinib. This may indicate that there are other tyrosine kinases that are capable of phosphorylating DGCR8. Therefore, it may also be possible that ABL mediated phosphorylation of DGCR8 is a small amount compared to the total phosphorylation of DGCR8, therefore making any perceived change in DGCR8 phosphorylation unresolvable by standard immunoprecipitation techniques.

Figures 3, 4C, 5A, and 5B are reprints of the figures as they appear in: "The kinase ABL phosphorylates the microprocessor subunit DGCR8 to stimulate primary microRNA processing in response to DNA damage." Tu, Chi-Chiang, Yan Zhong, Louis Nguyen, Aaron Tsai, Priya Sridevi, Woan Yu Tarn, and Jean

YJ Wang. *Science signaling* 8, no. 383 (2015): ra64. The thesis author was a co-author of this paper.

IV.

Materials and Methods

Cell culture and transfection

Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. 293T cells were grown to a confluence of 90% in a 6cm plate and transiently transfected with expression plasmids using GeneTran reagent following the manufacturer's instructions and incubated for the indicated time.

NIH 3T3 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin.

Induction of DNA damage

For doxorubicin and cisplatin treatments, cells were grown to a confluence of 70%. Doxorubicin treatment was at a concentration of 0.2ug/ml for the indicated time. Cisplatin treatment was at a concentration of 20uM for 24 hours.

Whole cell protein lysates and Western blotting

Whole cell protein lysates were created by lysing whole cells in cold lysis buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, 50mM NaF, 10mM BGP, 10mM pyrophosphate, 10mM orthovanadate, 1mM PMSF, 10% glycerol, and 1x protease inhibitor cocktail (Roche)). Lysates were then subject to sonication and centrifugation to pellet insoluble debris.

Protein lysates were separated on 6%, 10%, or 15% SDS-polyacrylamide gels, and transferred onto nitrocellulose membrane. Membranes were blocked by 5% milk or 5% bovine serum albumin in TBST (when blotting for phosphorylated proteins) for 1 hour prior to primary antibody incubation overnight. Membranes were washed in TBST, and animal-specific secondary antibody was added at a concentration of 1:2000 in 5% milk for 1 hour. Membranes were visualized using Pico enhanced chemiluminesence (ECL) reagent from Pierce and X-ray film.

Immunoprecipitation by FLAG

Cells being subject to FLAG immunoprecipitation were lysed in FLAG lysis buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 1% NP-40, 0.1% deoxycholate, 0.02% SDS, 3mM NaoV, 1mM PMSF, 1x protease inhibitor cocktail (Roche)). Lysates were then subject to sonication and centrifugation to pellet insoluble debris. Protein lysates were then incubated in FLAG antibody conjugated beads (Sigma) for 4 hours. Beads were then washed in FLAG wash buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 0.5mM EDTA, 0.1% NP-40, 0.01% Deoxycholate, 0.002% SDS, 3mM NaOV, 1mM PMSF) 3x. Proteins were then eluted using 2x Laemmli buffer for 15 minutes at 37 degrees C. The buffer was then aspirated and boiled at 95 degrees C for 10 minutes. Lysate was then resolved using SDS-polyacrylamide gels and subject to previously described western blotting techniques.

RNA Measurements

Total RNA was collected using Trizol reagent (Life Technologies). RNA was quantitated by the NanoDrop 1000 spectrophotometer and 1ug of RNA was used to synthesize complementary DNA by a cDNA synthesis kit (Applied Biosystems). RNA measurements by quantitative reverse transcriptase PCR were conducted using the ABI 7900HT fast real-time PCR system and the ABI StepOnePlus real-time PCR system. For miRNAs, U6 was used as the reference gene. For endogenous primary RNAs, upstream exons, and mRNAs, GAPDH was used as the reference gene. Quantitative analysis was done using comparative Ct values.

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