Nucleic Acids Research
Supplemental Information

NBS1-CtIP–Mediated DNA End Resection Suppresses cGAS Binding to Micronuclei

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Running title: NBS1 regulates cGAS binding to micronuclei

Keywords: NBS1, cGAS, CtIP, ATM, DNA damage response, micronuclei, end resection
Supplementary Figure Legends

Figure S1: MDC1, but not MRE11, co-localizes with cGAS in micronuclei.

A. Representative images show co-localization of MDC1 and cGAS in micronuclei. Bar graphs show the frequency of micronuclei harboring either MDC1 alone, cGAS alone, MDC1 and cGAS or neither in cells expressing DN-TRF2 and treated with doxycycline for 72 hours (right).

B. Representative images show no (left) or weak co-localization of MRE11 with cGAS in micronuclei. Bar graphs show the frequency of micronuclei harboring either MRE11 alone, cGAS alone, MRE11 and cGAS or neither in cells expressing DN-TRF2 and treated with doxycycline for 72 hours (right). Bar graph presents the mean and STDEV from three independent experiments.

Figure S2: NBS1 is recruited to both nuclear envelope-positive and -negative micronuclei and the increased proportion of cGAS-positive micronuclei in NBS1 knockdown and NBS cells is not due to micronuclei’s defective nuclear envelope coating.

A. NBS1 recruitment to micronuclei is independent of nuclear envelope coating. Bar graph shows the frequency of micronuclei containing either nuclear envelope marker (Lamin A/C) alone, NBS1 alone, Lamin A/C and NBS1 or neither in BEAS2B cells treated with 3 µM 6-thio-dG. Data in the bar graph present the mean and STDEV from three independent experiments.

B. Only a minor fraction of NBS1 co-localizes with RB1 onto micronuclei. Bar graph shows the percentage of micronuclei harboring RB1, NBS1, both or neither in BEAS2B cells treated with 3 µM 6-thio-dG. Data in the bar graph present the mean and STDEV from three independent experiments.

C. Increased proportion of cGAS-positive micronuclei in NBS1 defective cells is not due to micronuclei’s defective nuclear envelope coating. Bar graphs show the percentage of micronuclei harboring either Lamin A/C coating (NE) alone, cGAS alone, Lamin A/C and cGAS or neither in BEAS2B cells stably expressing shSCR- and doxycycline-inducible shNBS1 RNAs at 72 hours after 3 µM 6-thio-dG treatment. Bar graphs present the mean and STDEV from three independent experiments. Statistical analysis was performed using Student’s t-test.

Figure S3: Neither MRE11 nor its exonuclease activity play a role in micronuclear DNA end resection and cGAS recruitment to micronuclear DNA.

A-E. MRE11 deficiency and blocking its exonuclease activity increase the number of micronuclei, but not immune signaling. Western blot shows MRE11 expression in HT1080 cells stably expressing shMRE11 RNA and ALTD (MRE11-mutant) cells (A). Bar graph shows
frequency of micronuclei formation in MRE11-depleted (HT1080+shMRE11) and MRE11 exonuclease-inhibited (HT1080+mirin) cells at 72 hours after 3 μM 6-thio-dG withdrawal (B). Data in the bar graph present the mean and STDEV from 3-4 independent experiments. Bar graphs show the percentages of micronuclei harboring either cGAS, γH2AX, cGAS and γH2AX or neither in MRE11-depleted HT1080 cells (C) and Mirin-treated HT1080 cells (D) 72 hours after 3 μM 6-thio-dG treatment. Data in the bar graph represent STDEV from three to four independent experiments.

**E-G.** Expression of immune pathway genes is reduced in MRE11-defective cells. Bar graph shows expression of immune pathway genes in MRE11-proficient HT1080 cells (E), MRE11-depleted HT1080 cells (F), and mirin-treated HT1080 cells (G) at 72 hours after 3 μM 6-thio-dG treatment. Error bars represent the STDEV from three-five independent experiments.

**H.** Micronuclear DNA end resection is not affected by MRE11. Bar graph shows the percentage of micronuclei with either cGAS alone, BrdU signal alone, cGAS and BrdU signal or neither in HT1080-shSCR, HT1080+shMRE11 and HT1080+Mirin treated cells at 24 hours after exposure to 2.5 Gy IR. Data in the bar graph represent STDEV from three independent experiments. * P (range) <0.05; **** P<0.0001.

**Figure S4: Purification of human cGAS and its binding to double-strand break ended DNA substrate but not to DNA substrate harboring resected DNA ends.**

**A.** Coomassie brilliant blue stained SDS-PAGE shows cGAS elution from Histidine column.

**B.** Coomassie brilliant blue stained SDS-PAGE shows presence of cGAS after elution from Heparin column with 0.5-1M NaCl gradient.

**C.** Coomassie brilliant blue stained SDS-PAGE shows cGAS purity after elution from Superdex 200 column.

**D.** Schematic representation of three different DNA structures used for cGAS-DNA binding assay.

**E.** cGAS binds with double-stranded but not to end-resected DNA substrates. 5-10 μM cGAS was incubated with 25 fmol $^{32}$P labeled DNA substrates in the presence of absence of 100 base pairs cold double stranded DNA (competitor). DNA-Protein complex was resolved onto 5% native Poly acrylamide gel electrophoresis and the signal was detected by phosphor imaging.
Supplementary Tables

**Table S1:** List of primers used for cloning small hairpin RNAs and cGAS binding assay reported in this study.

**Table S2:** List of primary antibodies and their respective dilutions used for both western blotting (WB) and immunofluorescence staining (IF) reported in this study.

**Table S3:** List of primers used for qRT-PCR reported in this study.

Supplementary Results

**MRE11 does not influence cGAS recruitment to micronuclei:** Once NBS1 senses DSBs via its FHA-BRCT1/2 domains, it recruits multiple proteins, including MRE11,\(^1\) ATM,\(^2,3\) and CtIP,\(^4,6\) to these DSBs for chromatin remodeling, end-processing and downstream DDR signaling. Although \(\Delta\)MRE11-NBS1 expression in NBS cells did not alter the number of cGAS-positive micronuclei, a previous report indicated a role for MRE11 in sensing cytosolic DNA fragments.\(^7\) However, our results on the lack of co-localization between MRE11 and cGAS within the micronuclei and the cytosolic localization of MRE11 prompted us to investigate MRE11’s involvement in the accumulation of cGAS in micronuclei. First, we depleted MRE11 in HT1080 cells by using MRE11-specific shRNA (**Fig. S3A**). We found that depleting MRE11 did not alter the number of micronuclei that formed in response to 6-thio-dG treatment as compared with control group (**Fig. S3B**). Furthermore, the accumulation of cGAS in the micronuclei in shMRE11 cells was comparable to that of shSCR cells (**Fig. S3C**). Additionally, similar to a previous report,\(^7\) the expression of immune signaling genes was reduced in the absence of MRE11 (**Figs. S3E-G**).

Second, since MRE11 possesses both exonuclease and endonuclease activities, we used mirin to inhibit MRE11’s exonuclease activity to further show that blocking this activity does not augment cGAS accumulation in the micronuclei. As with MRE11 depletion, pre-treating cells with mirin and then with 6-thio-dG did not alter cGAS recruitment to the micronuclei as compared with DMSO-treated cells (**Fig. S3D**). Additionally, mirin treatment significantly reduced the expression of genes involved in immune pathways in response to 6-thio-dG treatment as compared with 6-thio-dG treated DMSO cells (**Fig. S3G**). So, MRE11 depletion prevents the induction of an inflammatory response (**Figs. S3E-G**) even though cGAS recruitment to micronuclei is not altered (**Fig. S3D**). Thus, cGAS recruited to a limited number of micronuclei in MRE11 depleted cells but does not trigger robust immune response.
Supplementary References

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Figure S2

(A) NBS1 alone, Lamin A/C alone, NBS1+Lamin A/C, NBS1+RB1, Neither

(B) NBS1 alone, RB1 alone, NBS1+RB1, Neither

(C) BEAS2B-shSCR, BEAS2B-shNBS1

Micronuclei (%)
A

HisTag (5 mL)

M 14 16 18 20 22 24 26 28 30 32 34 36 38 Fraction #

B

Heparin (1 mL)

W M 10 11 12 14 15 16 17 18 Fraction #

C

Superdex 200

M 27 28 29 30 31 Fraction #

Eluted as monomer

D

DSB end

5'-TACAGATCTACTAGTGATCTATGACTCTGATCTACATGATCTACA
3'-ATGTCTAGATGATCTAGATGACTGATAGACATGATCTAGATGTT

5' overhang

5'-TACAGATCTACTAGTGATCTATGACTCTGATCTACATGATCTACA
3'-ACATGATCTAGATGTT

3' overhang

5'-TGATACATGATCTACA
3'-ATGTCTAGATGATCTAGATGACTGATAGACATGATCTAGATGTT

E

DNA DSB End 5’-overhang 3’-overhang

cGAS - - - + - - - - +

Competitor - - - + - - - + - - - +

Figure S4

25 fmol DNA
| Serial # | Primer Name | Sequence (5’-3’) |
|----------|-------------|------------------|
| 1        | NBS1-shRNA1-F1 | CCGG CCGTCCCGAGTACAGGATTAAAACCTCGAGTTTTAAATCTGTACTGGGATGG TTTTT |
| 2        | NBS1-shRNA1-R1 | AATT AAAAAA CCATCCCCGACTGAGTTTAAACCTCGAGTTTTAAATCTGTACTGGGATGG |
| 3        | NBS1-shRNA2-F2 | CCGG CCTCTTGATGAACCATCTATTCTCGAGAAATAGATGGTTCATCAAGAAGG TTTTT |
| 4        | NBS1-shRNA2-R2 | AATT AAAAAA CCTCTTGATGAACCATCTATTCTCGAGAAATAGATGGTTCATCAAGAAGG |
| 5        | NBS1-shRNA3-F3 | CCGG GCTTATTTAGTCTGACTTTCTGAGAAACCTAGGGACTCTAAATAAGC TTTTT |
| 6        | NBS1-shRNA3-R3 | AATT AAAAAA GCTTATTTAGTCTGACTTTCTGAGAAACCTAGGGACTCTAAATAAGC |
| 7        | DSB End substrate | TACAGATCTACTGATCTAGTTATGAATGCTGTATCTACATGATCTACTACaaagagcagTGTAGATCATGTACAGATCATAGATCATAGATCTAGATCATAGATCTGTA |
| 8        | 5’-Resected substrate | TACAGATCTACTGATCTAGTTATGAATGCTGTATCTACATGATCTACTACaaagagcagTGTAGATCATGTACAGATCATAGATCATAGATCTAGATCATAGATCTGTA |
| 9        | 3’-Resected substrate | TGTACAGATCTACTACaaagagcagTGTAGATCATGTACAGATCATAGATCATAGATCTAGATCATAGATCTGTA |
| Sl. No. | Primary Antibodies | Catalog #  | Vendor         | Application (dilution) |
|--------|--------------------|------------|----------------|------------------------|
| 1      | Mouse monoclonal anti-γ-Tubulin (GTu88) | T6557      | Sigma          | WB (1:50000)           |
| 2      | Rabbit monoclonal anti-phospho-IRF3 (Ser396;4D4G) | 4947       | Cell Signaling | WB (1:500)             |
| 3      | Mouse monoclonal anti-cGAS           | 242363     | Abcam          | IF (1:100)             |
| 4      | Rabbit monoclonal anti-cGAS (D1D3G)  | 15102      | Cell Signaling | WB (1:200); IF (1:100) |
| 5      | Mouse monoclonal anti-MDC1 (MDC1-50) | 50003      | Abcam          | IF (1:400)             |
| 6      | Rabbit polyclonal anti-phospho-ATM (Ser1981; EP1890Y) | 81292      | Abcam          | WB (1:10000); IF (1:500) |
| 7      | Mouse monoclonal anti-phospho Stat1 (pSTAT1-Tyr 701; A-2) | 8394       | Santacruz      | WB (1:200)             |
| 8      | Mouse monoclonal anti-Lamin A/C (636)   | 7292       | Santacruz      | IF (1:300)             |
| 9      | Mouse monoclonal anti-NBS1            | NB100-221  | Novus Bio      | IF (1:100), WB (1:500) |
| 10     | Rabbit polyclonal anti-NBS1           | 3001       | Cell Signaling | IF (1:100)             |
| 11     | Rabbit polyclonal anti-NBS1           | NB100-143  | Novus Bio      | WB (1:500); IF (1:100) |
| 12     | Mouse monoclonal anti-MRE11 (12D7)    | 70212      | Gentex         | WB (1:1000); IF (1:1000) |
| 13     | Mouse monoclonal anti-ATM (2C1)       | 70103      | Gentex         | WB (1:5000)            |
| 14     | Mouse monoclonal anti-phospho-Histone H2AX (Ser139; JBW301) | 05-636     | Millipore      | IF (1:1000)            |
| 15     | Mouse monoclonal anti-RNF20           | 89007928   | Abnova         | IF (1:100)             |
| 16     | Rabbit Polyclonal CtIP                | 38016      | Abcam          | IF (1:100)             |
| 17     | Rabbit polyclonal phosphorylated RPA2  | NA         | homemade       | IF (1:100)             |
| 18     | Mouse monoclonal RPA2                 | NA         | homemade       | IF (1:100)             |
| 19     | Rat monoclonal anti-BrdU              | NB500-169  | Novus Biologicals | IF (1:100)               |
| 20     | Rabbit monoclonal anti-β-Actin (13E5) | 4970       | Cell Signaling | WB (1:1000)            |
| Serial # | Primer Name                | Sequence                     |
|---------|---------------------------|------------------------------|
| 1       | human IFNα Fwd (5'-3')    | AACTCCCTGATGAATGCGG          |
| 2       | human IFNα Rev (5'-3')    | TAGCAGGGGTGAGAGTCTTTT       |
| 3       | human IFNβ Fwd (5'-3')    | CAACTTGCTTGGATTCCCTACAAG    |
| 4       | human IFNβ Rev (5'-3')    | TATTCAGCCTCCCATTCAATTG      |
| 5       | human TLR9 Fwd (5'-3')    | CGCCCTGCACCCGCTGTCTCT       |
| 6       | human TLR9 Rev (5'-3')    | CGGGGTGCTGCCATGGAGAAG       |
| 7       | human IL1β Fwd (5'-3')    | CCACCACCTACAGCAAGGG         |
| 8       | human IL1β Rev (5'-3')    | GAACCTGGGCAGACTCAAA         |
| 9       | human CXCL10 Fwd (5'-3')  | AGGAACCTCCAGTCTCAGCA        |
| 10      | human CXCL10 Rev (5'-3')  | CAAATTTGCGCTTGCGGAAT        |
| 11      | human IFIT1 Fwd (5'-3')   | CCTCCTTGGGTTCGTCTACA        |
| 12      | human IFIT1 Rev (5'-3')   | GGCTGATATCTGGGTGCTCA        |
| 13      | human IFIT3 Fwd (5'-3')   | GAAGGAAGTGGGGCCGCTGTAAG     |
| 14      | human IFIT3 Rev (5'-3')   | GCCCTGCGGGCCATTTCTCACTACC   |
| 15      | human ISG56 Fwd (5'-3')   | TTGATGACGATGAAATGCTGA       |
| 16      | human ISG56 Rev (5'-3')   | CAGGTCACCAGACTCCAC          |
| 17      | human IL6 Fwd (5'-3')     | CCTTCCGTCCAGTTGCTTCT        |
| 18      | human IL6 Rev (5'-3')     | GCATTTGTGGTTGGGTCA          |
| 19      | human β-Actin Fwd (5'-3') | TCGTGCACAACGGCTCGAGATG      |
| 20      | human β-Actin Rev (5'-3') | CCAGCCAGGTCCAGACGGAT        |