5-Aza-2′-deoxycytididine causes replication lesions that require Fanconi anemia-dependent homologous recombination for repair

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ABSTRACT

5-Aza-2′-deoxycytididine (5-azadC) is a DNA methyltransferase (DNMT) inhibitor increasingly used in treatments of hematological diseases and works by being incorporated into DNA and trapping DNMT. It is unclear what DNA lesions are caused by 5-azadC and if such are substrates for DNA repair. Here, we identify that 5-azadC induces DNA damage as measured by γ-H2AX and 53BP1 foci. Furthermore, 5-azadC induces radial chromosomes and chromatid breaks that depend on active replication, which altogether suggest that trapped DNMT collapses oncoming replication forks into double-strand breaks. We demonstrate that RAD51-mediated homologous recombination (HR) is activated to repair 5-azadC collapsed replication forks. Fanconi anemia (FA) is a rare autosomal recessive disorder, and deaths are often associated with leukemia. Here, we show that FANCG-deficient cells fail to trigger HR-mediated repair of 5-azadC-induced lesions, leading to accumulation of chromatid breaks and inter-chromosomal radial fusions as well as hypersensitivity to the cytotoxic effects of 5-azadC. These data demonstrate that the FA pathway is important to protect from 5-azadC-induced toxicity. Altogether, our data demonstrate that cytotoxicity of the epigenetic drug 5-azadC can, at least in part, be explained by collapsed replication forks requiring FA-mediated HR for repair.

INTRODUCTION

DNA methylation is controlled by a family of DNA methyltransferases (DNMT), enzymes that catalyze the transfer of a methyl moiety from S-adenosyl-l-methionine to the 5-position of cytosines in the CpG dinucleotide to silence gene expression (1). When entering the cell, 5-aza-2′-deoxycytidine (5-azadC) is phosphorylated to 5-azadCTP and incorporated into DNA during replication. As a result of the chemistry of the methyltransferase reaction, the DNMT becomes covalently linked to DNA, in effect creating a protein–DNA cross-link (2–5). This results in depletion of soluble DNMT protein levels, which induces replication-dependent global demethylation and gene reactivation (6). Although there is a considerable amount of literature regarding the possible anti-cancer mode of action of 5-azadC, the molecular mechanism of cell death still remains unclear. There are two non-exclusive models: one involves the reactivation of silenced genes involved in cell growth, which is accompanied by cell cycle arrest and/or apoptosis (7). Another model emphasizes the importance of the formation of covalent DNMT–DNA adducts, which leads to DNA damage and cytotoxicity (3,8).

Epigenetic targets such as DNMT has become a promising new strategy for cancer treatments, especially hematologic malignancies such as myelodysplastic syndromes and acute myeloid leukemia (AML) (9,10). Many anti-cancer therapies are cytotoxicity based on their ability to cause DNA damage, e.g. ionizing radiation or chemotherapy drugs. The 5-azadC is a nucleoside analogue that when is incorporated into newly synthesized...
DNA, results in covalent trapping of DNMTs causing genome-wide protein–DNA cross links (2–5).

Fanconi anemia (FA) is a rare autosomal recessive disorder characterized by progressive pancytopenia, requiring bone marrow transplant, and associated congenital abnormalities, such as microcephaly, short stature, skeletal, cardiac and renal malformations. Mortality is associated with bone marrow failure and leukemia in the first 2 decades of life, with the majority of deaths occurring within 5 years after the onset of anemia (11,12). The disease is caused by mutations within a variety of genes associated with the FA pathway. Many genes have been linked in the pathogenesis of FA, and at least 15 genes, including a nine-gene FA nuclear core complex with associated downstream proteins, all function to promote genetic stability via repair of DNA following damage (13). One of these genes is the well-known breast cancer susceptibility gene BRCA2, while mutations in others such as PALB2, RAD51C or BRIP1 also pre-dispose to cancer. Germinal mutations of these genes are associated with dysregulation of cell cycle and apoptosis (14,15), and chromosomal instability (14). It has been postulated that disruption of the FA complex and associated pathways are related with chromosomal and telomere instability characterized by impaired DNA double-strand break (DSB) repair leading to the inactivation of tumor suppressor genes and activation of oncogenes (16). The FA pathway in normal cells is not constitutively active but is inactivated by genetic or epigenetic mechanisms in a variety of cancers, including breast, ovarian, head and neck, cervical, pancreatic and lung carcinomas, rendering the affected tumors potentially hypersensitive to DNA cross-linking agents (17).

Here, we report that 5-azadC induces DNA damage that depends on active replication, which suggests that trapped DNMT collapses with oncoming replication forks into DSBs. We demonstrate that RAD51-mediated homologous recombination (HR) is involved in the repair of such DSBs, a process that is defective in FA cells. This agrees with a reduced cell survival and a potentiation of chromosomal abnormalities. To strengthen this, we also show that proteasome inhibition sensitizes against 5-azadC in wild-type, but not in FA cells. Furthermore, we report a preferential protection in FA cells when non-homologous end joining (NHEJ) inhibitors were used.

MATERIALS AND METHODS

Chemicals

The 5-azadC, aphidicolin (APH), NU7026 and MG132 were purchased from Sigma and diluted in dimethyl sulfoxide except 5-azadC that was diluted in phosphate buffered saline (PBS) (10 mM sodium phosphate (pH 7.4), 140 mM NaCl and 3 mM KCl). All of them were aliquoted and stored at −80°C before use.

Cell culture

The parental Chinese hamster ovary cell line AA8 was purchased from the American Type Culture Collection (ATCC), USA. The isogenic FANCG-deficient Chinese hamster ovary mutant KO40, derived from AA8, was kindly provided by Dr. Fabrizio Palitti (Department of Agrobiology and Agrochemistry, University of Tuscia, Viterbo, Italy) and has been previously described (18). Cells were routinely maintained as monolayers in McCoy’s 5A media (LONZA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and the antibiotics penicillin (50 U/ml) and streptomycin (50 µg/ml). Cells were cultured at 37°C in an atmosphere containing 5% CO₂.

Colony formation

Cell survival following 5-azadC treatment was measured by clonogenic assay. AA8 and KO40 cells were plated at low density onto 10 cm Petri dishes. After 4 h, cultures were incubated for 24 h (two rounds of DNA replication approximately) in the presence of different concentrations of 5-azadC (1, 3.25, 7.5 and 15 µM). For combined experiments (5-azadC+ inhibitor), cells were treated for 24 h with the NHEJ inhibitor NU7026 (10 µM) or the proteasome inhibitor MG132 (0.1 µM), respectively. These doses were chosen on the basis of absence of cellular cytotoxicity. Then 5-azadC was discarded, and the cultures were further incubated for 24 h with each one of the inhibitors. After that, fresh media was added, and cells were allowed to grow from 7 to 10 days. Colonies were stained with methylene blue prepared in methanol (4 g/l). Surviving colonies made up of >50 cells per colony were counted. The data have been corrected according to cloning efficiencies of control cells.

Chromosome analysis

Exponential growing AA8 or KO40 cells were cultured for 24 h in the presence of different concentrations of 5-azadC (3.25, 7.5 and 15 µM). After that, cells were incubated with fresh media for 12 h before mitotic arrest. For combined experiments (5-azadC+APH), cells were treated for 12 h with 5-azadC. After that, cells were thoroughly washed and 0.5 µM of APH was added for a further 12 h. Then, APH was discarded, and cultures were allowed to recover in fresh media for 6 h until the addition of colcemid to arrest cells in metaphase. To prepare metaphase spreads, cells were treated with 2 × 10⁻⁵ M of colcemid for 2 h and 30 min. Cells were collected and incubated in hypotonic solution (0.075 M KCl) for 2 min, fixed in methanol: acetic acid (3:1) and dropped onto microscope slides. Slides were stained with 3% Giemsa/Sörensen’s buffer for 5 min and mounted in D.P.X. (Sigma). Two hundred complete metaphases were evaluated in each experimental point. Images were taken with a Nikon eclipse 50i microscope equipped with a Nikon DS-Fi1 camera and using the 100-fold magnification objective (numerical aperture 1.25) and the NIS Elements 3.0 acquisition software (Nikon).

To visualize sister chromatid exchanges (SCE), cells were incubated with 10 µM of bromodeoxyuridine (BrdU, Sigma) along with increasing doses of 5-azadC (0.25, 0.5 and 1 µM) for 24 h. Then, cells were washed and allowed to repair for 12 h before addition of colcemid. Differential staining of BrdU-substituted sister
chromatids was obtained by the fluorescence-plus-Giemsa technique as reported elsewhere (19).

**Immunofluorescence labeling and microscopy (Foci detection)**

Cells were seeded on coverslips the day before being treated with 5-azadC (0.25, 0.5 and 1 μM) for 24 h. After that, cells were washed with PBS and incubated for 30 s with cold 0.1% Triton X-100 in PBS to pre-extract soluble protein unlocated in foci. Afterwards, cells were fixed with 4% formaldehyde in PBS for 10 min at room temperature. The primary antibodies used were a rabbit polyclonal antibody α-RAD51 (H-92, Santa Cruz) or α-53BP1 (H-300, Santa Cruz) and a mouse monoclonal α-γH2AX (Upstate). Secondary fluorescent antibodies were an Alexa fluor 488-conjugated goat anti-mouse and an Alexa fluor 555-conjugated goat anti-rabbit (Invitrogen). DNA was stained with 100 nM of 4,6 diamidino-2-phenylindole for 15 min. Slides were mounted in anti-fade media (Vectashield). Immunofluorescence was observed using a Nikon eclipse 50i microscope with a 40-fold magnification objective. Cells with >10 foci were scored as positive. At least 200 nuclei were analyzed for each treatment. Micrographs were taken using a Leica AF6000 microscope equipped with a Leica DFC 350 FX camera and using the Application Suite Advanced Fluorescence acquisition software (Leica) and a 63-fold magnification objective (numerical aperture 1.4). Images were bright-contrast processed using Adobe Photoshop 5.0 software (Adobe).

**Statistical analysis**

For the determination of significance of the difference between the means, Student’s t-test was used. Statistical treatment and plotting of the results were performed using the Sigma Plot and MS Excel for Windows XP software. The results come from at least two independent experiments and are presented as mean ± standard deviation (SD) of the mean. Differences were considered significant when *P < 0.05 or **P < 0.01.

**RESULTS**

**5-azadC causes replication-dependent strand breaks resulting in chromatid breaks and radial fusion chromosomes**

It has been previously shown that cytotoxicity of 5-azadC to mammalian cells can be mediated through covalent DNMT-DNA adducts, which in turn cause DNA damage that activates ATR signaling (3,20). Here, we find that 5-azadC treatment produces γ-H2AX foci (Figure 1A and B), which has also been reported earlier (3). It is established that γ-H2AX foci can form also in the absence of DSBs (21), whereas 53BP1 foci formation are more strictly associated with DSBs. Here, we find that 5-azadC also induces 53BP1 foci (Figure 1A and C), suggesting that DSBs may be formed after 5-azadC treatments.

DSBs are highly toxic and may cause chromatid breaks if they persist into mitosis or radial-fusion chromosomes if chromatid breaks are aberrantly repaired. Figure 1D–G shows that control metaphases showed only low percentages of abnormalities, whereas 5-azadC treatment induced an increase in chromosomal aberrations, including chromatid breaks and radial chromosomes, which may explain toxic effects caused by 5-azadC.

Furthermore, we wanted to determine how 5-azadC may cause DSBs and chromatid breaks. A lack of induction of isochromatid breaks (data not shown) seems to indicate that 5-azadC behave as a typical S-phase dependent agent. In hypothesis, trapped DNMT may obstruct replication forks causing replication collapse and DSBs. It is well documented that replication-blocking DNA lesions can cause replication fork collapse and thereby lead to the formation of DNA DSBs (22). To test this, we determined chromosome damage by 5-azadC while transiently inhibiting DNA synthesis using APH, using a dose previously shown to completely inhibit replication progression in the same cells (23), to test whether the chromatid breaks were a result of collapsed replication forks. We found a decreased amount of chromatid breaks in agreement with this hypothesis (Figure 1H). Overall, our findings are in line with a hypothesis that 5-azadC-induced DSBs arising as a consequence of trapped DNMT collapses oncoming replication forks into DSBs.

**Repair of 5-azaC induced DSBs requires HR**

Our data suggest that DSBs arising as a consequence of 5-azaC incorporation are generated during the second round of DNA replication. It is well accepted that replication-induced DSBs are potent substrates for HR giving rise to RAD51-mediated SCEs formation (24–26). To test this directly, we treated cells with increasing doses of 5-azaC and scored SCEs or RAD51 foci formation. We found that the frequency of SCEs is increased after treatment with 5-azaC (Figure 2A). Furthermore, we also find an increase in RAD51 foci formation (Figure 2B and C), which altogether support a model where replication forks collapsed by DNMT–DNA adducts trigger HR for repair. Furthermore, cells deficient in BRCA2, a protein that interacts with RAD51 and promotes its loading to single-stranded DNA, are hypersensitive to the toxic lesions induced by 5-azaC (Supplementary Figure S1).

**FANC-mediated HR is required for 5-azaC survival**

The FA proteins are known to play a critical role in repair at obstructed replication forks, especially of crosslinks (27). Here, we tested 5-azaC sensitivity in the absence the FA core complex using the FANC mutant KO40 cell line (18). Results show that KO40 cells were more sensitive to 5-azaC treatment, with a significant decrease in cell survival to all doses tested compared with its isogenic and parental cell line AA8. The sensitization ranged from 2 to 10 times for the doses of 3.25 to 15 μM, respectively (Figure 3A). These results demonstrate that FANC-deficient cells are sensitive to 5-azaC-mediated cytotoxicity. Furthermore, using human fibroblasts deficient in FANCD2, a central key protein of the FA pathway, we
found that they were also sensitive to 5-azadC as compared with corrected cells (Supplementary Figure S2).

To test the molecular reason for the increased toxicity of FANCG-deficient cells, we determined the amount of DNA lesions induced by 5-azadC in KO40 and AA8 cells. Both AA8 and KO40 cells showed the same amount of increase of γ-H2AX and 53BP1 foci on 5-azadC treatment (Figure 3B and C), suggesting that the lesions are produced irrespectively of FA status.

Next, we wanted to assess whether the differences observed in survival could be the result of the HR repair defects in FANCG-deficient cells. Here, we found clear differences between AA8 and KO40 in the yield of SCEs induced by 5-azadC, where only the WT AA8 cells are able to induce SCE and the KO40 cells are defective in 5-azadC-induced HR (Figure 3D). The same results were obtained when we evaluate the residual foci formation of RAD51 as a marker of HR after 5-azadC treatment.
Overall, these data revealed that FA pathway promotes the repair of DNA lesions induced by 5-azadC by a process mediated by HR.

Next, we tested chromosome analysis on AA8 and KO40 cells cultured in the presence of 5-azadC. Control metaphases showed only low percentages of abnormalities (Figure 4A and B). Concerning 5-azadC treatment, there was a dose-dependent increase in chromosomal aberrations, including chromatid breaks and radial chromosomes for both AA8 and KO40 cells. However, only FANCG-deficient cells showed a dramatic increase in chromosome aberrations as compared with the parental cells AA8. Metaphases of KO40 treated with the highest dose of 5-azadC showed severe aberrations (Figure 4A and B). We also wanted to determine whether the increase in chromatid breaks and radial chromosomes were of the same origin, as the one occurring in wild-type cells, that is, derived following replication. To test this, 5-azadC-treated cells were washed and treated with APH, which prevents the subsequent collision of replication forks with DNMT–DNA adducts. We found that the 5-azadC-induced chromatid breaks and radial fusion chromosomes were reversed by preventing replication forks collide into the DNMT-trapped complex on DNA (Figure 4C and D).

Altogether, these data suggest that the FA pathway is involved in HR repair of replication-associated DSBs formed after collapse of replication forks that are obstructed by DNMT–DNA adducts. Failed repair leads to chromatid breaks and formation of radial chromosomes.

Proteasome inhibitor MG132 sensitizes wild-type, but not FA defective cells to 5-azadC

Previous reports have shown that proteasome function is required for activation of the FA pathway (28), as well as to catalyze HR (29). To test whether proteasome inhibition affects 5-azadC induced cell death, combined experiments were performed where AA8 and KO40 cells were co-treated with the proteasome inhibitor MG132 at non-toxic dose (Supplementary Figure S3). MG132 sensitized AA8 cells treated with 5-azadC to a similar level as observed in FA cells (Figure 5A). The sensitization effect was 2.5-fold across all doses used (Figure 5B). Interestingly, no evidence of sensitization was observed for KO40 cells, and hence an epistatic effect is observed.
between FANCG and the proteasome inhibitor MG132. This finding demonstrates that proteasome is required to promote cell survival after 5-azadC treatment. Also, the data point to that, directly or indirectly, proteasome and FA pathway work in the same pathway to promote survival. Overall, these data also strengthen the overall finding that FA-mediated HR is required for survival after 5-azadC treatment.

**DISCUSSION**

The 5-azadC (decitabine) and 5-azaC (azacitidine) are extensively used in myelodysplastic syndrome treatment and increasingly used in experimental treatment of AML (9,10). Both are inhibitors of DNMT, but the cytotoxic mechanism remains somewhat unclear and can involve both activation of apoptosis and DNA damage. Here, we confirm earlier findings that 5-azadC causes both activation of apoptosis and DNA damage. Here, we confirm earlier findings that 5-azadC causes differential induction of RAD51 foci formation in AA8 and KO40 cells after 24 h of treatment with increasing concentrations of 5-azaC. This finding demonstrates that proteasome is required to promote cell survival after 5-azadC treatment. Furthermore, the sensitization effect was increased with increasing doses (Figure 6B). Our findings are consistent with the interpretation given by others (32) that FA protects from the promiscuous action of NHEJ during the repair of lesions induced by crosslinkers. In this respect, the severe cytotoxicity that 5-azaC produced in KO40 cells can be significantly suppressed by inhibiting NHEJ. The 5-azadC is believed to have a more important role in the repair of two-ended DSBs (30,31). However, the relationship between HR and NHEJ is complex, and it has previously been demonstrated that the repair defect in FA cells reverts by inhibition of DNA-protein kinase and that the cells then are no longer sensitive to crosslinkers (32). The underlying mechanism is not entirely clear, but it is possible that inhibition of NHEJ prevents error-prone repair, which can be toxic.

Here, we used the DNA-protein kinase inhibitor NU7026 to test whether inhibition of NHEJ affects the response to 5-azadC. Interestingly, we show an increase in cell survival in both AA8 and KO40 cells after treatment with 5-azadC (Figure 6A) in the presence of a non-toxic dose of NU7026 (Supplementary Figure S3), compared with those cells that received only the treatment with 5-azadC. Also, we show that the increase in cell survival (represented as fold increase) was clearly higher for FANCG-deficient cells than for AA8 cells (Figure 6B). Furthermore, the sensitization effect was increased with increasing doses (Figure 6B). Our findings are consistent with the interpretation given by others (32) that FA protects from the promiscuous action of NHEJ during the repair of lesions induced by crosslinkers. In this respect, the severe cytotoxicity that 5-azaC produced in KO40 cells can be significantly suppressed by inhibiting NHEJ.

**DNA-protein kinase inhibition protects from the deleterious effect induced by 5-azadC**

It is well accepted that HR repairs a broader spectrum of lesions that occur at stalled replication forks, whereas DSBs are generally not repaired by HR. It is well accepted that HR repairs a broader spectrum of lesions that occur at stalled replication forks, whereas DSBs are generally not repaired by HR.

**Figure 3.** Cells deficient in the FA pathway are hypersensitive to 5-azadC. (A) Cell survival after a 24h treatment with increasing concentrations of 5-azadC. AA8 and KO40 cells were seeded on Petri dishes and allowed to attach for 4h; subsequently, they were treated with increasing concentrations of 5-azadC. Then media was changed and cells were allowed to form colonies. (B and C) Quantification of γ-H2AX and 53BP1 foci in AA8 and KO40 cells after 24h treatment with increasing concentrations of 5-azadC. (D) Differential induction of SCEs by 5-azadC in AA8 and KO40 cells. Cells were treated with 10μM BrdU together with increasing concentrations of 5-azadC (0.25–1μM) as described in ‘Materials and Methods’ section. (E) RAD51 foci formation in AA8 and KO40 cells after 24h of 5-azaC treatment. Data show the mean and the SD from 2–3 independent experiments. Differences were statistically significant (*P < 0.05, **P < 0.01 according Student’s t-test).
replication (34), which leaves an SCE (26). Our data strongly suggest that HR is activated to repair 5-azadC lesions as both RAD51 foci, and SCEs are induced by this treatment. This is further strengthened by the observation that KO40 cells, defective in FANCG, which is required for HR repair at collapsed replication forks (18), are hypersensitive to 5-azadC treatment and cannot trigger RAD51-mediated repair and fail to induce SCEs. We observe an increase in unrepaired chromatid breaks in FANCG defective cells, which is the logical consequence by failure to activate HR repair. We also observe an increase in radial chromosomes in FANCG defective cells, clearly demonstrating the link between unrepaired chromatid breaks and the formation of radial chromosomes. In absence of HR, it is highly likely that NHEJ will eventually fuse DSBs. If breaks occur at replication forks, only single DNA ends would be present and fusion with another end would result in formation of chromosome aberrations, such as radial chromosomes.

Altogether, our data point to a model to explain the effects of 5-azadC, where incorporated 5-azadC traps DNMT onto DNA, which becomes an obstacle to the second round of replication and results in a collapsed replication fork with a DSB (Figure 7). Such replication-associated DSB is normally repaired by RAD51-mediated HR, which results in an SCE. However, in absence of repair, chromatid breaks accumulate, and NHEJ fuses DNA ends that results in radial chromosomes, which will break during mitosis (Figure 7).

It is well established that proteasome inhibitors impair both the activation of FA pathway (28), as well as to catalyze HR (29). The likely mechanism is that the FA pathway requires a mono- ubiquitination of the FANCD2 and FANCI subunits to promote HR repair (35). Similarly, the HR pathway requires RNF8 mediated ubiquitination to promote HR repair (36). As proteasome inhibitors prevent degradation of ubiquitinated proteins, the level of free ubiquitin is rapidly depleted in cells, which will impair both FA and HR pathways. Here, we found that the proteasome inhibitor MG132 potentiate the toxic effects of 5-azadC treatment only in wild-type AA8 cells, but not in FANCG-defective cells. This can be explained by HR is impaired by MG132 in wild-type cells, which increase the sensitivity of 5-azadC to the same level as the FANCG-defective cells. As the proteasome inhibitor does not potentiate
FANCG-defective cells, it demonstrates that the sensitization is mediated through inactivation of the FA–HR pathway.

These results may have important clinical implications. Resistance is a major difficulty with 5-azadC treatments, and analogy can be made to melphalan resistance in multiple myeloma. Melphalan is a crosslinker and binds covalently to DNA and cause obstruction to oncoming replication forks, replication collapse and DSBs, much in the same matter as we demonstrate for 5-azadC. Furthermore, FANCG-mutated cells are sensitive to melphalan (37). Resistance to melphalan is demonstrated to be mediated by increased HR by overexpression of Fanconi’s anemia genes (38). Bortezomib is a proteasomal inhibitor used in first line in combination with melphalan to treat multiple myeloma (39). The mechanism of creating a clinical benefit with the combination of bortezomib and melphalan is likely mediated by preventing resistance through activation of the FA pathway to trigger HR repair (40). Here, we suggest that bortezomib may be useful in combination with 5-azadC, to prevent emerging...
resistance. Indeed, a recent phase I clinical study bortezomib was used in combination with 5-azadC in AML patients, and a complete remission was observed in 5 of 10 previously untreated patients (41), demonstrating that this may be an interesting future option that may be tested.

Fanconi’s anemia patients often develop AML, and treatment options are often limited owing to the intrinsic sensitivity to DNA damaging agents. As 5-azadC is a more modern and targeted treatment option, it may be suggested as an alternative treatment to these patients. In one reported case, a Fanconi’s anemia patient with an AML derived from healthy donor cells received complete remission after a 5-azaC treatment (42). Although we do not discourage, we would recommend cautious use of 5-azadC in treatment of AML in Fanconi’s anemia patients, as we show that cells defective in the Fanconi’s anemia pathway are hypersensitive to 5-azadC.

Here, we demonstrate that 5-azadC causes replication-associated DSBs, likely owing to DNMT–DNA adducts obstructing replication fork progression, which in turn trigger RAD51-mediated HR involving the Fanconi’s anemia pathway. This likely represent an important cytotoxic mechanism for 5-azadC. However, our data are not in disagreement with that 5-azadC may deplete DNMT and reduce cancer burden through activation of genes independently of DNA damage.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–3, Supplementary Methods and Supplementary References [43,44].

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