Structure of the sirtuin-linked macrodomain SAV0325 from *Staphylococcus aureus*

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Abstract: Cells use the post-translational modification ADP-ribosylation to control a host of biological activities. In some pathogenic bacteria, an operon-encoded mono-ADP-ribosylation cycle mediates response to host-induced oxidative stress. In this system, reversible mono ADP-ribosylation of a lipoylated target protein represses oxidative stress response. An NAD°-dependent sirtuin catalyzes the single ADP-ribose (ADPr) addition, while a linked macrodomain-containing protein removes the ADPr. Here we report the crystal structure of the sirtuin-linked macrodomain protein from *Staphylococcus aureus*, SauMacro (also known as SAV0325) to 1.75-Å resolution. The monomeric SauMacro bears a previously unidentified Zn^2+^-binding site that putatively aids in substrate recognition and catalysis. An amino-terminal three-helix bundle motif unique to this class of macrodomain proteins provides a structural scaffold for the Zn^2+^ site. Structural features of the enzyme further indicate a cleft proximal to the Zn^2+^ binding site appears well suited for ADPr binding, while a deep hydrophobic channel in the protein core is suitable for binding the lipoylated protein target.

Keywords: ADP-ribose; SauMacro; SAV0325; lipoyte; ADP-ribosylation; macrodomain; adenosine diphosphate ribose; lipolyation; oxidative stress; *Staphylococcus aureus*; X-ray crystallography

Introduction

The reversible post-translational transfer of adenosine diphosphate ribose (ADPr) moieties to proteins enables cells to dynamically control response to external stimuli. In bacteria, ADP-ribosyltransferases (ARTs) are the primary family of proteins responsible for ADP-ribosylation of target proteins.1,2 Sirtuin proteins also have established functions in diverse cellular processes such as aging, metabolic regulation and gene silencing.3 While they typically catalyze protein deacetylation reactions, members of the Sirtuin family including the macrodomain-linked sirtuins (SirTMs) have been shown to exhibit ADP-ribosylating activity.3–5 Both ARTs and SirTMs utilize a bound nicotamide adenosine dinucleotide (NAD°) cofactor, and in their reactions release nicotamide while covalently linking ADPr to a substrate.1–5 Substrates may be either a specific amino acid (usually a glutamate/aspartate or lysine) on the target protein, or in the case of some eukaryotic ARTs, another ADPr though a glycosidic ribose–ribose bond forming linear and branched polymers. Structurally conserved in all domains of life, ARTs are an ancient protein class with diverse modifying abilities and target proteins. Bacterial ARTs act as toxins, modifying host cell signaling proteins, resulting in cell death and disease.6–10 ARTs primarily transfer a single ADPr, a reaction referred to as mono-ADPribosylation (MARylation). In eukaryotes, ARTs have evolved into two
distinct classes, those that resemble bacterial, MARy-lating ARTs and those capable of labeling target pro-
teins with structurally and functionally diverse ADPr
polymeric chains.11,12 The latter enzymes are often
referred to as poly-ADPr polymerases (PARPs), and
control diverse cellular processes including transcrip-
tion and DNA repair through poly-ADPribosylation
(PARlyation).11–14
The existence and coordination of enzymes capa-
bale of both mono ADP-ribosylation and poly ADP-
ribosylation of target proteins paints a picture of a
complex and regulated network of ARTs that can
quickly and specifically alter cellular physiology
post-translationally.2 A number of enzymes complete
the ADPr modifying cycle by catalyzing the removal
of ADPr from poly-ADPr-targeted proteins. 15 Many
of these proteins contain a macrodomain fold, which
enables selective recognition of ADPr16 and discrimi-
nation between mono- and poly-ADPr. 15 Initially dis-
covered associated with peptidases in viruses 17
macrodomains have been found in all forms of life,
but it was not until crystal structures of macrodo-
main proteins from the thermophile
Archaeoglobus
flulgidus 16,18 and subsequently coronavirus 19 were
their ADPr binding functions understood. Possessing
a central six or seven stranded mixed β-sheet sand-
dwiched by four to five α-helices, the macrodomain
presents an L-shaped cleft that envelops the ADPr
moiety largely through conserved protein backbone
interactions, as well as aspartate-N6 adenosine and
asparagine-O2' ribose interactions. Macrodomain pro-
teins also function as O-acetyl-ADPr deacetylases,
removing the acetyl moiety from the common reaction
product of sirtuins.20 The macrodomain-containing
family includes the Poly-ADPr glycohydrolases
(PARGs),20–22 The catalytic functions of two addition-
al human macrodomain proteins capable of reversing
the glutamate-linked mono-ADPr, MacroD2,23,24 and
TARG1 (Terminal ADP-Ribose Glycohydrolase 1),25
have also been reported. Thus, the ADP-ribosylation
cycle, including MARylation and/or PARylation, can
be completed via macrodomain-mediated removal of
the terminal ADPr.
Recently, an operon-encoded reversible MARyla-
tion system was identified in the pathogenic bacteria
Staphylococcus aureus and Streptococcus pyogenes.5
This system regulates the pathogen response to host-
initiated oxidative stress through lipoate protein
ligase A (LplA2) transfer of a lipoate to a glycine
cleavage system H-like (GcvH-L) target protein [Fig.
1(A)]. At the center of this regulation is a MARylation/
deMARylation cycle, where a novel class of
macrodomain-linked sirtuins (SirTM) catalyzes the
NAD+-dependent transfer of a mono-ADPr to GcvH-
L, while a macrodomain protein removes the moiety
[Fig. 1(A)]. Rack et al. showed that only a lipoylated
GcvH-L could be MARylated by SirTM. Lipoylated
GcvH-L is also efficiently deMARylated by the macro-
domain (SauMacro for S. aureus and SpyMacro for S.
pyogenes). Altogether, the study points to the modifi-
cation of a shuttle protein (GcvH-L) with a redox-
active molecule (lipoate) under reversible control of a
sirtuin/macrodomain-catalyzed negative feedback
mechanism, where MARylated GcvH-L represses and
deMARlyated GcvH-L activates pathogen redox
defense.5 While the molecular basis of SirTM function
has been established, 5 the structure and function of
the SauMacro protein remains less well defined.
Here, we report the crystal structure of SauMacro
(a.k.a. Sav0325) to 1.75-Å resolution. Through
sequence and structural analysis, we demonstrate
that the SirTM-linked macrodomain protein family
consists of a core macrodomain fold decorated by a N-
terminal 3-helix bundle which scaffolds a novel Zn²⁺
coordination loop proximal to the ADPr binding site.
SauMacro is further characterized by a conspicuous
hydrophobic protein core cavity that is suitable for
engagement of the lipoylated target substrate.
Results and Discussion

Overall structure of the SAV0325 macrodomain

Crystallization of recombinant SauMacro was facilitated by limited trypsin digestion of full-length SauMacro immediately prior to crystallization. For structure solution, an unpublished structure of the Escherichia coli macrodomain fold protein (RCSB entry 1SPV) was successfully used as a molecular replacement search model. The asymmetric unit contains a single SauMacro molecule with continuous electron density in two segments corresponding to residues 1 to 22 (plus an N-terminal non-native "GSHMAS" sequence from the expression tag) and residues 30 to 263 of SauMacro [Fig. 1(B), Table I]. We hypothesize that trypsin cleavage occurred in the arginine rich disordered $\alpha_{10} - \alpha_{20}$ linker region (amino acids 23-30), and that this aided in crystallization.

The SauMacro macrodomain structure is characterized by the canonical $\alpha/\beta$ macrodomain fold (six-stranded mixed beta sheet ($\beta_{1}$–$\beta_{6}$) that is sandwiched by five alpha helices $\alpha_{1}$–$\alpha_{5}$), and an added N-terminal helical domain. Overall, the macrodomain core of SauMacro resembles other macrodomain structures, and is most closely related to MacroD2 (RMSD of 1.6 Å) [Fig. 1(C), Table II].

Three important differences distinguish SauMacro from other previously determined macrodomain structures. First, SauMacro contains an amino-terminal extension comprised of an antiparallel 3-helix bundle ($\alpha_{1}$–$\alpha_{3}$, residues 1–66), which is structurally distinct from the two amino-terminal helices found in MacroD2 and MacroD1 [Fig. 1(C), PDB: 2X47]. Other macrodomain structures, including human TARG1 (PDB: 4J5S)25 and AF1521,16,18 also

| Table I. Crystallographic Data Collection and Refinement Statistics |
|---------------------------------------------------------------|
| **PDB code** | SauMacro-Zn$^{2+}$ complex |
| **Data collection** | | |
| Wavelength (Å) | 1.0000 |
| Space group | P 21 21 21 |
| Cell dimensions | |
| a, b, c (Å) | 46.823, 47.853, 134.95 |
| z, β, γ (degrees) | 90, 90, 90 |
| Resolution (Å) | 50.0–1.75 (1.78–1.75) |
| Total reflections | 560,123 |
| $R_{merge}$ | 0.086 (0.345) |
| Completeness (%) | 98.83 (89.89) |
| Redundancy | 5.5 (2.8) |
| **Refinement** | |
| Resolution (Å) | 29.98–1.75 (1.812–1.75) |
| No. of reflections | 31085 (2784) |
| $R_{work}/R_{free}$ | 0.157/0.194 (0.206/0.251) |
| No. of nonhydrogen atoms | 2398 |
| Protein | 2077 |
| Ligand | 14 |
| Water | 307 |
| Average B-factors | 33.1 |
| Protein | 31.8 |
| Ligand | 37.7 |
| Water | 42.0 |
| Root mean square deviations | |
| Bond lengths (Å) | 0.008 |
| Bond angles (degrees) | 1.02 |
| Molprobity statistics | |
| All-atom clashscore | 1.2 |
| Ramachandran favored (%) | 98 |
| Ramachandran allowed (%) | 2 |
| Ramachandran outliers (%) | 0 |
| Overall score | 0.93 |

Statistics for the highest-resolution shell are shown in parentheses.

| Table II. Sequences and Crystal Structures Used in Multiple Sequence Alignments for This Study |
|---------------------------------------------------------------|
| **Abbreviation** | **Organism** | **Accession Code** | **Homology** |
|**Sequence alignments** | | |
| SauMacro | Staphylococcus aureus | 54042722 | — |
| SperMacro | Streptococcus pyogenes | 134271958 | 88/47 |
| CboMacro | Clostridium botulinum | 524018385 | 96/39 |
| EclMacro | Enterobacter cloacae | 571240966 | 66/40 |
| YmdB | Escherichia coli | 26107583 | 67/39 |
| CalMfs1 | Candida albicans | 238883620 | 72/49 |
| AteMfs1 | Aspergillus terreus | 115391435 | 84/39 |
|**Structure alignments** | | |
| MacroD2 | Homo sapiens | 4IQY | 23.5 |
| MacroD1 | Homo sapiens | 2X47 | 23.1 |
| Phosphatase (putative) | Escherichia coli | 1SPV | 22.5 |
| PARP14 | Homo sapiens | 3Q6Z | 21.0 |
| MacroH2A.2 | Homo sapiens | 2X97 | 20.4 |
| MacroH2A1.1 | Rattus norvegicus | 1YD9 | 20.1 |
| AF1521 | Archaeoglobus fulgidus | 2BFQ | 19.6 |

* GenBank and PDB codes are given for sequence and structural alignments, respectively.

b Percent coverage/identity and Dali reported Z scores with respect to SauMacro are reported for sequence and structural alignments, respectively.
lack these amino-terminal helices. Secondly, we identify a Zn\(^{2+}\)-binding site, with Zn\(^{2+}\) coordination mediated by an insertion loop between β2 and α1. This Zn-binding motif scaffolds the Zn\(^{2+}\) ion proximal to the presumed ADPr binding site. Lastly, the structure reveals a deep hydrophobic cavity in the SauMacro macrodomain that is proximal to the probable ADPr binding site.

**A novel macrodomain Zn\(^{2+}\) binding site**

The amino-terminal three-helix bundle is tightly packed against the β2-α1 connecting loop containing the Zn\(^{2+}\) [Fig. 2(A–C)]. Conservation of the N-terminal extension correlates with the conservation of the Zn\(^{2+}\)-binding motif. A BLAST search of SauMacro related proteins (Table II) reveals a distinct cutoff for conservation of the CxxxxHxC Zn\(^{2+}\)-binding sequence, proteins with high sequence homology to SauMacro also lack the Zn\(^{2+}\) binding motif. Thus the three-helix bundle N-terminal extension appears to provide an extended structural scaffold for the Zn\(^{2+}\)-binding β2-α1 insertion loop.

The proximity of the Zn\(^{2+}\)-binding motif to the predicted ADPr pocket implies that it may play roles in substrate binding and catalysis. The conserved residues C113, H118, and C120 mediate Zn\(^{2+}\)-coordination [Fig. 2(A)]. To validate the identity of the metal, we conducted X-ray fluorescence measurements on purified SAV0325 protein [Fig. 2(C)]. This analysis indicates that Zn is abundant in our protein purifications, consistent with Zn\(^{2+}\) modeled in the crystal structure. We also note that binding site geometry is consistent with a Zn\(^{2+}\) binding as confirmed by the “checkmymetal” (CMM) binding site validation server\(^{27}\) (http://csgid.org/csgid/metal_sites/). The loop containing the 113-CxxxxHxC-120 Zn\(^{2+}\)-binding motif appears to be an insertion relative to other known macrodomain structures, all of which replace this Zn\(^{2+}\)-binding sequence with a polyglycine linker. An inspection of the surface of SauMacro relative to other macrodomains containing ADPr reveals that the Zn\(^{2+}\)-binding motif does not occlude the canonical ADPr substrate-binding cleft. Interestingly, Asp55 of a symmetry related SauMacro molecule completes the tetrahedral coordination shell of the bound Zn\(^{2+}\) [Fig. 2(B)]. In the case of SpyGcvH-L, the MARylated residue is expected to be either Ast or Glu,\(^5\) so the observed Asp55-Zn\(^{2+}\) interaction might reflect a possible substrate-binding mode of the ADPr-ribosylated target. Thus, we hypothesize that SauMacro and related sirtuin-linked macrodomains use this Zn\(^{2+}\) site to coordinate substrate binding and/or electron movements during catalysis.

**ADPr binding site**

ADPr was added to the crystallization solution, however no evidence for bound ligand was observed in our electron density maps. Notably, the loop between α4 and β6 is found in a conformation that partially occludes the predicted ADPr binding site, in a mode reminiscent of apo MacroD1. The CASTp server identifies a large contiguous solvent-accessible surface pocket in proximity to the Zn\(^{2+}\) site [Fig. 3(A–D)]. This pocket comprises over 1400 Å\(^2\) of surface area, and can be divided into two main sections connected via a narrow tunnel: one adjacent to the Zn\(^{2+}\) and the others containing the conserved acidic residue D93. Mapping the ADPr from the MacroD2 structure (PDB: 4I3Y) onto this surface places the terminal ribose (ribose\(^{−}\)) in the Zn\(^{2+}\)-adjacent pocket and the adenosine near D93. Electrostatic surface analysis implicates the surface around the Zn\(^{2+}\) pocket as electronegative, while the surface that would presumably contact the adenosine as electronegative [Fig. 3(B)]. Taken together, we infer that the identified pocket could accommodate the ADPr, consistent with SauMacro de-ADP-ribosylation function.\(^5\)

Next, we structurally aligned a number of similar macrodomains predicted by the DALI server to have similar structural features (Z score > 19) [Table II, Fig. 3(C)]. All of the compared structures contain an ADPr or ADPr-like bound substrate molecule in a similar spatial orientation on the protein surface. In considering the structurally similar macrodomains, many of the residues involved in substrate recognition are conserved in SauMacro [Fig. 3(C,D)]. Important electrostatic interactions include Asp93, which could hydrogen bond to adenine N6 amino group, as well as N107 and D122, both of which would contact the O2' hydroxyl of the terminal ribose. Substrate-bound macrodomains contain an aromatic residue that provides π-stacking interactions with the adenosine base; in SauMacro, F249 may perform this role [Fig. 3(D)]. C209, as well as the hydrophobic residues I94, I121, I187, and I211 line the pocket that could accommodate ADPr. G214 is a well-conserved residue, and the presence of a bulky side chain at this position would likely occlude ADPr phosphate binding. Some backbone rearrangements of SauMacro would be necessary to prevent clashes with ADPr, especially the loop connecting β5 and α4. Overall, the surface properties of this cleft, strong sequence homology to known ADPr-macromdomain complexes, and the previous report that SauMacro acts as an ADPr hydrolase\(^5\) strongly implicate that ADPr binds in the predicted pocket.

**Predicted lipoate binding site**

In addition to binding and hydrolyzing ADPr, SauMacro, and other related macrodomains occupying LpA2 and SirTM-containing operons are expected to recognize lipoate. Efforts to co-crystallize SauMacro
with lipoate were unsuccessful and our structure does not contain lipoate. However, we identify a conspicuous deep hydrophobic pocket that both spatially and electrostatically may accommodate the redox-active molecule [Fig. 4(A)]. This pocket is part of the surface identified by CASTp and resides in close proximity to the proposed ADPr binding site [Fig. 4(B)]. One face of the pocket contains...
residues 169-TVGPQ-173, which is highly conserved among macrodomains with high sequence homology to SauMacro [Fig. 2(D)]. The pocket is lined with highly conserved hydrophobic residues A105, I121, L187, F208, and I211, as well as Y191, which we predict would stack against the lipoate dithiolan ring. At the bottom of the pocket are two cysteines on the same face of helix α3, C190, and C194, which would lie within disulfide bonding distance to the two thiolates of the lipoate. Finally, the carboxyl face of the molecule, which would be covalently attached via an amide bond to a lysine of the target protein, extrudes out from the hydrophobic pocket, in potentially close contact to the conserved residue Q173.

The loop connecting β5 and α4 adopts three different conformations among the MacroD2, MacroD1, and SauMacro structures [Fig. 4(B)]. MacroD2 (PDB: 4IQY) and MacroD1 (PDB: 2X47), which are both ADPr-binding proteins, have very similar overall folds (RMSD of 0.85 Å over 210 Cα’s), and this loop represents the most significant structural deviation between the two structures. Additionally, MacroD2 was determined in complex with ADPr, while MacroD1 is a ligand-free structure. Jankevicius et al. noted that an apparent 13 Å shift (5.3 Å for Cα positions) would be required to bring F272 in MacroD1 from an “open” conformation to the closed conformation occupied by Y190, the structurally homologous residue in MacroD2, stacks against the
In SauMacro, the corresponding residue is likely F216, and its loop adopts an intermediate position, ~10 Å (3.4 Å for Cα atoms) away from Y190 in MacroD2 [Fig. 4(B)]. SauMacro F216 is less likely to adopt the same closed conformation as Y190, as this position might occlude binding to the lipoate.

The close juxtaposition of the Zn-binding site, ADP-ribose pocket and the proposed hydrophobic lipoate-binding cavity suggests that the ADPr and lipoylation sites on the target GcvH-L proteins should be within close three-dimensional proximity of one another. The distance between the aspartic acid ligand bound in our structure (the proposed Asp-ADPr binding site) to the solvent accessible region of the lipoate binding site is ~8 Å [Fig. 4(C)]. Overall this geometry closely correlates with the spacing of the known GcvH-L ADPr modification (D27 of GcvH-L) and lipoylation (K56 of GcvH-L) sites [Fig. 4(C)].

Conclusions

The structure of SauMacro reported here reveals the first atomic details of a sirtuin-linked macrodomain. As a recently discovered class of oxidative-stress response regulators in pathogenic bacteria, the biochemical and biomedical relevance of these molecules are only beginning to be understood. Our structure suggests that SauMacro is a unique macrodomain protein with a novel Zn\(^{2+}\) binding insertion loop relative to other macrodomains. A three-helix bundle provides the structural scaffolding for this site, and sequence homology implies that this amino-terminal structure may be conserved for related macrodomains in bacteria and fungi. Through structural homology and analysis, we propose potential binding sites for ADPr and lipoate on SauMacro. Future co-crystal structures of SauMacro in complex with these substrates, or with the GcvH-L target protein, and in concert with biochemical analysis, will provide the foundation for potential
novel antimicrobials targeting this stress response pathway.

Materials and Methods

Protein expression and purification

N-terminally 6x His-tagged SauMacro (SAV0325) was expressed in from pET28a in E. coli Rosetta 2 (DE3) (Novagen). Terrific broth media was inoculated with saturated cell cultures and allowed to grow at 37°C until OD_{260}=1.5, at which point the temperature was lowered to 15°C, induced with 0.2 mM IPTG and further incubated overnight. Cells were pelleted, resuspended in 20 mM Tris pH 7.5, 150 mM NaCl, 10 mM Imidazole, sonicated and clarified by centrifugation. Affinity chromatography was accomplished by flowing clarified cell lysate over Ni-NTA resin. Bound protein was eluted by addition of imidazole and further purified by size-exclusion chromatography using a Superdex 75 column (GE Healthcare) equilibrated in 20 mM Tris pH 7.5, 500 mM NaCl, and 0.1% βME. Prior to crystallization a purified SAV0325 was subjected to limited trypsin digest at room temperature for 1 hr at a ratio of 650:1 (w/w) SauMacro to trypsin. Trypsinized protein was resolved by additional size-exclusion purification, followed by a final cation-exchange purification eluted in 20 mM Tris pH 7.5, 175 mM NaCl, and 0.1% βME. The resulting fractions from the single peak from the ion-exchange purification were pooled and concentrated to 25 mg/mL for crystallographic screening.

Crystallization and data collection

Crystals were grown at 4°C using the sitting-drop vapor diffusion method, by mixing SauMacro (25 mg mL⁻¹ protein plus 1 mM ADP-ribose) with 0.1 M CHES pH 9.5, 15% (v/v) ethanol in a 1:1 ratio (250 nL: 250 nL) using a Mosquito robot (TTP Labtech, Hertfordshire, UK). The resulting rod shaped crystals were cryoprotected in a solution of mother liquor supplemented with 26% (v/v) ethylene glycol and flash frozen in liquid nitrogen for subsequent data collection. Diffraction data were collected to 1.75 Å on a MAR 225 CCD detector at Southeast Regional Collaborative Access team (SER-CAT) 22-BM beamline at the Advanced Photon Source, Argonne National Laboratory. Data were indexed, integrated, and scaled using HKL2000. The data were processed in P2_1_2_1 space group, with unit cell dimensions of a = 46.823, b = 47.853, c = 134.950, α = β = γ = 90° (also see Table I). X-ray fluorescence scans [Fig. 2(C)] on ammonium sulfate precipitated SAV0325 were performed and analyzed at SER-CAT 22-BM.

Structure determination and analysis

Crystalllographic phases were determined by molecular replacement using 1SPV as a search model in Phaser. Subsequent rounds of automated model building in PHENIX and manual model building in Coot with refinement in PHENIX yielded the final model. Water molecules were placed automatically using PHENIX and checked manually in Coot. Significant positive density was found in between residues C113, H118, and C120, indicative of a metal ion coordination site. Based on ionic distances and coordination geometry, the metal was assigned as Zn²⁺. Although the protein crystallization solution was supplemented with ADP, no definitive difference electron density corresponding to this ligand was observed. Model validation was carried out automatically with Molprobity. Data collection and refinement statistics are detailed in Table I. PyMOL was used for structure-sequence conservation figures, while all other molecular graphics were produced with UCSF Chimera. Solvent accessible surface pockets were calculated using the CASTp server. To visualize electrostatic surfaces, the SauMacro coordinates were prepared for Poisson-Boltzman calculations using PDB2PQR and the calculations were performed with APBS.

Sequence and structural alignments

Homologous sequences were manually selected from a DELTA-BLAST search of the SauMacro protein sequence. These sequences were aligned using the MultAlign server and visualized with ESPript. Proteins with similar structural homology to SauMacro were identified with the Dali server and subsequent sequence alignments and figures were prepared with ENDscript. All sequences used in this study are listed in Table II.

PDB accession codes

Coordinates and structure factors have been deposited in the Protein Data Bank as PDB entry 5KIV.

Acknowledgments

C.D.A. expressed and purified recombinant SauMacro, as well as performed the trypsinolysis and crystallization. R.S.W. collected diffraction data and determined the SauMacro crystal structure; G.K.F., B.D.W. and R.S.W. refined and interpreted the structure and homology data. G.K.F., C.D.A. and R.S.W. wrote the manuscript. The authors thank A. Moon and S. Andres for comments and I. Ahel for the gift of the SauMacro expression vector. The authors declare no competing financial interest. We thank L. Pedersen of the NIEHS collaborative crystallography core for help with data collection, and M. Schellenberg for conducting fluorescence scans. Use of the Advanced Photon Source was supported by the U. S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. W-31-109-Eng-38. Our studies are supported by the US National Institute of Health Intramural Program: US National Institute of Environmental Health Sciences (NIEHS), 1Z01ES102765 (R.S.W.).

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