Glycoconjugate J (1989) 6:489-498

Chemical Evidence for Covalent Linkages of a Semi-synthetic Glycoconjugate Vaccine for *Haemophilus influenzae* Type B Disease

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Received April 5/August 30, 1989.

Key words: chemical analysis, saccharide-protein linkages, H. influenzae type b glycoconjugate vaccine, H. influenzae type b oligosaccharide-CRM_{197} vaccine, semi-synthetic glycoconjugate vaccine

We have defined the nature of the covalent linkages in a *Haemophilus influenzae* type b oligosaccharide-CRM_{197} conjugate vaccine, designated HbOC. The conjugate was acid hydrolyzed to release a novel amino-acid derivative, *N*ε-(2-hydroxyethyl)lysine (OHEt-Lys), identifiable with an amino-acid analyzer. This amino-acid derivative was formed by reduction of Schiff bases formed between *H. influenzae* type b oligosaccharides (HbO) and the lysyl ε-amino groups of CRM_{197} (a non-toxic, cross-reactive variant of diphtheria toxin), followed by acid hydrolysis of HbOC. Quantification of OHEt-Lys per CRM_{197} molecule allowed the determination of a covalency ratio, a useful parameter for evaluating the stoichiometry and consistency of HbOC preparations. Covalent association between HbO and CRM_{197} was also demonstrated by the coincidence of immunoreactivity of gel-electrophoresed HbOC on a Western blot probed with anti-CRM_{197} and anti-saccharide antisera.

Haemophilus influenzae type b (Hib) is a human pathogen, causing a number of diseases including meningitis, sinusitis, cellulitis, bacteremia, pneumonia, and otitis media in young children [1]. A vaccine composed of the type b capsule, a polymer of repeating [-3)-β-D-Rib*f*-(1-1)-ribitol-5-(PO_4-] units, ("PRP", structure **1**, Fig. 1) has been shown to be effective in inducing protective serum antibodies against Hib disease in children over 18 months of age [2-4]. However, the type b polysaccharide, considered as a T-cell independent antigen, is non-immunogenic in younger children, who are at risk to Hib disease; moreover, it fails to induce a boostable immune response upon re-immunization [5]. As an approach to overcome these limitations, covalent attachment of PRP to protein carriers has been undertaken by several laboratories [6-12]. These new semi-synthetic glycoconjugate vaccines, which display the immunological properties of T-cell dependent antigens, have recently been shown to induce enhanced, as well as boostable immune responses to PRP in young children [13, 14] and adults [15, 16].

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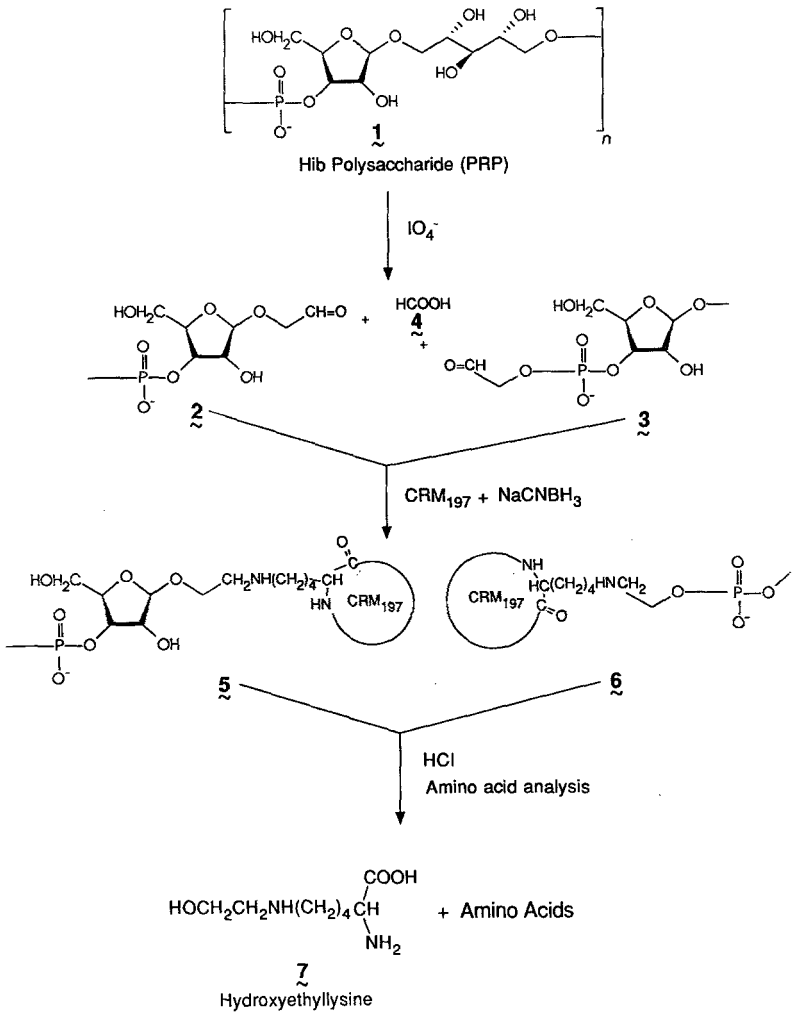

Figure 1. Reaction pathway leading to the formation of *N*ε-(2-hydroxyethyl)lysine.

The semi-synthetic glycoconjugate vaccines, made so far, are not generic, but differ markedly in chemical design as well as in immunological properties [17-19]. Most Hib glycoconjugates have been prepared using the capsular PRP polysaccharide covalently linked to various carrier proteins (tetanus toxoid [9], diphtheria toxoid [8], and meningococcal outer membrane protein [11]) by means of exogenous spacer molecules. In contrast to these, a glycoconjugate vaccine based on using Hib oligosaccharides (HbO), rather than the intact capsular polysaccharide, has been developed [10, 13]. This oligosaccharide-based conjugate vaccine, designated HbOC, is comprised of HbO with an average degree of polymerization of 20 repeat units (Dp ≈20) coupled covalently to the variant (non-toxic) diphtheria toxin, CRM_{197}, *via* reductive amination. Unlike other Hib glycoconjugates, the synthesis of HbOC does not necessitate the use of exogenous spacers (see reaction scheme of Fig. 1) to effect covalent coupling between saccharides and protein. In human infants, as

Table 1. Degree of HbO substitution per CRM molecule in HbOC vaccines as measured by Nε-(2-hydroxyethyl)lysine content.

Samples	OHEt-Lys/Ala ratio	Covalency ratio (OHEt-Lys/CRM)
LL-5-68	0.283	11.31
LL-5-17	0.241	9.64
LL-6-46A	0.287	11.49
LL-6-46B	0.250	9.98
LL-6-46C	0.232	9.31
WW-2-8	0.235	9.40

in laboratory animals, HbOC has been shown to induce primary and boostable anti-PRP antibody response [10, 13, 14]. Recently, HbOC has been licensed for use in children, 18 months to 5 years of age, and is known by its trade name, HibTITER™.

To achieve the enhanced immunogenicity and stability and to impart T-cell dependence to the Hib saccharide, the saccharide to protein carrier bond must be covalent. The aim of this study is to prove the covalent linkage of the saccharide-protein by direct chemical analysis of acid-hydrolyzed HbOC. The synthesis of HbOC predicts certain neo-lysinyl derivatives could be formed by reductive amination (Fig. 1). One of these is Nε-(2-hydroxyethyl)lysine (OHEt-Lys) which could be quantitatively detected by an amino-acid analyzer and could be used as a 'quality control' parameter of HbOC. Covalent association between HbO and CRM_{197} was further demonstrated by SDS-PAGE/Western blot analysis of HbOC.

Materials and Methods

Materials

HbOC products were prepared by reductive amination as described in European patent application PCT/US87/01020 and [10]. The HbOC lots in Table 1 were synthesized under a given set of reaction parameters, e.g. pH, temperature, ionic strength, initial saccharide to protein ratios, and were analyzed to have a final saccharide/protein ratio of 0.5 ± 0.2 as determined by the pentose [20] and Lowry [21] colorimetric assays. Specific rabbit polyclonal antibodies made to CRM_{197} and to PRP were prepared by the Immunobiologics Services Department, Praxis Biologics, Inc., USA. Polyclonal anti-CRM_{197} serum was produced by immunizing a rabbit with 10 μg CRM_{197} (Praxis Biologics, Inc.) in complete Freund's adjuvant and boosted with the same amount in incomplete Freund's adjuvant four weeks later. The anti-CRM_{197} serum was collected one week after the booster immunization and stored at -20°C. Polyclonal serum, containing anti-PRP antibodies, was produced by vaccinating a rabbit three times, two weeks apart, with 18 μg of conjugate consisting of PRP saccharides linked to the F-protein of respiratory syncytial virus. The anti-PRP antibody-containing serum was collected two weeks after the last immunization and was demonstrated to react with capsular PRP polysaccharide by both ELISA and Western immunoblot assays (data not shown).

The amino-acid derivative, Nε-(2-hydroxyethyl)lysine was synthesized by reductive alkylation of Nα-acetyl-L-lysine with glycoaldehyde as described [22]. Similarly, Nε-(2,3-dihydroxypropyl)lysine was synthetically prepared by reductive alkylation of Nα-acetyl-L-lysine with glyceraldehyde.

Experimental General Procedures for Amino-acid Analysis

HbOC vaccine solutions containing approximately 60 μg protein (*via* Lowry assay) were evaporated to dryness in 4.0 ml glass vials *in vacuo* using a Savant Speed Vac Concentrator (Model SVC100H). To the dried sample, 100 μl of 6 M HCl (constant boiling) was added and the vial was flushed with nitrogen, capped tightly, and placed in a hydrolysis block set at 150°C for 90 min. After cooling to room temperature, the cap was removed and the solution was evaporated to dryness in a vacuum centrifuge to remove HCl. The dried hydrolysate mixture was redissolved in 200 μl of 0.2 M sodium citrate buffer pH 2.2 before application on the amino-acid analyzer constructed as described earlier [23]. The amino-acids were separated by ion exchange chromatography and detected by color produced upon heating with ninhydrin. The quantity of amino-acids is directly proportional to the absorbance reading when reacted with ninhydrin. To obtain the covalency ratio, OHEt-Lys/CRM, the peak area of OHEt-Lys was related to alanine peak area and multiplied by a factor of 40 (there are 40 alanines per CRM molecule). Alanine was chosen as the internal standard because it is acid stable and well resolved from other amino-acids and unaffected by the conjugation process.

SDS-PAGE and Western Blot of HbOC

HbOC samples were electrophoresed on 5.0% stacking gel and 10% polyacrylamide resolving gel by the method of Laemmli [24]. The gels were stained with silver nitrate according to the procedure of Tsai and Frasch [25]. The HbOC samples from other gels were transferred electrophoretically to nitrocellulose sheets as described by Towbin *et al.* [26]. The Western blots were probed with rabbit anti-CRM or anti-PRP polyclonal antisera. Antigen-antibody complexes were identified with peroxidase conjugates as previously described [27].

Results

Chemical Analysis of Nε-(2-Hydroxyethyl)lysine in HbOC Vaccines

The synthesis of HbOC *via* reductive amination, followed by acid hydrolysis, can lead to the potential formation of two unique amino-acid derivatives, Nε-(2-hydroxyethyl)lysine and Nε-(2,3-dihydroxypropyl)lysine. The latter derivative would be derived from an incomplete cleavage of vicinal hydroxyls (ribitol moiety) during periodate oxidation treatment of PRP (structure **1**, Fig. 1) to generate oligosaccharides (HbO). Of these two possible neoLys derivatives, only Nε-(2-hydroxyethyl)lysine was found in acid hydrolyzed HbOC. The reaction pathway leading to the formation of Nε-(2-hydroxyethyl)lysine is depicted in Fig. 1. Briefly, Hib capsular polysaccharide (Fig. 1, structure **1**) is fragmented by controlled periodate oxidation to generate bivalent HbO of an average chain length of 15-

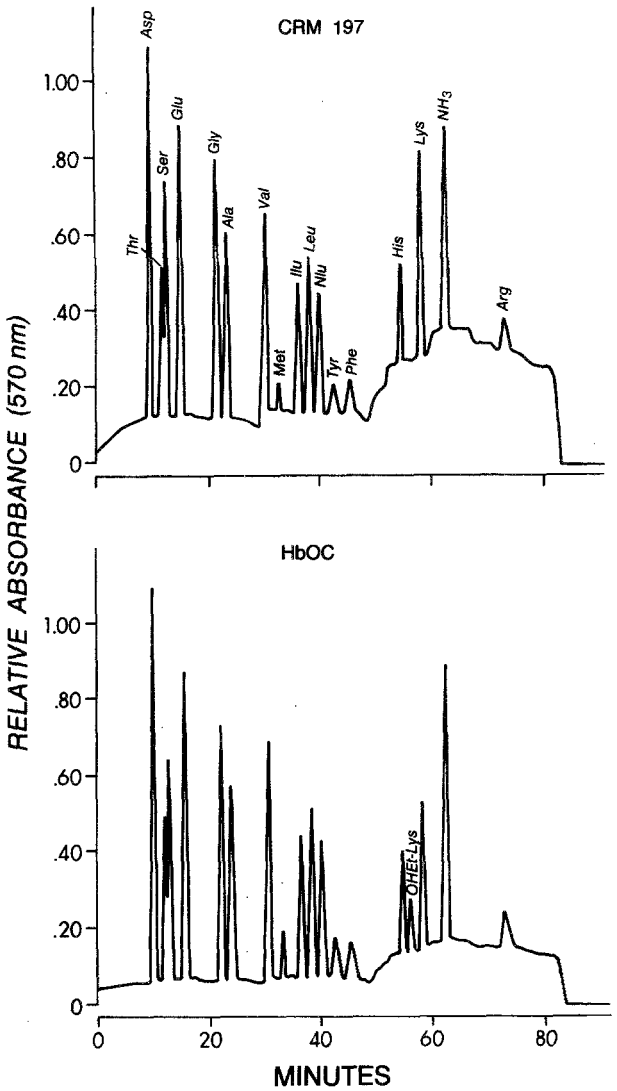

Figure 2. Amino-acid chromatograms of acid hydrolysates of CRM_{197} (top panel A) and HbOC (bottom panel). Hydroxyethyllysine eluted between the histidine and lysine peaks with a retention time of 54.67 min in HbOC. OHEt-lys is not detected in CRM_{197}. Nlu is an abbreviation for norleucine which is used as an internal standard.

30 repeat units. Two different reducing end groups as depicted in structures **2** and **3**, respectively, as well as formic acid (structure **4**) are generated (Fig. 1). The reducing end groups, **2** and **3**, located on each end of the oligosaccharide fragment, react primarily with ε-amino groups of lysine residues in CRM_{197} to form Schiff bases, which upon reduction with $NaBH_3CN$, give rise to stable secondary amine bonds linking the saccharide and protein moieties as depicted by structures **5** and **6**. Acid hydrolysis of structures **5** and **6** would release OHEt-Lys.

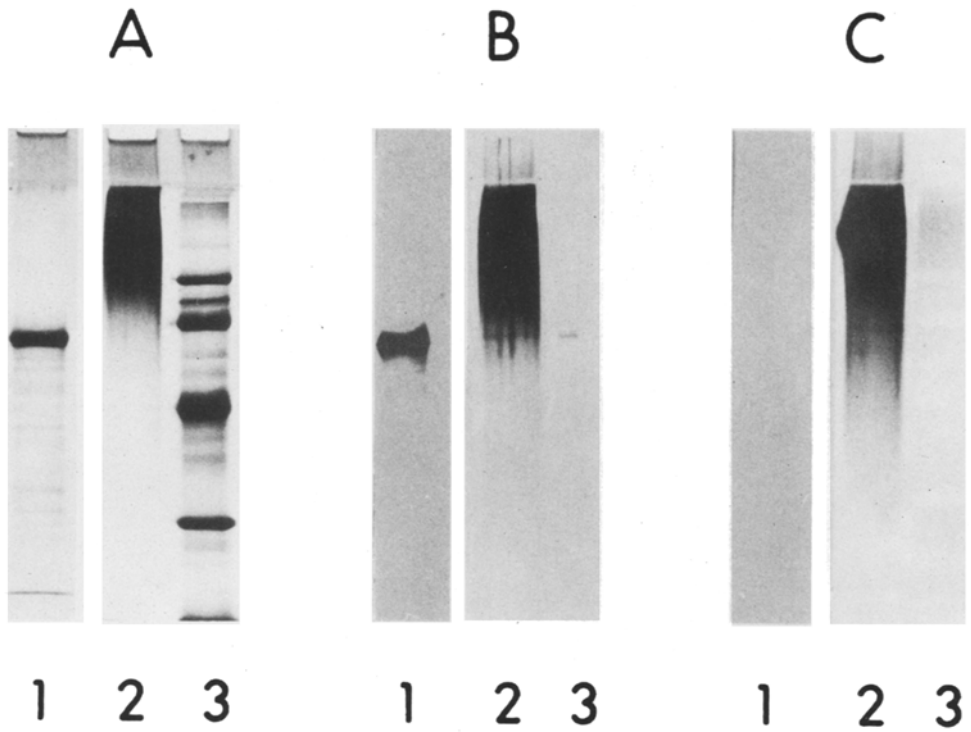

Figure 3. SDS-PAGE/Western blot analysis of HbOC lot WW-2-8. Panel A is silver stained gel; panel B is immunoblot probed with anti-CRM antisera; panel C is immunoblot probed with anti-PRP antisera. Lane 1, CRM_{197}; lane 2, HbOC; lane 3, low molecular weight protein standards were from Bio-Rad Lab, Richmond, CA, USA.

The detection of *N*ε-(2-hydroxyethyl)lysine (OHEt-Lys) in the acid hydrolysate of HbOC offers proof of covalent linkage between HbO and CRM protein. As indicated in Fig. 2 (bottom panel), *N*ε-(2-hydroxyethyl)lysine eluted between the histidine and lysine peaks in the amino-acid chromatogram of HbOC. The elution position of OHEt-Lys was confirmed using synthetic OHEt-Lys. No OHEt-Lys peak was detected in the control chromatogram of CRM_{197} (Fig. 2).

The OHEt-Lys was quantitatively related to the alanyl content of CRM_{197} to yield the covalency ratio, OHEt-Lys per CRM molecule, which reflects the degree of saccharide substitution. As shown in Table 1, the covalency ratios for six HbOC products, made under similar reaction conditions, were very close, e.g. 9.3 to 11.5, thus indicating consistency in the synthesis of HbOC. The number of OHEt-Lys groups is consistent with the number of HbO molecules linked to the CRM_{197} [4-5] as determined by orcinol (pentose) and Lowry (protein) analyses assuming each end of the HbO is covalently attached to the protein. Since the maximal level of saccharide substitution is 40, i.e., 39 lysyl ε-amino groups and one NH_2-terminus per CRM molecule, the covalency ratio (Table 1) indicated that about 25% of the available amino groups were substituted with HbO. Presumably, these sites are surface-exposed and accessible for Schiff base formation. Amino-acid analysis of HbOC

revealed reduction in lysinyl content of ca. 25%, which corresponded approximately to the amount of OHEt-Lys detected. No other additional unusual peaks, e.g. *N*ε-(2,3-dihydroxypropyl)lysine, the other possible neoLys derivative, or loss of any other amino-acid residue were observed in the chromatogram. *N*ε-(2,3-Dihydroxypropyl)lysine, prepared synthetically, was observed to elute in front of the histidine peak in the amino-acid chromatogram (data not shown).

Analysis of HbOC via SDS-PAGE and Western Blots

Fig. 3A shows the silver stained band patterns of CRM_{197} (lane 1) and HbOC (lane 2) on SDS-gel. As depicted, HbOC electrophoresed with a slower mobility than CRM_{197}, evidently due to higher molecular weight due to covalent attachments of HbO to CRM_{197}. When transferred to nitrocellulose paper, both CRM_{197} and HbOC reacted with anti-CRM antisera (Fig. 3B). When probed with anti-PRP antisera, only HbOC was detected. The coincidence in the immunoreactive patterns of HbOC to both anti-CRM (Fig. 3B) and anti-PRP (Fig. 3C) antisera, strongly support covalent association between HbO and CRM_{197}.

Discussion

HbOC has been recently licensed for human use in the United States to protect against *Haemophilus influenzae* diseases in children of 18 months or older. The identity and purity of HbOC, known as HibTITER™, is well-defined chemically. This study was undertaken to define the nature of the covalent bond between HbO and CRM_{197} and to determine whether the covalent linkage is quantifiable.

The chemistry of reductive amination used to prepare HbOC creates a unique amino-acid derivative readily assayable by amino-acid analysis. As shown in Fig. 1, the saccharides are stably joined to the protein carrier through two linkages as represented in structures **5** and **6** (derived, respectively, from structures **2** and **3**). Upon acid hydrolysis both conjugate structures, **5** and **6**, released *N*ε-(2-hydroxyethyl)lysine (structure **7**), as well as the other constituent amino-acids of CRM_{197} (Fig. 1). Conceivably, another unique amino-acid, *N*ε-(2,3-dihydroxypropyl)lysine, would be formed if the periodate oxidation of the vicinal hydroxyls in the ribitol moiety of PRP (structure **1**) was incomplete. However, no such derivative was found in the acid hydrolysate of HbOC.

The identification of OHEt-Lys provides the most convincing evidence of covalent protein-carbohydrate linkages in HbOC vaccine. The amount of OHEt-Lys found in the acid hydrolysate of HbOC approximated the 25% reduction of lysinyl residues in CRM_{197}. The identity of OHEt-Lys was confirmed by chemical synthesis [21]. Quantification of OHEt-Lys with respect to an internal amino-acid, alanine, of CRM_{197} enables calculation of the covalency ratio, which indicates the number of sites of attachment of saccharide chains to the polypeptide chain. The narrow range of covalency ratios shown in Table 1 provided further confirmation that the six HbOC lots were prepared in a consistent manner. Different covalency ratios for HbOC can be obtained, however, when the reaction parameters (initial saccharide/protein ratio, pH, reaction time, temperature) used for the conjugation reaction are altered (data not shown).

Amino-acid analysis has been used to demonstrate covalency in another Hib glycoconjugate vaccine consisting of PRP linked to a meningococcal membrane protein through a 'bigeneric' spacer [10] and in neoglycoproteins generated by direct reductive amination of disaccharides on to proteins [28].

Other potential analytical methods, i.e. gel permeation chromatography and gel electrophoresis, which demonstrate molecular weight differences between reactants and products, have been recently used to support covalency of lipopolysaccharide-protein conjugates [29, 30]. When using these separation techniques, one must be cognizant that an increase in molecular weight of the product could be due to strong non-covalent aggregation of the reactants.

Prior to the results obtained by amino-acid analysis of HbOC (*vide supra*), we utilized the Western blot technique in conjunction with SDS-PAGE to provide supporting evidence for covalent saccharide-protein linkages in HbOC. Under SDS-PAGE denaturing conditions, the protein and carbohydrate components would co-migrate if they are covalently linked; a conjugate held together by non-covalent binding would be expected to dissociate into individual protein and saccharide components. On entering the SDS-gels, HbOC migrated as a broad diffuse band with a slower mobility than CRM_{197} (M_r 66,000, Fig. 3); thus, the shift to the higher molecular weight region of the gel denoted covalent attachment of saccharides on to CRM_{197}.

Glycoproteins, in general, give broad diffuse bands on SDS-PAGE due to aberrant hydrodynamic properties, microheterogeneity in carbohydrate composition, and less binding of SDS per polypeptide weight compared to water soluble proteins [31]. The Western blots demonstrated coincidence of immunoreactivity with both anti-CRM and anti-PRP antisera, thus further supporting covalent association between CRM_{197} and Hib oligosaccharides. Free, unlinked HbO was not detected in Western blots due to its non-retention on SDS-gels during electrophoresis. Since the transferred HbOC reacted with both anti-PRP and anti-CRM_{197} antisera, the antigenic determinants of the saccharide and protein carrier were not destroyed by the reductive amination process used for conjugation.

In conclusion, we have provided proof of the covalent linkage between PRP oligosaccharides and CRM_{197} in HbOC by identifying a unique amino-acid, OHEt-Lys, formed by the reductive amination reaction. Quantification of OHEt-Lys by amino-acid analyzer affords the covalency ratio, a useful index to gauge consistency of HbOC preparations (Table 1). In addition, covalency was demonstrated by the coincidence of anti-PRP and anti-CRM immunoreactivity with HbOC on Western immunoblot. The principles of these analytical techniques can be applied to investigate the structure/immunogenicity of other semi-synthetic glycoconjugates.

Acknowledgements

We are grateful to Sunny Kuratana and David Kirkley for their technical assistance in performing the SDS-PAGE/Western blot analyses. We acknowledged Dace Madore and Gary Zlotnick for helpful comments and discussions.

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