Supplemental materials

Glycosyl linkage analysis of recombinant Fap1 proteins

Materials and Methods:

**Protein sample preparation.** The recombinant Fap1 proteins modified by Nss (+Nss) and not modified by Nss (-Nss) were purified as described in the materials and methods of the article. The 1mg samples were dialyzed using a Tube-O-Dialyzer (4.0 kDa cut-off membrane; G BioSciences) against nanopure water at 4°C for about 22 hours to remove salts and other contaminants. Nanopure water was replaced three times during the entire dialysis period.

**Release of O-linked glycans by β-elimination.** O-linked glycans were cleaved from the samples by β-elimination procedure. Briefly, 250 µL of 50 mM NaOH was added to each of the samples and then checked for pH. Upon determination that the pH was basic, another 250 µL of 50 mM NaOH containing 19 mg of sodium borohydride was added to each of the samples, vortexed, and incubated overnight at 45°C. The incubated samples then were neutralized with 10% acetic acid and desalted by passing through a packed column of Dowex resins and then were lyophilized. Dried samples were cleaned of borate with methanol:acetic acid (9:1) under a stream of nitrogen gas before permethylation.

**Per-O-methylation of O-linked glycans.** Released O-linked glycans were permethylated and evaluated by mass spectrometry (Anumula and Taylor, 1992) to verify if the derivatization was carried to completion. The glycans were dissolved with dimethylsulfoxide and then methylated with NaOH and methyl iodide. The reaction was quenched with water and per-O-methylated glycans were extracted with methylene chloride.

**Glycosyl Linkage Analysis.** For determination of glycosyl linkages, partially methylated alditol acetates (PMAAs) were prepared from the released permethylated O-linked glycans. Briefly, permethylated glycans were hydrolyzed with 2N trifluoroacetic acid (TFA) at 100°C for 4 h, followed by reduction with NaBD₄. The latter-freed hydroxyls were acetylated with acetic anhydride/pyridine (1:1, v/v) at 100 °C for 15 min.

**Gas Chromatography-Mass Spectrometry (GC-MS).** The PMAAs were analyzed on a Hewlett Packard 5890 GC interfaced to a 5970 MSD (mass selective detector, electron impact ionization mode). The separation was performed on a 30 m EC 1 bonded phase fused silica capillary column (Altech). Electron impact mass spectra were obtained under the following conditions: oven temperature, 80 °C (2 min) → 180 °C (20 °C/min) → 240 °C (4 °C/min); detector temperature, 280 °C; inlet temperature, 250 °C.

Supplemental Figure Lengends:

S Fig.1. Glycosyl linkage analysis of recombinant glycosylated Fap1. Fap1 glycosylated by Gtf1, Gtf2 and Nss, Fap1ΔRII (+Nss) (A) or by Gtf1 and Gtf2 only Fap1ΔRII(-Nss) (B) were subjected to β-elimination, the released glycans were per-O-methylated and subjected to GC-MS analysis.
S Fig. 1A. GC chromatogram of PMAAs of released O-linked glycans from Fap1ΔRII(+Nss).

Legend:
- Meth - methyl group
- Ac - acetyl group
**Results:**

Terminal glucose, Terminal HexNAcitol with methyl or acetyl group at C1 position, and 6-linked HexNAcitol with methyl or acetyl group at C1 position were observed in Fap1ΔRII(+Nss). With the exception of terminal glucose, all glycosyl linkages were also observed in Fap1ΔRII(-Nss). These results support the conclusion that Nss is responsible for the transfer of glucosyl residue to GlcNAc-modified recombinant Fap1.