The role of C-terminus of GEN1 Holliday junction resolvase in somatic cells

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Abstract

Successful chromosome segregation depends on the timely removal of DNA recombination and replication intermediates that interlink sister chromatids. Holliday junction resolvases are required for the resolution of the recombination intermediates to ensure proper chromosome segregation at mitosis. In human cells, the GEN1 protein, a member of the XPG/Rad2 family of structure-selective endonucleases, is specialized for the cleavage of Holliday junction recombination intermediates. By analogy to another member of XPG/Rad2 called EXO1, GEN1 has been hypothesized to be an acceptor for post-translational modification and play regulatory roles in somatic cells. So far, our data show that the C terminus of GEN1 protein has crucial functions in somatic cells. However, the exact function of the C terminus is unknown. Hence, the aim of this study is to identify the role of the C terminus of GEN1 protein by investigating post-translational modifications of GEN1.

Introduction

Holliday Junctions are DNA intermediates that form during homologous pairing and strand exchange. The removal of HJs is crucial for chromosome segregation in cell division. One pathway of processing HJs involves HJ resolution by structure-selective endonucleases such as the MUS81-EME1-SLX1-SLX4-XPF-ERCC1 (SMX)[1-5] and GEN1[6,7], which produces CO and NCO nick-duplex DNA products. MUS81-EME1 and SLX1-SLX4-XPF-ERCC1 form a complex at the prometaphase[4,5], while GEN1 only gains access to DNA when nuclear membrane breaks[8]. These resolvases play a critical role in chromosomal segregation[9,10].

GEN1 is a member of the Rad2/XPG family of nucleases. The N-terminal fragment of human GEN1 (GEN1 1-527), containing the nuclease active site, does Mg^{2+}-dependent incision of 5′ flaps, replication forks, and nicked or intact HJ structures.[11,12] The C terminus is believed to play regulatory roles, but the details are yet to be known.[12] The C terminus of human EXO1, another member of Rad2/XPG family, contains multiple phosphorylation sites that, in response to RF blockage, serve to regulate the stability of EXO1 in vivo.[13,14] By analogy to EXO1, it may be an acceptor for post-translational modification.

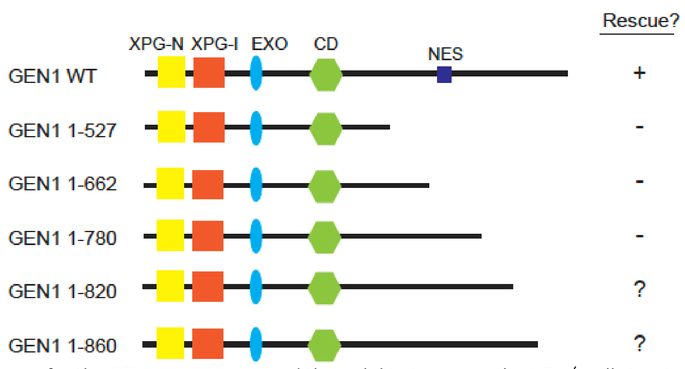
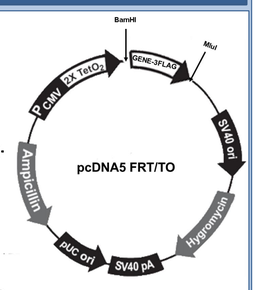


Figure 1: *A schematic diagram for the GEN1 truncations and their ability to rescue the GEN$^{-/-}$ cells treated with siMUS81*

To determine the exact role of the C terminus of GEN1, we will first generate a series of truncations of GEN1 (*Fig 1*) to find the functional region of GEN1 by rescuing GEN1$^{-/-}$ cells. Then posttranslational modifications will be investigated and various mutants of GEN1 will be generated to find exactly where the modification occurs.

Methods and Materials

Plasmids: The GEN1 truncation constructs (GEN1$^{1\text{-}820}$ &GEN1$^{1\text{-}860}$) were generated by PCR and cloned in-frame into a modified pcDNA5 FRT/TO vector (MluI site upstream of CMV is mutated, Mlu1 site followed by a 3x FLAG tag in fame). Sequencing was done to check the success of cloning.



Transfection: Lipofectamine 2000 was used to transfect 293 cells with the clonedplasmids extracted using Neucleobond Xtra midi-prep kit (MACHERY-NAGEL).

Stable Line Generation: 293 GEN1 KO cells were transfected with prepared plasmids and Flp-Recombinase Expression Vector, pOG44 using PEI. Hygromycin was used to select the cells.

Western Blotting: 10% acrylamide gels were made for PAGE. Proteins were detected using the following primary antibodies: mouse anti-FLAG and mouse-anti-α-tubulin.

Results

C-terminus of GEN1 plays crucial role in GEN1 activity. GEN1$^{-/-}$ cells were treated with siMUS81 and siControl and rescued with GEN1 WT and GEN1 N terminus fragment. While the WT could rescue the MUS8 depleted cells, majority of the cells rescued with GEN1 1-527, even when overexpressed, displayed G2 arrest (Fig. 2b). The clonogenic cell survival assays also showed that cells lacking the C terminus of GEN1 had much lower survival rate than cells with it (*Fig 2c*). Western Blotting was used to make sure that the expression levels were suitable (Fig. 2a).

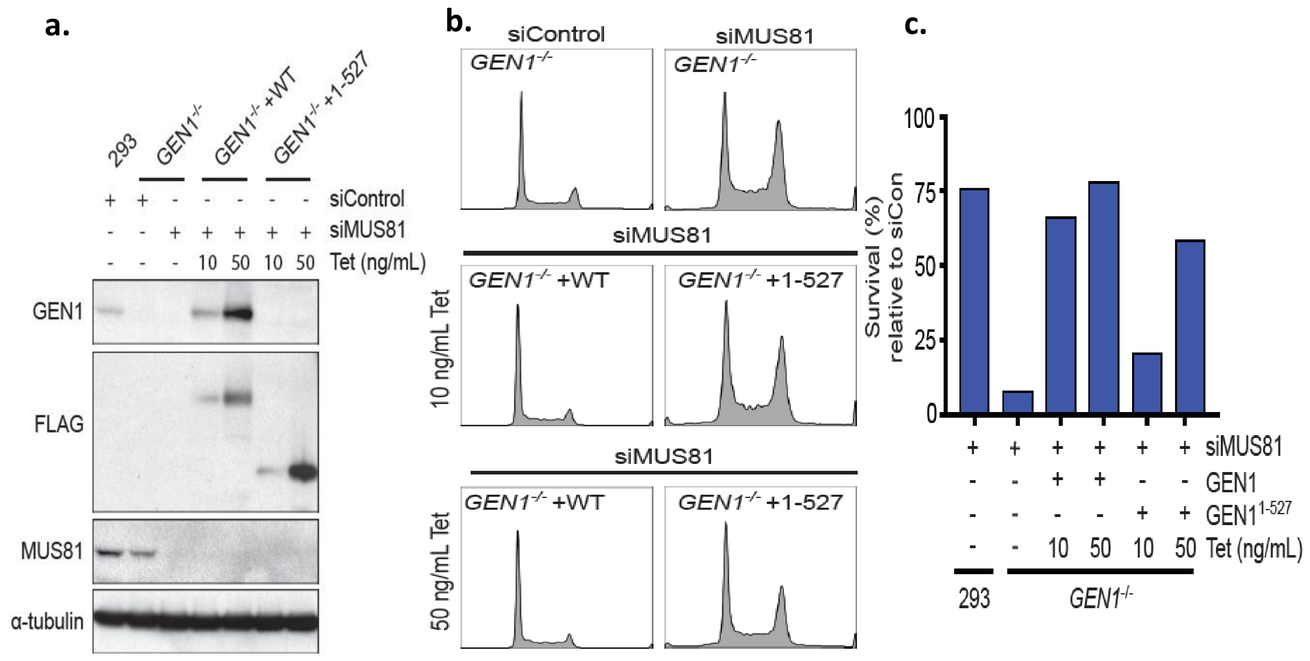


Figure 2[##]. C terminus of GEN1 plays crucial role in GEN1 activity. a) Western blotting to check for the expression and overexpression of GEN1, Mus81 and α-tubulin using (GEN1 and FLAG for GEN1), MUS81 and alpha-tubulin antibody respectively. b)GEN1$^{-/-}$ cells were treated with control siRNA and MUS81 siRNA. FACS results show their DNA content distributions. c) Clonogenic cell survival assays was carried out on 293 cells and GEN1$^{-/-}$ cells treated with MUS81 siRNA. Complementation via stable expression of GEN1 and GEN1 1-527.

A series of GEN1 truncations were generated to confirm the functional region in the C-terminus of GEN1. GEN1 1-662 and GEN1 1-780 truncations were generated and they were used to complement GEN$^{-/-}$ cells treated with siMUS81. Complementation with these two truncations also showed similar levels of G2 arrest as GEN1 1-527, i.e. the N terminus (*Fig 3b*).

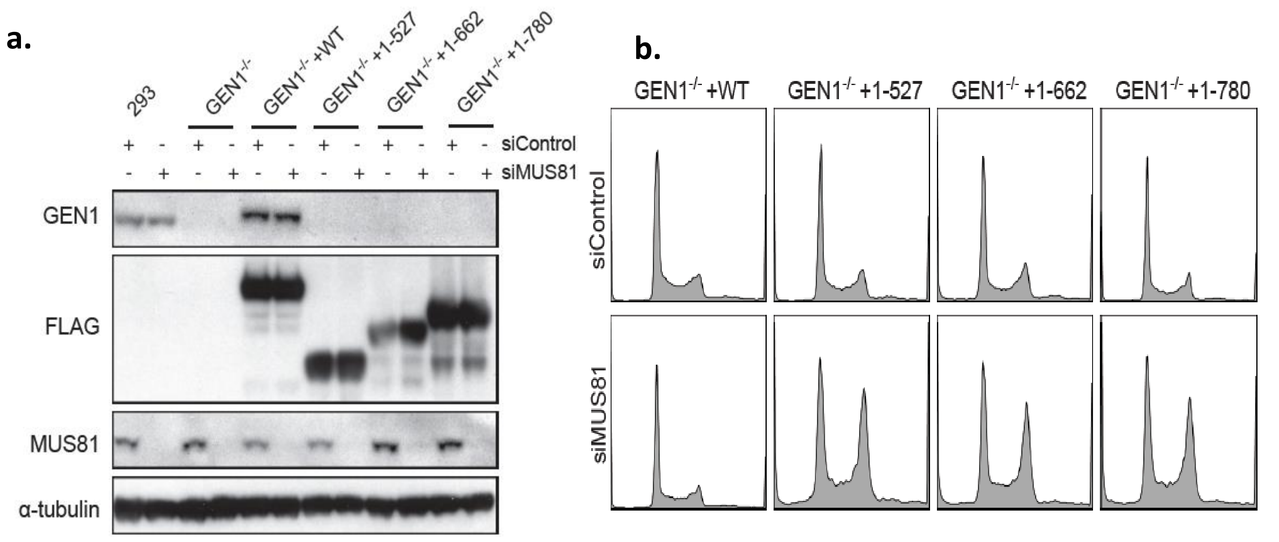


Figure 3[##]. A series of truncations of GEN1 was generated to confirm the functional region in the C-terminus of GEN1. 293 and GEN1 -/- were treated with siMUS81 and siControl a) Western Blot to check the overexpression of GEN1 truncations using the antibodies as indicated. b) FACS results show their DNA content distributions.

Discussion

Our study, so far, indicates that the major functional region of GEN1 resides further in the C terminus than 780th amino acid. Therefore, we generated two more truncations, 1-820 and 1-860 of GEN1. Their expressions were checked in 293 cells (*Fig 4)* and stable lines of GEN$^{-/-}$ + GEN1 1-820 and GEN1$^{-/-}$ + GEN1 1-860 are being generated at the moment. Going on, the post translational modifications of GEN1 in the C terminus will be investigated.

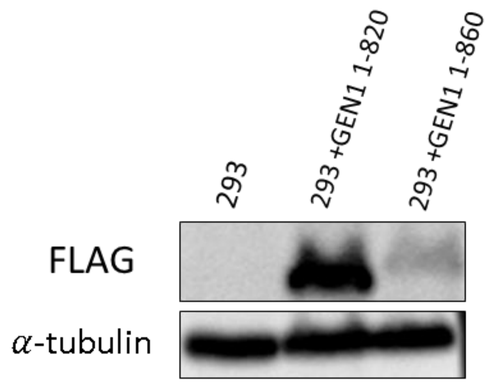


Figure 4. Western Blot to check for the expression of GEN1 1-820 and GEN1 1-860 truncation in 293 cells

Reference and Acknowledgements

Acknowledgements

Data of Figure 2 and Figure 3 are obtained from Dr.Gary Chan with his permission. All rights of the data belong to Dr. Chan.

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