Regulation of Hypermethylation in EBV-Associated Gastric Carcinomas Through EBNA1 and DNMT1

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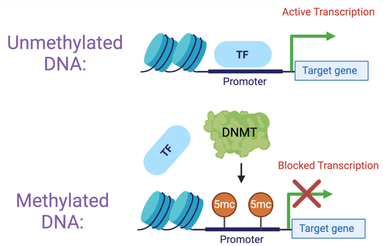
Abstract

Infecting more than 95% of the population, Epstein-Barr Virus (EBV) is linked to numerous cancer types, but the development of gastric cancer is the most common malignancy resulting from EBV infection. All EBV-associated tumors express EBV nuclear antigen-1 (EBNA1), a viral protein necessary to replicate and maintain the viral genome. EBV-associated gastric carcinoma (EBVaGCs) are the most methylated of all human malignancies. EBV-induced hypermethylation is known to silence tumor suppressor and cell cycle genes, therefore promoting cancer development. However, it is unclear how EBV induces hypermethylation in gastric cancers. Our goal is to identify if EBNA1 is necessary for increased methylation in gastric cancers. We have observed that EBNA1 interacts with DNA-methyltransferase-1 (DNMT1), a key enzyme in maintaining DNA methylation. DNMT1 may be partially binding on the DNA-binding domain of EBNA1. Furthermore, the expression of EBNA1 increased global methylation of EBV-negative gastric carcinoma cells. Our findings also indicate that reducing DNMT1 expression through siRNA knockdown significantly decreased methylation levels in EBV-positive cells. Uncovering the role of EBNA1 in EBVaGCs may open new opportunities towards the development of anti-EBNA1 treatments in patients afflicted with EBVaGCs.

Introduction



- Epstein-Barr virus-associated gastric carcinoma (EBVaGCs) are the most methylated of all human malignancies.[1]
- It has been established EBV has a causal role in the pathogenesis of these cancers.[2]
- Epstein-Barr Virus Nuclear Antigen 1 (EBNA1) is a viral protein present in all EBV infections and EBV tumors.[3]

Research Questions

- Does the presence of EBNA1 without EBV infection impact the methylation phenotype in gastric cancer cells?
- Does EBNA1 associate with the DNMTs?

Results

Do DNMTs Facilitate Hypermethylation in EBV+ Cells?

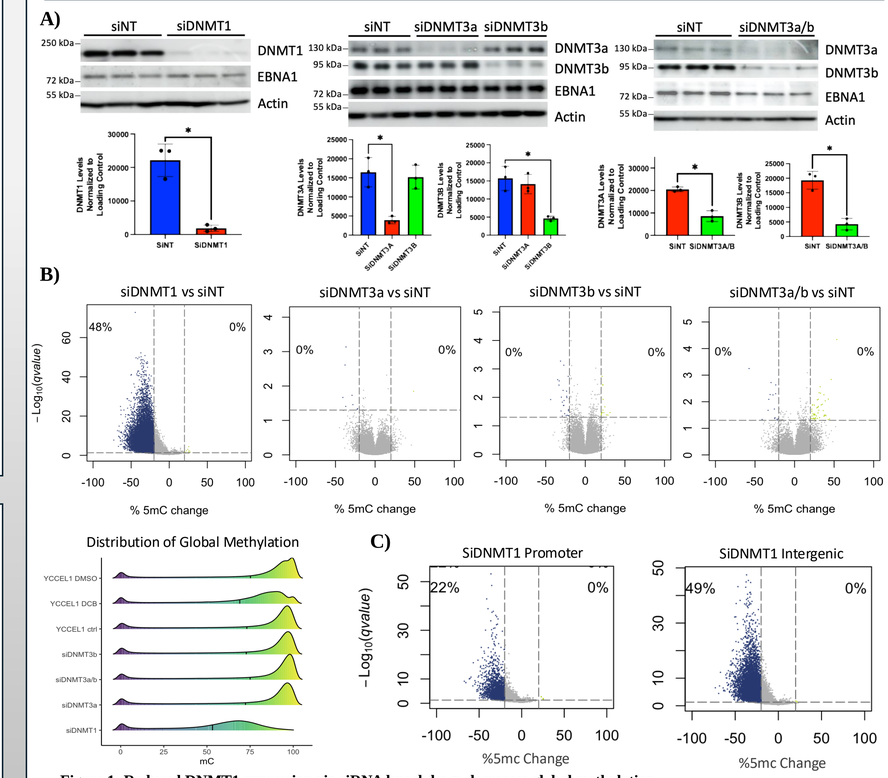


Figure 1: Reduced DNMT1 expression via siRNA knockdown decreases global methylation
A) Western blot analysis and densitometry of DNMT Levels in YCCEL1 (EBV+) following siRNA knockdowns of DNMTs. **B)** DNA methylation analysis using reduced representation bisulfite sequencing (RRBS) of YCCEL1 cells following siRNA knockdowns of DNMTs. C) DNA methylation analysis of promoter and intergenic regions via RRBS in YCCEL1 cells have undergone siRNA knockdown of DNMT1.

Does EBNA1 Expression Increase Global Methylation?

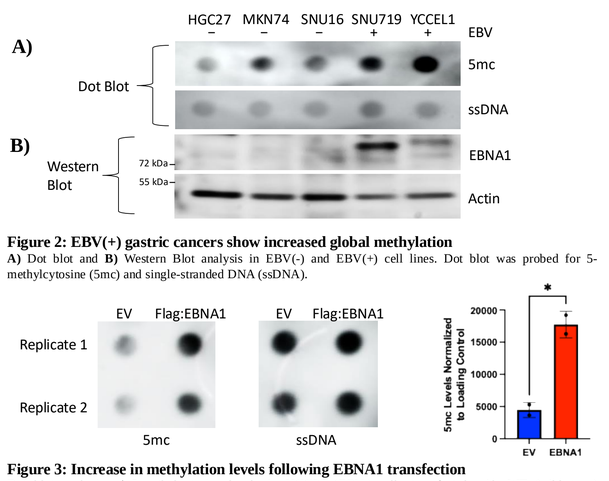


Figure 2: EBV(+) gastric cancers show increased global methylation
A) Dot blot and **B)** Western Blot analysis in EBV(-) and EBV(+) cell lines. Dot blot was probed for 5-methylcytosine (5mc) and single-stranded DNA (ssDNA).

Figure 3: Increase in methylation levels following EBNA1 transfection
Dot blot analysis of 5-methylcytosine levels in HGC27 (EBV-) cells transfected with WT (wild type) Flag:EBNA1. EBNA1 levels were normalized to the loading control, ssDNA.

Is there an Interaction Between EBNA1 and DNMTs?

A)

Protein	Fold Change (EBNA1 vs EV)	P-value
EBNA1	123.37	4.5573×10^{-7}
USP7	86.10	1.3001×10^{-7}
DNMT1	**13.01**	$\mathbf{3.4765 \times 10^{-4}}$

B)

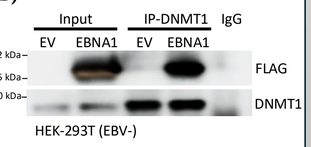


Figure 4: Increase in methylation levels following EBNA1 transfection
A) Immunoprecipitation of EBNA1 followed by liquid chromatography-tandem mass spectrometry shows fold change differences in binding affinity to EBNA1 in EBNA1-associated proteins. **B)** Co-immunoprecipitation (Co-IP) of HEK293T (EBV-) cells transfected with WT Flag:EBNA1.

What Sequence of EBNA1 is the Binding Site for DNMT1?

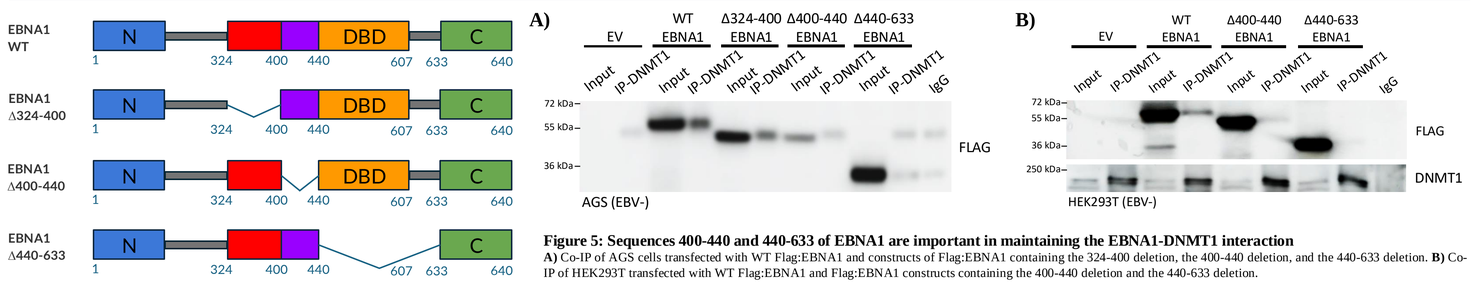
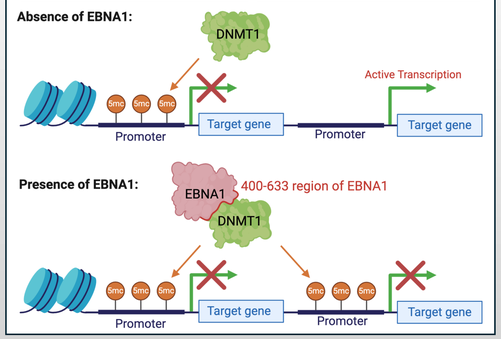


Figure 5: Sequences 400-440 and 440-633 of EBNA1 are important in maintaining the EBNA1-DNMT1 interaction
A) Co-IP of AGS cells transfected with WT Flag:EBNA1 and constructs of Flag:EBNA1 containing the 324-400 deletion, the 400-440 deletion, and the 440-633 deletion. **B)** Co-IP of HEK293T transfected with WT Flag:EBNA1 and Flag:EBNA1 constructs containing the 400-440 deletion and the 440-633 deletion.

Conclusion

- DNMT1 is responsible for roughly half of global methylation in EBV+ cells
- EBNA1 expression may contribute to elevated global methylation
- EBNA1 interacts with DNMT1
  - Binding occurs towards the C-terminal region of EBNA1

Working Model



Future Directions

- Continue mapping the EBNA1 DNMT1 interaction
- Mutational analysis using DNMT1 constructs.
- Analyze which genes are methylated by DNMT1
  - Tumor suppressor genes?
- RNA-seq analysis of viral and host genes methylated by DNMT1

Acknowledgements

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References

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