

Deleting *STX16* exon 4 to understand the genetic mechanisms underlying pseudohypoparathyroidism-1B and *GNAS* imprinting

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INTRODUCTION

Autosomal dominant pseudohypoparathyroidism type-1b is characterized by renal parathyroid hormone resistance, with resultant hypocalcemia and hyperphosphatemia. This disorder is associated with an isolated loss of methylation at *GNAS* exon A/B and most patients carry maternal microdeletions in the neighboring *STX16* gene. (Fig. 1) The shortest deletion overlap is a 1.2-kb region spanning *STX16* exon 4 (Fig. 1) and thought to harbor a cis-acting element regulating *GNAS* A/B methylation. However, ablation of the orthologous mouse region does not recapitulate the patient findings and no functional data exists supporting the association between the *STX16* gene and *GNAS* imprinting. The A/B methylation is established in the female germline and maintained throughout the development in somatic cells. The aim of the study was to investigate the role of the 1.2-kb *STX16* region in the maintenance of A/B methylation.

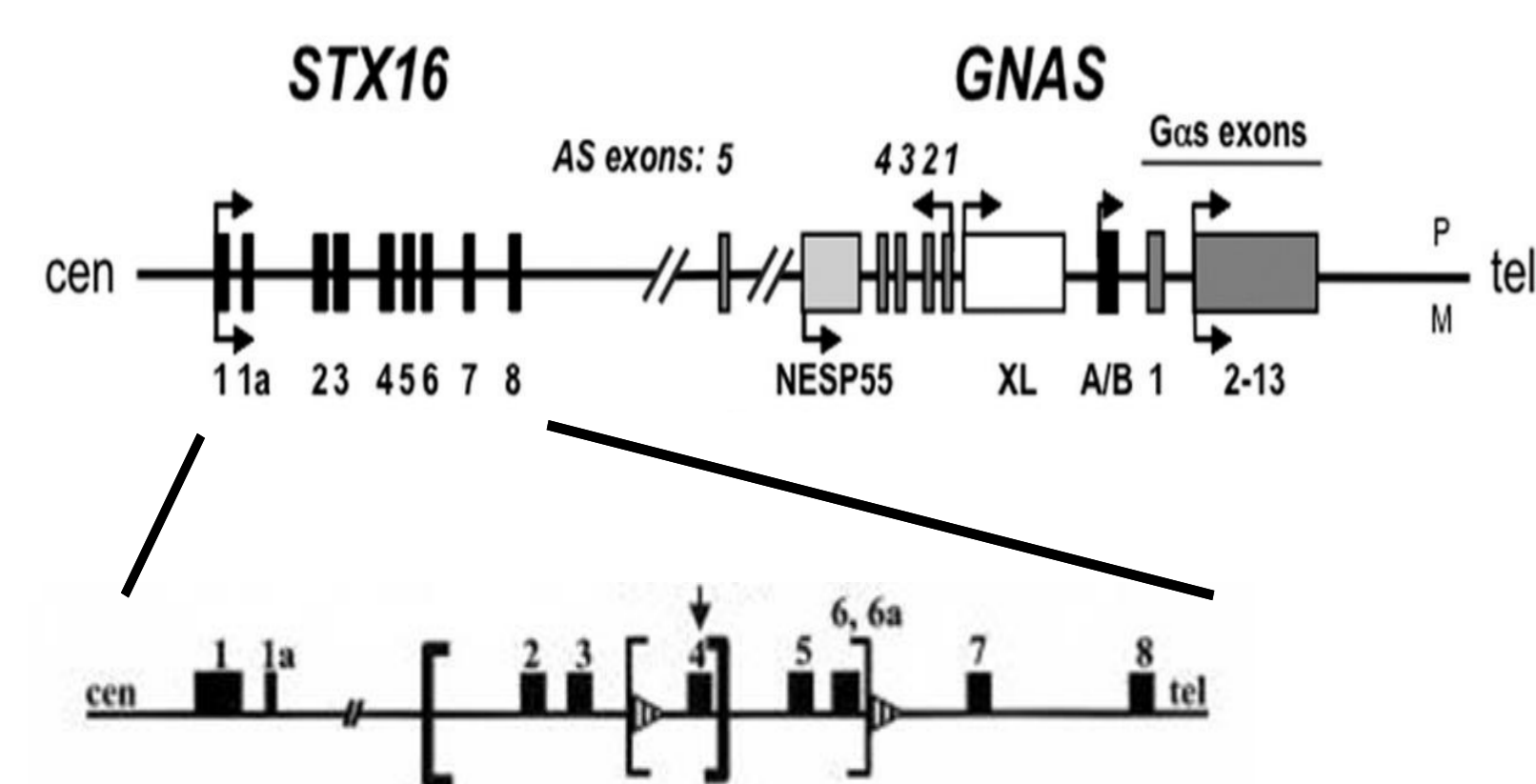


Figure 1. Schematic presentation of the human *STX16* gene and *GNAS* locus. The 4.4-kb deletion (thick brackets) removes exons 2-4; the 3-kb deletion (thin brackets) removes exons 4-6. The 1,286-bp overlapping region is in between the thin and thick brackets.

METHOD

- Cell Line**
HCT116 cells, Human Colorectal Cancer Cell Line (from ATCC) were cultured in McCoy's media at 37°C. DNA was extracted by using DNAeasy Kit.
- CRISPR/Cas9**
Cas9 and guide-RNAs were introduced to HCT116 cells by lentiviral vectors and transduced cells were enriched by blasticidin (cas9) and puromycin (sgRNA). www.portals.broadinstitute.org sgRNA tool was used for guide-RNA design. The CRISPR/Cas9 editing results were analyzed on the TIDE tool (Tracking of Indels by Decomposition).
- TOPO® TA Cloning® Kit & Sanger Sequencing**
TA cloning kit was used to sequencing of the PCR products containing the deletion. Sanger Sequencing was done at MGH DNA Core.
- Bisulfite Treatment**
EZ DNA Methylation-Gold™ Kit is used for bisulfite conversion of GC rich cell DNA.
- Methylation Status Analysis**
MS-MLPA, Multiplex ligation-dependent probe amplification is obtained from MRC Holland.
HinfI enzyme, NEB was used for Combined Bisulfite Restriction Analysis.

RESULTS

HCT116 cells, a near-diploid human cell line derived from colorectal carcinoma, were employed as a somatic cell line. To delete 1.2-kb region, we used CRISPR/Cas9 and suitable guide-RNAs targeting upstream and downstream of exon 4. We first generated HCT116 cells stably expressing Cas9 and introduced the guide-RNAs into those cells by lentiviral delivery and selected the highest efficiency guide-RNAs, upstream 1 and downstream 4 by using TIDE analysis (Table 1). After antibiotic enrichment of the transduced cells, the presence of a 2.1-kb deletion spanning *STX16* exon 4 and flanking intronic regions was confirmed by PCR (Fig.2), followed by TA-cloning and Sanger sequencing of the products (Fig. 3).

Table 1. TIDE Analysis of sequences from the targeted regions. R²: Goodness of fit a measure for the reliability of the estimated values.

SgRNA & Sequencing Primer	UP1 F	UP1 R	UP2 F	UP2 R	UP3 F	UP3 R	UP4 F	UP4 R	DOWN1 F	DOWN1 R	DOWN2 F	DOWN2 R	DOWN3 F	DOWN3 R	DOWN4 F	DOWN4 R
Efficiency %	78.1	1.2	77.2	0.8	38.3	38.2	44.4	2.3	17.9	25	26.4	34.6	36.1			
R ²	0.82	0.01	0.82	0.01	0.96	0.97	0.75	0.02	0.98	0.98	0.93	0.98	0.97			

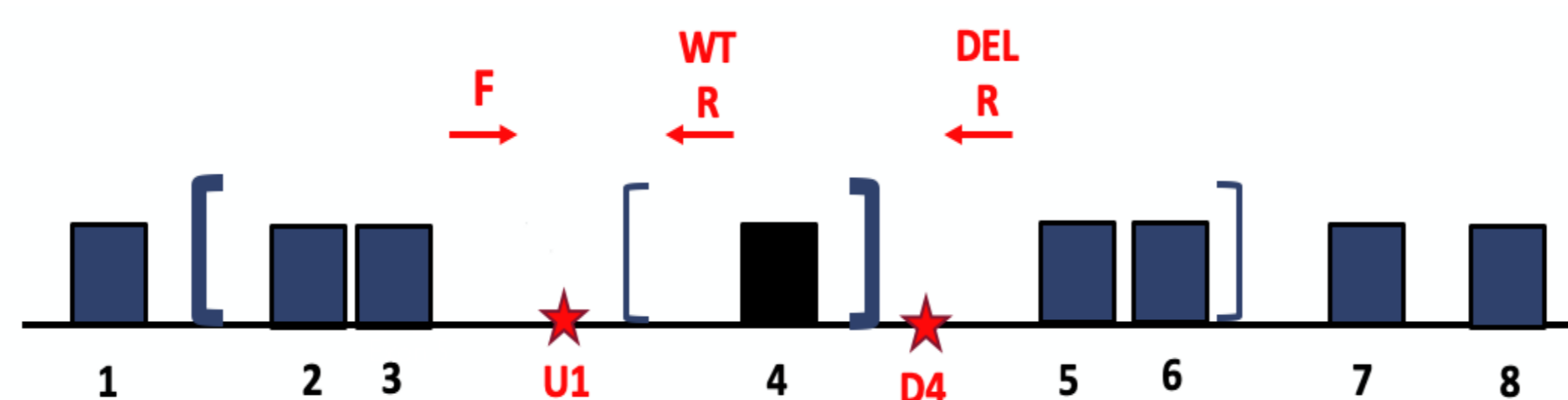


Figure 2A. Forward and Reverse WT & Deletion Primers

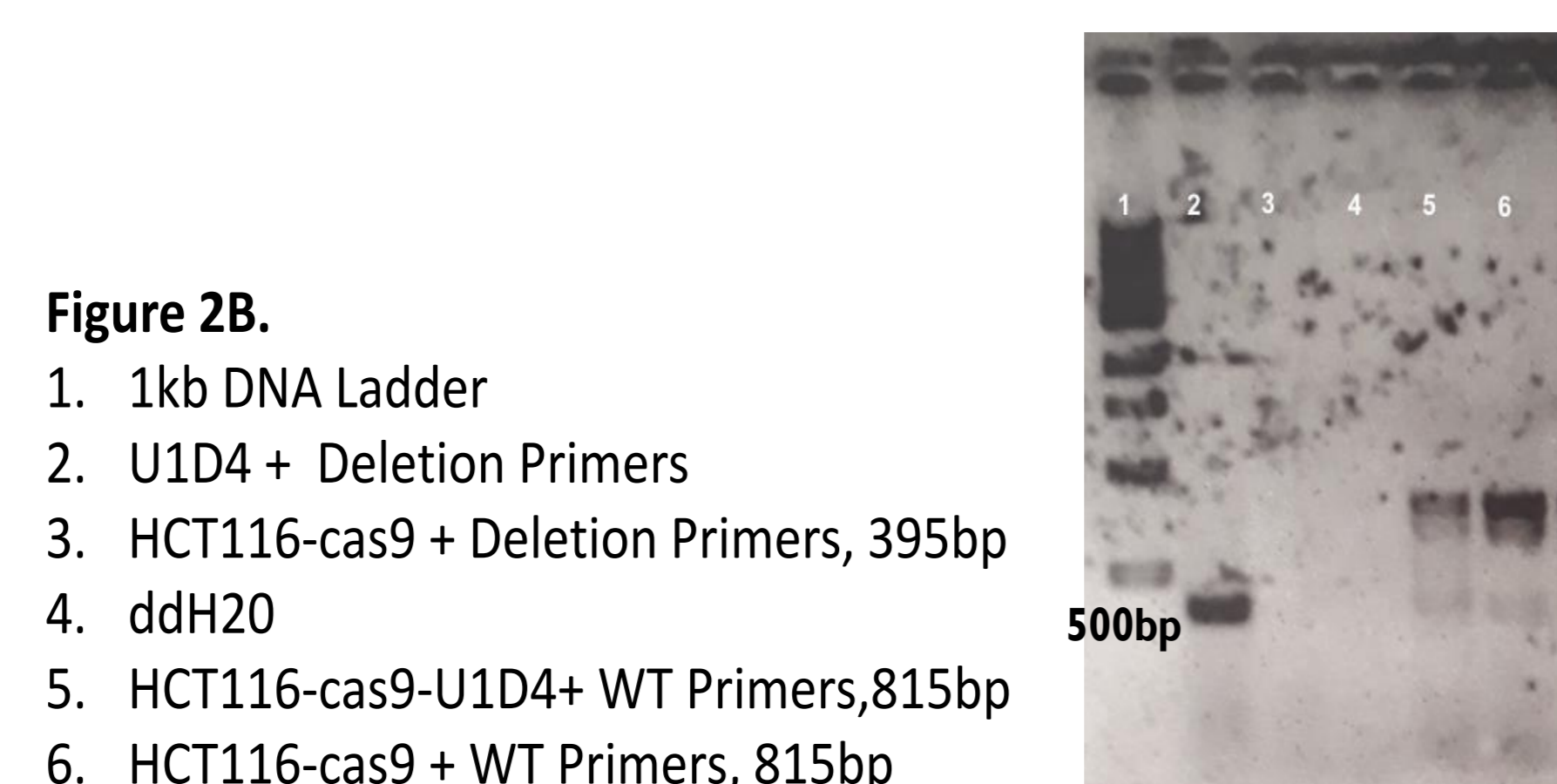


Figure 2B.

- 1kb DNA Ladder
- U1D4 + Deletion Primers
- HCT116-cas9 + Deletion Primers, 395bp
- ddH2O
- HCT116-cas9-U1D4+ WT Primers, 815bp
- HCT116-cas9 + WT Primers, 815bp

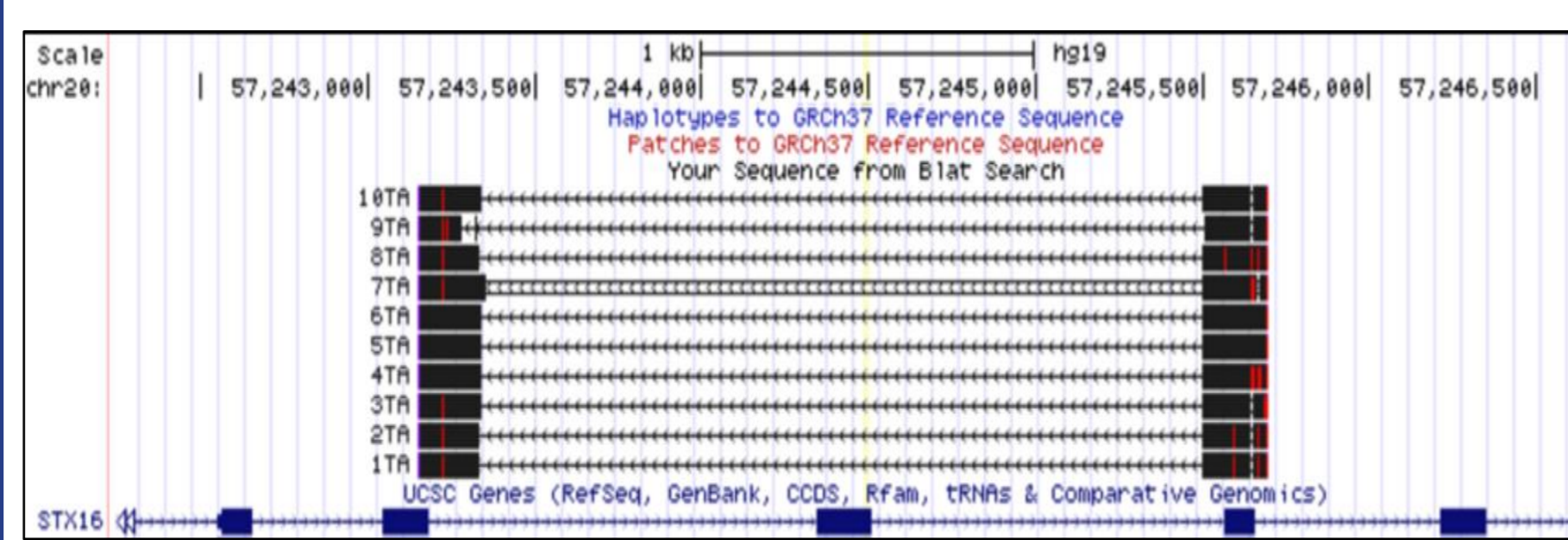


Figure 3. UCSC Database, *STX16* Exon 4, TOPO® TA Cloning® of PCR product from cells transduced with the U1 and D4 sgRNAs (U1D4 cells; from Fig. 2B). The sequencing indicates a deletion of ~2.1kb comprising exon 4.

MS-MLPA & COBRA

Multiplex ligation-dependent probe amplification (MLPA) was performed in non-clonal cells, comparing results from the control cells expressing CAS9 alone and the U1D4 cells. The results indicated a modest reduction in the *STX16* exon 4 copy number (compare Fig.4B and 4C), suggesting that the deletion was present only in a subset of cells. Our transcript based is on the NM_003763.6 reference transcript (Bastepe et al, 2003), while the MS-MPLA Protocol uses the NM_001001433.2 reference transcript. Thus, exon 5 corresponds to exon 4, which is included in the shortest region of deletion overlap. Methylation specific-MLPA analysis indicated no differences between U1D4 and control cells regarding the methylation status at *GNAS* exon A/B (Fig.4E, 4F). The absence of alterations at the A/B DMR was confirmed by performing combined bisulfite restriction analysis (Fig.5).

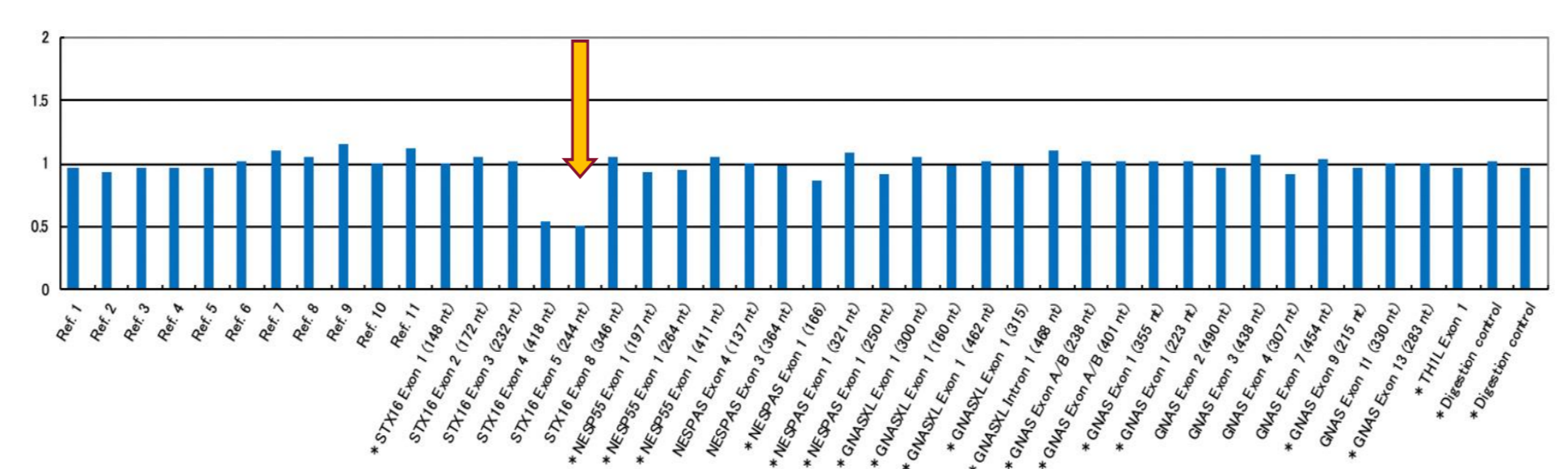


Figure 4A. MLPA, Copy Number, human 3 kb *STX16* deletion

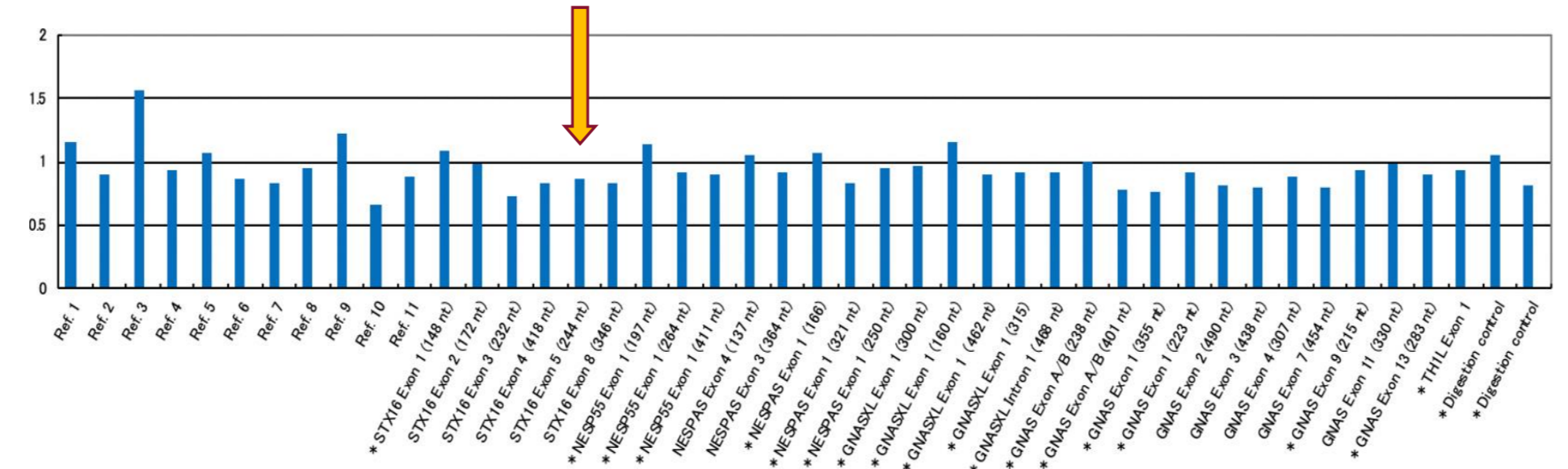


Figure 4B. MLPA, Copy Number, HCT116 cells stably expressing cas9

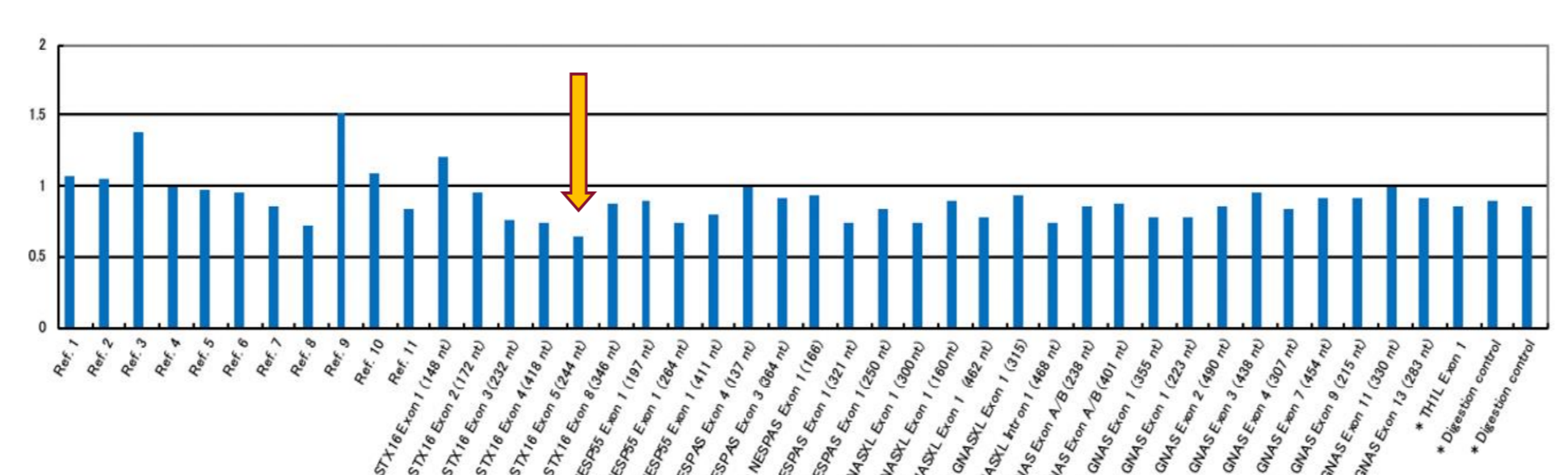


Figure 4C. MLPA, Copy Number, HCT116-cas9-U1D4 cells

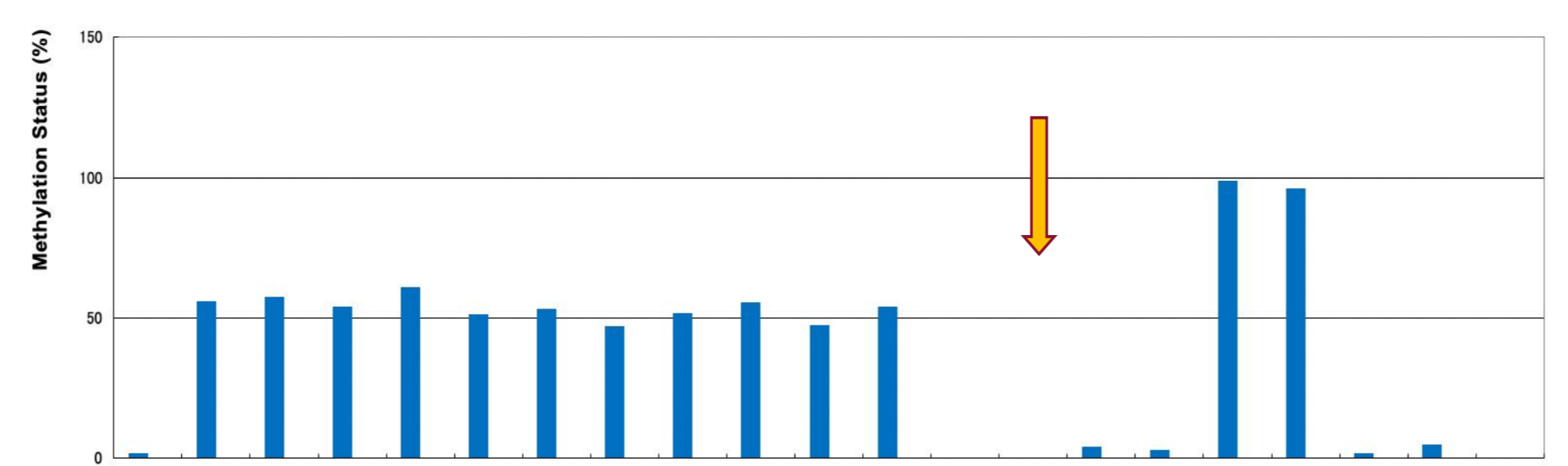


Figure 4D. MS-MLPA, Methylation Status, Human 3kb *STX16* Deletion

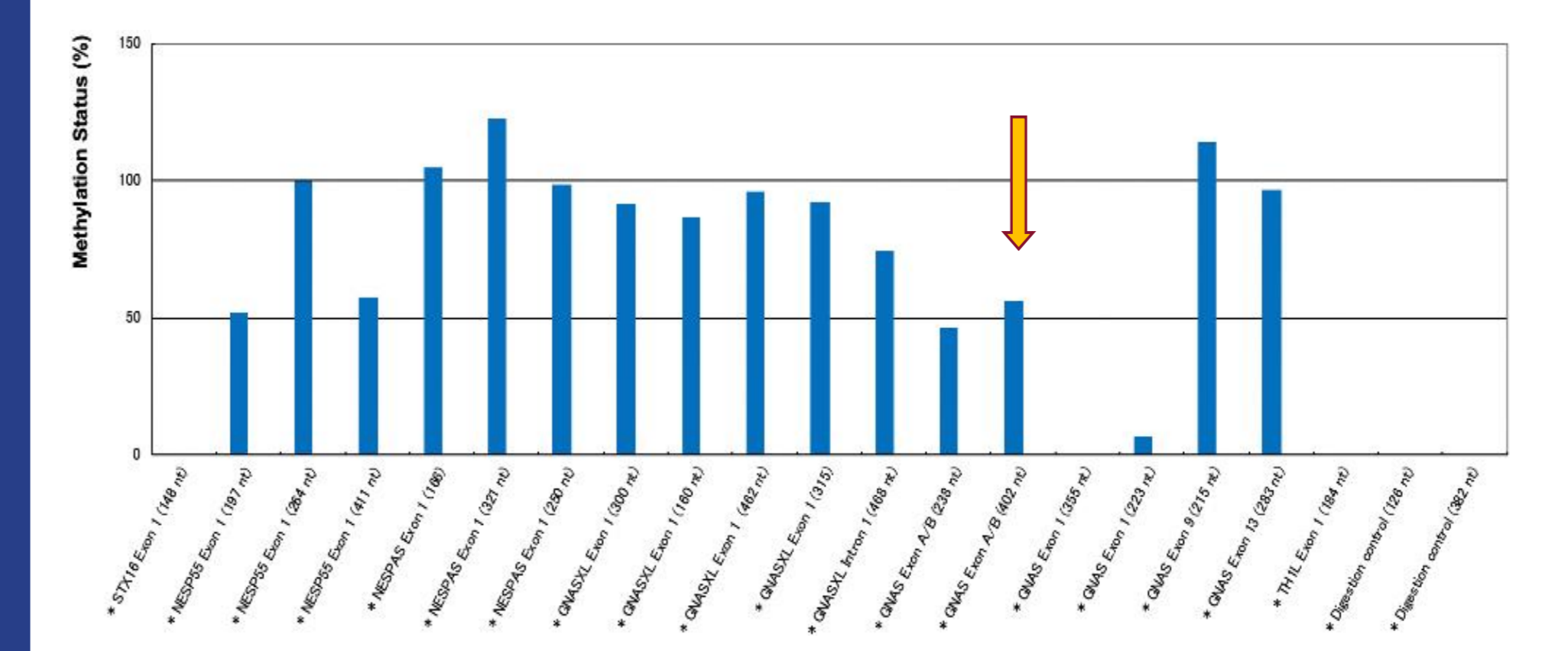


Figure 4E. MS-MLPA, Methylation Status, HCT116-cas9, cell line control

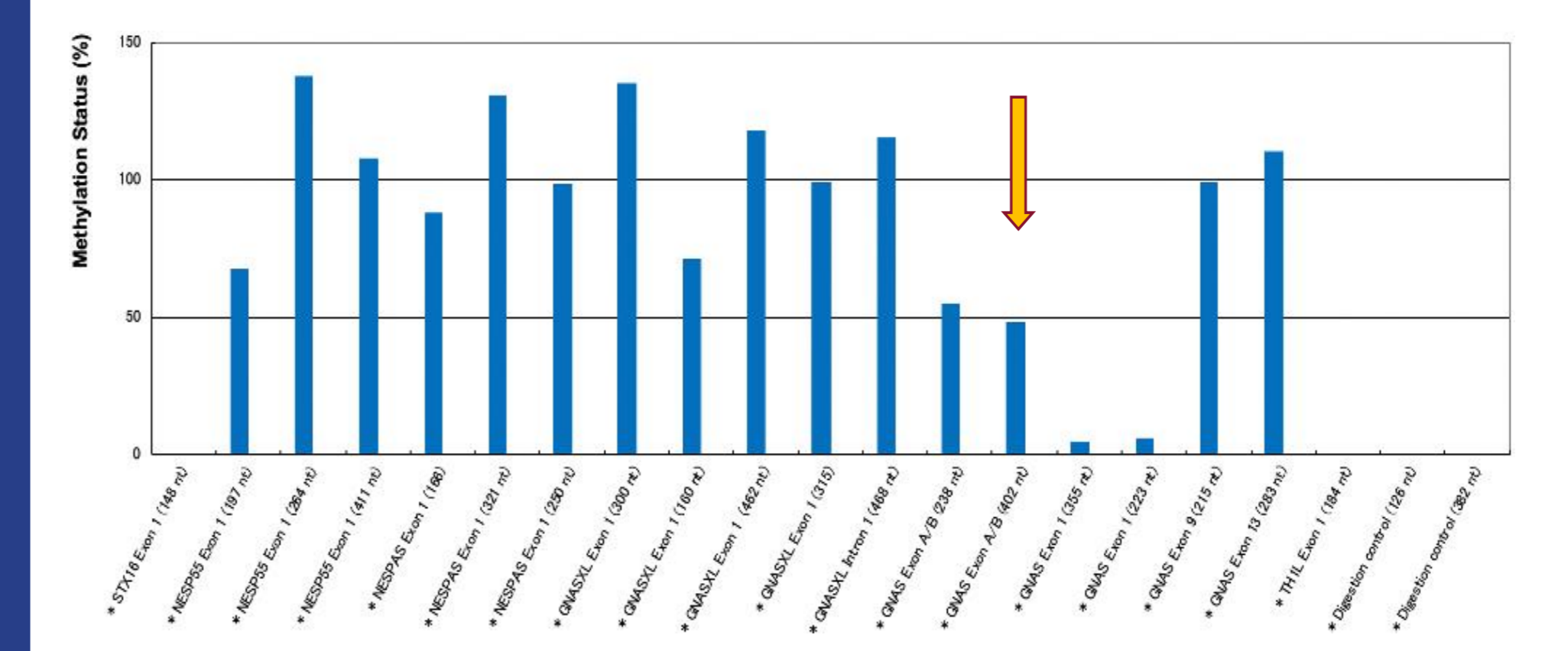


Figure 4F. MS-MLPA, Methylation Status, HCT116-cas9-U1D4 cells

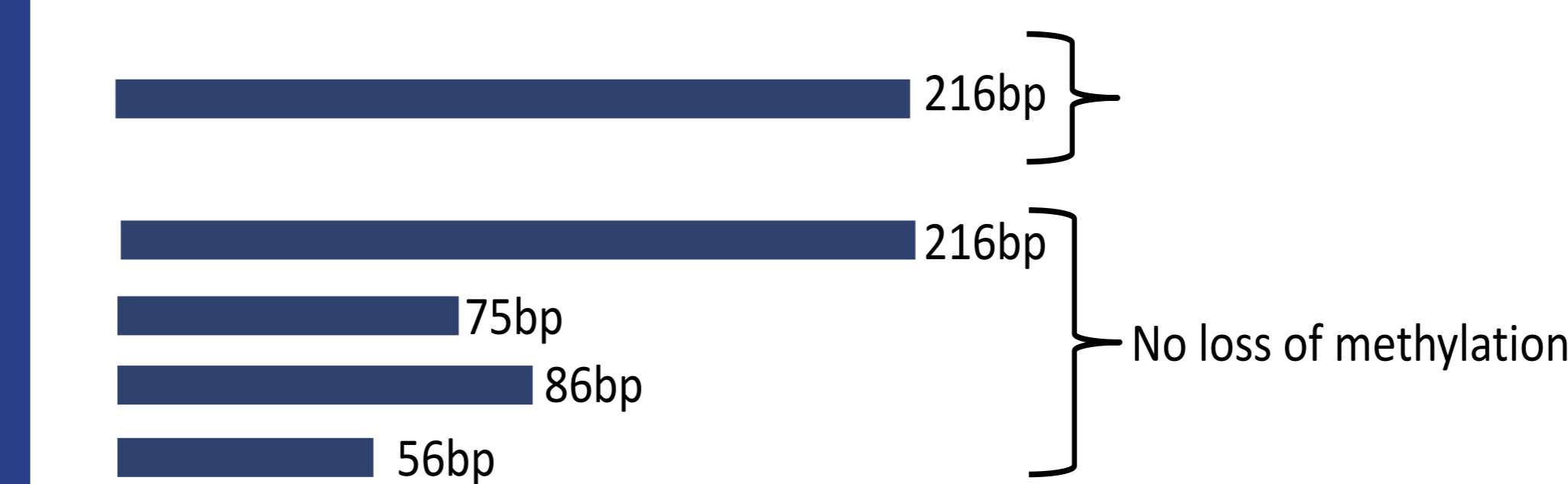


Figure 5A. HinfI digestion, expected band sizes.

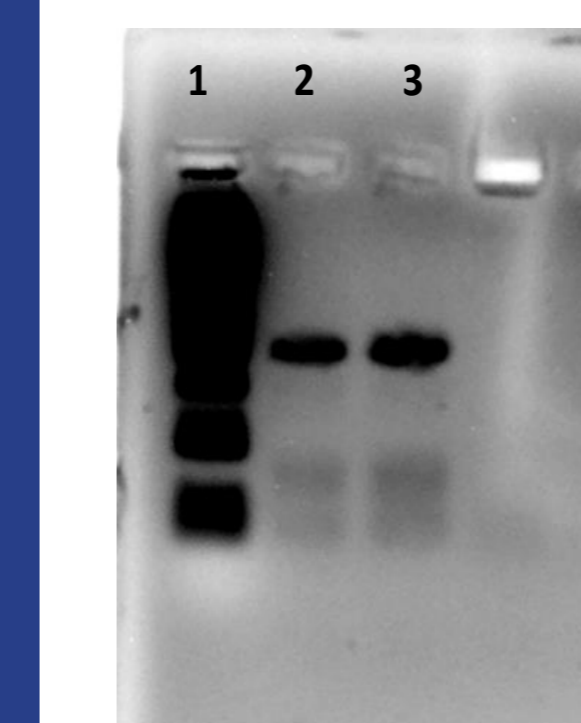


Figure 5B. Combined Bisulfite Restriction Analysis, HinfI.

- 50bp DNA Ladder
- HCT116-cas9-U1D4 cells
- HCT116-cas9 cells

SINGLE CELL CLONING

HCT116-cas9-U1D4 cells were sorted by FACS to obtain single cell colonies. The clonal cells were screened by PCR for the presence of the 2.1-kb deletion, revealing two clones in which a robust PCR amplicon was amplified (Fig.6). Sanger sequencing of the products was performed to examine the deletion breakpoints (Fig.7A,7B). To determine HCT116 cells have the homozygous or heterozygous deletion, we designed WT primers targeting the non deleted allele. (Fig. 7C)

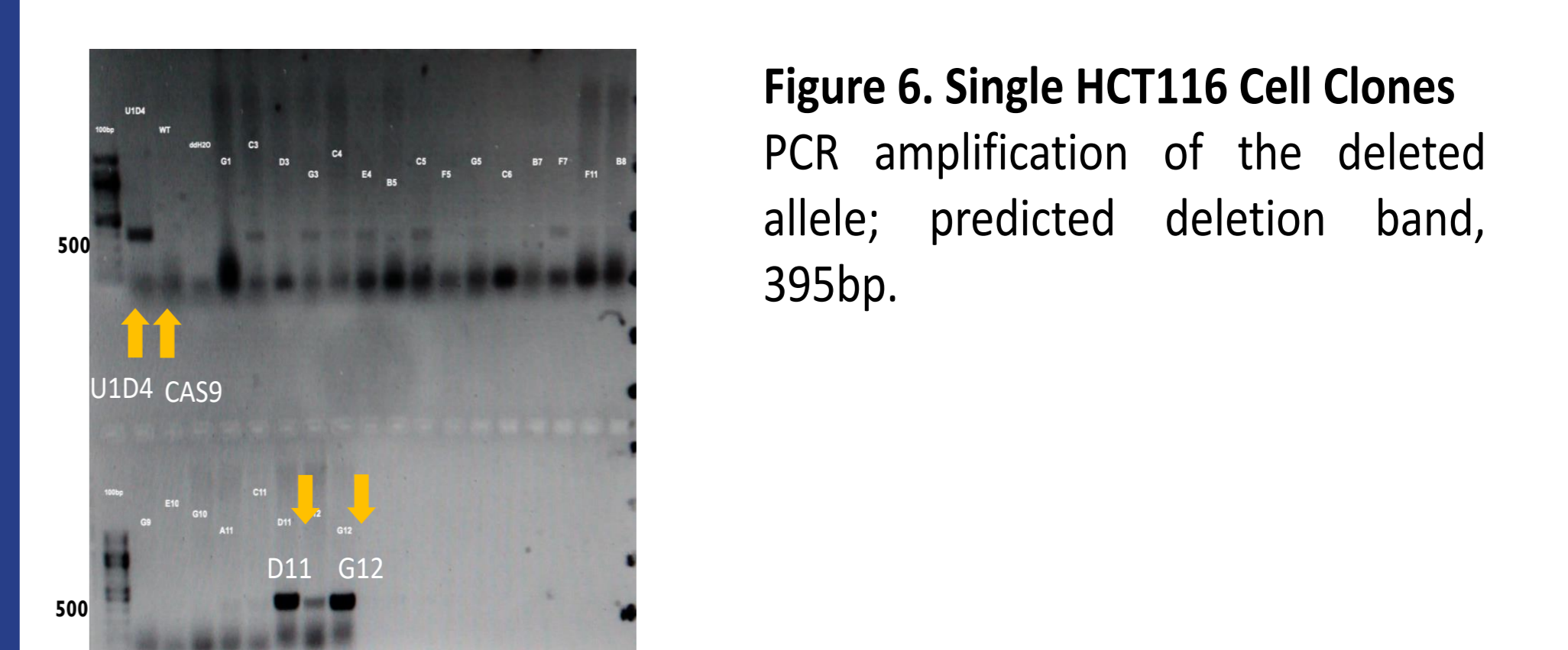


Figure 6. Single HCT116 Cell Clones PCR amplification of the deleted allele; predicted deletion band, 395bp.

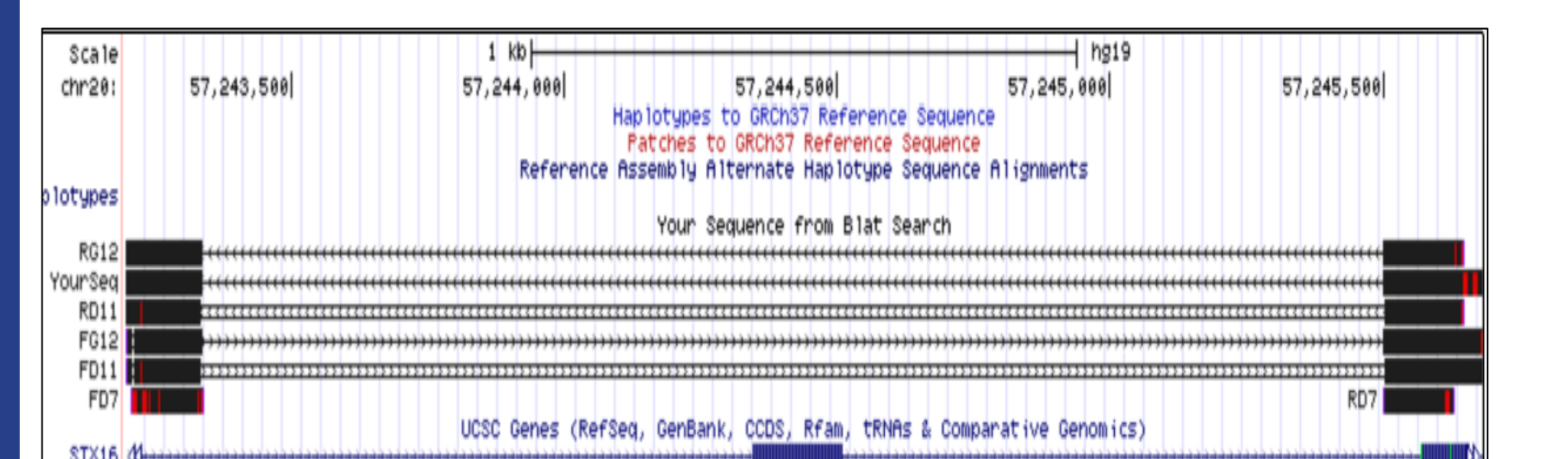


Figure 7A. UCSC Database, Sanger Sequencing of single cell clones D11 & G12

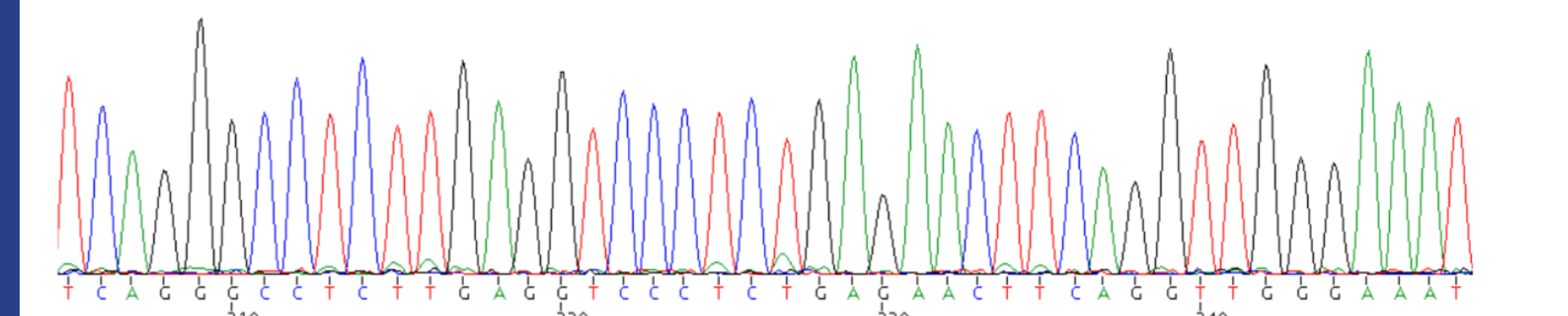


Figure 7B. D11 Sanger Sequencing Trace data lacks any evidence for two differently deleted alleles, suggesting that the deletion may be heterozygous.

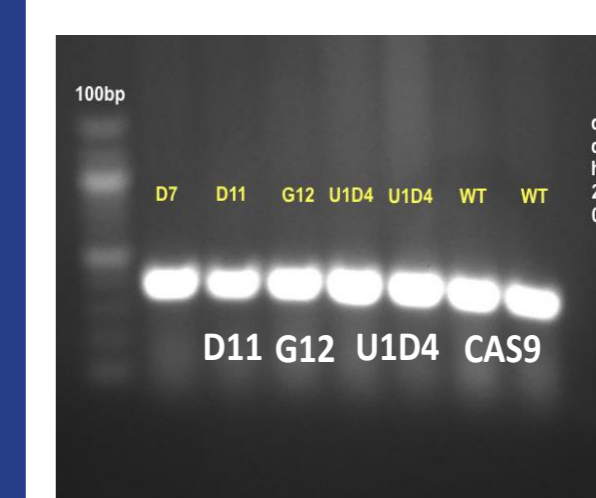


Figure 7C. PCR amplification of the wildtype allele by using primers specific for the undeleted region. The data suggests that single cell clones D11 and G12 have the *STX16* exon 4 deletion in the heterozygous state.

CONCLUSION:

Our initial findings suggest that the putative cis-acting element within *STX16* may not regulate the maintenance of exon A/B methylation in somatic cells; however, this conclusion needs to be confirmed by isolating single cell clones in which both *STX16* alleles are deleted (i.e. homozygous deletion). We are introducing the same deletion into human ES cells, so that we can determine its impact on *GNAS* imprinting during early embryonic development.

REFERENCES & ACKNOWLEDGEMENT

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