Supporting Information Appendix

Reversible epigenetic down-regulation of MHC molecules by Devil Facial Tumour Disease illustrates immune escape by a contagious cancer

Hannah V. Siddle[1], Alexandre Kreiss[2], Cesar Tovar[2], Chun Kit Yuen[1], Yuanyuan Cheng[3], Katherine Belov[3], Kate Swift[4], Anne-Maree Pearse[4], Rodrigo Hamede[5], Menna E. Jones[5], Karsten Skjødt[6], Gregory M. Woods[2] and Jim Kaufman[1,7]

1. *Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK*
2. *Menzies Research Institute Tasmania, School of Medicine, University of Tasmania, 17 Liverpool Street, Hobart, Tasmania 7000, AUSTRALIA*
3. *Faculty of Veterinary Science, University of Sydney, Sydney, NSW 2006, AUSTRALIA*
4. *Animal Health Laboratory, Department of Primary Industries and Water, 165 Westbury Road, Prospect, Launceston, Tasmania 7250, AUSTRALIA*
5. *School of Zoology, University of Tasmania, Private Bag 5, Hobart, Tasmania 7001, AUSTRALIA*
6. *Department of Cancer and Inflammation, University of Southern Denmark, Winsloewparken 21, 5000, Odense C, DENMARK*
7. *Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK*

Table of Contents

Supplementary Material

Supplementary Materials and Methods

Cell Culture

DFTD (1426, 4906 and C5065) cell lines were cultured at 35^{o}C and 5% CO_2 in "complete medium" composed of RPMI 1640 (Invitrogen) with 10% heat inactivated foetal bovine serum (FBS, Invitrogen), L-glutamine (2 mM, PAA) and kanamycin (100 μg/ml, PAA). A devil fibroblast cell line was cultured in DMEM (high glucose, PAA) with 10% FBS (Invitrogen), penicillin/streptomycin (0.1 units/ml penicillin and 0.1 mg/ml streptomycin, PAA) and L-glutamine (2 mM, PAA). Cells were split 1:3 every 72 hours. CHO cells were cultured in Ham's F12 media with 5% penicillin/streptomycin (0.1 units/ml penicillin and 0.1 mg/ml streptomycin, PAA), 10% FBS (Invitrogen) and L-glutamine (2 mM, PAA) at 37 ºC with 5% CO_2.

Reverse-transcriptase quantitative PCR (RT-qPCR)

RT-qPCR was carried out using the Absolute Blue Sybr Green Fluorescein qPCR mix (Thermo Scientific) with primers at 250 nM each and the following cycling conditions; 95^{o}C for 15 min, followed by 40 cycles of 95^{o}C for 15 s, 59^{o}C for 30 s and 72^{o}C for 30 s. A number of housekeeping genes were trialled, including 28S ribosomal RNA (28S), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), TATA-binding protein (TBP), ribosomal protein L13a (RPL13A) and hypoxanthine phosphoribosyltransferase 1 (HRPT1), but RPL13A gave the most consistency with equal amounts of cDNA across the samples. The tumour cDNA samples were tested in triplicate with no-DNA controls for all master mixes. Fibroblast cDNA at four dilutions (1, 0.25, 0.04 and 0.008) was used to create a standard curve for the amplification of RPL13A, MHC class I and β_2-microglobulin (β_2m) in each RT-qPCR experiment.

PCR conditions for rapid amplification of cDNA ends (RACE) PCRs

A 5' RACE PCR was carried out to amplify the 5' end of MHC class I with a 5' RACE primer (GeneRacer, Invitrogen) and MHC class I reverse primer 5'GTCGTAGGCGAACTGAAG 3'. 3' RACE PCR was carried out using a 3' RACE primer and MHC class I forward primer 5' CAGATTTCCCGAGTGGAC 3'. Similarly, 5' and 3' RACE PCRs were carried out to amplify β_2m transcripts using forward (5'TGTGCATCCTTCCCTACCTGGAGG 3') and reverse (5' CATTGTTGAAAGACAGATCGGACCGC 3') β_2m specific primers in exon 2 of β_2m and either a 5' or 3' RACE primer. The optimal RACE PCR conditions were as follows: 1 x buffer, 1.6 mM $MgCl_2$, 200 μM dNTP, 0.5 μM RACE primer, 2 μM reverse/forward primer and 0.3 μl of Taq polymerase (Expand High Fidelity Taq, Roche). PCR cycles were performed with initial denaturation at 94^{o}C for 3 min, followed by 30 cycles of 94^{o}C for 30 s, 65^{o}C for 30 s, and 72^{o}C for 2 min, and a final extension at 72^{o}C for 10 min.

β_2m expression and purification

Bacterial colonies containing the pET22b$^+$- β_2m construct were cultured to OD_{600} = 0.6 and recombinant β_2m expression was induced with 1 mM IPTG. Inclusion bodies were dissolved in 8 M urea, and β_2m was purified using a Hi-Trap nickel affinity column (BD Biosciences) according to the manufactures instructions. The expression of HIS-tagged β_2m was confirmed using western blot as described below, using an anti-HIS antibody (Roche cat no. 04905318001) according to the manufacturer's instructions. The denatured β_2m was refolded overnight at 4°C in a refold buffer (100 mM Tris-HCl pH 8.2, 400 mM arginine, 2 mM EDTA, 0.5 mM oxidized glutathione and 5 mM reduced glutathione) and purified by size exclusion chromatography using the AKTA FPLC system with an S75 10/30 Superdex column (Amersham).

Specificity of MHC class I and β_2m antibodies

The specificity of the TD50 antibody was tested in three independent experiments. First, a full length devil MHC class I transcript (Saha*01, a previously published full length class I transcript (NCBI accession: EF591089)) was amplified using the following primers (F – 5' GCGGAGACCCGGGCGGGCTCTTACCCATACGATGTTCCA 3' and R - 5' TACTTCTAGATTATTTGGCTGTCAGAGAGACATCTGACCCC 3') and cloned into the pcDNA 3.1 expression vector (Invitrogen) using standard molecular biology procedures. The resulting construct was sequenced in both directions with primers T7 and bovine growth hormone (BGH) to ensure no errors were introduced during amplification or cloning. DFTD cells were then transiently transfected using Fugene transfection reagent according to the manufacturers instructions (Promega) with either the MHC class I- pcDNA 3.1 construct or no DNA (a mock transfection as a control). DFTD cells were incubated for 30 h at 35^{o}C before cells were harvested and a cell lysate was prepared as described in the main text. A western blot was performed as described in the main text using the antibody TD50. Second, a western blot was performed using fibroblast cell line lysate (prepared as described in the main text) and probed with either the TD50 antibody or with the TD50 antibody pre-blocked by adding 2 mg of the immunisation peptide (GGKGGDYVPAAGN) and incubating on ice for 30 min before use as a probe. Third, fibroblast cell line lysate (prepared as described in the main text) was deglycosylated using peptide-N glycosidase F (PNGase F; NEB) according to the manufacturer's instructions. Untreated and deglycosylated fibroblast lysates were then probed on a western blot with TD50 as described in the main text.

Supplementary Figures

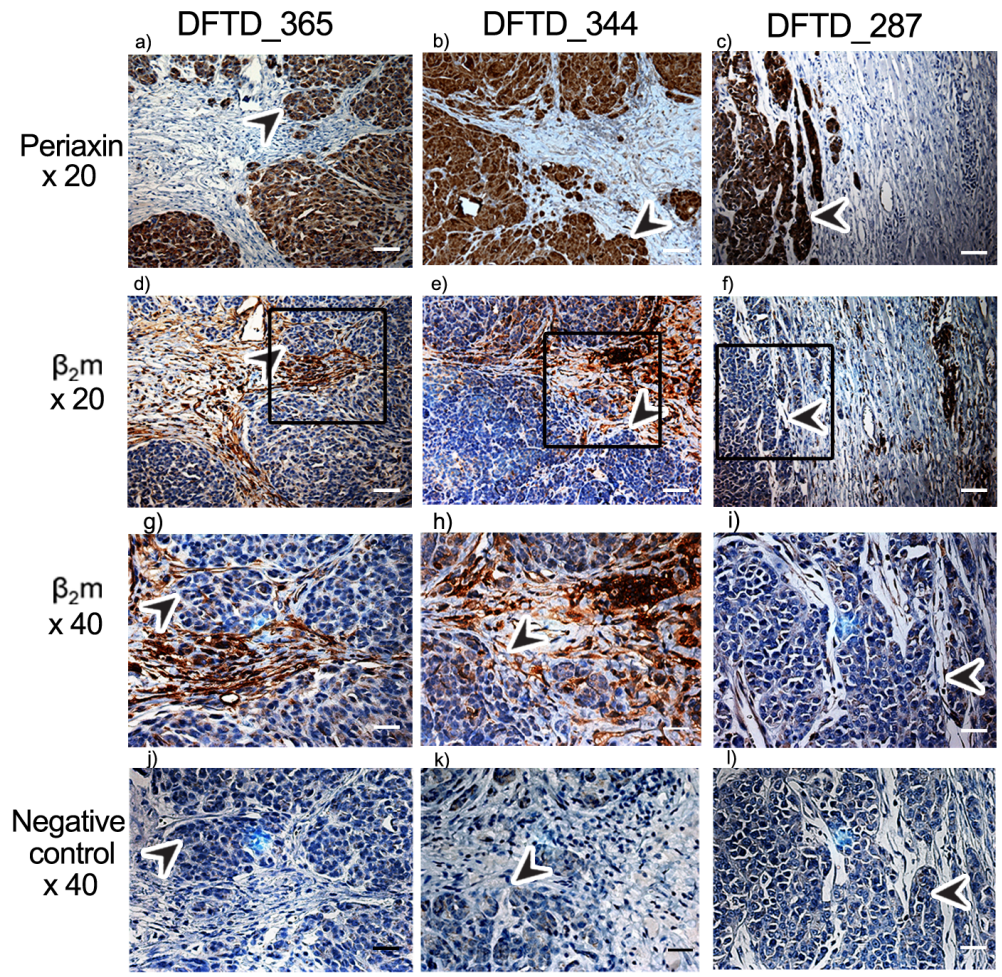

Figure S1. Additional DFTD biopsies stained for periaxin and β_2m. IHC on serial sections of primary DFTD biopsies from wild devils (DFTD_365, DFTD_344 and DFTD_287) stained for; **a - c)** periaxin, a marker specific for DFTD cells, at 20 x magnification, **d - f)** β_2m at 20 x magnification, **g - i)** β_2m at 40 x magnification, **j - l)** negative controls stained with pre-immune rat serum at 40 x magnification. Boxes indicate areas shown at 40 x magnification and arrow heads indicate similar positions in the serial sections, pointing towards DFTD cells. Positive cells for each marker are stained brown, nuclei are stained blue. Scale bars are 50 μm for 20 x magnification and 20 μm for 40 x magnification.

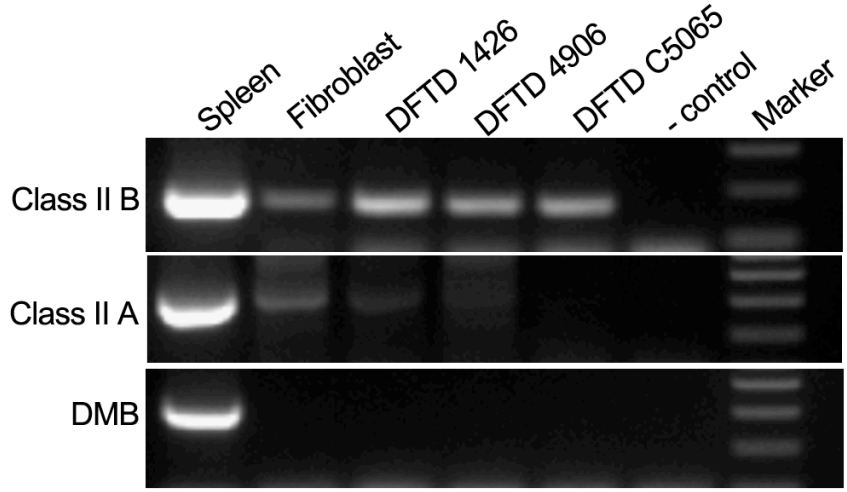

Figure S2. RT-PCR amplification of MHC class II genes. RT-PCR amplification of MHC class IIB, MHC class IIA and DMB from RNA from spleen, fibroblast cell line and DFTD cell lines. A no-cDNA negative control was included in each experiment. Markers show that amplicons are between 100 and 300 bp.

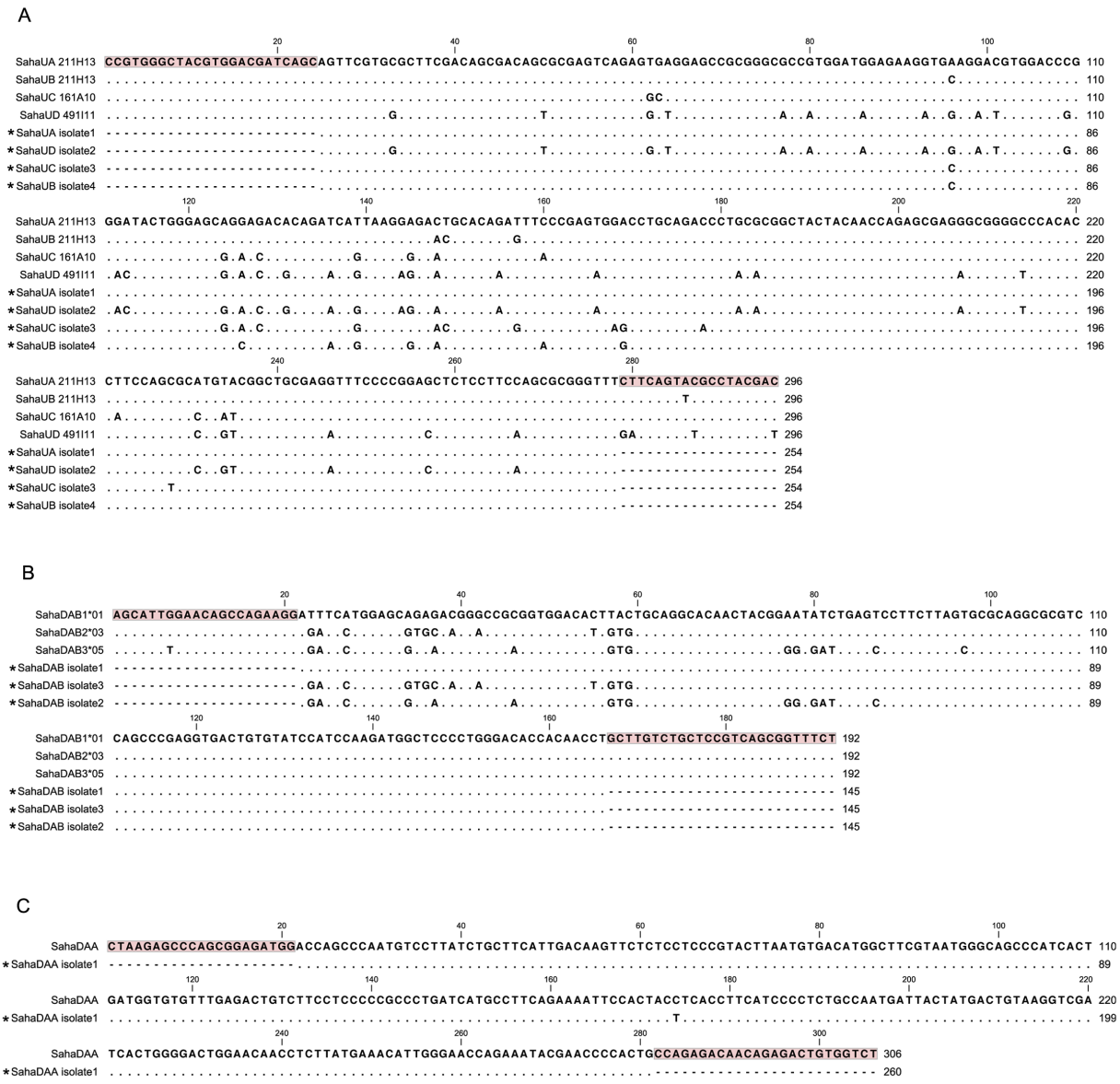

Figure S3. Alignments of MHC class I, class II B and class II A sequences amplified during RT-PCR. A) Nucleotide alignment comparing sequences from four previously described devil MHC class I genes (including the polymorphic loci, *SahaUA*, *UB* and *UC*, and the nonpolymorphic *UD*) with class I sequences isolated from DFTD cells during RT-PCR. The top four sequences are derived from BACs described by Cheng et al. (2012) (Accession numbers – 211H13 FQ482146.2, 161A10 FQ482138.3, 491I11 FQ482142.7). The bottom four sequences were isolated from DFTD cells as described for Figure 2 of this paper, and are marked with a star. Primer sites are shown in red. **B)** Nucleotide alignment comparing sequences from three previously described devil MHC class II B genes from BACs described in Cheng et al. (2012) with class II B sequences isolated from DFTD cells during RT-PCR, as described for Figure S2 of this paper. The top four sequences are previously described MHC class II B genes with the following accession numbers, SahaDAB1*01 – EF591102.1, SahaDAB2*03 – EF591104.1, SahaDAB3*05 – EF591105.1. The bottom four sequences were isolated from DFTD cells and are marked with a star. Primer sites are shown boxed in red. **C)** Nucleotide alignment comparing the single devil class II A gene (Accession number FQ790241:10943-10728) with a single class II A sequence isolated from devil spleen, as described for Figure S2 of this paper. Primer sites are shown in red.

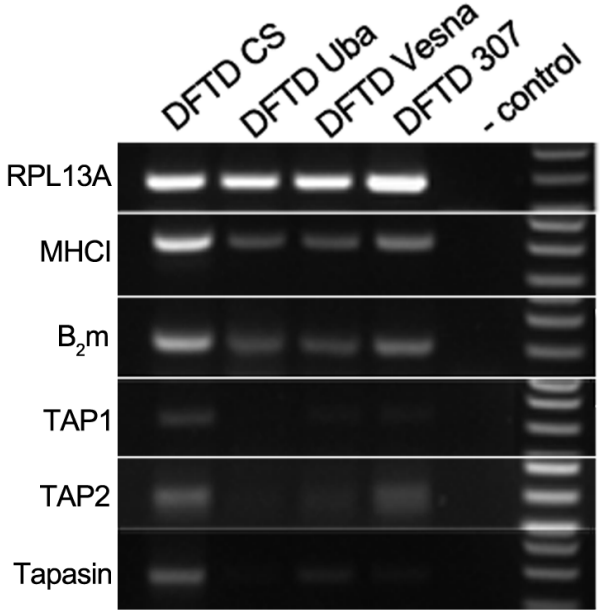

Figure S4. RT-PCR experiments on additional DFTD biopsies. RT-PCR amplification of RPL13A, MHCI, β_2m, TAP1, TAP2 and tapasin from RNA of primary DFTD biopsies from wild devils (DFTD_CS, DFTD_UBA, DFTD_Vesna, DFTD_307). A no cDNA negative control was included in each experiment. Markers show that amplicons are between 100 and 300 bp.

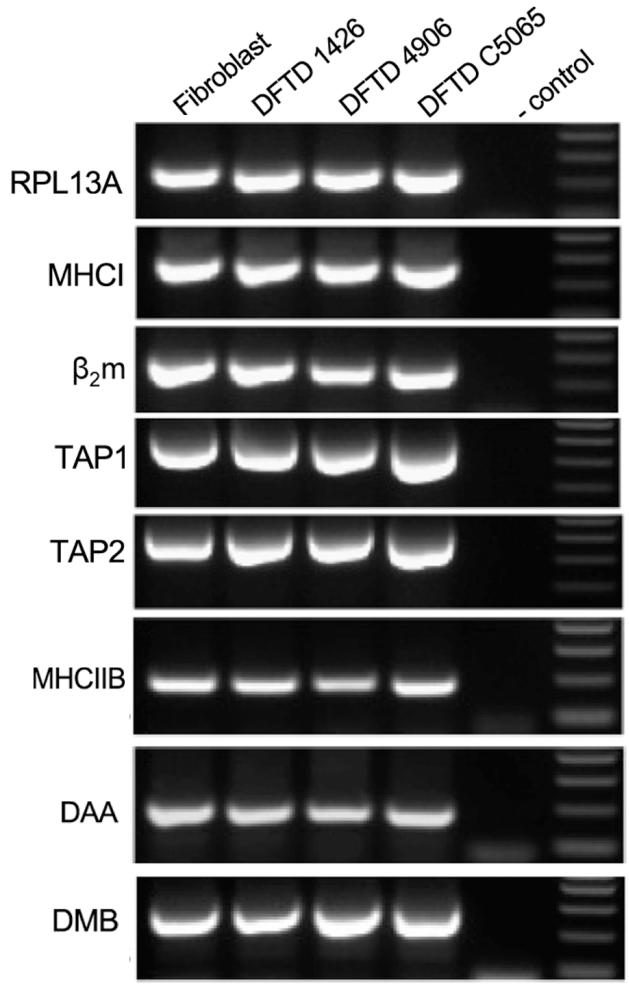

Figure S5. Antigen processing genes are not deleted from the genome of DFTD cells. PCR amplification of RPL13A, MHC class I, β_2m, TAP1 and TAP2, MHC class IIB, MHC class IIA and DMB from DNA of DFTD and fibroblast cells. A no DNA negative control was included in each PCR. Markers show four bands at 400, 300, 200 and 100 bp.

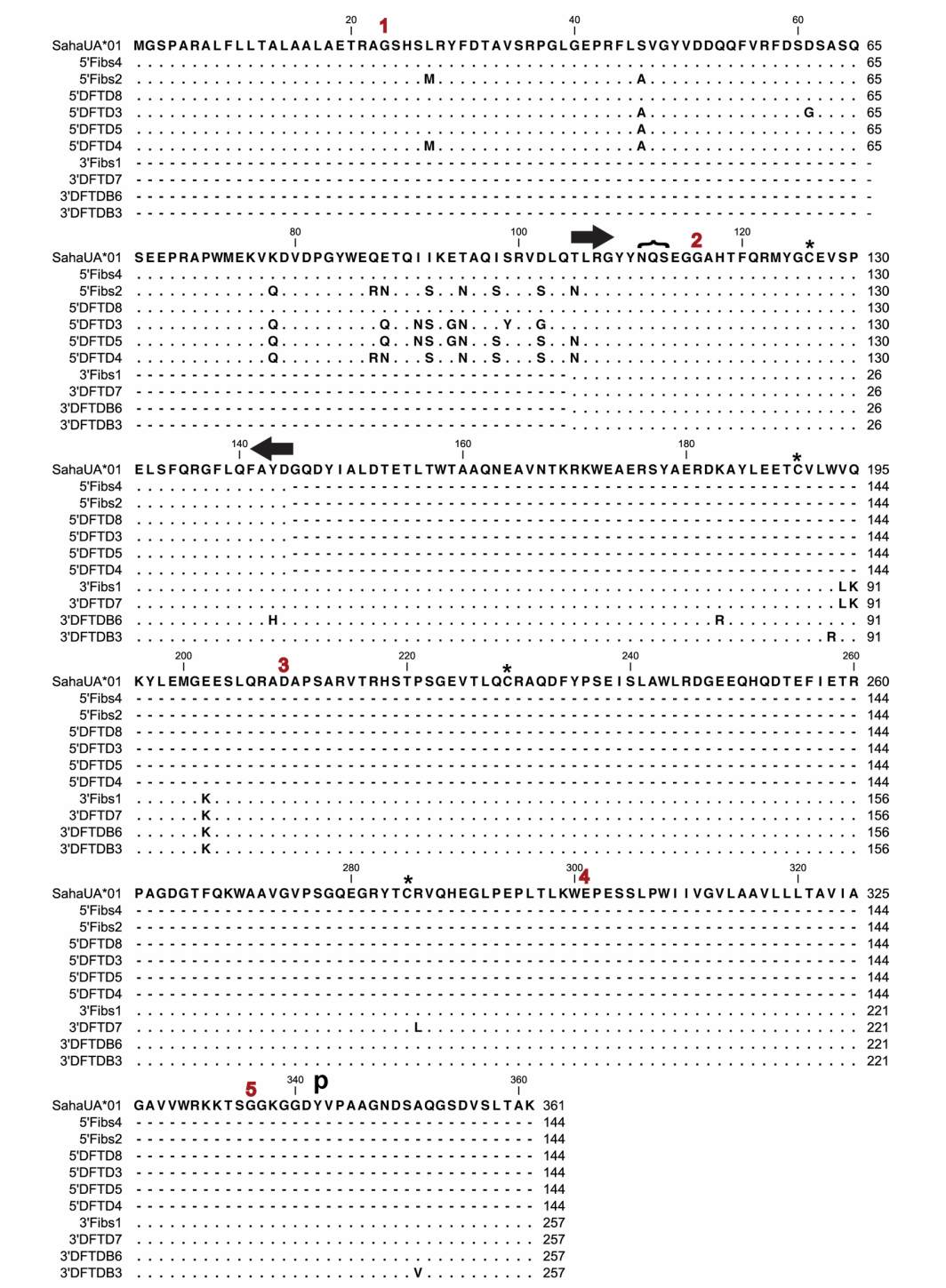

Figure S6. Amino acid alignment of MHC class I transcripts isolated by 3' and 5' RACE PCRs from DFTD cells (4906) and fibroblast cells. Transcripts are aligned to Saha*01, a previously published full length MHC class I transcript (NCBI accession: EF591089). Sequences isolated from fibroblast cells are designated by 5'Fibs or 3'Fibs. Sequences isolated from DFTD cells are designated 5'DFTD or 3'DFTD. The black arrows indicate the sites of the reverse and forward primers used for 5' and 3' RACE respectively. The 5' and 3' transcripts could not be aligned using overlapping sequence due to the amplification of multiple loci and alleles, so 5' and 3' transcripts are given separately in the alignment. The first amino acid of each domain is given by a numeral in grey. A glycosylation site is indicated by brackets, asterisks indicate cysteines expected to be in disulphide bridges, and p indicates a phosphorylation site.

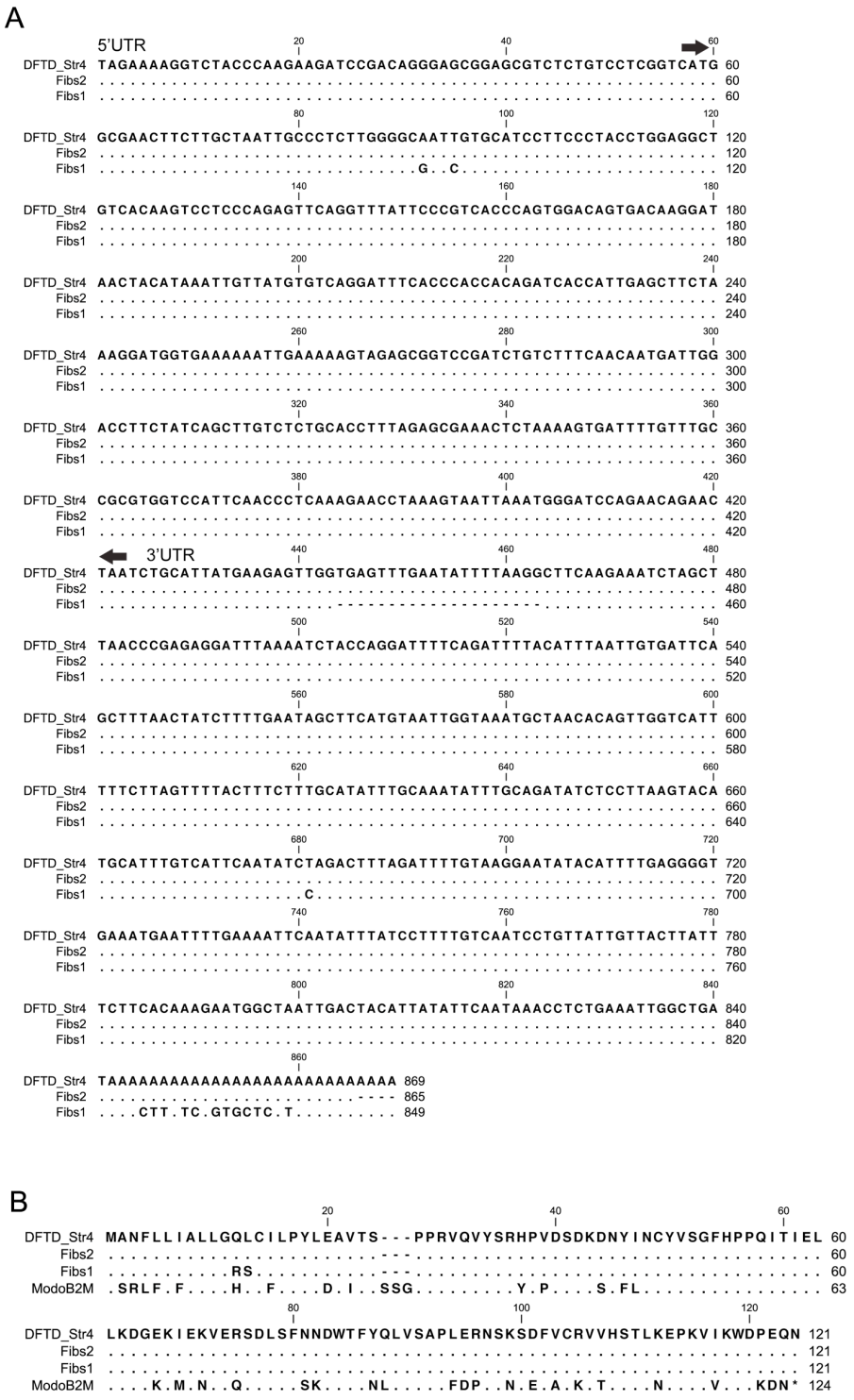

Figure S7. Alignment of full length β2m transcripts from DFTD (4906) and fibroblast cells. **A)** Nucleotide alignment of β_2m from DFTD (DFTD_str4) and fibroblast cells (Fibs1 and Fibs2), with the black arrows indicating start and stop codons, **B)** amino acid alignment of β_2m from DFTD (DFTD_str4), fibroblast cells (Fibs1 and Fibs2) and the grey short-tailed opossum (*Monodelphis domesticas*, ModoB2m).

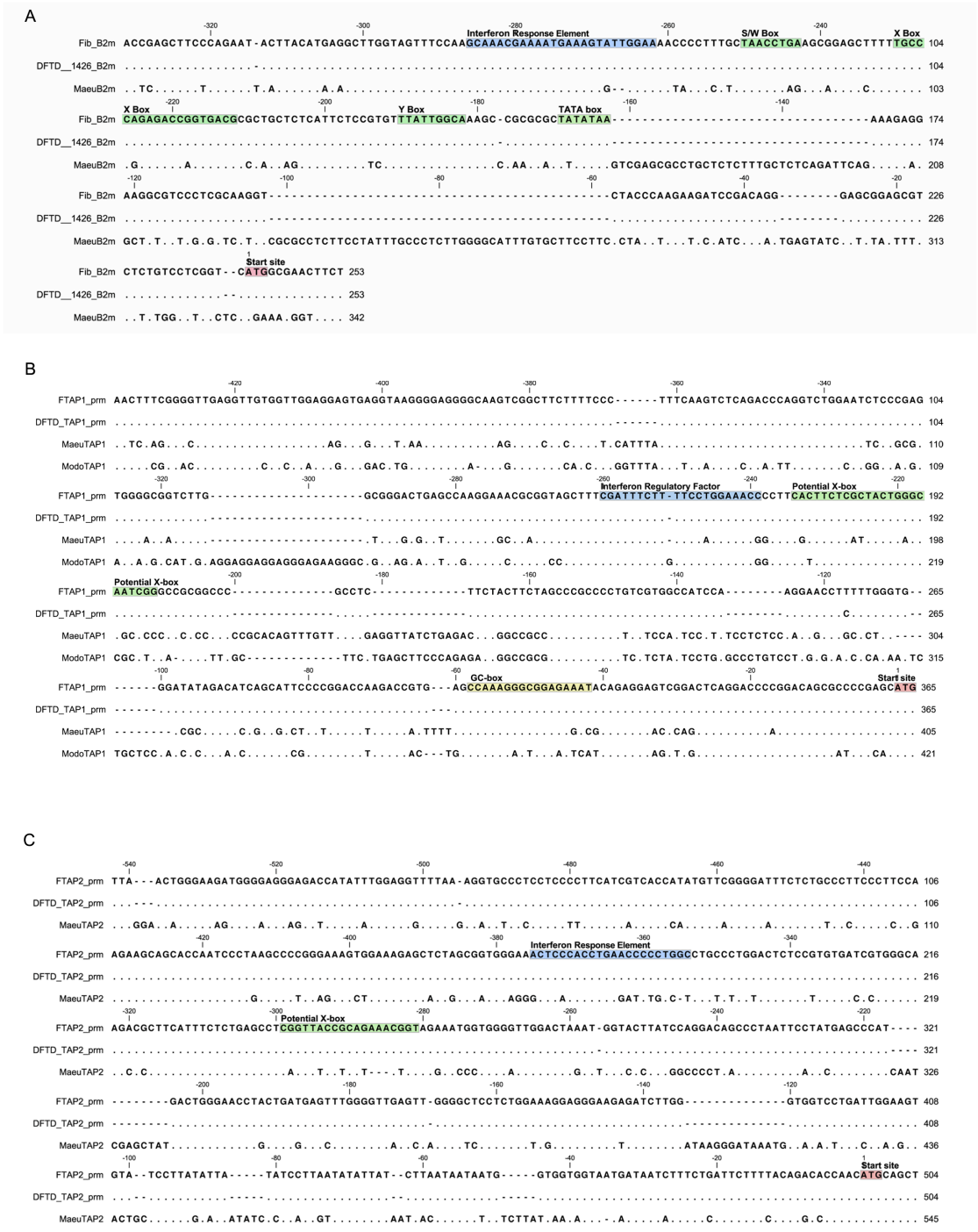

Figure S8. Nucleotide alignment of the β2m, TAP1 and TAP2 promoter regions from DFTD (1426). A) Nucleotide alignment of the β_2m promoter region from devil fibroblast (Fib_B2m), DFTD (DFTD_1426_B2m) and tammar wallaby (*Macropus eugenii,* MaeuB2m). A putative IRE site, S, X and Y boxes, TATA motif and start codon for the devil β_2m are indicated. **B)** Nucleotide alignment of 362 base pairs upstream from the transcription start site for TAP1 from devil fibroblast (FTAP1_prm), DFTD (DFTD_TAP1_prm), tammar wallaby (MaeuTAP1) and opossum (ModoTAP1). A putative IRE site, potential X box, GC box and transcription start site are highlighted. **C)** Nucleotide alignment of 496 base pairs upstream from the transcription start site for TAP2 from devil fibroblast (FTAP2_prm), DFTD (DFTD_TAP2_prm) and tammar wallaby (MaeuTAP2). A putative IRE site and transcription start site, X box and transcription start site are highlighted.

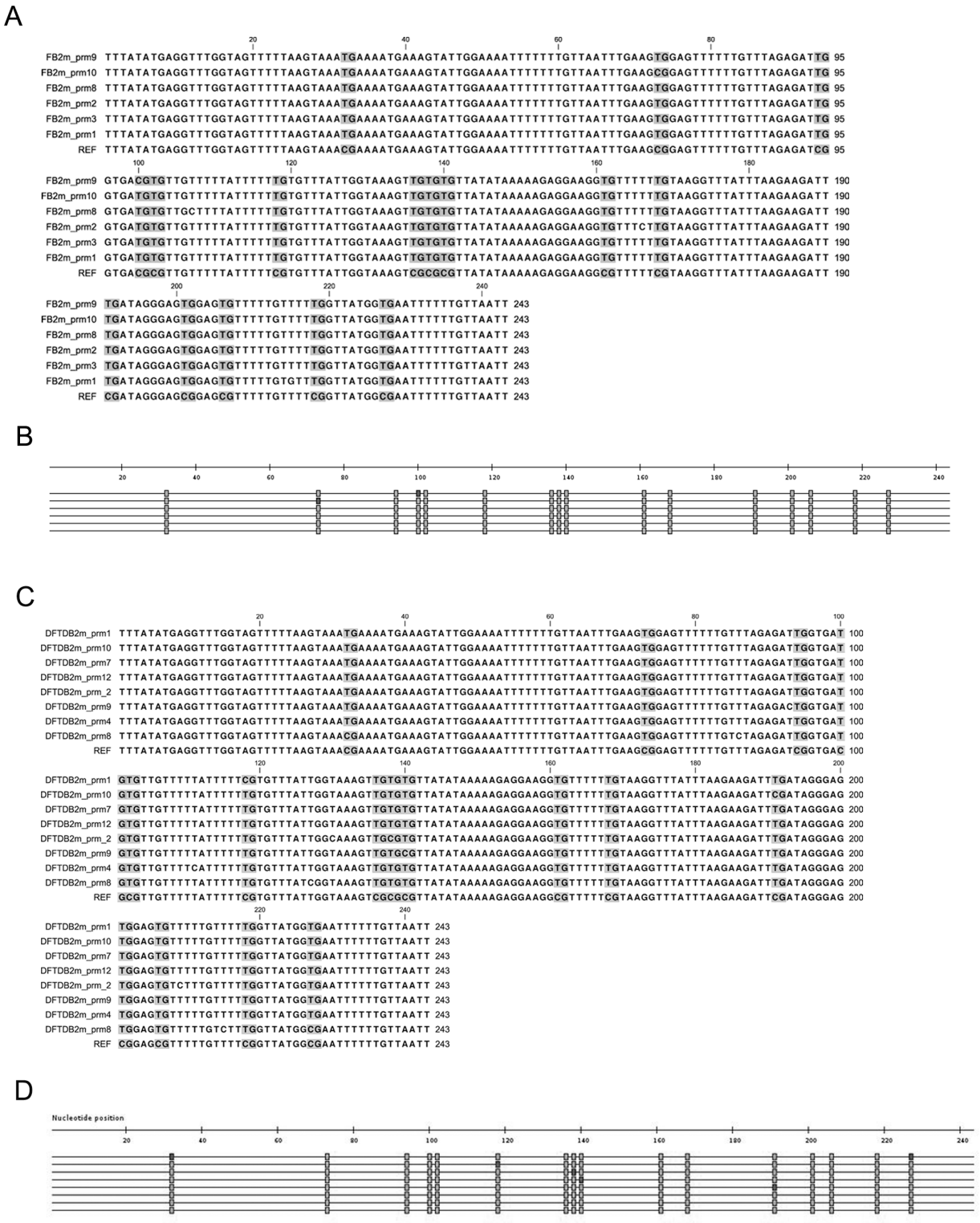

Figure S9. Bisulphite sequencing of the β_2m promoter from DFTD cells and fibroblast cells. A) Nucleotide alignment of bisulphite-treated fibroblast DNA from the β_2m transcription start site to 243 bp upstream with CpG sites highlighted in grey, **B)** Schematic of the methylation pattern of the fibroblast β_2m promoter. Each CpG site is shown as a box, methylated CpG sites are represented by dark grey boxes, **C)** Nucleotide alignment of bisulphite-treated DFTD DNA from the β_2m transcription start site to 243 bp upstream with CpG sites highlighted in grey, **D)** Schematic of the methylation pattern of the DFTD β_2m promoter. Each CpG site is shown as a box, methylated CpG sites are represented by dark grey boxes.

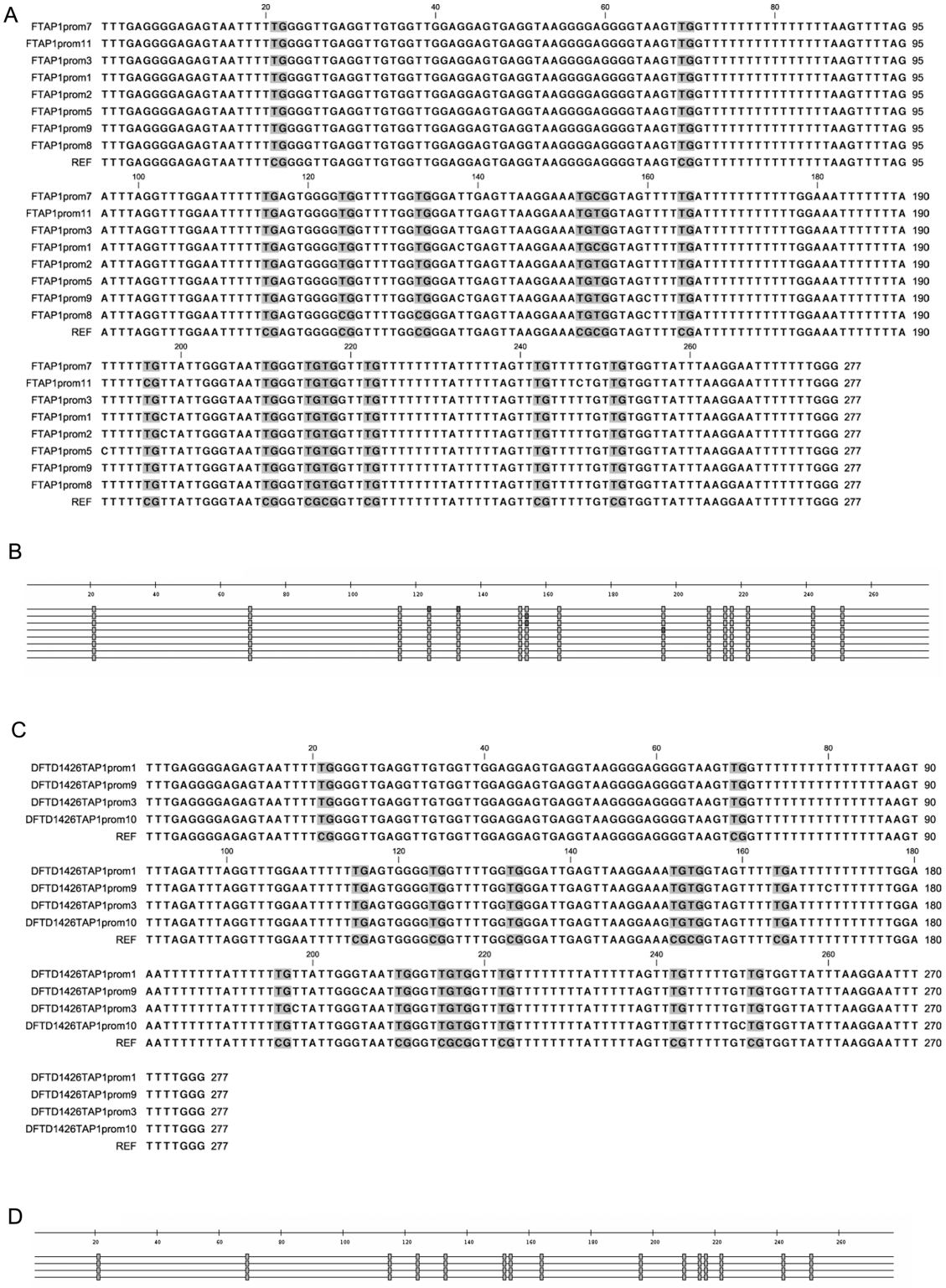

Figure S10. Bisulphite sequencing of the TAP1 promoters in DFTD cells and fibroblast cells. A) Nucleotide alignment of bisulphite-treated fibroblast DNA from the TAP1 transcription start site to 277 bp upstream with CpG sites highlighted in grey, **B)** Schematic of the methylation pattern of the fibroblast TAP1 promoter. Each CpG site is shown as a box, methylated CpG sites are represented by dark grey boxes, **C)** Nucleotide alignment of bisulphite-treated DFTD DNA from the TAP1 transcription start site to DNA 277 bp upstream with CpG sites highlighted in grey, **D)** Schematic of the methylation pattern of the DFTD TAP1 promoter. Each CpG site is shown as a box, methylated CpG sites are represented by dark grey boxes.

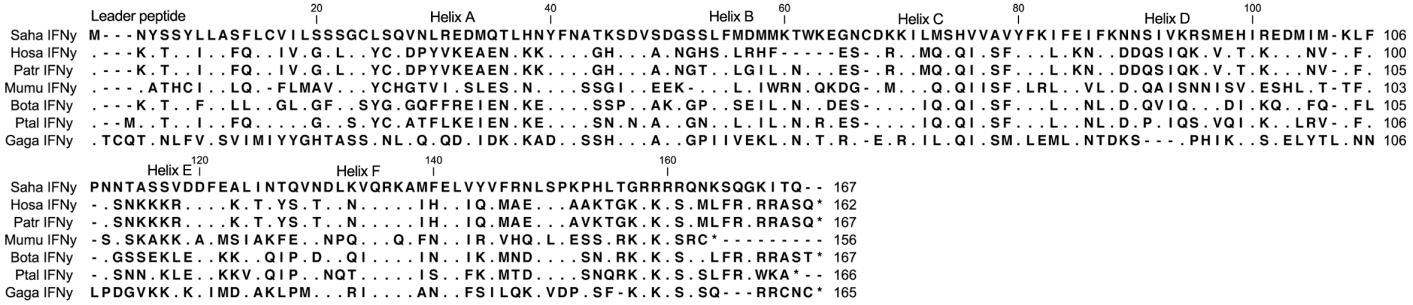

Figure S11. Amino acid alignment of IFNγ sequences from Tasmanian devil and other species. Amino acid alignment comparing the devil IFNγ sequence used for expression of protein with IFNγ sequences from other vertebrate species. Saha – Sarcophilus harissi (Tasmanian devil), Hosa – Homo sapien (human), Patr - Pan troglodytes (chimpanzee), Mumu – Mus musculus (mouse), Bota – Bos tauros (cow), Ptal - Pteropus alecto (black flying fox, a megabat), Gaga – Gallus gallus (chicken). Identical residues are represented as a dot, gaps are represented as a dash and stop codons (when present in the alignment) are represented by a star. Helices are indicated by text.

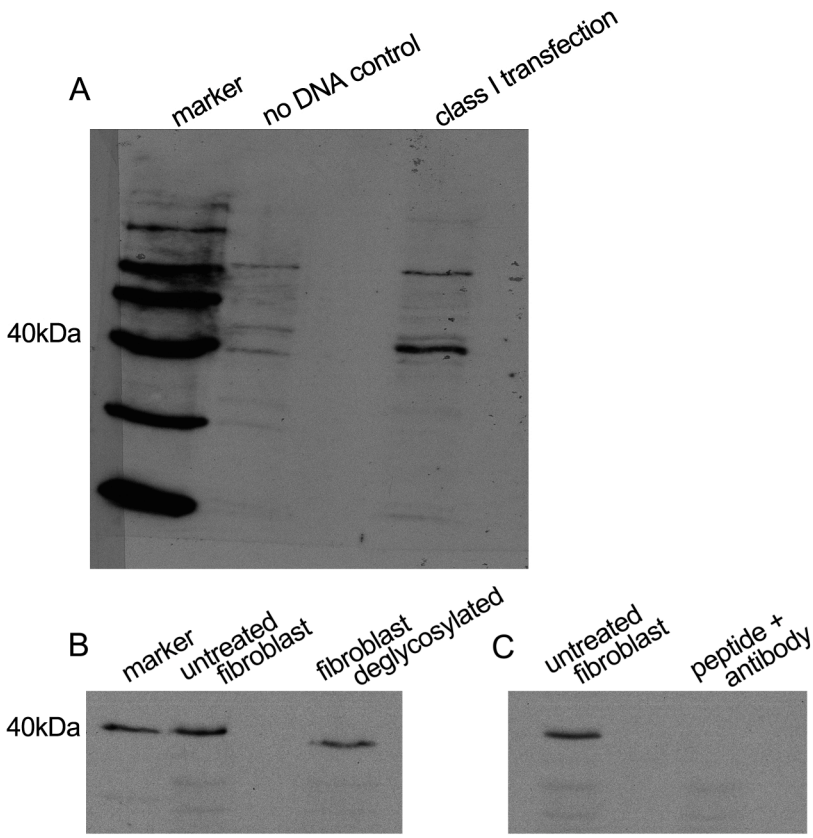

Figure S12. Western blots illustrating specificity of the MHC class I monoclonal antibody. A) Western blot on whole cell detergent lysates from DFTD cells either transfected with an MHC class I construct (*Saha-UB*) or mock transfected with no DNA, and probed with MHCI-mAb clone TD50. A specific band is present around 40 kDa only in the transfected DFTD cells. **B)** Western blot on whole cell detergent lysates from fibroblast cells either untreated or deglycosylated with Peptide N-glycosidase F and then probed with MHCI-mAb antibody clone TD50. A band is present around 40 kDa in the untreated fibroblast cell lysates, which drops to around 37 kDa in the deglycosylated lysate (indicating that the protein recognised has a single N-linked glycan as expected based on devil class I sequences). **C)** Western blot on whole cell detergent lysates from fibroblast cells probed with MHCI-mAb clone TD50 directly or pre-blocked with the peptide used for mouse immunisations. A band is present around 40 kDa in the untreated fibroblast cell lysates, but is not present when the MHCI-mAb is blocked with the immunogen (indicating that specific Ab in the tissue culture supernatants is reacting with the protein in the lysate).

Supplementary Tables

Supplementary Table 1. Primers used for RT-PCR on cDNA

GENE	**SEQUENCE (5'-3')**
RPL13A F	CCCCACAAGACCAAGCGAGGC
RPL13A R	ACAGCCTGGTATTTCCAGCCAACC
MHClex1F	CCGTGGGCTACGTGGACGATCAGC
MHClex2R	GTCGTAGGCGAACTGAAG
TAP2ex6F	TGTGGGCTAAGGCAGATTCTGG
TAP2ex7R	ATTCCCAGGAGGAGCTAAGCG
TAP1ex2F	ACAGACTGGATCCTGCAGGATGAAG
TAP1ex4R	GAGACGTGATAGCACCTGTTTGG
B2Mex1F	TGTGCATCCTTCCCTACCTGGAGG
B2Mex2R	CATTGTTGAAAGACAGATCGGACCGC
Tapasin ex3F	AGCCTCTTGCAGCTGTCTCAGTCC
Tapasin ex4R	TGGCCACCCAGTCCTGGAGTCAC
CIITAF	ACCCTTGTCCAACTTGGTTGTGTTACC
CIITAR	ACCAGGCTACAAAGGTCCTCTACATCC
Class IIb2F	AGCATTGGAACAGCCAGAAGG
Class IIb3R	AGAAACCGCTGACGGAGCAGACAAGC
Class IIa3F	CTAAGAGCCCAGCGGAGATGG
Class IIa4R	AGACCACAGTCTCTGTTGTCTCTGG
DMBF	CGAATATTGCATCTCCTTCAACAAGG
DMBR	ATAGAAGCCCCATACATAACAGGCC

Supplementary Table 2. Primers used for PCR on DNA

GENE	**SEQUENCE (5'-3')**
MHClex1.1F	GGAGCCGCGGGCGGCGTGG
MHClex1.1R	CCTGGCTCTGGTTGAAGTAGCC
TAP2 ex5F	TGTGGGCTAAGGCAGATTCTGGCAGGG
TAP2 ex6R	TCCCAGGAGGAGCTAAGCGTGG
TAP1 ex7F	TGCCTCAGTATCAGCACCGGTATCTGC
TAP1 ex8.1R	CTTCCATGACCTCCTCTAGCTCTGG
B2M ex2.1F	CTCCCAGAGTTCAGGTTTATTCCC
B2M ex2.1R	AGGTTCTTTGAGGGTTGAATGGACC
Tapasin ex3F	AGCCTCTTGCAGCTGTCTCAGTCC
Tapasin ex4R	TGGCCACCCAGTCCTGGAGTCAC
CIITAF	ACCCTTGTCCAACTTGGTTGTGTTACC
CIITAR	ACCAGGCTACAAAGGTCCTCTACATCC
Class IIb2.1F	GACATAGCCCAGAGCACTTCACG
Class IIb2.1R	GCCTGCGCACTAAGAAGGACTCA
Class IIa2.1F	CCTCCCGTACTTAATGTGACATGGC
Class IIa2.1R	TCATAAGAGGTTGTTCCAGTCCCC
DMBF	CGAATATTGCATCTCCTTCAACAAGG
DMBR	TGACTGGCACAATCCTGGAGTCC
B2m promoter F	ATCGTGCCTAAGCGTTTGAGAAGGCGA
B2m promoter R	AGGAGAAGCCGGCCAGTGGAGC
TAP1 promoter F	TCTGAGGGGAGAGCAACTTTCGGG

TAP1 promoter R	GTTGGAGAGATGCGAGAGACTTCG
TAP2 promoter F	GAGCACTCAGTACTAGGAAGCGA
TAP2 promoter R	AAGCAGGATCAGGTCAGTCAGG

Supplementary Table 3. Primers used for bisulphite sequencing

GENE	SEQUENCE (5'-3')
B2m F	ATTTATATGAGGTTTGGTAGTTTTTAAGTA
B2m R	CAAAATAAAACAATTAACAAAAAATTC
TAP1 F	AAGTTTTTTTGAGGGGAGAGTAATT
TAP1 R	ACCCAAAAAAATTCCTTAAATAACC

Supplementary Table 4. Amplification reactions and conditions for PCR

GENE	BUFFER	$MgCl_2$ (mM)	dNTPS (μM)	PRIMERS (mM)	ENZYME	TEMPLATE	ANNEALING TEMP
RT-PCR							
RPL13A	1x	3	200	0.6	Phusion (NEB)	100	60°C
class I	1x	3	200	0.6	Phusion (NEB)	100	60°C
B2m	1x	3	200	0.6	Phusion (NEB)	100	60°C
TAP1	1x	3	200	0.4	Phusion (NEB)	200	61°C
TAP2	1x	3	200	0.2	Phusion (NEB)	200	59°C
Tapasin	1x	3	200	0.6	Phusion (NEB)	100	61°C
CIITA	1x	3	200	0.4	Phusion (NEB)	200	60°C
Class IIB	1x	3	200	0.5	Phusion (NEB)	200	61°C
DMB	1x	3	200	0.5	Phusion (NEB)	200	62°C
Class IIA	1x	3	200	0.4	Phusion (NEB)	200	61°C
DNA PCR							
28S	1x	1.4	200	0.6	Taq (Invitrogen)	100	60°C
Class I	1x	1.4	200	0.6	Taq (Invitrogen)	100	60°C
B2m	1x	1.4	200	0.6	Taq (Invitrogen)	100	60°C
TAP1	1x	1.4	200	0.6	Taq (Invitrogen)	100	60°C
TAP2	1x	1.4	200	0.6	Taq (Invitrogen)	100	60°C
Amplification of promoters							
B2m	1x	1.4	200	0.6	Taq (Invitrogen)	100	60°C
TAP1	1x	1.4	200	0.6	Taq (Invitrogen)	100	60°C

TAP2	1x	1.4	200	0.6	Taq (Invitrogen)	100	60°C
Amplification of bisulphate treated DNA							
B2m	1x	2	200	0.6	Expand HF (Roche)	100	58.5°C
TAP1	1x	2	200	0.6	Expand HF (Roche)	100	58.5 °C

Supplementary Table 5. Antibodies

ANTIBODY	CLONE OR CAT NUMBER	SUPPLIER (WHERE APPLICABLE)
Periaxin	HPA 001868	Sigma
β-actin	Clone AC-15 (A1978)	Sigma
CD3ε	A0452	Dako
MHC class I	TD50	NA (this paper)
B2m	polyclonal	NA (this paper)