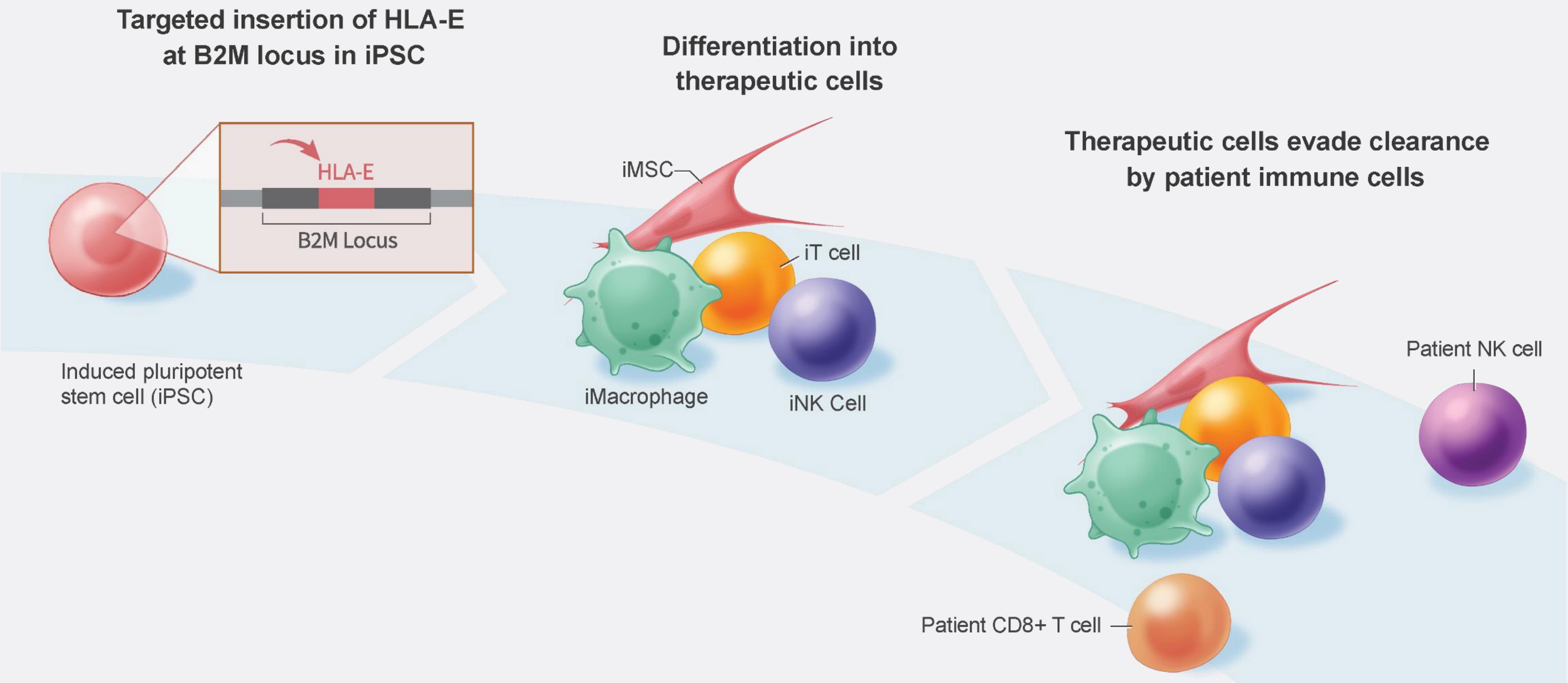
Targeted Insertion of HLA-E at the B2M Locus of mRNA-Reprogrammed iPSCs Facilitates the Development of Allogeneic Cell Therapies with Enhanced Safety Features

Elizabeth Belcher, Raven Dance Hinkel, Christopher B. Rohde, Matthew Angel, Kyle M. Garland
Factor Bioscience Inc., Cambridge, MA

Summary

Modulation of human leukocyte antigen (HLA) elements for evasion of clearance by both CD8+ T cells and NK cells has been explored as a strategy to improve *in vivo* persistence of exogenous cell therapies. We previously applied our gene-editing mRNA, UltraSlice™, for high-specificity editing and inactivation of β2 microglobulin (B2M), a key component of all HLA class I molecules that can trigger CD8+ T cell-mediated immune rejection. However, B2M$^{-/-}$ cells can be susceptible to elimination by host NK cells, which recognize cells that lack HLA class I. Here, we report the development of an mRNA-engineered iPSC line designed to evade both CD8+ T cells and NK cells by expressing a minimally-immunogenic B2M-HLA-E fusion protein in lieu of the endogenous B2M, while mimicking the inducible expression behavior of B2M. Using UltraSlice™, we optimized conditions for the insertion of a flexible linker-containing HLA-E transgene upstream of the stop codon in the native B2M gene. The resultant iPSCs express the fusion protein after stimulation and do not express HLA-A, HLA-B, or HLA-C, all HLA class I molecules associated with CD8+ T cell immunogenicity.



Conclusions

- Insertion of a single-stranded DNA repair template at the B2M locus is enhanced through codon optimization of the donor sequence.
- This work demonstrates the feasibility of generating iPSC-derived allogeneic cell therapies that have inherent potential to reduce the manufacturing complexities of autologous and donor-derived cell therapies (e.g., scalability, batch-to-batch consistency, cost, etc.)
- There are numerous potential therapeutic applications for cells derived from B2M-HLA-E iPSCs, as such cells will have immune-evasive properties and should therefore exhibit prolonged *in vivo* persistence.

1 Targeted Insertion of HLA-E

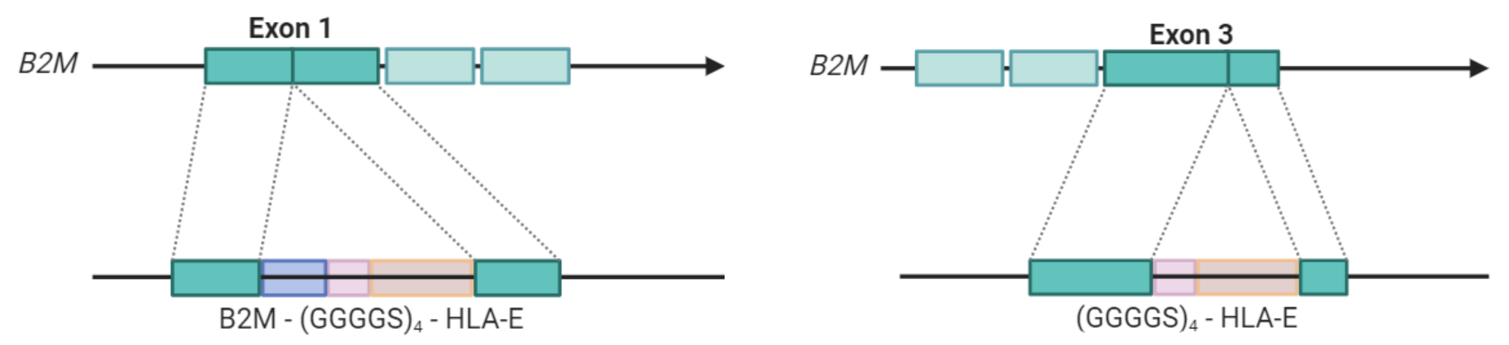


Figure 1. Strategies for inserting HLA-E into B2M locus. Targeting exon 1 utilizes a donor template that contains the B2M sequence in addition to the flexible (GGGGS)4 linker and HLA-E sequence. Targeting exon 3 only utilizes the linker and HLA-E sequence in the donor template, as it inserts after the endogenous B2M coding sequence and just before the stop codon.

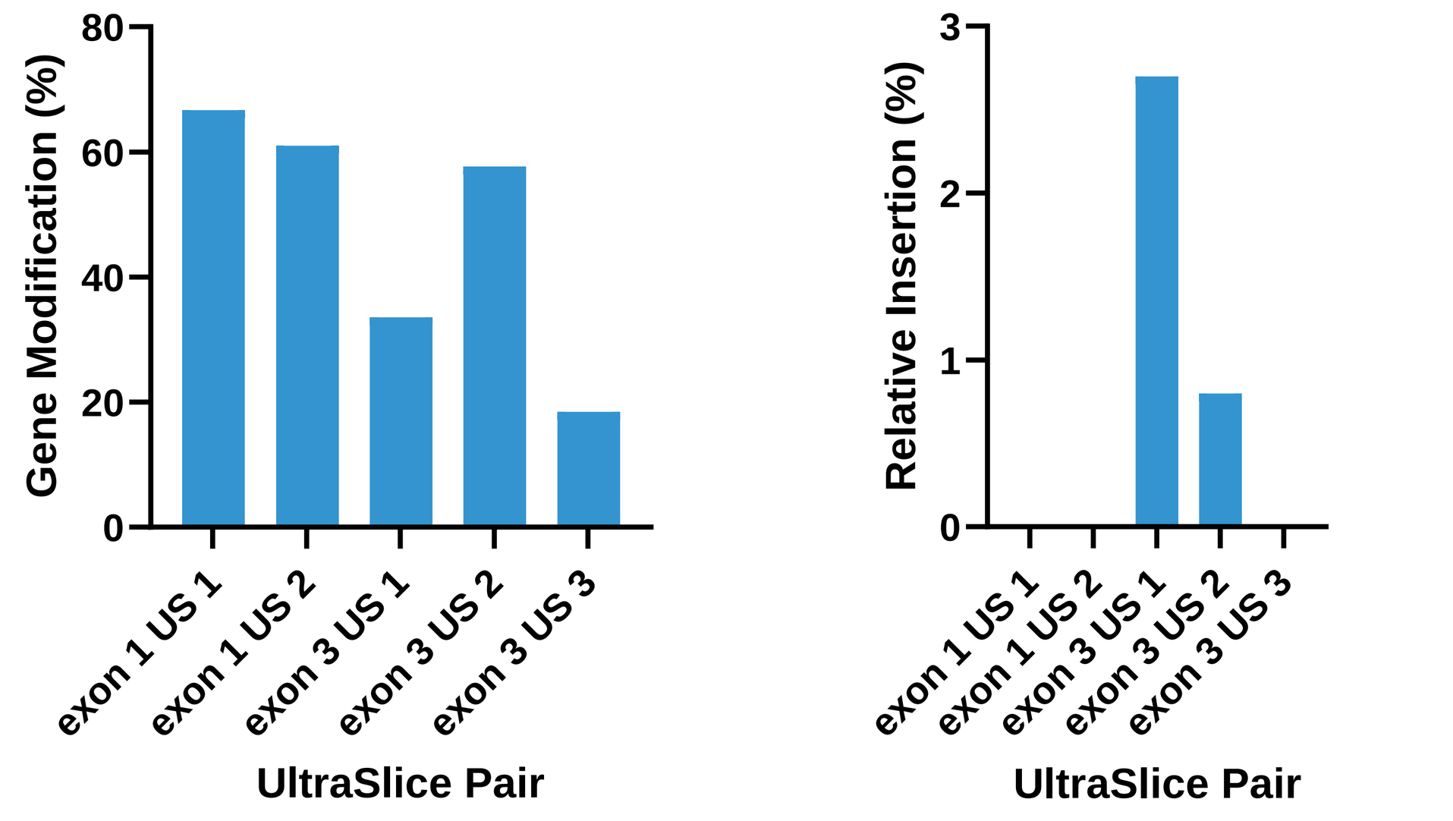


Figure 2. Screening UltraSlice pairs for activity at B2M locus. UltraSlices targeting exon 1 had higher gene modification, while UltraSlices targeting exon 3 were more efficient at inserting the donor template. UltraSlices targeting exon 3, specifically pair 1, were selected for further cell line development.

2 Transgene Insertion and Optimization of Linker Sequence

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                              Homology arm, B2M exon 3            (GGGGS)4 Linker                                          HLA-E sequence
Initial Linker(83.3% GC): TGTCTTTTTCATAGATCGAGACATGGGCGGGGGAGGCTCCGGGGGAGGCGGCTCCGGCGGCGGGGGAAGTGGCGGGGGAGGATCCGGTTCTCACTCCTTGAAGTATTT
               Clone C3: TGTCTTTTTCATAGATC--------------------------------------------------------------------GGTTCTCACTCCTTGAAGTATTT
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Figure 3. Sequencing data from transgene insertion using initial donor template. Insertion of the initial donor template, which has a GC content of 83.3%, resulted in truncated insertion and a loss of 68 nucleotides, including part of B2M exon 3 as well as the flexible linker. This led to the development of donor templates with modified linker sequences comprising reduced GC content via codon optimization.

	Initial Linker	Mod Linker 1	Mod Linker 2
% GC	83.3	71.7	61.7
ΔG	-5.28	-1.51	1.16

Figure 4. GC contents and Gibb's energies for linker sequences. Linker sequences were codon optimized to reduce GC content, which also reduced the Gibb's free energy of each. Calculated using IDT OligoAnalyzer™.

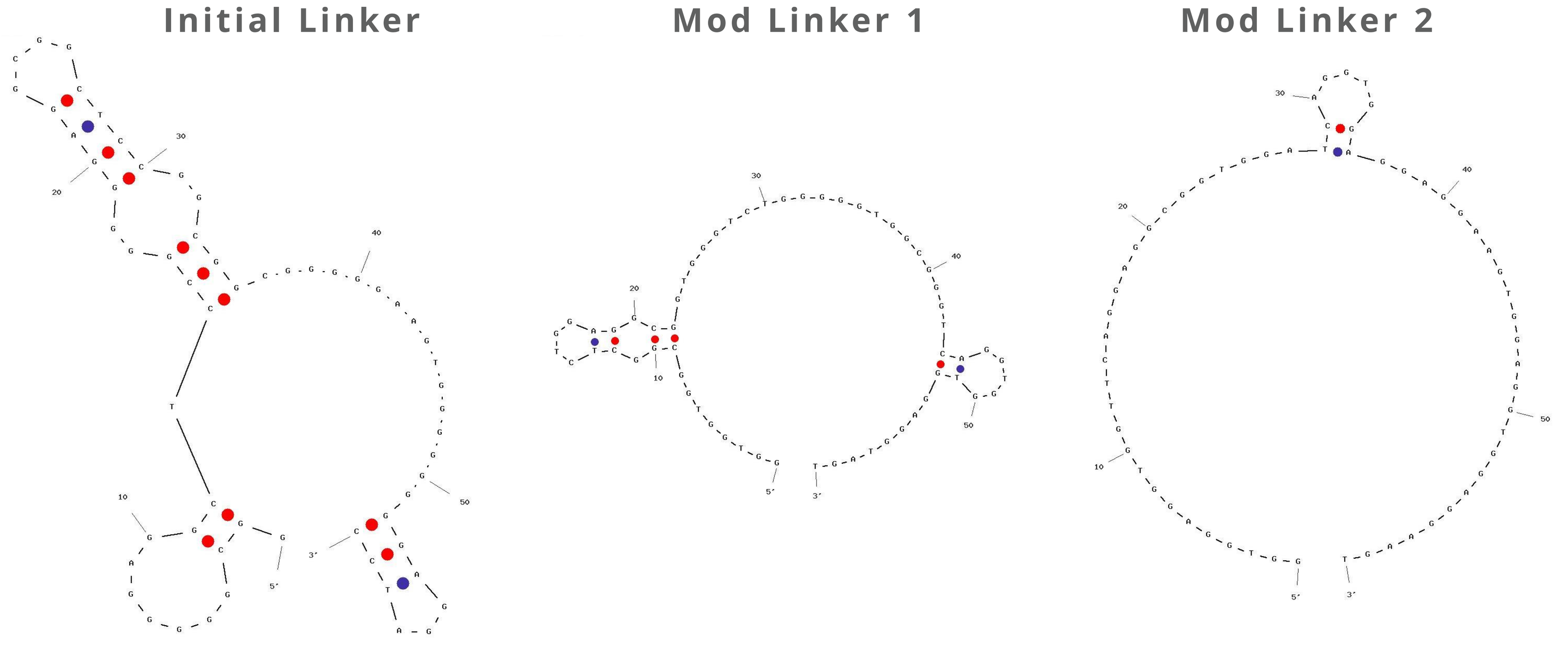

Figure 5. Theoretical secondary structures formed by linker sequences. Representative figures for secondary structure formation of each flexible linker variant. As the GC content decreased, fewer hairpin structures were predicted to form. Images generated using IDT OligoAnalyzer™.

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                                Homology arm, B2M exon 3            (GGGGS)4 Linker                                   HLA-E sequence
Mod Linker 2 (61.7% GC): TGTCTTTTTCATAGATCGAGACATGGGTGGAGGTGGTTCAGGAGGCGGTGGATCAGGTGGAGGAGGAAGTGGAGGTGGAGGAAGTGGTTCTCACTCCTTGAAGTATTT
            Clone F11: TGTCTTTTTCATAGATCGAGACATGGGTGGAGGTGGTTCAGGAGGCGGTGGATCAGGTGGAGGAGGAAGTGGAGGTGGAGGAAGTGGTTCTCACTCCTTGAAGTATTT
            Clone F13: TGTCTTTTTCATAGATCGAGACATGGGTGGAGGTGGTTCAGGAGGCGGTGGATCAGGTGGAGGAGGAAGTGGAGGTGGAGGAAGTGGTTCTCACTCCTTGAAGTATTT
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Figure 6. Sequencing data from transgene insertion using modified donor templates. A modified donor template comprising a linker sequence with reduced GC content enabled complete bi-allelic insertion, as seen with clones F11 and F13.

3 Improved Insertion with Reduced GC Content Linker

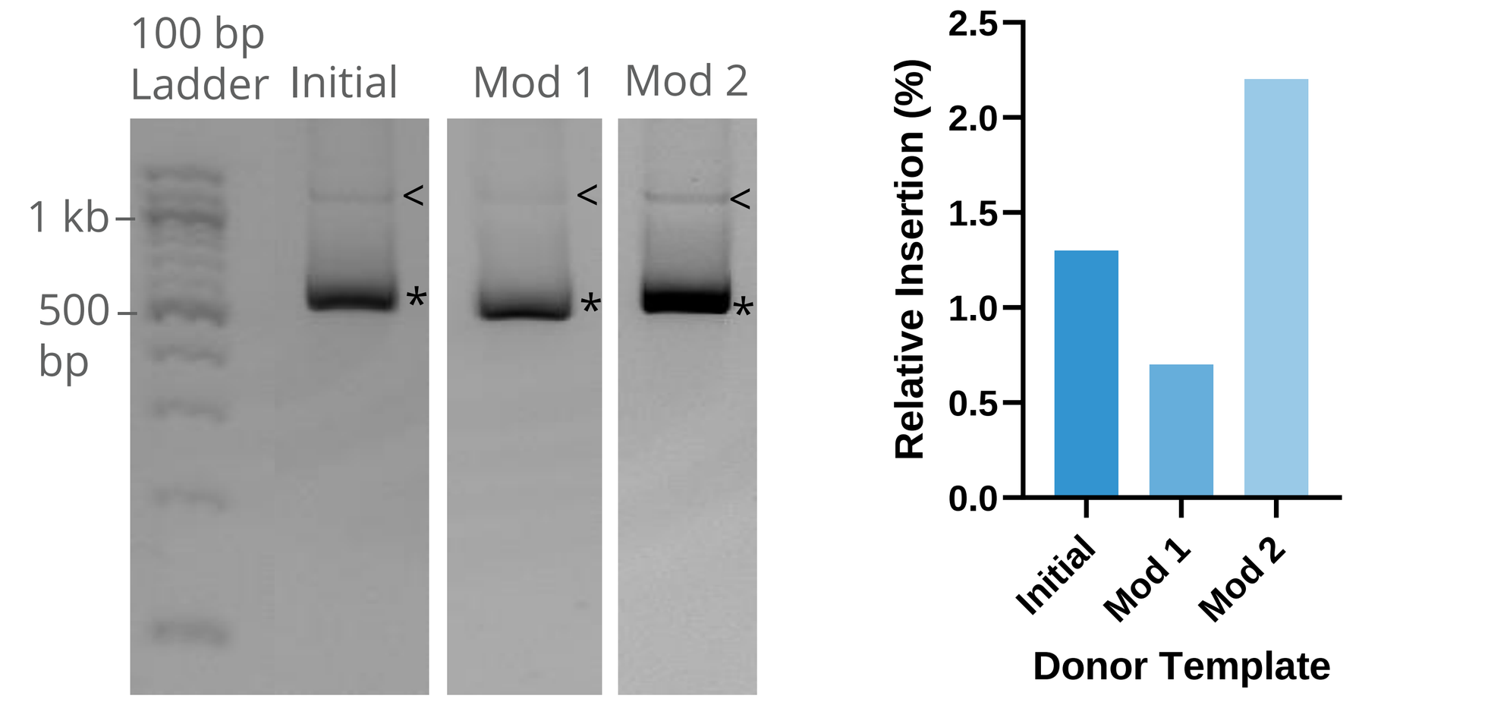

Figure 7. Insertion rates of donor templates into iPSCs. iPSCs were electroporated (1140 V, 20 ms, 2 pulses) with B2M exon 3-targeting UltraSlices and each of the donor templates. Mod Linker 2 exhibited the highest relative insertion. Wildtype band (*) at 611 bp, inserted band (<) at 1682 bp.

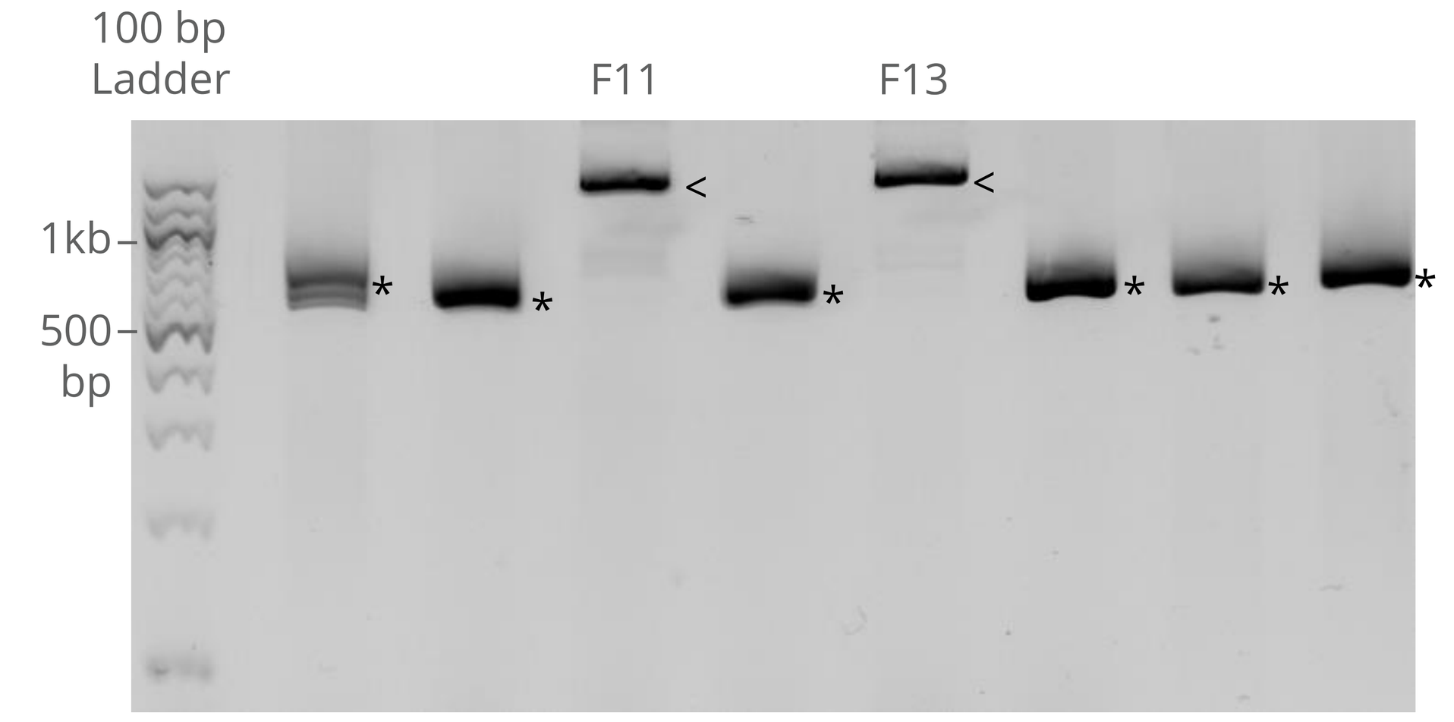

Figure 8. Single iPSC colonies with biallelic insertion. After electroporation, edited iPSCs were single cell deposited, then single colonies were harvested and analyzed for insertion of transgene. Colonies F11 and F13 demonstrate full bi-allelic insertion of the B2M-HLA-E repair template containing mod linker 2 (<), while the other colonies present wildtype bands (*).

4 Inducible Transgene Expression

HLA-E expression is dependent on the B2M promoter. iPSCs were stimulated with 50 ng/mL of IFNγ for 72 hours before analysis via flow cytometry and simple western for B2M and HLA expression.

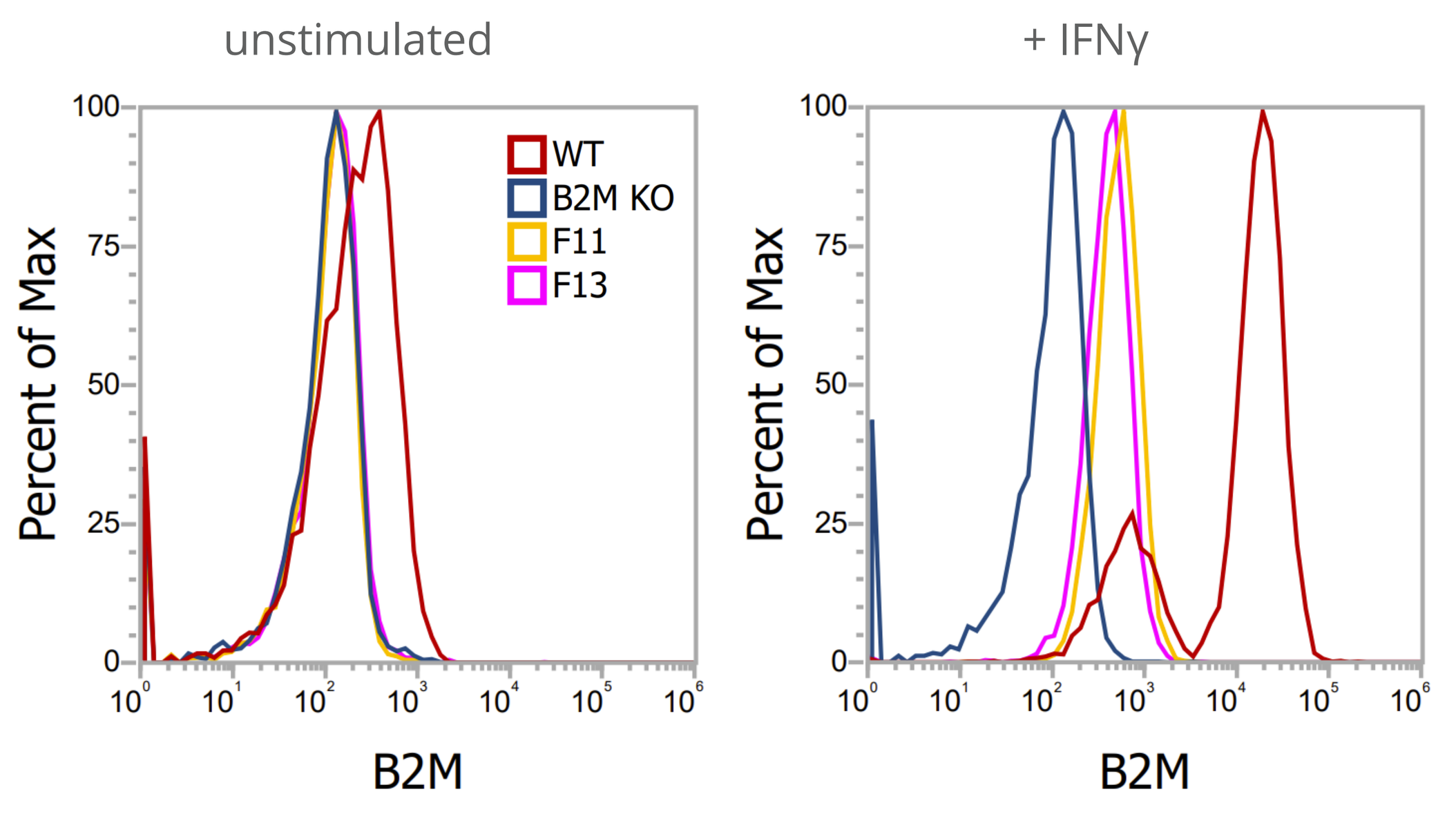

Figure 9. B2M expression of iPSCs. WT iPSCs express low levels of B2M without stimulation; B2M expression increases with IFNγ stimulation. B2M-HLA-E inserted cells express low levels of B2M after stimulation, indicating gene expression is still inducible by stimuli. B2M KO cells do not express B2M.

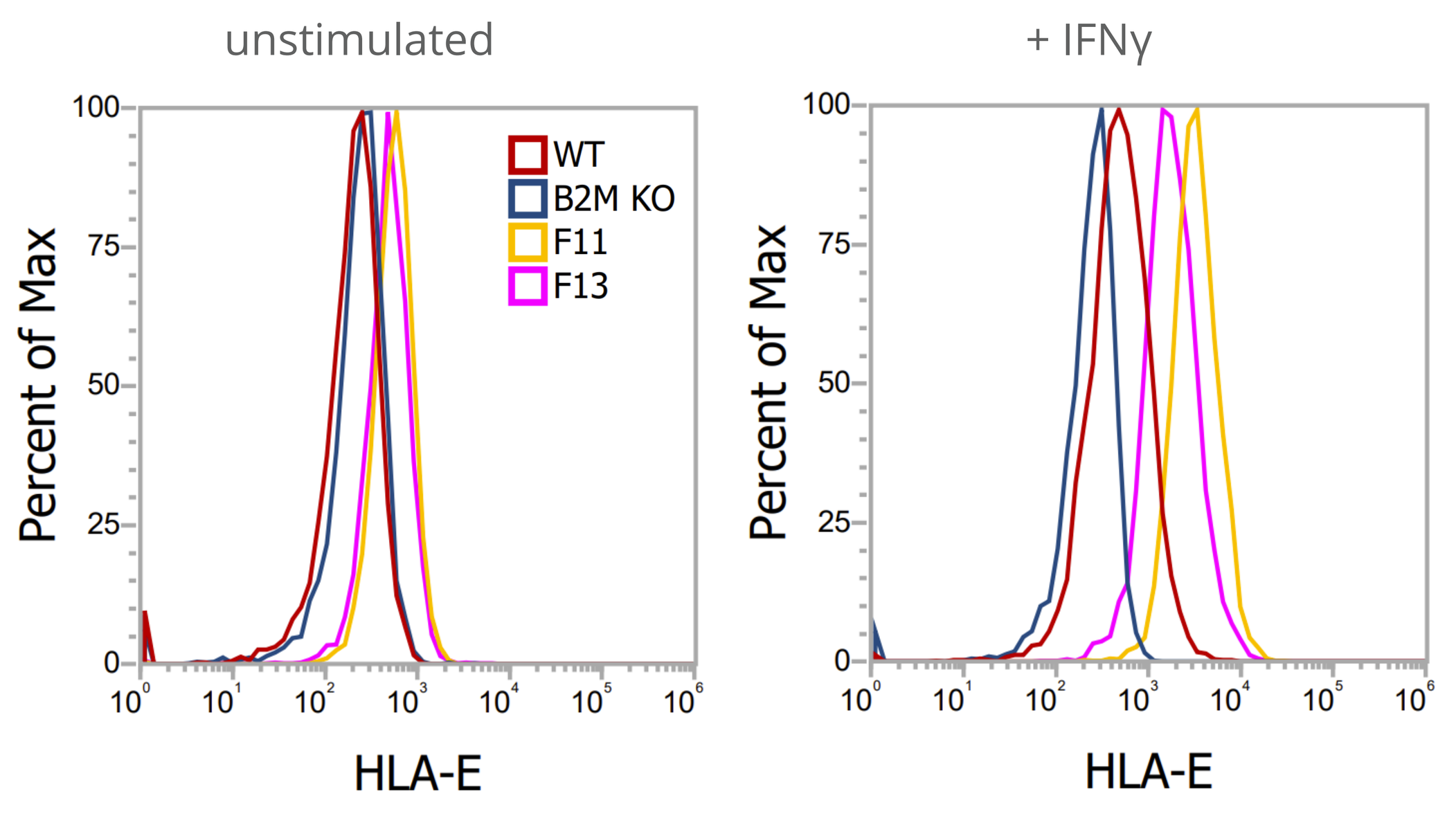

Figure 10. HLA-E expression of iPSCs. B2M-HLA-E inserted iPSCs express low levels of HLA-E unstimulated. HLA-E expression increases after IFNγ stimulation, indicating expression is linked to stimuli. WT iPSCs express low levels of HLA-E when stimulated, while B2M KO iPSCs do not express HLA-E.

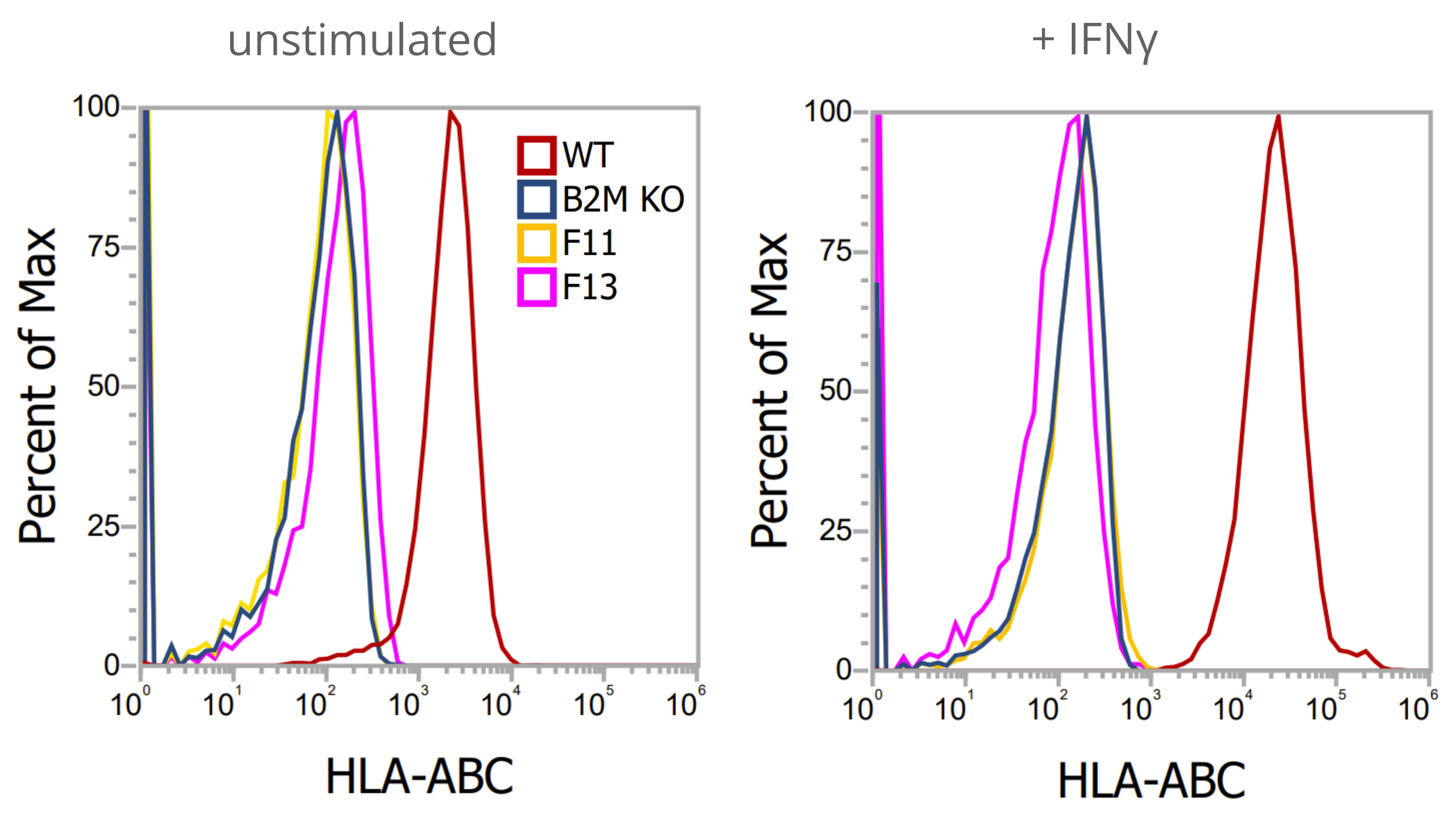

Figure 11. HLA-ABC expression of iPSCs. Only WT iPSCs express HLA-ABC, which is upregulated after stimulation with IFNγ. F11 and F13 exhibit complete loss of HLA-ABC, which is known to be dependent on B2M expression. B2M KO iPSCs also do not express HLA-ABC.

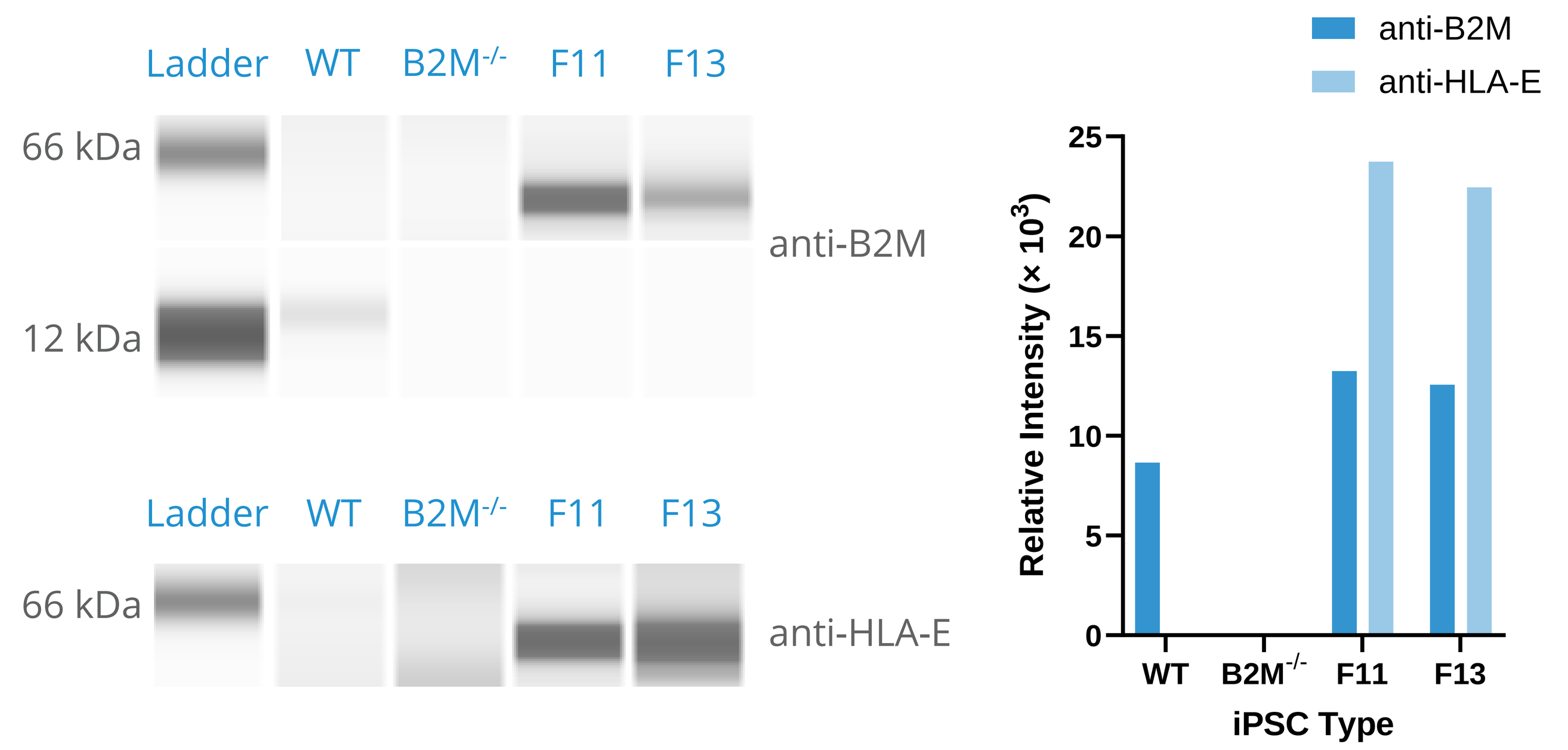

Figure 12. JESS Western of iPSCs. WT iPSCs express B2M only at its theoretical molecular weight (12 kDa), while edited iPSCs express a fusion B2M-HLA-E protein detectable using both B2M and HLA-E antibodies at its theoretical molecular weight (62 kDa). B2M KO iPSCs do not express either protein.