SUPPLEMENTAL FILES

A multi-enhancer *RET* regulatory code is disrupted in Hirschsprung disease

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SNP ID	Risk Allele	Risk Allele Frequency in old cases (n=220)	Risk Allele Frequency in new cases (n=235)	Risk Allele Frequency in 1000G (n=503)	Risk Allele Frequency gnomAD (n=9400)
rs788263	G	0.63	0.59	0.39	0.39
rs788261	C	0.63	0.59	0.39	0.39
rs788260+	A	0.63	0.59	0.39	0.39
rs1547930	G	0.87	0.85	0.76	0.77
rs7069590	T	0.94	0.84	0.75	0.78
rs2435357	T	0.67	0.66	0.25	0.29
rs12247456	G	0.83	0.84	0.67	0.69
rs7393733	G	0.83	0.83	0.67	0.69
rs2506024+	A	0.82	0.82	0.59	0.60

Supplemental Table S1: Case and control frequencies of the risk allele for 10 HSCR associated SNPs showing allelic difference in reporter assays and within CREs regulating *RET* gene expression. The new case allele frequencies were obtained from a genotyping study of short segment HSCR cases in 235 European ancestry individuals (Kapoor et .al 2021) and the new control frequencies were obtained from 9,400 European ancestry individuals in gnomAD (Karczewski et al. 2020). There was no overlap betwwen new and old cases and controls.
+ The SNPs rs788260 and rs2506024 are perfect proxies (r^2 =1) for rs2506030 and rs2505441 respectively which were not genotyped in our new assays.

Enhancer	Percentage of cells with no deletion	Percentage of cells with 1-3 bp deletion	Percentage of cells with >3 bp deletion
E2	30	52	18
E4	68	22	10
E5	100	0	0
E14	69	20	11
E26	42	24	34
E27	72	15	13
E28	63	13	24
RET-7	80	5	15
RET-5.5	60	7	33
RET+3	61	20	19
E2+E4	71	22[a]	7[a]
E26+E27+E28	65	18[a]	17[a]
R3+E26 +E27+E28	75	15[a]	10[a]

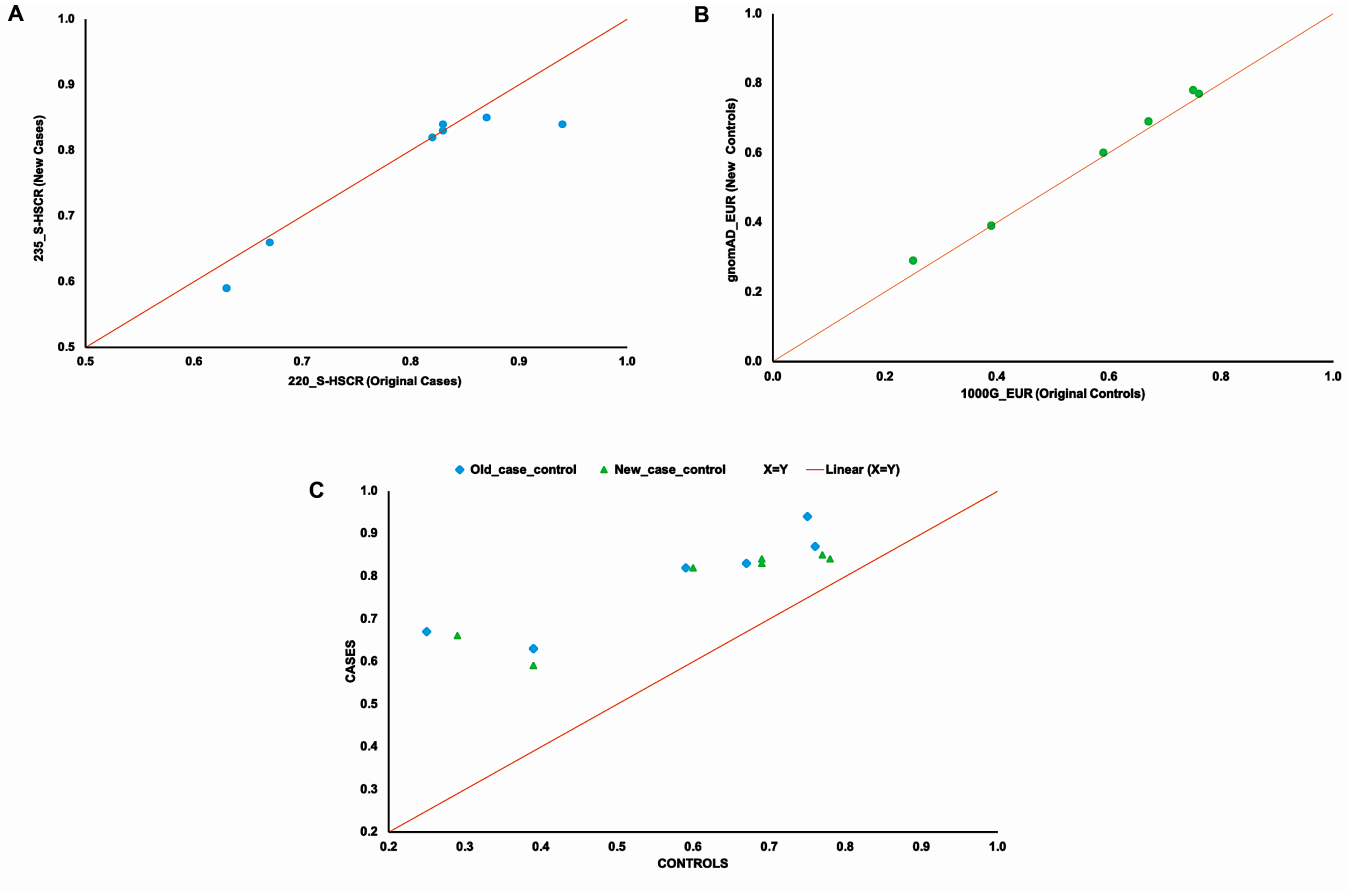
Supplemental Table S2: Percentage of cells carrying deletions of various length calculated using *Inference of CRISPR Edits (ICE)* tool (Hsiau et al., 2019). Nucleotides surrounding the HSCR associated polymorphisms in all enhancers were successfully deleted except for enhancer 5. [a] The number represents the precent of deletion for pool of cells for each independent enhancer region.

Enhancer	Guide RNA Sequence
E2	TAAACCGTTAAGTAATGACC
E4	TGGGACTCATCTGTGCACGG
E5	AGTGAGCCAGCAAAGTGAAA
E14	CAGCCCGCCTCACTGCTCCA
E26	GTGCTGAGCAAACAAGCCTG
E27	GGCACCCTGTAAGAGTGAGG
E28	AGCCCCTGCCTCCACAGACA
RET-7	AGAAACCAGCAGAGCCACAT
RET-5.5	GTGAGAAGCTGAAAGAGTGT
RET+3	ACCCTTACATGGTCATCCAC

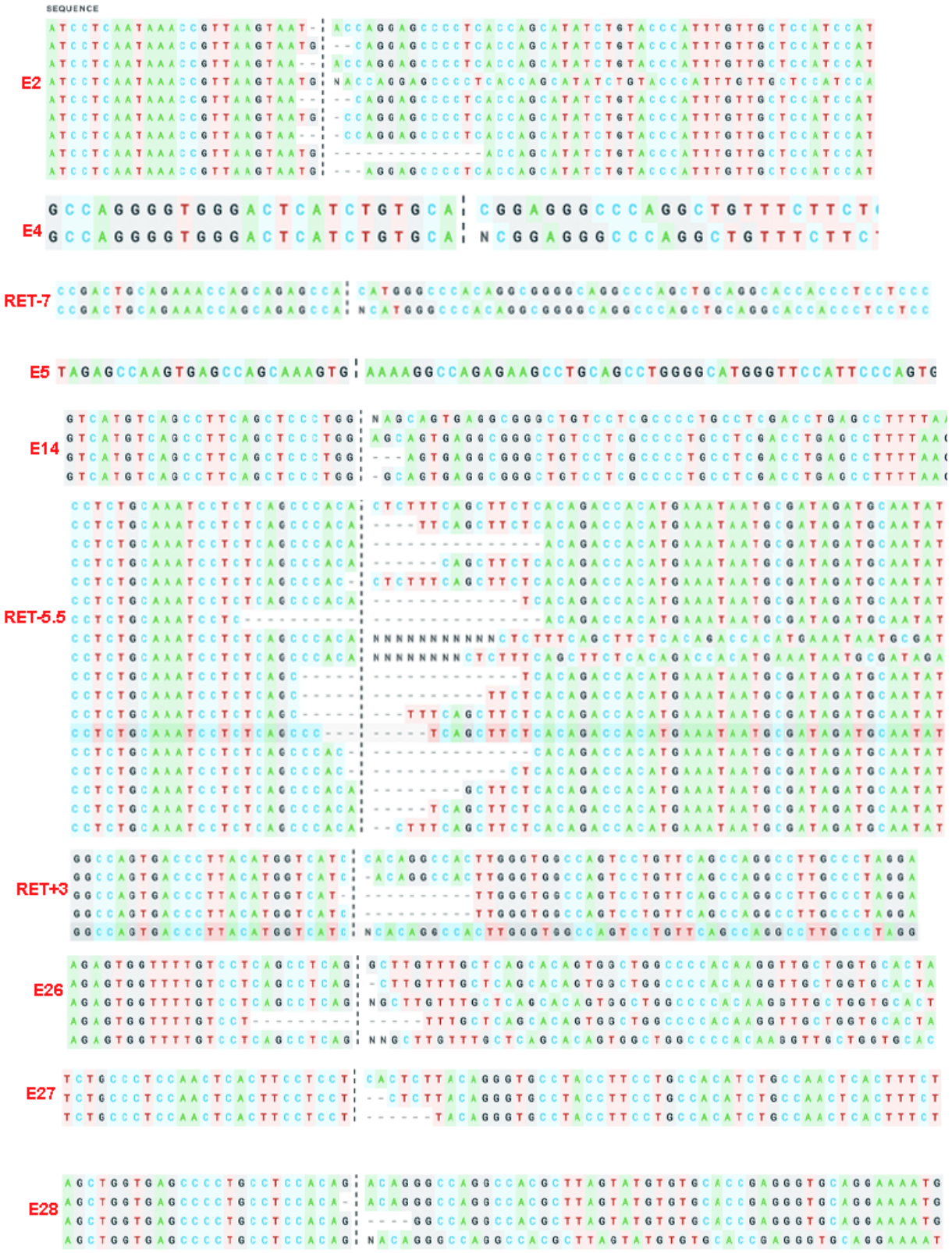
Supplemental Table S3: Sequences of the individual guide RNA targeting the HSCR associated polymorphisms in the 10 enhancers.

Enhancer	Forward Primer	Reverse Primer	Amplicon Size
E2	CTTCAAACCGCACCCGCTCCTT	AGTGACGTCCTGCCTGCATCCT	283
E4	AGCCTGAATCCAGCCACCAGGT	CTGCCCTGGGAGAGTGTCCACA	417
E5	CAGCCAGCCCTCAGCACATGTC	AGAGTGACCTGCCAGCTCCCTG	525
E14	AGCAGGCCTCTTGCTCAGAGCT	AGTGGGAGAGGCAGTCAGCTGG	538
E26	TGCAGGTGGTCCTACCCGAGTG	GGAGTGTGAAAGGGCTGGGCAC	339
E27	GTGCCCAGCCCTTTCACACTCC	CCCCACCTCACTGCTCAGGTCA	421
E28	TCGCATGAGGGGTTCTGCAGGA	CCATTACACACCACGGGGGCAC	512
RET-7	CCCCTGCTCCGTGGAACCCTAA	CCTGGCAGTGCTCAACAGACGG	455
RET-5.5	AGCCTGTGATGCTGGGCTCTCA	GCCAGAGCTGAGCTGGGAGACT	349
RET+3	CTGGGGTAGGGCGAAAATGGGC	ACCCAGCCTTCTGCTCTGAGGG	537

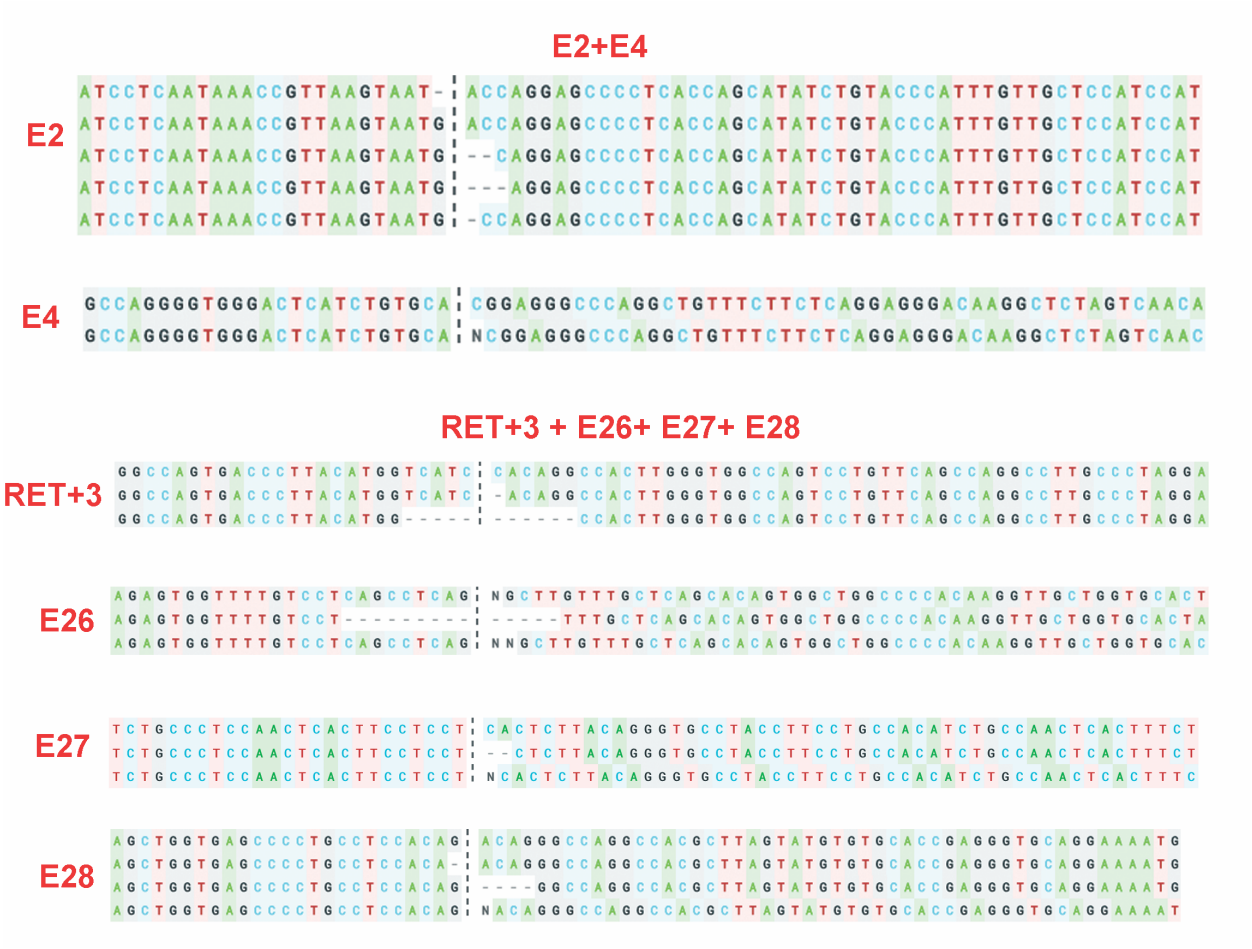
Supplemental Table S4: PCR primer sequences used for Sanger sequencing to detect CRISPR induced insertions and deletions surrounding the HSCR associated polymorphisms in the 10 enhancers.



Supplemental Figure S1: Scatter plots comparing the allele frequencies of the risk allele for 10 HSCR associated functional SNPs in (A) original and new S-HSCR cases and (B) 1000 genome and gnomAD controls. (C) Scatter plot comparing case-control allele frequencies for both old and new, shows increase of all risk allele frequency in cases compared to controls.



Supplemental Figure S2: Individual deletion of enhancer regions confirmed by Sanger sequencing of cells after single guide transfection. There are variable lengths of deletions in all our enhancer regions except E5 which did not have any insertion/deletion (INDEL)



Supplemental Figure S3: Multi enhancer deletion of enhancers E2+E4 and enhancers RET+3 +E26+E27+E28 confirmed by Sanger sequencing of cells after multiple guide transfection into the cells.