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Linking genetic variation with epigenetic profiles in Sjögren's syndrome.

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Abstract

DNA methylation represents an important regulatory event governing gene expression that is dysregulated in Sjögren's syndrome (SjS) and a number of autoimmune/inflammatory diseases. As disease-associated single-nucleotide polymorphisms (SNPs) have relevance in controlling DNA methylation, 94 non-HLA SjS-SNPs were investigated, among them 57 (60.6%) with widespread effects on 197 individual DNA methylation quantitative trait loci (meQTL) were selected. Typically, these SNPs are intronic, possess an active promoter histone mark, and control *cis*-meQTLs located around transcription start sites. Interplay is independent of the physical distance between SNPs and meQTLs. Using epigenome-wide association study datasets, SjS-meQTLs were characterized (41 genes and 13 DNA methylation CpG motifs) and for the most part map to a pro-inflammatory cytokine pathway, which is important for the control of DNA methylation in autoimmune diseases. In conclusion, exploring meQTLs represents a valuable tool to predict and investigate downstream effects of genetic factors in complex diseases such as SjS.

Key words: Sjögren's syndrome; SNP; meQTL; pro-inflammatory cytokine pathway

1 Introduction

Primary Sjögren's syndrome (SjS) is a systemic autoimmune disease with female predominance. It is characterized by epithelitis that affects exocrine glands, mainly lachrymal and salivary glands, and results in progressive loss of secretory function (Ramos-Casals, Brito-Zeron et al. 2012, Mavragani and Moutsopoulos 2014, Ramos-Casals, Brito-Zeron et al. 2015). Clinical manifestations include xerophthalmia and xerostomia, but also systemic manifestations. Patients have a 20- to 40-fold increased risk of developing lymphoma (Christodoulou, Kapsogeorgou et al. 2010, Tobon, Renaudineau et al. 2010, Ramos-Casals, Brito-Zeron et al. 2012, Ramos-Casals, Brito-Zeron et al. 2015, Mingueneau, Boudaoud et al. 2016). Histological evaluation shows focal and peri-epithelial T and B cell infiltration in exocrine glands together with characteristic circulating autoantibodies, including anti-sicca syndrome type A (SSA/Ro) and type B (SSB/La) antibodies (Christodoulou, Kapsogeorgou et al. 2010, Tobon, Renaudineau et al. 2010, Capaldo, Carvajal Alegria et al. 2016, Mingueneau, Boudaoud et al. 2016, Bowman 2018).

Approximately 120 million single nucleotide polymorphisms (SNPs) have been reported in the human genome, and among them approximately one hundred can increase the risk of developing SjS (Farh, Marson et al. 2015, Roadmap Epigenomics, Kundaje et al. 2015). The pathophysiological contribution of genetic factors in SjS is estimated to be around 35% according to the familial clustering analyses (Reveille, Wilson et al. 1984, Brito-Zeron, Baldini et al. 2016). However, the genetic impact of individual risk alleles is usually modest (Odds ratios < 2). Exceptions are variants in the human leucocyte antigen (HLA) cluster that result in moderately increased Odds ratios of 2-3 (Cruz-Tapias, Rojas-Villarraga et al. 2012, Konsta, Le Dantec et al. 2015). Another key challenge in understanding the impact is that up to 90% of genetic risk variants map to non-coding regions of the human genome, and only a subset co-localize with regulatory regions (Farh, Marson et al. 2015, Konsta, Le Dantec et al. 2015). These "regulatory SNPs" can control gene expression through direct or indirect effects on the recruitment and/or assembly of the transcriptional machinery. Direct effects are mediated when SNPs are present within proximal gene regulatory regions close to the transcription start site (TSS) where they control transcription factor (TF) binding (Farh, Marson et al. 2015, Roadmap Epigenomics, Kundaje et al. 2015). Indirect consequences result from SNPs in cell-specific distal regulatory elements that

can be associated with altered DNA methylation leading to the description of methylation quantitative trait loci (meQTL)(Banovich, Lan et al. 2014, Brito-Zeron, Baldini et al. 2016). Associations between genetic risk variants and meQTLs reveal *cis*-regulatory effects when SNPs and meQTLs are located within the same locus, usually at a short distance (<1Mb). However, *trans*-effects have also been reported when SNPs and meQTLs are more distal, for example on separate chromosomes(Ding, Gudjonsson et al. 2010). *Cis*-regulatory effects explain transcriptional variance for a majority of genes, underscoring the importance of adequate control of *cis*-genetic variation in epigenome-focused explorations(Montgomery and Dermitzakis 2011, Chen, Ge et al. 2016).

Methylation QTL studies, which test associations between genetic variants and epigenetic profiles therefore provide powerful approaches to annotate putative consequences of disease-associated risk alleles on epigenetic marks. The aim of this study was to associate genetic risk variants with epigenetic profiles (meQTL) in SjS by establishing a database of SNPs associated with meQTL. Analysis was completed by integration of data from epigenome wide association studies (EWAS) obtained from SjS patients.

2 Materials and Methods

1.1 Genetic variant selection and characterization

To link genetic variants with the epigenetic profile in SjS, we identified disease-associated SNPs(Konsta, Le Dantec et al. 2015) based on the publicly available genome wide association studies (GWAS) catalogue (<https://www.ebi.ac.uk/gwas/>), a common resource of SNP-trait associations (Figure 1). Non-HLA SNPs were subjected to subsequent analysis to determine (i) their position on the human genome 38 (hg38) using the dbSNP tool (<https://www.ncbi.nlm.nih.gov/snp/>); (ii) the location of variants (transcription start site [TSS], exon, intron, 3'-UTR, and intergenic region) using the HaploReg v4.1 tool(Ward and Kellis 2012) and the variant effect predictor tool (VEP, <https://www.ensembl.org/Tools/VEP>); and (iii) their functional relevance that was manually completed requesting HaploReg-V4.1 and SNP nexus(Dayem Ullah, Oscanoa et al. 2018) datasets based on information from the ENCODE and Roadmap Epigenome Programs in immune cells(Bernstein, Stamatoyannopoulos et al. 2010, Davis, Hitz et al. 2018). The SNP2TFBS database(Kumar, Ambrosini et al.

2017) was selected to test whether promoter or enhancer SNPs affect TF binding, the CTCFBSDB 2.0 database to explore for CTCF-binding factor (CTCF)(Ziebarth, Bhattacharya et al. 2013), and the miRdSNP database(Bruno, Li et al. 2012) to test 3'UTR SNPs capacity to bind microRNAs.

1.2 Integration of meQTL and EWAS data in SjS

The mQTLdb database using peripheral blood from 3,948 individual controls across human life course was used for the SNPs-meQTL analysis(Gaunt, Shihab et al. 2016). In addition, we compared the generated meQTL database with EWAS data obtained from SjS patients' peripheral blood mononuclear cells (PBMCs), circulating CD4⁺ T cells, circulating CD19⁺ B cells, and long-term cultured salivary gland epithelial cells (SGEC)(Altorok, Coit et al. 2014, Imgenberg-Kreuz, Sandling et al. 2016, Miceli-Richard, Wang-Renault et al. 2016, Charras, Konsta et al. 2017). For target genes that overlap in both the meQTLs database and EWAS reports, reactome pathway enrichment analysis and protein-protein-interaction (PPI) analysis were conducted using STRING V11.0(Szklarczyk, Gable et al. 2019). For SNPs located in or around the interferon regulatory factor 5 (*IRF5*) and the Protein Kinase N1 (*PKN1*), SNPs in strong linkage disequilibrium (LD) ($r^2 \geq 0.8$) were selected using 1,000 genome phase I datasets(Ward and Kellis 2012). In addition, for these SNPs we used data from the Genotype-Tissue Expression (GTEx) project, V8 RNA sequencing data across 948 individuals and information regarding expression (e)QTLs, and splicing (s)QTLs (<https://gtexportal.org>).

1.3 Statistical analysis

Continuous data are described as the mean \pm standard error of the mean (SEM). One-way ANOVA followed by Tukey's test was used for multiple comparisons; $p < 0.05$ was considered significant. Statistical analyses were performed using GraphPad Prism 7.0a (La Jolla, CA).

3 Results

3.1 Non-HLA SjS-risk variants and their association with methylation quantitative trait loci (meQTL)

Data obtained from GWAS were used to update our previously published list of SjS-SNPs(Konsta, Le Dantec et al. 2015). Based on this update, performed in September 2019, we identified 94 non-HLA SjS-associated SNPs corresponding to 63 genes

(Supplementary Table 1). The majority of SNPs were found to be located in non-coding regions, mostly introns (54/94, 57.4%), and include one SNP within a splice donor site (rs2004640), and others in intergenic regions (23/94, 24.5%) (Figure 2A). Of the remaining SNPs, SNPs were located close to the TSS and, among these one has the capacity to provide an additional transcription factor (TF) binding site (rs77571059), and two have the capacity to disrupt TF recruitment, such as rs79407237 affecting interferon regulatory factor 1 (*IRF1*) and rs1800629 affecting the myeloid zinc finger 1 (*MZF1*) binding. Five SNPs are located in exons and represent missense substitutions (mutations). We identified 3 SNPs in 3'UTRs that affect micro-RNA target sites (rs10954213, rs3024498 and rs3733346).

Since genetic variation may affect DNA methylation, thereby providing insights into complex disease phenotypes, the mQTLdb tool was used to link SNPs with methylation marks (Gaunt, Shihab et al. 2016). As presented in Figure 2B, 54/94 (57.4%) Sjs-associated SNPs were associated with meQTLs located in *Cis*-and/or in *trans*, corresponding to 197 individual meQTLs. In contrast to Sjs SNPs, *cis*-meQTLs were more frequently retrieved within TSS (60.8%, Figure 2A), where CpG islands are more frequent. Associations between genomic locations of Sjs-associated SNPs and *cis*-meQTLs were tested further, with the higher associations were observed between intronic Sjs-associated SNPs and TSS *cis*-meQTLs (Figure 2C). Similar genomic area locations were observed between Sjs-associated SNPs and their related *trans*-meQTLs (Figure 2D).

3.2 Linking genetic risk factors and meQTL locations

Sjs-associated SNPs' capacity to control TSS *cis*-meQTLs was related to their capacity to impact *cis*-meQTLs over long physical distances (mean distance: 109 ± 14 kb). Effects were even more pronounced considering intergenic SNPs when compared to all other Sjs-SNPs (Figure 3A). We further tested the capacity of Sjs-associated SNPs to control several meQTLs. As many as 28 independent meQTLs associated with one SNP (rs2569190) with a meQTL/SNP ratio of 2.6 ± 0.5 for each Sjs-SNP. Again, Sjs-associated SNPs' capacity to control meQTLs was statistically independent from the genomic location of the risk factor as tested by determining the *cis*- and *trans*-meQTL/SNP position ratios (Figure 3B/C). Altogether, this indicates that Sjs-associated SNPs' capacity to control qualitatively and quantitatively meQTLs is independent from the SNPs' genomic location.

3.3 Functional activity of genetic risk factors and meQTL location

According to the ENCODE and epigenetic road map programs (Bernstein, Stamatoyannopoulos et al. 2010, Davis, Hitz et al. 2018), the control of DNA methylation may be attributed to the chromatin functional activity at individual SNPs. Accordingly, we ascertained functional activity at Sjs-associated SNP in immune-cells based on histone and CTCF annotation (Figure 4A). H3K4me3 and H3K4me1 (histone 3 lysine 4 tri/mono-methylation) are the predominant histone modifications found in nucleosomes flanking promoter and enhancer elements, respectively, that are active when associated with H3K27Ac (histone 3 lysine 27 acetylation). When present in the gene body, H3K36me3 (histone 3 lysine 36 trimethylation) is associated with active gene transcription. CTCF (CCCTC-binding factor) is a zinc finger protein transcription factor that can bind to transcriptional insulator elements, blocking communication between enhancers and promoters, and its binding is disrupted by DNA methylation.

We found that SNPs associated with active promoter marks (H3K4me3 and H3K27Ac, 14% of all SNPs) were strongly associated with *cis*-meQTL in TSS (26.5% of all meQTLs), exons (7.6%), and introns (14.2%) but this was not the case for 3'UTRs and intergenic meQTLs (Figure 4B). The predominant role of Sjs-associated SNPs with active promoter histone marks on meQTLs was further supported by the observation that these SNPs exhibited higher ratios of *cis*-meQTL ($p=0.003$) and *trans*-meQTLs ($p=0.127$). However, statistical significance was not reached for *trans*-meQTLs (Figure 4C/D). Of note, the 13 Sjs-SNPs associated with active promoter marks were located within TSS (2/7), introns (10/54), and 3'UTRs (1/4), which supports again their independence from the genomic location.

3.4 Integration with EWAS data obtained in Sjs

To better understand the pathophysiological role of meQTLs in Sjs and their association with differentially methylated CpG (DMC) motifs reported in Sjs, the list of 105 genes obtained from the 197 meQTLs was compared with the data from Sjs EWAS available from the literature (Figure 5A). We found that 41 genes overlap between the meQTL database and EWAS datasets. Subsequently gene functional annotation, reactome pathway analysis and protein-protein interaction (PPI) were conducted using STRING V11.0 to investigate the functions and predominant pathways of meQTL target genes. Reactome pathway enrichment analysis

demonstrated enrichment in 13 signaling pathways, including “cytokine activation of the immune system” ($p=9.5 \times 10^{-5}$); IFN-gamma signaling ($p=0.01$); and IL-4/IL-13 signaling ($p=0.02$), the “adaptive immune system” ($p=0.01$), “effects of phosphatidylinositol-4,5-bisphosphate (PIP2) hydrolysis” ($p=0.001$), and “genes regulated by an elevation of the cytosolic calcium level” (Figure 5B). With the analysis of the PPI network using a high confidence ratio of 0.700 (Figure 5C), a total of 23 genes were found to be an important node corresponding to a pro-inflammatory cytokine and NF-kappa B signaling pathways (HSA-1280215 and HSA-5357956, $p < 10^{-15}$) to be controlled by SjS-SNPs through altered DNA methylation (Figure 5C).

Comparing DMCs, analysis revealed that 13 meQTLs belong to DMCs retrieved from experiments conducted in PBMCs, peripheral CD19⁺ B cells, peripheral CD4⁺ T cells, and long-term cultured SGENs (Table 1). With the exception of cg04864179 (*IRF5*), that was retrieved in both PBMC and CD19⁺ B cells data sets, associations were specific and two genomic regions, one covering *PKN1* and Prostaglandin E Receptor 1 (*PKN1/PTGER1*), and another one covering *IRF5*, Transportin 3, Small Cajal body-specific RNA 17 (*IRF5/TNPO3/SnoU12*) were retrieved at least twice. Therefore, these regions were explored further.

3.5 The *PKN1/PTGER1* locus

Two out of three previously reported SjS-associated SNPs were linked with meQTLs in the *PKN1/PTGER1* locus. We identified 4 additional SNPs in high LD (≥ 0.8) to update the meQTL list. Information regarding the impact of the minor variant on DNA methylation was recorded (beta value) from mQTLdb as well as effects of SjS-SNPs on transcription (eQTLs) and splicing (sQTLs) using the GTExPORTAL (Figure 6). Two groups of SNPs were highlighted: One composed of 5 members, including the SjS risk allele rs3786654 (*PKN1*), which was associated with 3 meQTLs including one in the second exon of *PTGER1* (cg19243826), and two meQTLs present in the intergenic region between *PKN1* and *PTGER1*. For this group, a negative effect of the minor allele was reported on (i) DNA methylation; (ii) *PTGER1/GIPCI/C19orf53* gene expression; and (iii) splicing of *GIPCI*. For the second group with only one member, the intronic SjS-associated risk allele rs2241362 (*PKN1*), an opposite effect was observed as the minor allele was associated with a positive effect on DNA methylation, *PTGER1* gene expression, and splicing of *GIPCI* transcripts.

Only one meQTL (cg19243826) was affected by Sjs-associated SNPs from groups I and II and exhibited a positive correlation between DNA methylation and gene expression. As a negative correlation is usually observed, this supports the presence of a regulatory element at that location. Accordingly, the presence of a binding site for the insulator and negative regulator CTCF was tested within the 50 bp of cg19243826 and CTCF binding in this area was retrieved based on ChIP-Seq experiments performed during the ENCODE project (data not shown).

3.6 The *IRF5/TNPO3* locus

Analysis was repeated for the *IRF5/TNPO3/SnoU12* locus showing that (i) 7/14 (50%) DMCs associated with Sjs-SNPs were located within the transcription start sites of the *IRF5* gene, which possesses four TSS referred to as 1A-D (Graham, Kyogoku et al. 2007); (ii) 3/15 (21.4%) DMCs were located within *TNPO3* locus; and (iii) 3/15 (21.4%) DMCs were present in *trans* at chromosomes 1, 17 or 19 (Figure 7).

Based on the genomic location of SNPs and the impact of SNP minor alleles on DNA methylation (β value), eQTLs and sQTLs, three main groups of SNPs were defined. For the first group, corresponding to SNPs located within the *IRF5* locus, the minor allele was associated with (i) DNA methylation within TSS-1D (cg12816198), a pseudo promoter of the *IRF5* locus (Clark, Read et al. 2013); (ii) DNA demethylation at TSS-1A/C of the *IRF5* locus; (iii) *IRF5* overexpression; (iv) an increase of the *IRF5* splice variants; but (v) without effect on *TNPO3* and *RP11-309L24* gene expression although SNP group I meQTLs were detected in the *TNPO3* locus. Group II was related to SNPs present within the distal part of *IRF5*, *TNPO3* and *SnoU12* loci. Impact on the *IRF5* locus was limited to meQTLs located on TSS-1C plus an effect reported on two *trans*-meQTL located on chromosomes 17 (cg17652174) and 19 (cg24748945), respectively.

In contrast to groups I and II, the minor allele of group III (5 SNPs including exon 1B-exon 2 splice polymorphism rs2004640 and 4 SNPs in linkage disequilibrium with the *IRF5* 3'UTR early polyadenylation signal rs10954213) presented opposite effects on DNA methylation status at pseudo-TSS-1D (demethylation) and at TSS-1C (hypermethylation). This mediates resting conditions for *IRF5* transcripts and splice variant repression, while increasing *RP11-309L24* pseudo-gene expression.

4. Discussion

Characterizing the multifaceted contribution of genetic and epigenetic factors to the pathophysiology and clinical phenotypes of autoimmune/inflammatory conditions are major challenges. In this study we investigated links between disease-associated genotypes and epigenetic profiles in SjS integrating available datasets from targeted genotyping approaches, GWAS, and EWAS. *In silico* analysis of 94 SjS-associated non-HLA risk-factors was highlighted by the finding that 85.1% of selected risk variants map to promoter, enhancer, transcription, and insulator regulatory elements located outside coding regions of genes. Among these, SjS risk variants associated with histone marks of active promoters (H3K4me3 and H3K27Ac) were the most effective in controlling DNA methylation at multiple sites. DNA methylation, in turn, regulates transcription by controlling TF binding, or its repression through CTCF recruitment and, in some cases, RNA splicing as exemplified for the *IRF5/TNPO3/SnoU12* and *PKNI/PTGER1* loci. Last but not least, the cross-talk between genetic and epigenetic modifications impacts predominantly a pro-inflammatory cytokine pathway known to be important for the control of DNA demethylation in autoimmune diseases.

Using GWAS approaches, a long list of non-HLA loci, with relatively low impact (Odds ratio: ≤ 2.0), that are involved in a wide variety of innate and adaptive immune processes, have been described in SjS. These include the type I IFN regulated genes *IRF5* and *PKNI* (Lessard, Li et al. 2013, Li, Zhang et al. 2013, Song, Chen et al. 2016, Taylor, Wong et al. 2017). Other genetic associations have been found for SjS and lies with the regulation and activation pathways of B and T cells (e.g. *CXCR5*, *BLK*), and signaling to NF-kappa B (e.g. *TNIP1*). However, very little is known about how the genetic structure of the disease affects the normal biological activity and/or is involved in the molecular pathophysiology of the disease. In this study, and to provide arguments for these questions, we expanded the list of SjS-related SNPs and identified 94 non-HLA SNPs affecting 63 genes. As described in other autoimmune/inflammatory conditions (Farh, Marson et al. 2015), a majority of SjS-associated genetic risk variants map to immune-cell regulatory elements containing histone marks for active promoters and enhancers, active transcription of genes, and insulators. Regulatory SNPs can interfere with the epigenetic network independent of their orientation and/or distance from the TSS.

DNA methylation plays an important role in tissue specific silencing of genes and/or repetitive elements. This is (mainly) attributed to methylation of CpG islands, genomic regions that contain CpG dinucleotide repeats and co-localize to the TSS in up to 70% of human genes (Illingworth and Bird 2009, Fali, Le Dantec et al. 2014). From a mechanistic point of view, the repressive effect of DNA methylation is linked to the recruitment of methylated DNA binding proteins (e.g. MBD2), their association with repressive complexes (e.g. histone deacetylase) and, as a consequence, inhibition of TF recruitment (Boyes and Bird 1992). A regulatory role of DNA methylation outside CpG islands and throughout the gene body (in “shores” and “shelves”) has also been described and they can act: i) as cell-specific and alternative TSS when demethylated and associated with the promoter H3K4me3 histone mark (Garaud, Le Dantec et al. 2009, Maunakea, Nagarajan et al. 2010), ii) as splicing regulators through the recruitment of the methyl-CpG-binding protein MeCP2 (Maunakea, Chepelev et al. 2013), and iii) through the control of chromatin organization and gene expression through the recruitment of CTCF, a DNA binding partner (Wang, Maurano et al. 2012). In this study, analysis was exemplified by two loci *PKNI/PTGER1* and *IRF5/TNPO3/snoU12*. Polymorphism in *PKNI* of SjS patients is associated with increased germinal center formation (Reksten, Johnsen et al. 2014), and *PKNI* knock-down in mice impacts lymphocyte egress from lymphoid organs by controlling cytokine pathways (Wang, Wang et al. 2008, Mashud, Nomachi et al. 2017). In addition to *PKNI*, this study highlights the prostaglandin receptor *PTGER1* because SjS-associated SNPs in *PKNI* are effective in controlling DNA methylation, transcription and splicing of the *PTGER1* locus, possibly through the recruitment of CTCF at cg19243826. Increased prostaglandin A2 expression is characteristic of SjS patients (Tishler, Yaron et al. 1996) but implications of the receptor *PTGER1* is currently unknown.

Regarding the second locus explored, *IRF5* is under the control of alternate promoters and a total of four alternate first exons/TSS have been reported (Clark, Read et al. 2013). However, alternate transcripts do not impact the protein sequence since the (translational) start codon is located in exon 2. In PBMC and SGEC from SjS patients with *IRF5* risk alleles, increased expression of *IRF5* and other type I interferon-induced genes, including *MX1* and *IFITM1*, were reported (Miceli-Richard, Gestermann et al. 2009). The most common *IRF5* risk allele (OR: 1.5-2.0) associated

with SjS is a CGGGG insertion-deletion polymorphism (rs77571059) located -64bp from the TSS-1A, which confers an additional binding site for the transcription factor signaling protein (SP-)1(Sigurðsson, Goring et al. 2008, Nordmark, Kristjansdottir et al. 2011). The methylation level of the CGGGG motif is low (<15% in PBMCs) and did not differ between SjS patients and controls(Gestermann, Koutero et al. 2012), supporting independence from DNA methylation, an assertion that was further substantiated by the absence of meQTLs linked with rs77571059. The second SjS risk allele in *IRF5* (rs2004640) is present in the 3' end of exon 1B (TSS-1B), within a splice donor site. It contributes to the production of a new transcript when the minor allele (T) is present. The third SjS risk factor in *IRF5* (rs10954213) is present in the 3'UTR and creates an early polyadenylation site resulting in stable mRNA resistant to degradation(Rullo, Woo et al. 2010). Both rs2004640 and rs10954213 were referred to as group III with DNA methylation at TSS-1C, *IRF5* transcription and splicing repression, as reported for the SjS protective allele rs13242262(Clark, Read et al. 2013). This supports a protective mechanism involving DNA methylation at TSS-1C to counterbalance the exacerbated effects of rs2004640 and rs10954213 on *IRF5* at the RNA level. Moreover, this study provides an additional explanation for the long list of *IRF5/TNPO3*-associated autoimmune/inflammatory conditions. Indeed, up to 46 SNPs control DNA methylation at cg04864179 and cg00140447, the former being characterized in two independent EWAS studies(Imgenberg-Kreuz, Sandling et al. 2016, Miceli-Richard, Wang-Renault et al. 2016). As a consequence, it's not the SNP alone that defines DNA methylation, but the balance between protective and risk alleles for one meQTL that is important, which opens new perspectives. Last but not least, *IRF5* affects multiple target genes, which is in agreement with our observation that several *trans*-meQTL were associated with *IRF5/TNPO3* polymorphisms(Wang, Sandling et al. 2013).

The first argument that links pathogenic SNPs with DNA methylation was provided by reports of significant associations between SjS-associated genetic risk factors and DMCs from lymphocytes and SGECS(Renaudineau and Ballestar 2016, Charras, Konsta et al. 2017). The second argument came from the description of meQTLs and reports of significant associations between genetic variants and DMCs in SjS, indicating that SjS risk factors potentially affect DNA methylation-sensitive pathways at target CpG sites(Imgenberg-Kreuz, Sandling et al. 2016, Charras, Konsta et al.

2017). However such assertion derives from data obtained from long-term cultured SGEC and peripheral blood lymphocytes, which could not exclude that *in situ* variables may be different if *ex vivo* salivary gland epithelial cells and lymphocytes were used. The third argument was delivered by the description of a histone-guided mechanism of DNA methylation, particularly by H3K4me3(Guo, Wang et al. 2015, Sharifi-Zarchi, Gerovska et al. 2017), through the recruitment of the ten eleven translocation (TET) enzymes that oxidize 5-methylcytosines (5mCs) for demethylation(Deplus, Delatte et al. 2013). Altogether, these arguments are in agreement with our observation that SjS-associated risk factors that are accompanied by active promoter marks (H3K4me3) are more effective in controlling meQTLs when compared to SNPs associated with histone marks of enhancers (H3K4me1) or active transcription (H3K36me3). As a consequence, studying links between genetic risk and disease-associated meQTLs may help to develop mechanistic insights into altered pathways leading to DNA methylation dysregulation in complex diseases such as SjS.

DNA methylation is not only controlled by genetic variants, but also by environmental factors. The influence of environmental factors is amplified in autoimmune/inflammatory diseases as established in disease-discordant monozygotic twins(Fraga, Ballestar et al. 2005, Javierre, Fernandez et al. 2010). In SjS, reduced global DNA methylation and increased DNA hydroxymethylation was reported in salivary glands and linked with disease-associated lymphocyte infiltration(Konsta, Charras et al. 2016, Konsta, Le Dantec et al. 2016, Lagos, Carvajal et al. 2018). Cellular interactions with infiltrating B cells are suspected of being pathognomonic based on co-culture model experiments and on the observation that salivary gland epithelial cell DNA demethylation can be reversed when treating patients with the B cell depleting agent rituximab(Thabet, Le Dantec et al. 2013). Moreover, the Jak/STAT and TNF/TNF-R signaling pathways control DNA demethylation through induction of TET enzymes that confer DNA demethylation(Cole, Quach et al. 2016, Charras, Arvaniti et al. 2019). The Jak/STAT pathway can be either activated by pro-inflammatory cytokines such as type I interferons that are predominantly produced by infiltrating monocytes in minor salivary glands(Brkic, Maria et al. 2013) or by epithelial cells themselves in response to oxidative stress(Cole, Quach et al. 2016, Lagos, Carvajal et al. 2018). We previously demonstrated that this can be reversed

with the use of JAK inhibitors(Charras, Arvaniti et al. 2019). As a consequence strong arguments support the concept that TET enzymes could act at the cross-road between genetic and environmental factors based on the control of TETs by H3K4me3(Deplus, Delatte et al. 2013) and by the pro-inflammatory Jak/STAT and TNF/TNF-R pathways, respectively. Another argument for this hypothesis is related to the characterization of a pro-inflammatory cytokine pathway as a major node controlled by SjS-associated SNPs through altered DNA methylation. This observation is in agreement with the pro-inflammatory cytokine-related epigenetic signature reported in autoimmune diseases including SjS, systemic lupus erythematosus, and rheumatoid arthritis(Nakano, Whitaker et al. 2013, Ulf-Moller, Asmar et al. 2018, Carnero-Montoro, Barturen et al. 2019) .

In conclusion, this study establishes a new approach that links genetic and epigenetic profiles in the pathogenesis of SjS. Results reveal a strong association between genetic risk, meQTL, and DNA methylation controlling *cis*- and *trans*-regulatory events. This approach may represent a valuable tool to unveil pathogenic mechanisms in autoimmune/inflammatory diseases, to stratify patients based on molecular events, and to select and/or propose future therapeutic options. However, additional work needs to be done to confirm experimentally the cell specificity, interplay with the pro-inflammatory cytokine signaling pathway and TETs, the functional role of the characterized regulatory SNPs and their association with meQTL.

Disclosures

The authors have no conflicts of interest relevant to this manuscript.

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Abbreviations: SjS: Sjögren's syndrome, GWAS: Genome wide association studies, QTL: quantitative trait loci, HLA: human leucocyte antigen, SNPs: single nucleotide polymorphisms, IFN: interferon, EWAS: epigenome wide association studies, SGECs: salivary gland epithelial cells, IRF5: IFN-regulatory factor 5, STAT4: signal transducer and activation of transcription 4, PBMCs: peripheral blood mononuclear cells, SGECs: salivary gland epithelial cells, DMCs: differentially methylated CpGs, eQTL: expression quantitative trait loci, meQTL: methylation quantitative trait loci, BRD1: Bromodomain 1, RA: rheumatoid arthritis.

Table 1: DNA methylation CpG motifs (DMC) retrieved from both methylation quantitative trait loci (meQTLs) associated with Sjögren’s risk alleles, and from cell-specific epigenome-wide association studies (EWAS) conducted in patients with Sjögren’s syndrome. **Abbreviations:** Chr: chromosome; CD4: CD4+ T cells; CD19: CD19+ B cells; PBMC: peripheral blood mononuclear cells; SGEC: salivary gland epithelial cells; TSS: transcription start site; *MAPKAPK2*: MAP kinase-activated protein kinase 2, *NLRP3*: NOD-, LRR- and pyrin domain-containing protein 3, *OR2B11*: Olfactory receptor 2B11, *LINC01117*: Long Intergenic Non-Protein Coding RNA 1117, *DGKQ*: Diacylglycerol Kinase Theta, *LTA*: Lymphotoxin-alpha, *BAK1*: BCL2-antagonist/killer 1, *HLA-C*: Human leucocyte antigen-C, *IRF5*: Interferon regulatory factor 5, *B3GNTL1*: beta-1,3-N-acetylglucosaminyltransferase like 1, *PTGER1/GIPCI*: Prostaglandin E Receptor 1/PDZ domain containing family member 1.

DMC	Chr	position	location	Loci	CD4	CD19	PBMC	SGEC
cg10336193	1	206848389	intergenic	<i>MAPKAPK2</i>	0	+	0	0
cg22494876	1	247618161	intergenic	<i>NLRP3-OR2B11</i>	0	0	+	0
cg22386008	2	177737177	intergenic	<i>LINC01117</i>	0	+	0	0
cg22718636	4	961658	intronic	<i>DGKQ</i>	+	0	0	0
cg21699330	6	31540313	intronic	<i>LTA</i>	0	0	+	0
cg19869469	6	33561266	TSS	<i>BAK1</i>	0	0	0	+
cg10409680	6	31239320	intronic	<i>HLA-C</i>	+	0	0	0
cg12816198	7	128577593	TSS	<i>IRF5</i>	0	+	0	0
cg13914531	7	128579876	TSS	<i>IRF5</i>	0	0	+	0
cg04864179	7	128579964	TSS	<i>IRF5</i>	0	+	+	0
cg17652174	17	81008971	Intronic	<i>B3GNTL1</i>	0	+	0	0
cg19243826	19	14584926	exonic	<i>PTGER1/GIPCI</i>	0	0	0	+
cg20742389	19	14591033	intronic	<i>PTGER1/GIPCI</i>	0	+	0	0

Figures

Figure 1 : Summary of bioinformatic analysis conducted. **A-** Out of the 94 non-HLA Sjögren's syndrome (SjS) single nucleotide polymorphisms (SNPs) were selected and among them 54 SNPs were associated with methylation quantitative trait loci (meQTLs) using the GWAS catalog (<https://www.ebi.ac.uk/gwas/>) and mQTLdb (<http://www.mqtl.org/>) tools, respectively. SNPs and meQTLs were annotated according to their genomic position and to their respective distance. The functional relevance of SNPs in immune cells was collected following manual annotation using HaploReg v4.1, SNP nexus and VEP tools (<https://pubs.broadinstitute.org/mammals/haploreg;> <https://www.snp-nexus.org;> <https://www.ensembl.org/Tools/VEP>). **B-** Comparison of the generated meQTL database with epigenome-wide association studies (EWAS) obtained from SjS patients to define SjS-meQTLs genes and DNA methylation CpGs (DMC). **C-** The SjS-meQTL gene dataset was tested for reactome pathway enrichment and for protein-protein interaction network (<https://string-db.org>). **D-** From the SjS-meQTL DMC data set 10 loci were highlighted and among them the two main loci were further analyzed to test SjS SNP/meQTLs influence on transcription and RNA splicing (<https://gtexportal.org>). The bioinformatic webtools are indicated.

Figure 2: Identification of epigenetically controlled genetic risk factors for Sjögren's syndrome and relation with single nucleotide polymorphisms (SNP) genomic location. **A-** Genomic location of the 94 non-human leucocyte antigen (non-HLA) SjS-SNPs, annotated to 63 genes, the 211 *cis*-meQTLs, and the 32 *trans*-meQTLs. **B-** Proportion of SjS-risk factors associated with *cis*- and/or *trans*-methylation quantitative trait loci (meQTL). **C-** Heatmap showing that SjS-SNPs are predominantly associated with *cis*-meQTL located in a transcription start site (TSS: 54.5% of all *cis*-meQTLs), and the higher association is retrieved with intronic SjS-SNPs (36.0% of all *cis*-meQTLs). **D-** SjS SNPs and associated *trans*-meQTL are located at the same genomic location, although not on the same chromosome. **Abbreviations:** 3'UTR: 3' untranslated region; IG: intergenic.

Figure 3: Distance and quantitative interplay between SjS-associated SNPs and meQTLs are independent from SNP genomic location. **A-** The importance of SNP genomic location on its distance from *cis*-meQTLs is tested, by definition *cis*-

meQTLs are located in an area ranging from 1bp to 1Mb from SNPs. Longer distances are reported for SNPs located within intergenic areas when compared to others (ANOVA test with Tukey's correction). **B-** The number of *cis*-meQTLs associated per SNP is statistically independent from the genomic location of the risk factor, results are expressed as ratios. **C-** The number of *trans*-meQTLs associated per SNP according to its genomic location. **Abbreviations:** SNP: single nucleotide polymorphism; TSS: transcription start site; 3'UTR: 3' untranslated region.

Figure 4: Functional activity at single nucleotide polymorphisms (SNPs) is critical for its association with meQTLs. **A-** Chromatin states used to define functional activity at SNPs, adapted from Ernst & Kellis³⁴. The prevalence is reported. **B-** Heatmap showing *cis*-meQTL genomic location according to Sjs-associated SNP functional annotation variations. The stronger activity on *cis*-meQTLs is reported for Sjs-SNPs located in active promoters. **C-** *Cis*-meQTL number per SNP according to its functional activity results are expressed as ratios. **D-** *Trans*-meQTL number per SNP. **Abbreviations:** SNP: single nucleotide polymorphism; TSS: transcription start site; 3'UTR: 3' untranslated region; IG: intergenic.

Figure 5: Search for pathophysiological pathways controlled by Sjs-associated SNPs through altered DNA methylation. **A-** Venn diagram to compare genes obtained from the Sjs-associated SNP meQTL gene database and EWAS gene data (<http://bioinformatics.psb.ugent.be/webtools/Venn/>). **B-** Reactome pathway enrichment of the 41 Sjs-SNP meQTL genes reveals 13 statistically significant pathways. The number of genes per pathway is indicated as well as the Log10 of p values. **C-** Protein-protein-interaction analysis of the 41 Sjs-SNP meQTL genes using STRING V11.0. Network nodes represent proteins and edges represent protein-protein association (a high confidence score of 0.7 was used)

Figure 6: Genetic control of DNA methylation and gene expression at the *PKN1/PTGER1/GIPC1* locus (chromosome 19p13.12). Genomic organization of the 12 *cis*-meQTLs controlled by Sjögren's syndrome (Sjs) risk factors at PKN1 locus (n=2 in bold, rs3786654 and rs2241362). To complete this analysis, 4 single nucleotide polymorphisms (SNPs) in strong linkage disequilibrium ($r^2 > 0.8$) with Sjs risk factors were added. Collected from the mQTLdb, the beta value reflects SNP minor allele up- (>0.2) or down-regulation (<-0.2) on DNA methylation, and a semi-

quantitative colored scale was used. According to the impact of SNP minor allele on transcription (eQTL) and splicing (sQTL) in *cis*, SNPs were subdivided in two groups (I and II). Methylation levels (eQTL), splicing rate (sQTLs) and methylation levels (meQTLs) are positively correlated in each group at cg19243826, which is in agreement with the presence of a CTCF binding site controlled by SNP-meQTLs at cg19243826 (CTCFBSDB 2.0 database and ENCODE).

Figure 7: Genetic control of DNA methylation and gene expression at the *IRF5/TNPO3* locus (Chromosome 7q32.1). Genomic organization of the 11 *cis*-meQTLs and 3 *trans*-meQTLs controlled by Sjögren's syndrome (SjS) risk factors at *IRF5/TNPO3* locus (in bold). To complete this analysis, single nucleotide polymorphisms (SNPs) in strong linkage disequilibrium ($r^2 > 0.8$) with the 6 SjS risk factors were added (in bold). Collected from the mQTLdb, the beta value reflects SNP minor allele up- (> 0.2) or down-regulation (< -0.2) on DNA methylation, and a semi-quantitative colored scale was used. According to the impact of the SNP minor allele on transcription (eQTL) and splicing (sQTL) in *cis*, SNPs were subdivided in three groups (I negative effect limited to *IRF5*, II negative effect on *IRF5/TNPO3/RP11-309L24*, and in blue group III positive effect on *IRF5* and *TNPO3*). Methylation levels (eQTL) and splicing rate (sQTLs) are negatively correlated with methylation levels (meQTLs) in each group, supporting the concept that DNA methylation is a repressive epigenetic mark at *IRF5* transcription start sites (TSS)-1D/B/C. *IRF5* is under the control of four alternative promoters (TSS-1A to TSS-1D).

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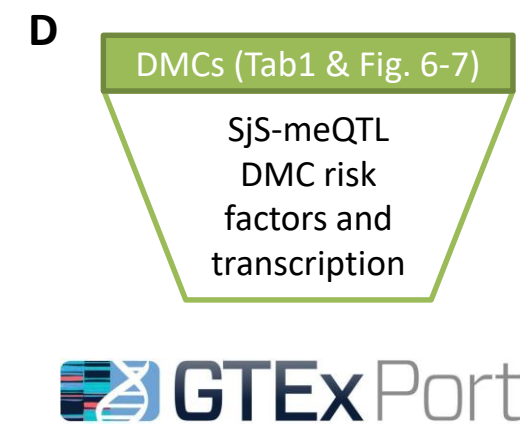
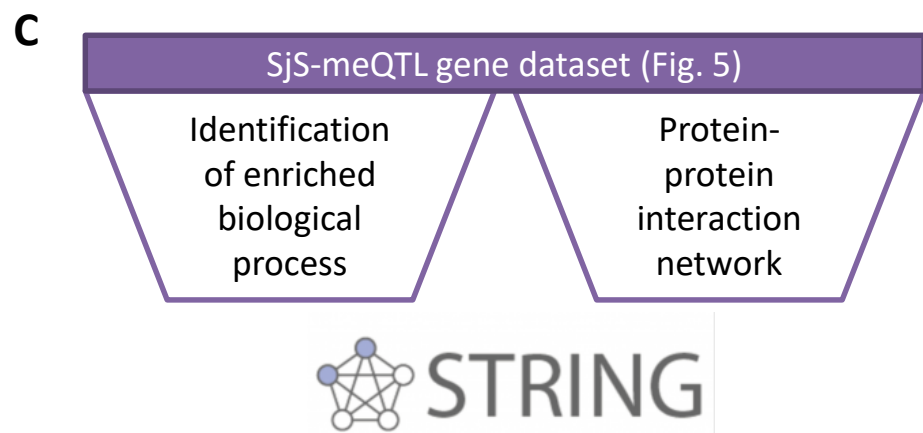
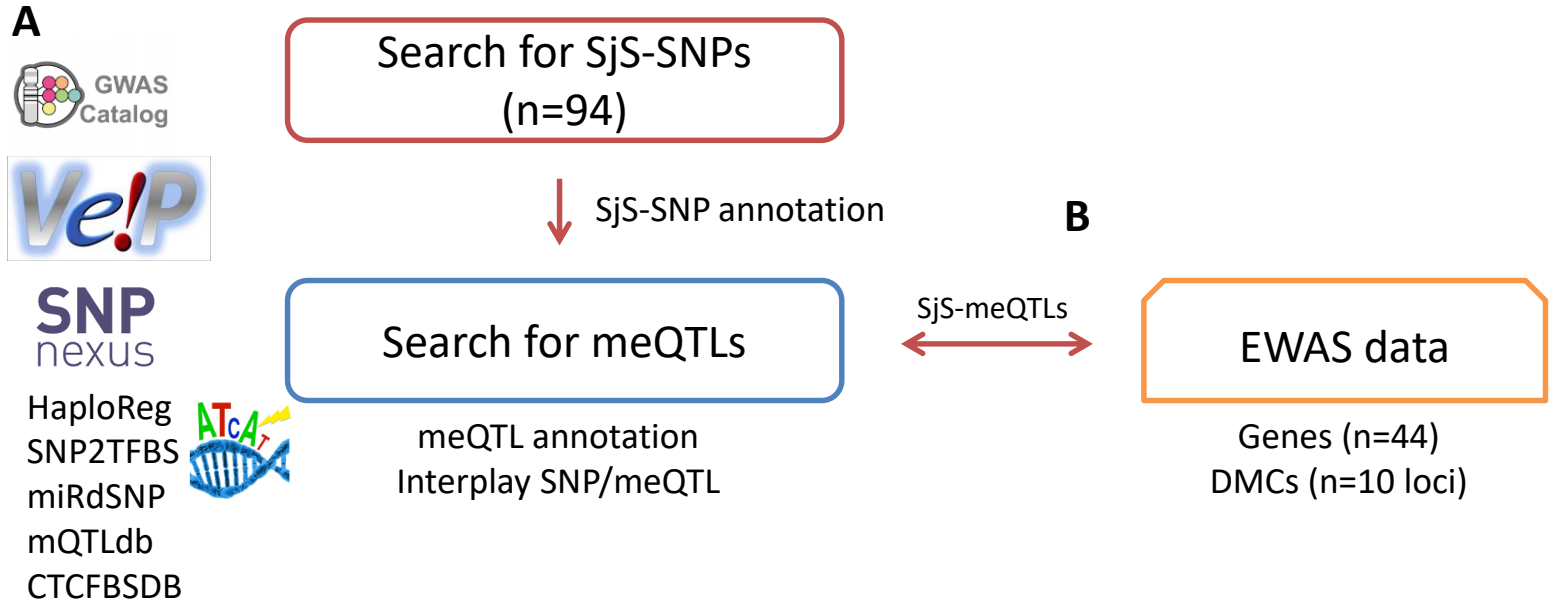
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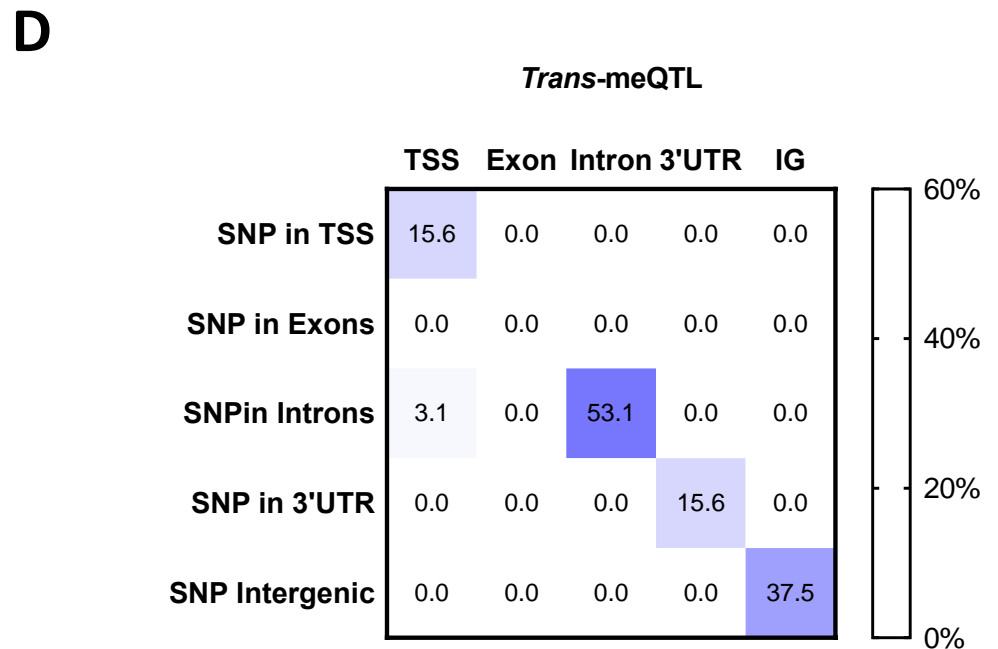
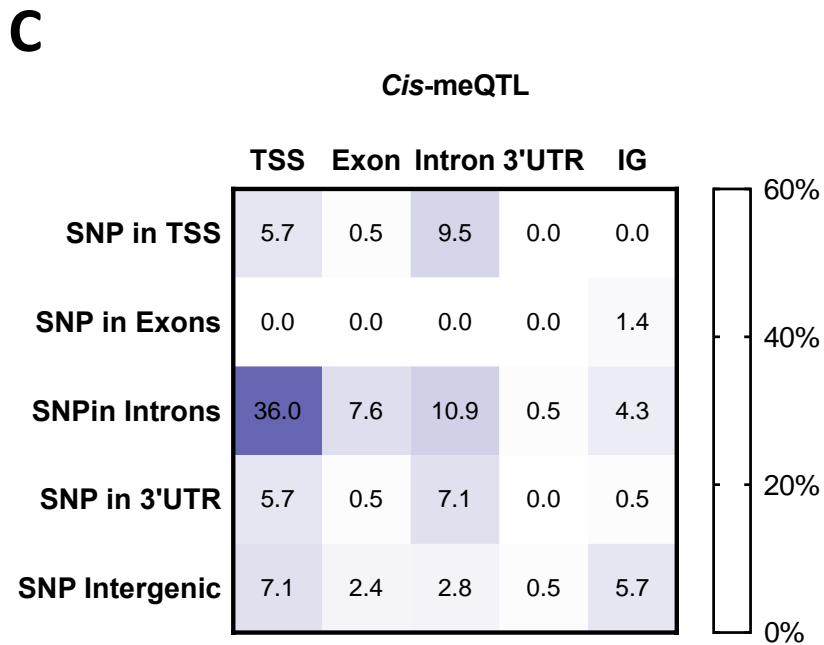
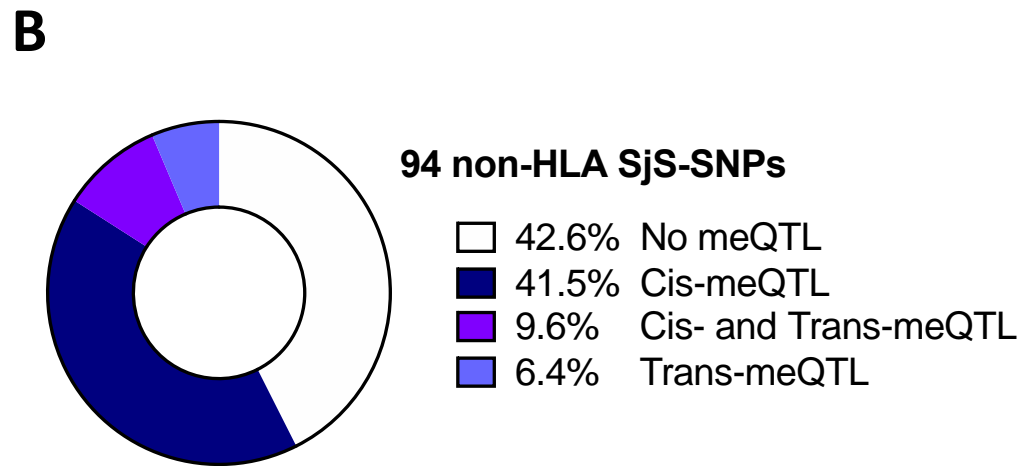
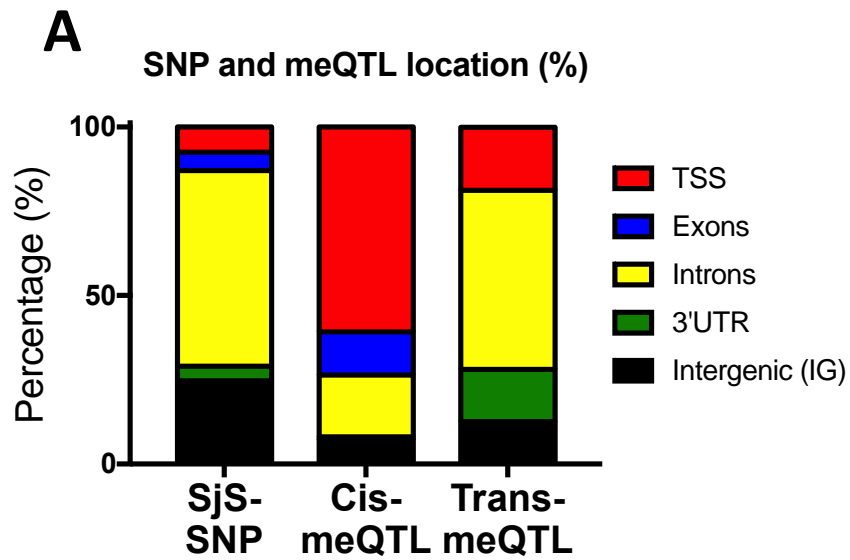
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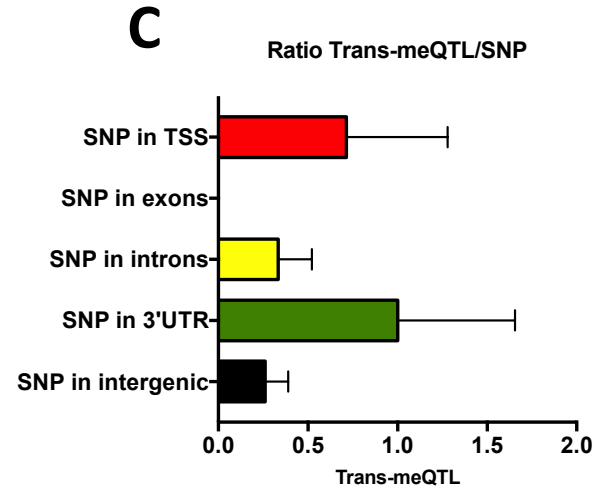
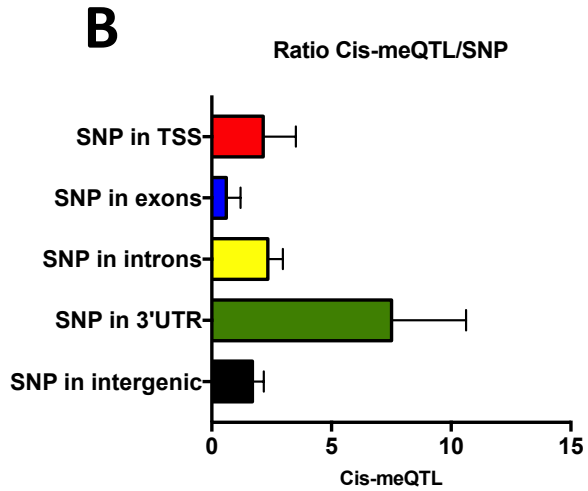
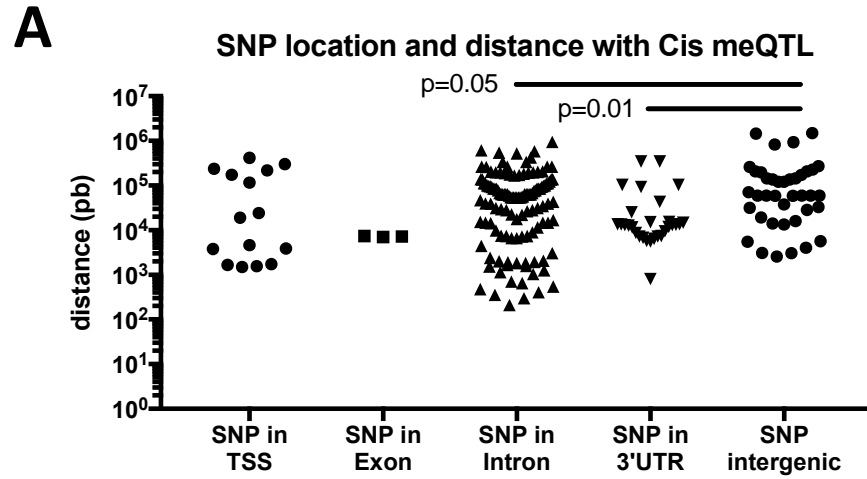
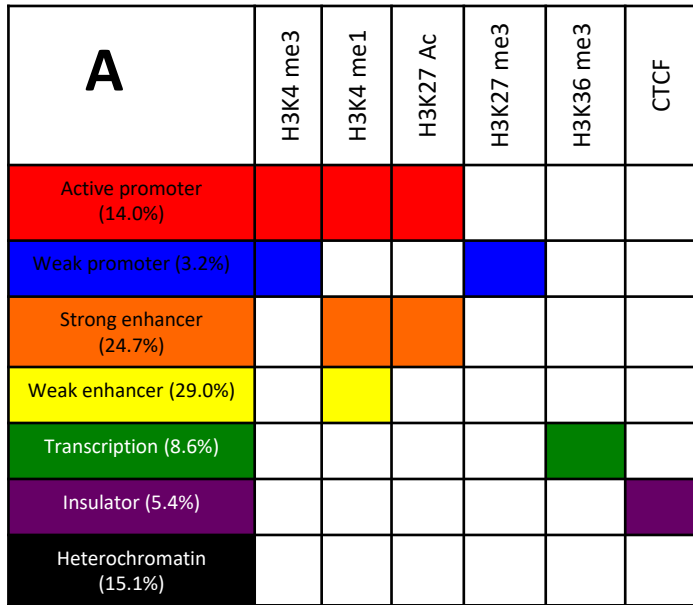
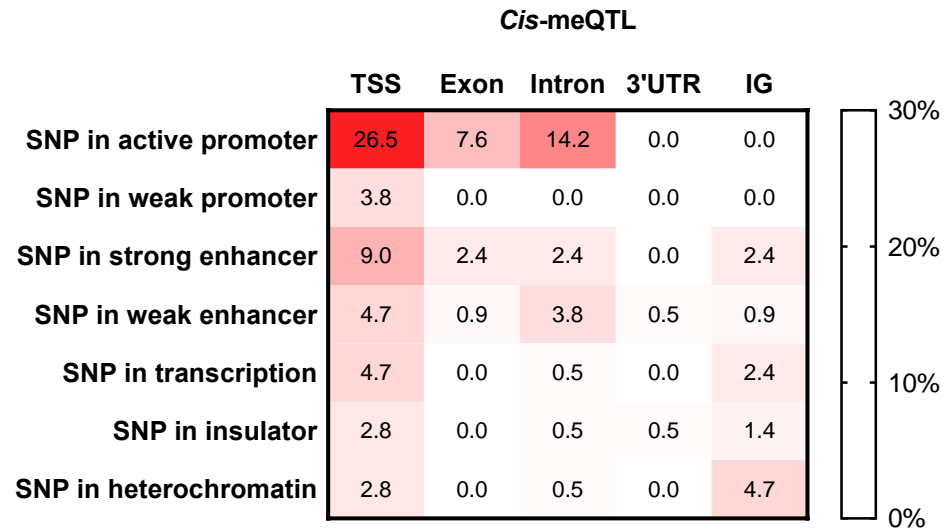


Figure 4

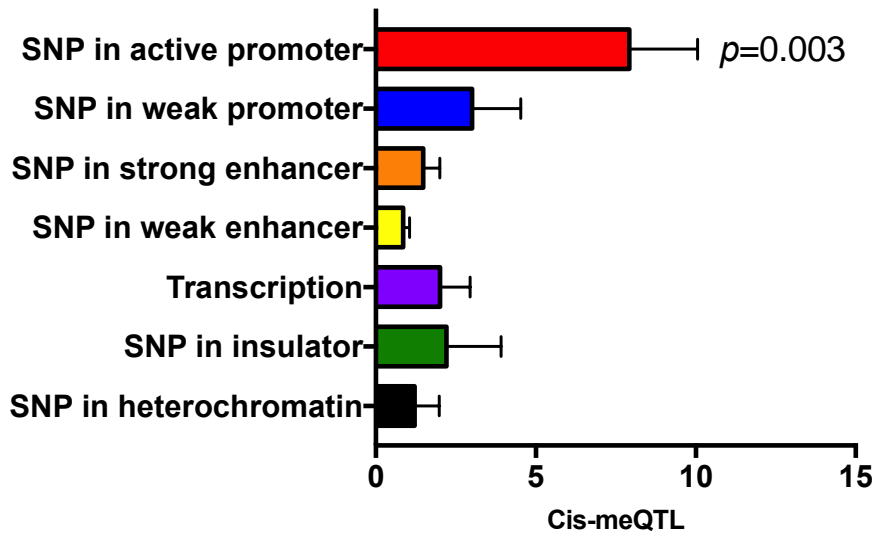


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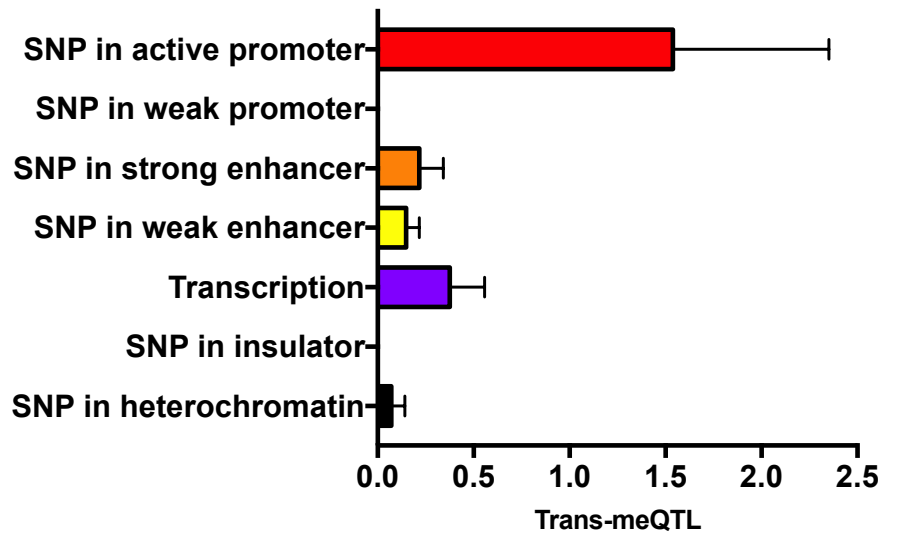
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Ratio Cis-meQTL/SNP

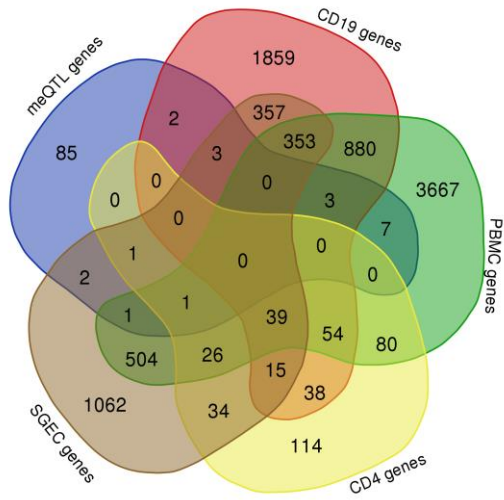


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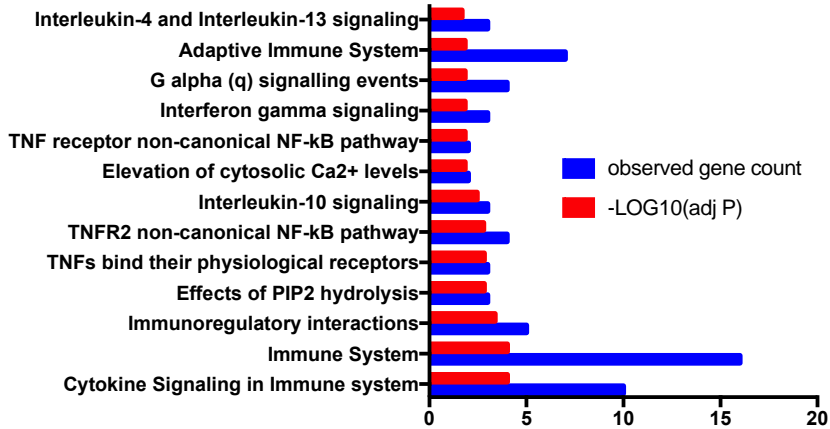
Ratio Trans-meQTL/SNP



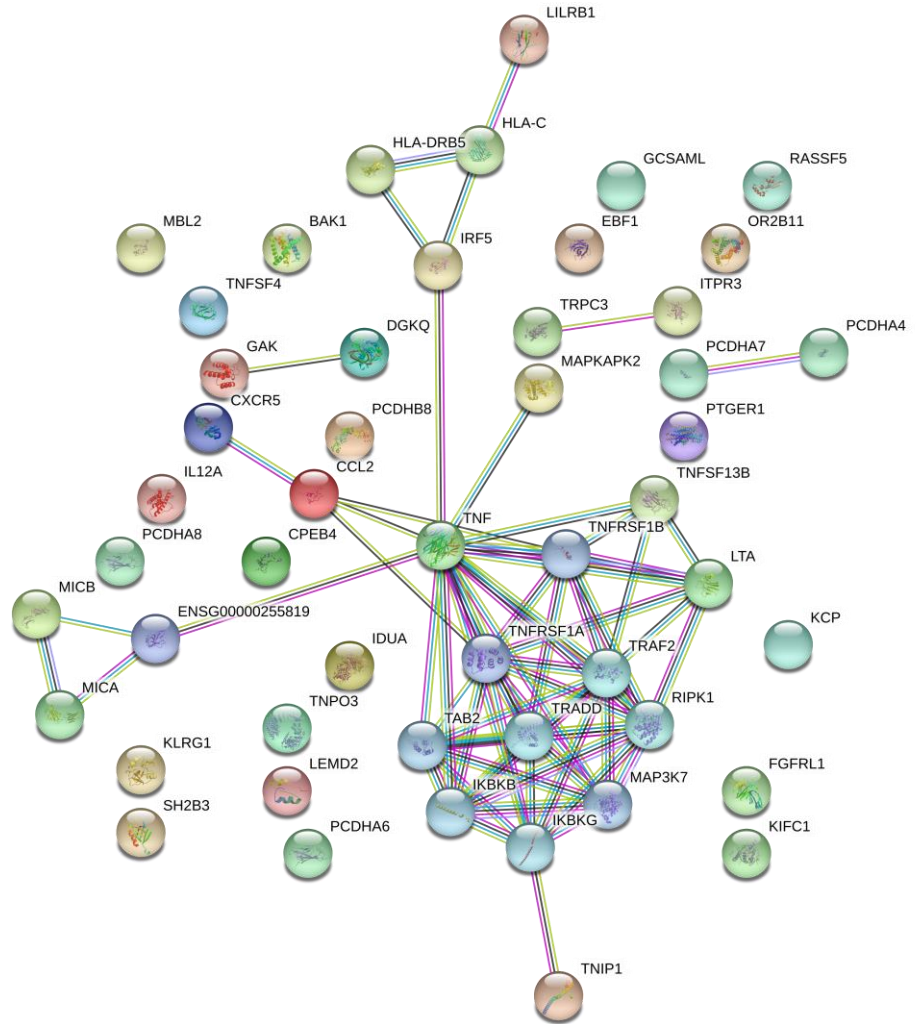
A



B



C



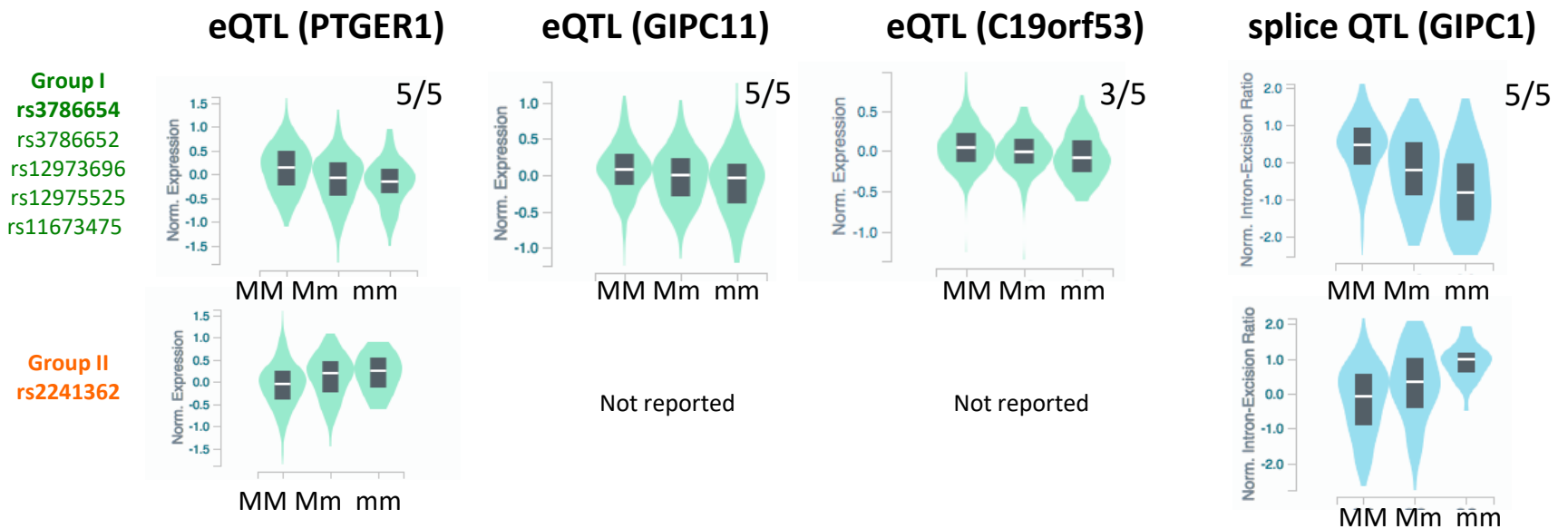
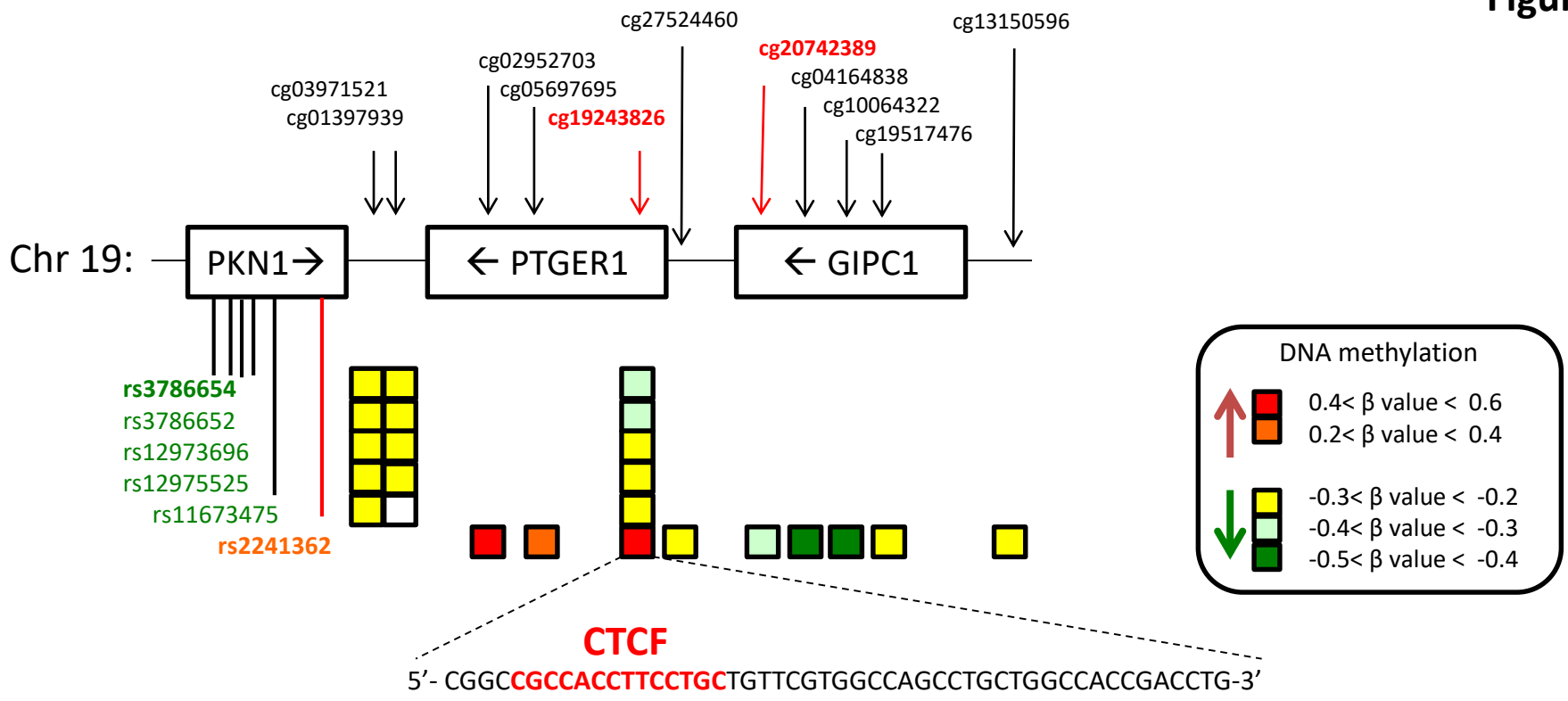


Figure 7

