# The *JAK2* 46/1 haplotype influences PD-L1 expression

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## Running Title

PD-L1 expression as a driver of MPN inherited risk.

## Key Points

- PD-L1 expression is increased in 46/1 haplotype carriers.
- 3D chromatin structure differs between *JAK2* haplotypes and non-coding elements in the *JAK2* loci regulate both *JAK2* and *PD-L1* expression.

## Abstract

Although described more than a decade ago, the mechanism by which the *JAK2* 46/1 haplotype increases the risk of developing myeloproliferative neoplasms (MPN) remains unexplained. Inflammation and immunity are linked to MPN development and thus could be relevant to the mechanism by which 46/1 mediates its effect. Here, we show that PD-L1 expression is elevated in 46/1 haplotype both in healthy carriers and CD34+ cells from MPN patients. Using circular chromosome conformation capture (4C-seq) we observe that *PD-L1* and the neighboring *PD-L2* loci physically interact with *JAK2* in a manner that differs between 46/1 and non-risk haplotypes. Finally, we show through CRISPR/Cas9 genome editing that a non-coding region within *JAK2* intron 2 influences both *JAK2* and *PD-L1* expression. We suggest that increased *PD-L1* expression may be one of the mechanisms by which 46/1 leads to an increased inherited risk of developing MPN.

## Introduction

Classic Philadelphia-negative myeloproliferative neoplasms (MPN) are clonal hematopoietic stem cell (HSC) disorders characterized by the proliferation of one or more cell lineages[1]. A key component in their pathogenesis is aberrant cytokine pathway signaling, caused in most cases by coding mutations in *JAK2*, *CALR* and *MPL*[1].

The great majority of MPN cases are sporadic (with no familial aggregation), however several common inherited genetic variants have been associated with an increased inherited risk of developing an MPN[2,3]. Of these, the *JAK2* 46/1 haplotype, a >200Kb region at 9p24.1 which includes the *JAK2, INSL4* and *INSL6* genes, is probably the most relevant as it explains around 28% of the population attributable risk of developing an MPN[4]. This is a common haplotype, as it is present in 50% of the population, but it increases up to around 70% in MPN patients carrying *JAK2* coding mutations. This haplotype was described more than a decade ago but, despite its obvious importance and clinical associations (i.e. 46/1 is associated with higher increase rate of JAK2 V617F allele burden in polycythemia vera or lower constitutional symptoms -and better survival- in myelofibrosis)[5,6], the mechanism by which it exerts its effects remains unclear[4,7,8]. Two non-exclusive hypotheses have been proposed, the hypermutability of *JAK2* on the 46/1 haplotype and the 'fertile ground' hypothesis in which *JAK2* V617F is proposed to have a selective advantage if it is acquired on 46/1[3,9].

Although not part of the 46/1 haplotype, the gene encoding CD274/PD-L1 (programmed death-1 receptor ligand, hereafter referred to as *PD-L1*) is also located at 9p24.1, about 320kb proximal to *JAK2*. This is of interest because *PD-L1* is overexpressed in *JAK2* V617F-mutated MPN cases, potentially leading to immune escape[10]. Furthermore, *PD-L1* is expressed on disease-initiating MPN stem cells and the degree of *PD-L1* expression in CD34+ cells has been linked to *JAK2* V617F mutational burden[11,12]. Since both the presence and mutational burden of *JAK2*

V617F is strongly associated with 46/1[4,7,8,13] we aimed to explore if there was any relationship between 46/1 and *PD-L1* expression, driven by mechanisms such as the existence of cis-regulatory elements within the region or structural alterations to chromatin, independent of *JAK2* V617F status.

## Methods

### Patients and samples

Peripheral blood was collected from healthy blood donors (age range 18-70 years old, normal blood counts, no relevant diseases) after informed consent was obtained. Frozen CD34+ cells from JAK2 V617F positive MPN patients were obtained from the Hospital Universitario 12 de Octubre Biobank. This study was conducted according to the biomedical research guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Hospital Universitario 12 de Octubre (Nº 16/096). TaqMan SNP Genotyping Analysis (C_31941696_10, Thermofisher) was used for patients haplotyping (See Supplemental Material)

### PD-L1 protein levels determination

PD-L1 expression in leukocyte populations and cell lines (K562 and HEL) was studied by flow cytometry of viable cells by staining with PD-L1 PE conjugated antibody (Biolegend (San Diego, CA, USA) on a FACSCanto II cytometer (BD Biosciences, San Jose, CA, USA). For CD34+ cells from MPN patients, PD-L1 ELISA was used (Thermo Scientific, ref. BMS2327). For further details, see Supplemental Material.

### Circular Chromosome Conformation Capture: 4C-seq

4C technology allows the identification of the interactions that take place between a defined genomic region (viewpoint) and the rest of the genome. The protocol we used is described in the Supplemental Material, but in brief we designed primers to study 7 viewpoints around and within the 46/1 *JAK2* haplotype selected by the

presence of markers of regulatory activity. 4C-seq was performed as previously described[14] on $10^7$ granulocytes purified from peripheral blood by Ficoll gradient (Ficoll-Paque® PLUS, GE Healthcare (2 nullizygous for the 46/1 haplotype and 2 homozygous for the 46/1 haplotype). Quantification of the interactions between each viewpoint and PD-L1 and PD-L2 regions (chr9:5447958-5470872 and chr9:5507588-5570246, respectively) were performed by calculating the sum of the normalized reads counts for each sample within the regions of interest. To study significant contacts, the frequency of captured sites per window was used to fit a distance decreasing monotone function and Z-scores were calculated from its residuals using a modified version of FourCSeq[15]. Significant contacts were considered in cases where the Z-score was >2 in both replicates and deviated significantly (adjusted p value <0.05) from its normal cumulative distribution in at least one of the replicates. After data analysis, processed reads and interactions were visualized at the WashU EpiGenome Browser (https://epigenomegateway.wustl.edu/).

Cell culture and transfection and CRISPR experiments

K562 and HEL cell lines were cultured in RPMI supplemented with 100 U/mL penicillin-streptomycin (Gibco, 15140122), 2 mM glutamine (Gibco, 25030123), and 10% (v/v) fetal bovine serum (Gibco, 10270106). For genome editing, K562 cells were transfected with the CRISPR/Cas9 gene editing-tool as described[16]. 5 x $10^5$ K562 cells were plated in MW6 and transfected with 1.25 µg of each of the plasmids using lipofectamine 3000 following manufacturer's instructions. For HEL cells, 5 x $10^6$ cells were electroporated with 35ug each of the plasmids using the Bio-Rad Gene Pulser II with the following conditions: 975µF y 264mV and 22ms. Forty-eight-hours after transfection, single GFP+ cells were sorted using a FACSAria Fusion (BSC II). Clones were grown and DNA was extracted and genotyped using QuickExtract Solution (Lucigen). For HEL cells, two rounds of electroporation were performed to obtain a high deletion efficiency. K562 cells were treated with 10ng/ml of human IFN-gamma overnight (PEPROTECH; Ref. 300-02). Guides and primers used for genotyping are described in Supplemental Table 4. For quantitative PCR methods, see Supplemental Material.

Statistics

Student's t-test or one-way ANOVA (GraphPad PRISM 8.4.3) were used for independent samples depending on the experimental design.

## Results and Discussion

To explore the possibility that PD-L1 expression is influenced by *JAK2* haplotype status, we performed flow cytometry to measure PD-L1 levels in different peripheral blood cell populations from a cohort of healthy individuals that had been selected as either homozygous or nullizygous for 46/1 (n=37 each). As shown in Figure 1A, there is a clear tendency towards overexpression of PD-L1 in all cell-types in 46/1 homozygotes, which was statistically significant for natural killer lymphocytes and monocytes. We also studied PD-L1 levels in CD34+ cells from JAK2 V617F+ MPN patients (n= 11 and 9 for 46/1 nullizygous and homozygous, respectively) and observe a clear tendency towards higher expression in 46/1 homozygous carriers (Figure 1B).

As the *PD-L1* locus lies in close proximity to the *JAK2* gene and the 46/1 haplotype block, we hypothesized that transcriptional regulatory elements located within the haplotype might physically interact with *PD-L1* and influence its expression. Therefore, we selected 7 regions (viewpoints, VP) within or in the immediate vicinity of the haplotype (see Supplemental Material for details) to map long-distance chromatin interactions through circular chromosome conformation capture followed by deep sequencing (4C-seq). Using neutrophils from 46/1 nullizygous (n=2) and 46/1 homozygous (n=2) healthy donors, we found that the *JAK2* locus has contacts with both *PD-L1* and *PD-L2* loci. Furthermore, the 3D chromatin interactions between the *JAK2* and PDL1/2 regions were different between 46/1 homozygotes and 46/1 nullizygotes (Figure 2).

Finally, we aimed to identify putative transcriptional cis-regulatory elements present in the *JAK2* haplotype that could be involved in the regulation of *PD-L1*. We selected a 12kb region located in *JAK2* intron 2 based on the location of strongly linked 46/1 SNPs (E_i2, Figure 3A and Supplemental Material), and deleted it with CRISPR-Cas9 in the myeloid cell-line K562. Correctly deleted cells, both heterozygous and homozygous, together with non-deleted controls (Supplemental Figure 3A), were isolated and the expression of *JAK2*, *PD-L1*, and *RIC1* (a gene neighboring the *PDL1/2* locus with minimal *JAK2* contacts; Figure 2) were measured. Expression of *PD-L2* is barely detectable in myeloid cells, so we did not include it in the analysis. Deletion of E_i2, either one or two copies, led to a strong reduction of *JAK2* mRNA levels, and *PD-L1* expression was also reduced but only when both alleles were deleted (Figure 3B). *RIC1* expression did not change in either heterozygous or homozygous deleted clones. As endogenous expression of *PD-L1* in K562 cells is low, we increased its levels by stimulating cells with interferon gamma[17,18] (Supplemental Figure 3B), observing the same reduction in *JAK2* and *PD-L1* expression in the deleted cells (Figure 3C). In addition, analysis of protein levels by flow cytometry also showed a decrease of PD-L1 in the homozygous clone. (Supplemental Figure 3C). Finally, we decided to study the effect of E_i2 deletion in the presence of the *JAK2* V617F mutation. For this, we used the HEL cell-line, that contains multiple copies of *JAK2* by amplification of the genomic region[19]. We quantified the degree of deletion of *JAK2* in multiple clones (Supplemental Figure 3D, E) and observed that highly edited HEL cells present lower levels of PD-L1 protein (Supplemental Figure 3F). The low levels of *PD-L1* in K562 might also explain the paradoxical observation that heterozygous deletion leads to higher levels of *PD-L1* compared to controls (Figure 3B, middle panel), that is surely caused by increased variability due to low expression. Thus, our data identifies a regulatory element located in the *JAK2* haplotype that influences *PD-L1* expression.

It has been shown that only a minority of patients with clonal hematopoiesis of indeterminate potential associated with the *JAK2* V617F mutation develop an MPN and that inflammation/immunity as well as constitutional genetics plays a central role in MPN progression[20–23]. In this context, the PD1/PD-L1 axis has been

implicated in playing a key role in MPN development after acquisition of *JAK2* V617F[10]. We have shown that the *JAK2* 46/1 haplotype influences *PD-L1* expression levels and that this may be mediated by interacting transcriptional regulatory elements within the *JAK2* locus. Further investigations are needed to confirm our hypothesis that increased *PD-L1* expression is part of the mechanisms by which 46/1 leads to an increased inherited risk of developing MPN.

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## Authorship Contributions

GC-T. designed the study, performed experiments, analyzed and interpreted the data and wrote the manuscript with the support of MM, JM-L, RA and NCPC. RR participated in 4C experiments and analysis. WJT performed the analysis to identify 46/1 SNPs. AL assisted with sample processing and FACS studies. JV, MT, AJC and AM participated in the molecular characterization of *JAK2* haplotypes interactions with *PD-L1* expression. RG-V performed and analyzed PD-L1 ELISA.

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## Figure Legends

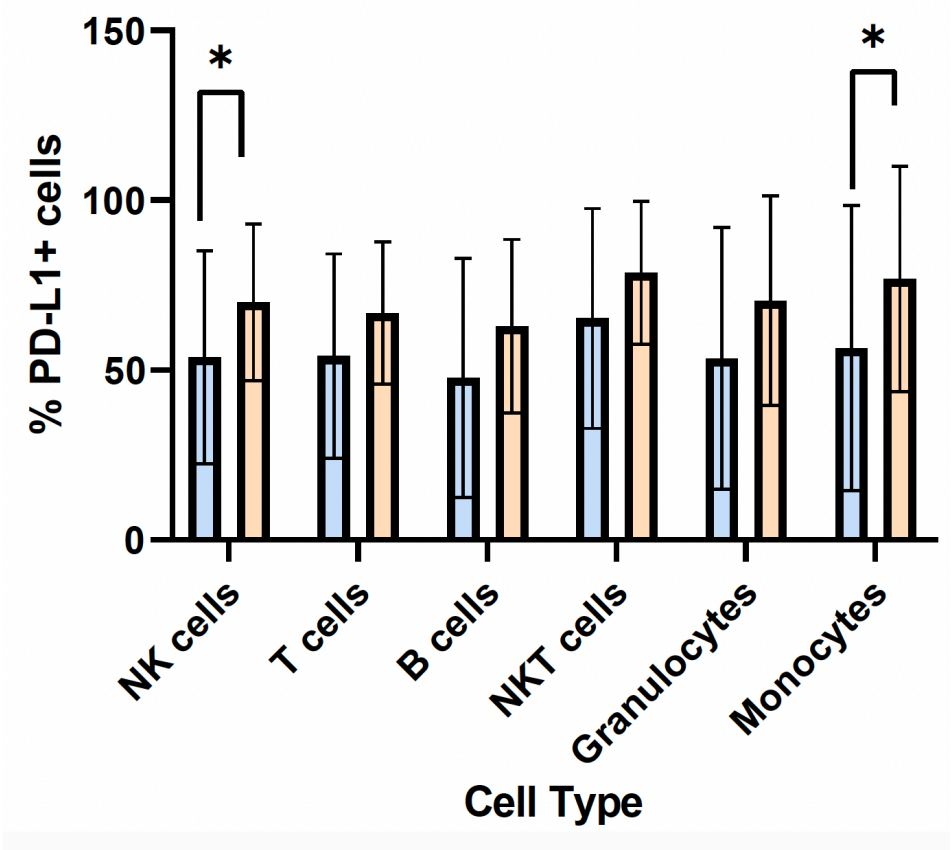
**Figure 1. *JAK2* 46/1 haplotype influences PD-L1 expression levels.** A, The percentage of PD-L1 expressing cells, measured by flow-cytometry, in different peripheral blood cell populations from healthy donors 46/1 nullizygous (blue) and the 46/1 homozygous (orange), n=37 each. *, p-value < 0.05 (non-parametric Kruskall-Wallis test). B, PD-L1 protein levels, determined by ELISA and expressed as PD-L1/Total Protein (pg/µg) in CD34+ cells obtained from bone marrow of JAK2+ MPN patients 46/1 nullizygous (blue) and 46/1 homozygous (orange), n=11 and n=9, respectively. Error bars indicate standard error of the mean (SEM). B, B lymphocytes; NK, natural killer cells; NKT, natural killer T-cells; T, T lymphocytes.

**Figure 2. Chromatin contacts of *JAK2* with the *PD-L1* locus may differ between the non-risk and the 46/1 haplotype** Visualization of a 1.6 megabase region from human chromosome 9, spanning the *JAK2* and *PDL1/2* loci (hg19 chr9:4,300,000-5,900,000). Chromatin interactions established from the 7 distinct genomic regions or viewpoints (VP2, 3, 4, 8, 10, 5, 6, genomic coordinates in Supplemental Table 2), shown as spider plots, for both 46/1 nullizygous (green and light green, n=2) and 46/1 homozygous (blue and light blue, n=2) healthy individuals are shown from top to bottom, showing differences between them around *PD-L1/2* loci. Regions corresponding to the 46/1 haplotype and the *PDL-1/2* loci are boxed. Black horizontal arrows below the UCSC browser view indicate the position of all genes in the region, and the name of those referred to in this study are included. On the right of each spider plot, normalized read counts (or contacts) are represented for both *PD-L1* (red) and PD-L2 (blue) loci. As indicated, left column for each loci represents 46/1 homozygous and right column 46/1 nullizygous.

**Figure 3. *JAK2* haplotype regulates *PD-L1* expression as shown by CRISPR-Cas9 deletion.** A, Genomic locus of *JAK2*, indicating the region of intron 2 that was deleted to test its regulatory activity (hg19 chr9: 5,006,961-5,018,796) based on the location of strongly linked 46/1 SNPs (see Supplemental Material and

Supplemental Table 3). B, Expression of *JAK2* (left), *PD-L1* (middle) and RIC1 (right) in control (grey), heterozygous (yellow) and homozygous (blue) intron 2 element (E_i2) deleted K562 clones. C, Expression of *JAK2* (left), *PD-L1* (middle) and *RIC1* (right) in control (grey), heterozygous (blue) and homozygous (red) intron 2 element (E_i2) deleted K562 clones treated overnight with 10ng/ml of Interferon gamma. In B and C, mRNA levels were normalized using *GAPDH* as an endogenous control, and data was analyzed by one-way ANOVA followed by Tukey's multiple comparison test; P-value < 0.05 (*), 0.01 (**), 0.005 (***), 0.001 (****).

A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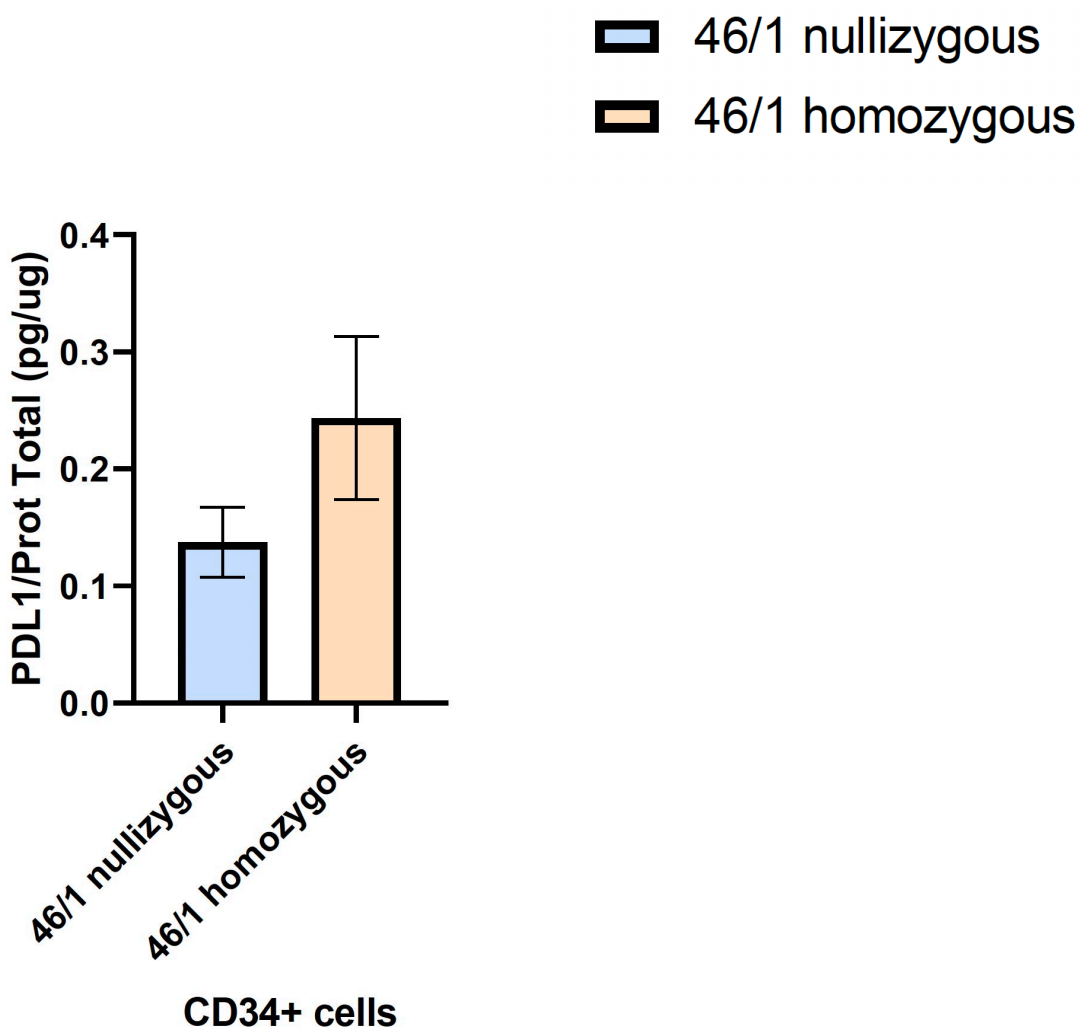
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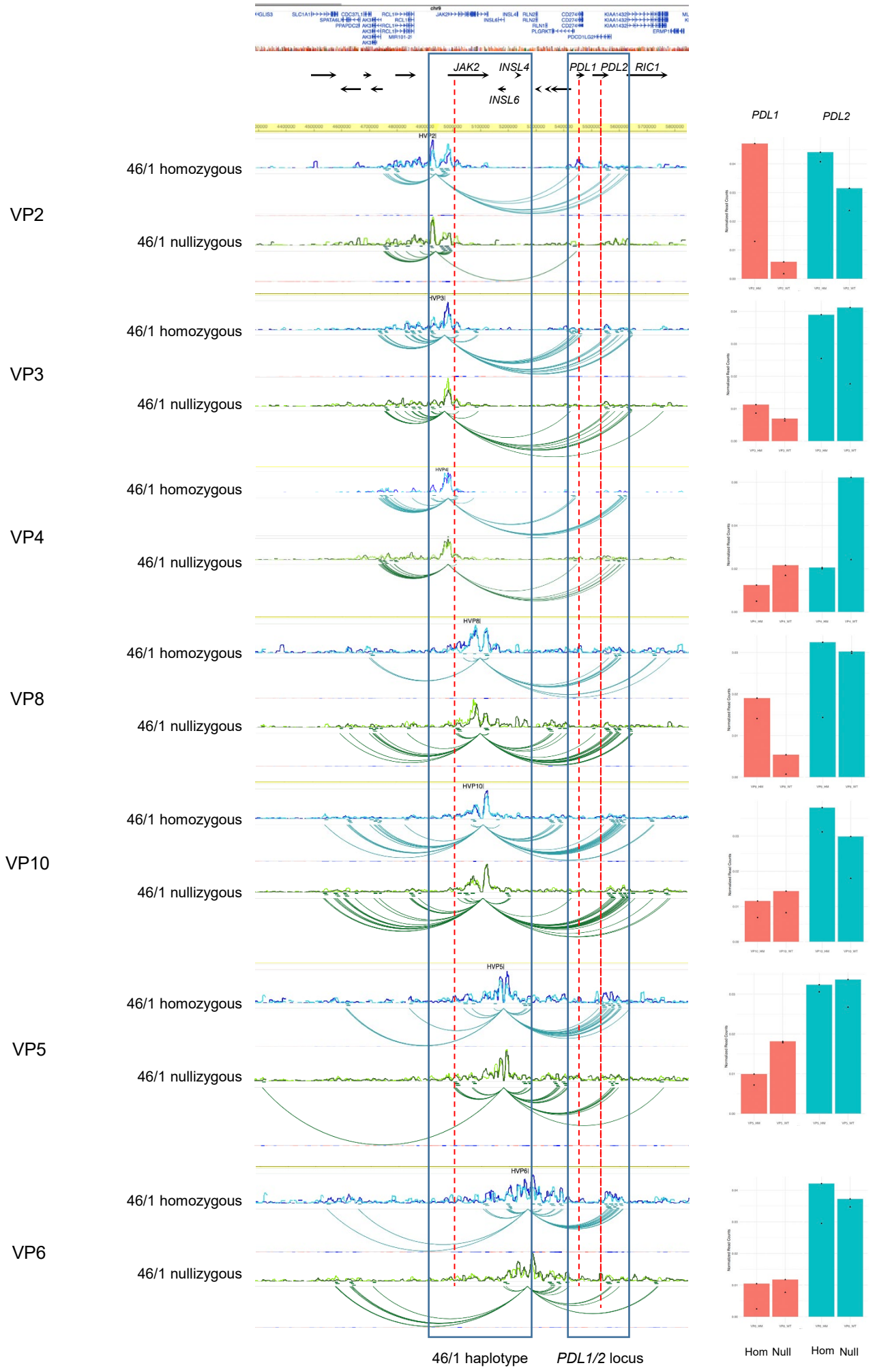
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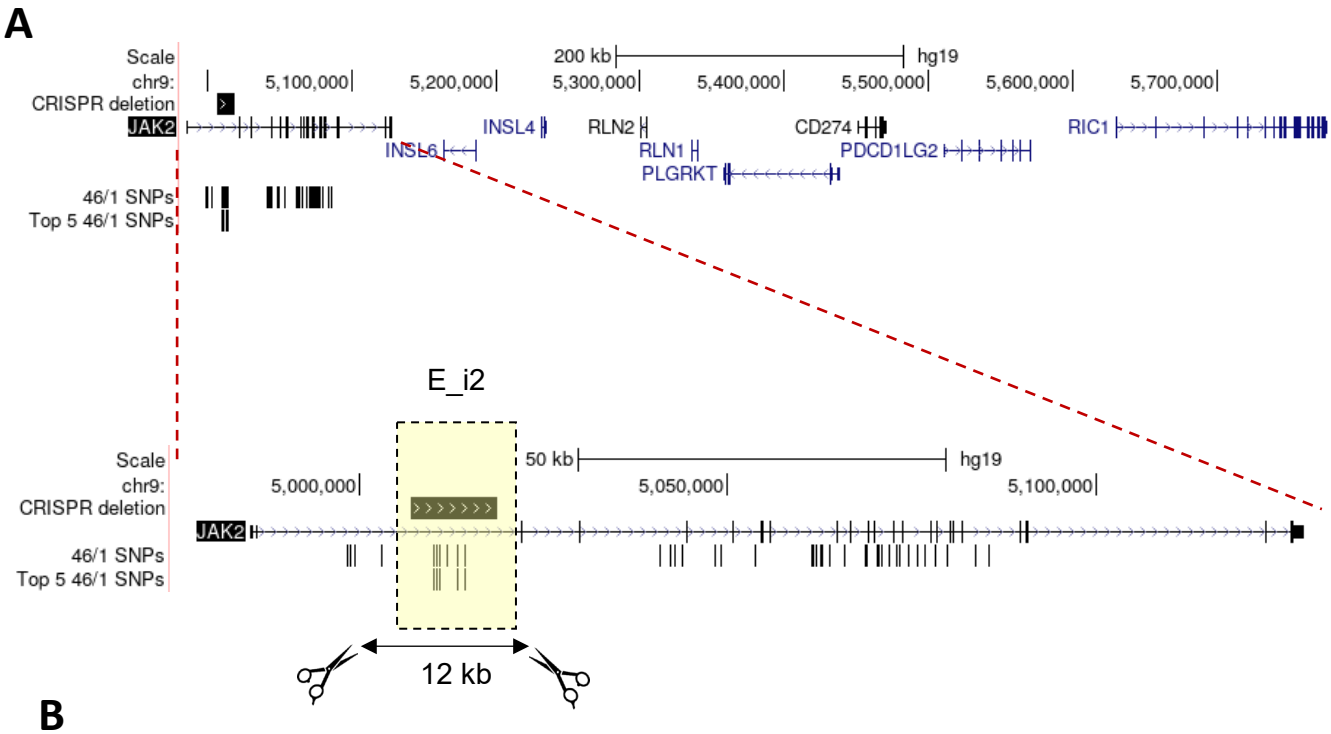
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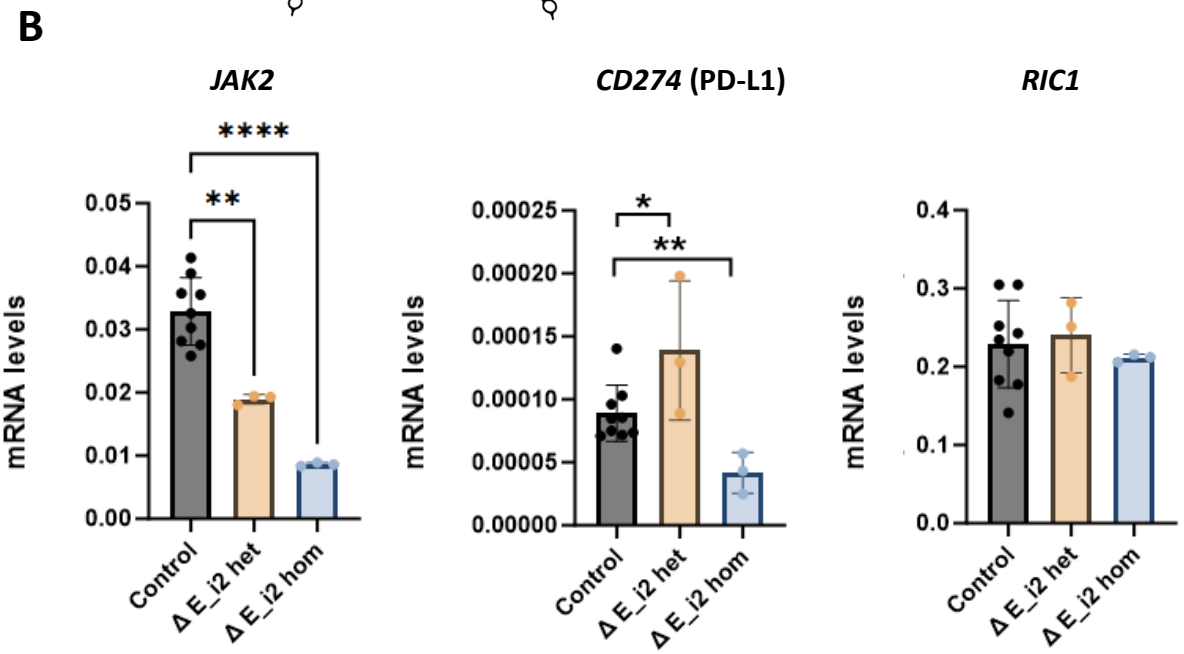
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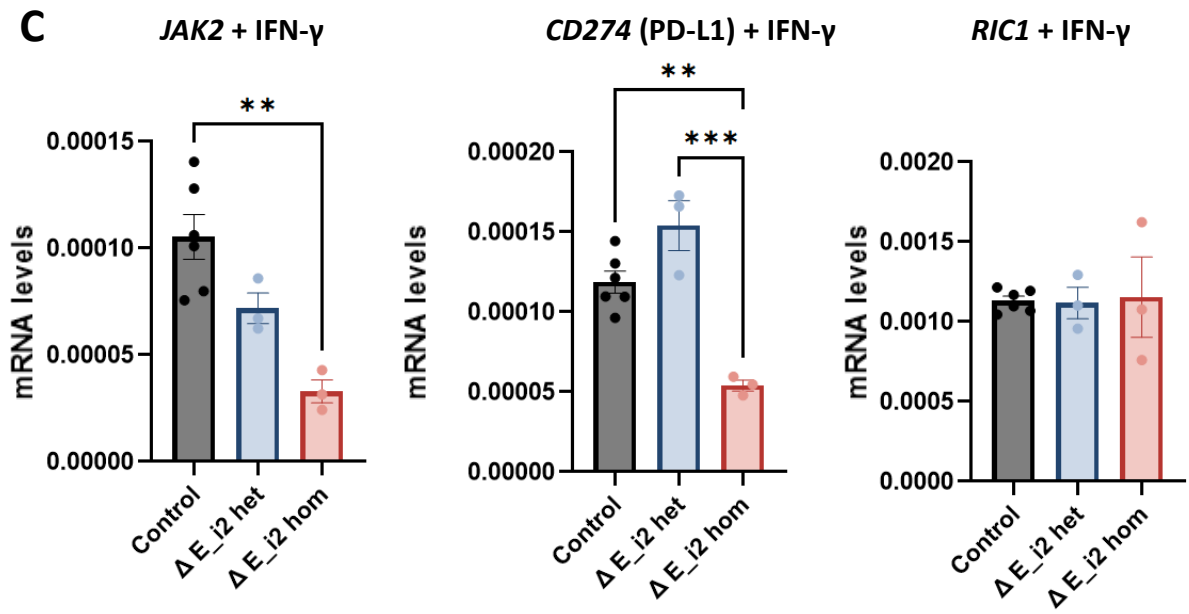
C
JAK2 + IFN-γ
CD274 (PD-L1) + IFN-γ
RIC1 + IFN-γ
mRNA levels
Control
Δ E_i2 het
Δ E_i2 hom