Mediator kinase inhibition suppresses hyperactive interferon signaling in Down syndrome

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Abstract

Hyperactive interferon (IFN) signaling is a hallmark of Down syndrome (DS), a condition caused by trisomy 21 (T21); strategies that normalize IFN signaling could benefit this population. Mediator-associated kinases CDK8 and CDK19 drive inflammatory responses through incompletely understood mechanisms. Using sibling-matched cell lines with/without T21, we investigated Mediator kinase function in the context of hyperactive IFN in DS. Activation of IFN-response genes was suppressed in cells treated with the CDK8/CDK19 inhibitor cortistatin A, and this occurred through suppression of IFN-responsive transcription factor activity. Moreover, we discovered that CDK8/CDK19 affect splicing, a novel means by which Mediator kinases control gene expression. Kinase inhibition altered splicing in pathway-specific ways and selectively affected IFN-responsive gene splicing in T21 cells. To further probe Mediator kinase function, we completed cytokine screens and untargeted metabolomics experiments. Cytokines are master regulators of inflammatory responses; by screening 105 different cytokine proteins, we show that Mediator kinases help drive IFN-dependent cytokine responses at least in part through transcriptional regulation of cytokine genes and receptors. Metabolomics revealed that Mediator kinase inhibition altered core metabolic pathways, including broad up-regulation of anti-inflammatory lipid mediators. Elevated levels of lipid mediators persisted at least 24hr after Mediator kinase inhibition, and many identified lipids serve as ligands for nuclear receptors (e.g. PPAR, LXR) or G-protein coupled receptors (GPCRs; e.g. FFAR4). Notably, ligand-dependent activation of these GPCRs or nuclear receptors will propagate anti-inflammatory signaling pathways and gene expression programs, and this mechanistic link suggests that metabolic changes caused by CDK8/CDK19 inhibition can durably and independently suppress pro-inflammatory IFN responses. Collectively, our results establish that Mediator kinase inhibition antagonizes IFN signaling through transcriptional, metabolic, and cytokine responses, with implications for DS and other chronic inflammatory conditions.

Introduction

Down syndrome (DS) is relatively common in the human population (ca. 1 in 700 live births)(de Graaf et al. 2017) and results from trisomy of chromosome 21 (T21). T21 manifests in myriad ways, including an increased propensity for autoimmune and neurological disorders, as well as elevated incidence of leukemia (Hasle et al. 2000; Antonarakis 2017; Antonarakis et al. 2020). Notably, T21 also results in chronic immune dysregulation associated with hyperactivation of interferon (IFN) signaling (Sullivan et al. 2016; Araya et al. 2019; Waugh et al. 2019).

The chronic, hyperactive IFN response in DS can be attributed, at least in part, to the fact that four IFN receptors are encoded on chromosome 21: IFNAR1 and IFNAR2 for Type I IFNs (e.g., IFNβ, IFNγ), IFNGR2 for IFNγ, and the Type III subunit IL10RB for IFNλ, which also serves as a subunit of the IL10 receptor. Numerous pathologies associated with DS, including autoimmune-related disorders, are considered direct consequences of chronic IFN pathway activation (Madan et al. 2006; Marild et al. 2013; Araya et al. 2019; Waugh et al. 2019; Bush et al. 2021). For these reasons, hyperactive IFN signaling lies at the heart of DS pathophysiology (Waugh et al. 2023), and therapeutic strategies to dampen IFN responses are being tested in clinical trials (NCT04246372, NCT05662228).

We recently demonstrated that the Mediator kinases (CDK8 and its paralog CDK19) are drivers of inflammatory responses to the universal cytokine IFNγ (Steinparzer et al. 2019). This discovery has implications for DS, because hyperactive IFN signaling underlies many DS symptoms and because Mediator kinases are promising targets for molecular therapeutics, in part due to low toxicity of a selective inhibitor in mouse models (Pelish et al. 2015). Many studies have linked Mediator kinase activity to immune system function and inflammatory responses (Bancerek et al. 2013; Chen et al. 2017; Johannessen et al. 2017; Yamamoto et al. 2017; Guo et al. 2019; Steinparzer et al. 2019; Hofmann et al. 2020; Freitas et al. 2022), and CDK8/CDK19 inhibition can suppress autoimmune disease in animal models (Akamatsu et al. 2019). Mediator kinases target the STAT transcription factor (TF) family (Bancerek et al. 2013; Poss et al. 2016) and activation of JAK/STAT pathway TFs (e.g. STAT1, IRF1) is blocked upon Mediator kinase inhibition in IFNγ-stimulated human or mouse cells (Steinparzer et al. 2019). Collectively, these results suggest that Mediator kinase inhibition could mitigate chronic, hyperactive IFN signaling in T21.

Mediator is a 26-subunit complex that regulates RNA polymerase II (RNAPII) transcription genome-wide (Richter et al. 2022). Mediator is recruited to specific genomic loci through interactions with sequence-specific, DNA-binding TFs, and Mediator interacts with the RNAPII enzyme at transcription start sites. Through these distinct interactions, Mediator enables TF-dependent control of RNAPII function. CDK8 or CDK19 can reversibly associate with Mediator as part of a 4-subunit "Mediator kinase module" that contains additional subunits MED12, MED13, and CCNC (Luyties and Taatjes 2022).

Mediator kinases have not been studied in the context of DS, and a goal of this project was to define their roles in the context of IFNγ signaling, using donor-derived cell lines. We also sought to address other fundamental questions regarding Mediator kinase function that remained largely unexplored. For instance, it is not known whether Mediator kinases impact pre-mRNA splicing that is coupled to RNAPII transcription, despite evidence that CDK8 and/or CDK19 phosphorylate splicing regulatory proteins (Poss et al. 2016). Furthermore, metabolic changes are at least as important as transcriptional changes in driving biological responses (Rinschen et al. 2019; Zhu and Thompson 2019), but how Mediator kinases impact metabolism in human cells remains incompletely understood (Zhao et al. 2012; Galbraith et al. 2017). Finally, cytokines serve as key drivers of IFNγ responses, and despite links between CDK8/CDK19 function and inflammation (Chen et al. 2017; Steinparzer et al. 2019; Freitas et al. 2022), it is largely unknown whether Mediator kinases impact cytokine levels.

Using a combination of approaches, we have identified novel and diverse mechanisms by which Mediator kinases control IFNγ responses under both basal and IFNγ-stimulated conditions. Beyond anticipated effects on RNAPII transcription, our results indicate that Mediator kinases act in part by governing TF activation and suggest that they selectively control gene expression through pre-mRNA splicing; furthermore, regulation of cytokine and metabolite levels contributes to CDK8/CDK19-dependent control of pro-inflammatory IFNγ signaling. Taken together, our findings identify Mediator kinases as therapeutic targets that could mitigate immune system dysregulation in individuals with DS.

Results

Experimental overview: transcriptomics, metabolomics, and cytokine screens

The goals of this project were to compare and contrast transcriptional and metabolic changes in the following experimental contexts: i) T21 vs. D21, ii) ±IFNγ stimulation, iii) ±Mediator kinase inhibition, and iv) IFNγ stimulation + Mediator kinase inhibition. For T21 vs. D21 comparisons, we selected lymphoblastoid cells from the Nexus Biobank that were derived from siblings matched in age (3- or 5-year old) and sex (male). We used the natural product cortistatin A (CA) to inhibit CDK8 and CDK19 (Pelish et al. 2015). CA is the most potent and selective Mediator kinase inhibitor available (Clopper and Taatjes 2022); for example, kinome-wide screens showed no off-target kinase inhibition even at 1 μM, a concentration 5000-times greater than its measured K_D of 0.2 nM (Pelish et al. 2015). Throughout this project, we used CA at a concentration of 100 nM, to minimize potential off-target effects. Immunoblots confirmed CA-dependent inhibition of STAT1 S727 phosphorylation, a known CDK8 substrate (**Figure 1 – figure supplement 1A**).

Prior to completion of RNA-seq experiments, we probed the timing of the IFNγ response at IFN target genes GBP1 and IRF1 by RT-qPCR (**Figure 1 – figure supplement 1B**). Based upon these results, we chose the 4-hour time point for RNA-seq (see Methods). An overview of the RNA-seq experiments is shown in **Figure 1A**, with data for all genes across all conditions in **Figure 1 – source data 1**.

Large-scale, untargeted metabolomic analyses were completed 24h post-IFNγ stimulation in D21 and T21 cells. This time point was chosen to approximate a "steady state" following IFNγ treatment and to allow time for metabolic adaptations to occur; this time point was also consistent with prior metabolite analyses in IFN-treated cells (Wang et al. 2018; Lee et al. 2021b). Moreover, the 24h time point complements the 4h RNA-seq data, in that transcriptional changes identified at 4h might contribute to subsequent metabolic changes. An overview of the metabolomics experiments is shown in **Figure 1A**, with raw data for all identified metabolites across conditions in **Figure 1 – source data 2**.

Down syndrome has been described as a "cytokinopathy" based upon evaluation of blood plasma from individuals with or without T21. Arrays screening 29 different cytokines showed elevated levels in Down syndrome individuals (Malle et al. 2023). Based upon these and other results, we sought to directly measure cytokine levels to assess IFNγ and CA-dependent effects. Therefore, we completed a series of cytokine screens (n=105 different cytokines) in T21 and D21 cells (**Figure 1 – figure supplement 1C**), using the same experimental design as the metabolomics experiments (basal conditions, +CA, +IFNγ, or +CA+IFNγ). Biological replicate cytokine screens were completed for each condition after 24h treatment; measurements for all cytokines in both replicates can be found in **Figure 1 – source data 3**.

The T21 transcriptome, metabolome, and cytokine levels are consistent with hyperactive IFN signaling

For RNA-seq, biological triplicate experiments were completed, and a normalization (Hunter et al. 2023) was performed to account for the potential 1.5-fold expression differences from chromosome 21 in T21 cells (Hwang et al. 2021). Comparison of gene expression in T21 vs. D21 cells revealed massive differences, as expected (**Figure 1 – figure supplement 1D; Figure 1 – source data 1**). Gene set enrichment analysis (GSEA)(Subramanian et al. 2005) revealed significant up-regulation of inflammatory pathways (e.g. IFNγ, IFNα, Complement, TNFα) in T21 vs. D21 cells (**Figure 1 – figure supplement 1E; Figure 1 – source data 4**), consistent with prior reports (Sullivan et al. 2016; Sullivan et al. 2017; Malle et al. 2023). Furthermore, the "upstream regulators" identified from Ingenuity Pathway Analysis (IPA) (Kramer et al. 2014) predicted activation of numerous inflammatory markers in T21 cells (e.g. IFNγ, IFNα, TNF, TGFβ1, NFκB), based upon the RNA-seq results (**Figure 1 – figure supplement 1F; Figure 1 – source data 5**). Negatively enriched pathways in T21 cells (GSEA Hallmarks, **Figure 1 – figure supplement 1E, Figure 1 – source data 4**) reflected proliferative gene expression programs (e.g. MYC targets, G2M checkpoint); in agreement, we observed slower growth rates for T21 cells compared with their D21 counterparts.

Untargeted metabolomics experiments identified and quantified over 600 biochemicals representing every major metabolic pathway (**Figure 1 – source data 2**). We observed evidence that inflammatory pathways were activated in T21 cells under basal conditions (IPA results, **Figure 1 – figure supplement 1G**; **Figure 1 – source data 5**), and 196 biochemicals increased and 214 decreased in T21 vs. D21 (**Figure 1 – figure supplement 2A, B; Figure 1 – source data 2**). Pathways related to fatty acid transport, growth, and energy metabolism were reduced in T21 cells (vs. D21; **Figure 1 – figure supplement 1G**). Supporting these general trends, the metabolomics data revealed T21-specific changes in glycolysis, nucleotide and lipid biosynthesis, and other fundamental cellular processes (**Figure 1 – figure supplement 2B; Figure 1 – source data 2**). Metabolic distinctions between T21 and D21 cells will be described in more detail later, in the context of IFNγ treatment and/or Mediator kinase inhibition. An overview of metabolic pathways that are relevant for this study is shown in **Figure 1B**.

As with the RNA-seq and metabolomics data, the cytokine screen results implicated chronic activation of the IFN response in T21 cells under basal conditions; for example, levels of many cytokines were elevated in T21 vs. D21 cells (**Fig 1C**, left column). Furthermore, treatment with IFNγ appeared to "normalize" cytokine levels in T21 vs. D21 cells (**Fig 1C**, right column), such that many cytokines were detected at roughly equal levels in IFNγ-stimulated T21 and D21 cells.

T21-specific transcriptional, metabolic, and cytokine changes are reflected in population-level data

For practical reasons, the transcriptomics, metabolomics, and cytokine screening experiments completed here precluded parallel examination of additional genetically distinct donor-derived T21 and D21 cell lines. A basic observation from past studies was that the transcriptomes of T21 individuals are not only tissue- and cell type-specific, but also reflect individual-to-individual differences within the same cell types (Sullivan et al. 2016; Hwang et al. 2021). However, we hypothesized that the general trends in our sibling-matched T21 vs. D21 lines would show some commonality with large-scale cohort studies.

We analyzed RNA-seq data from whole blood samples from individuals with DS (n=304) or euploid (i.e. D21; n=96) controls (Waugh et al. 2023), generated by the Human Trisome Project (www.trisome.org, NCT02864108). The data revealed upregulation of the IFNγ response, Complement, cytokine production, and other inflammatory pathways in T21 individuals (vs. D21; **Figure 1 – figure supplement 2C; Figure 1 – source data 4**). Approximately ten percent of differentially expressed genes were shared between the Human Trisome Project cohort study (T21 vs. D21; whole blood transcriptomes from individuals varying in age and sex) and our RNA-seq

results from sibling-matched T21 and D21 lymphoblastoid cell lines (**Figure 1 – figure supplement 2D**), consistent with individual-to-individual variation within the human population (Sullivan et al. 2016; Hwang et al. 2021). Notably, the shared "core sets" of genes reflected prominent trends in our T21/D21 RNA-seq comparisons. For example, up-regulated genes represented cytokine production and other inflammatory pathways, whereas down-regulated genes involved nucleotide and fatty acid metabolism, splicing, GPCR signaling, and proliferation. These pathway trends are well-represented in our RNA-seq and metabolomics data (e.g. **Figure 1 – figure supplement 1E, G; Figure 1 – source data 6**) and additional data supporting these T21-specific alterations will be described in more detail later.

A recent analysis of blood plasma cytokine levels, measuring 29 cytokines from DS individuals (n=21) or age-matched euploid controls (n=10) identified elevated levels of approximately 10 to as many as 22 of these 29 cytokines (Malle et al. 2023). In agreement, at least 13 of the 22 cytokines (59%) were elevated in our T21 vs. D21 comparison (red font in **Fig 1C**). Collectively, the RNA-seq and cytokine data from cohorts of DS individuals indicate that the two model cell lines evaluated here, derived from age- and sibling-matched D21 and T21 individuals, broadly reflect population-wide trends.

Mediator kinase inhibition tempers T21 inflammatory pathways under basal conditions

We next asked whether Mediator kinases influenced the up-regulation of IFNγ signaling in T21 vs. D21 cells. We first describe how Mediator kinase inhibition impacted T21 or D21 cells under basal conditions.

Transcriptome: As shown in **Figure 2A, Figure 2 – figure supplement 1A-D,** and **Figure 1 – source data 4**, CA caused similar changes to gene expression programs in T21 and D21 cells, although some T21-specific differences were evident (**Figure 2 – figure supplement 1C, D**). CA treatment up-regulated 445 genes in D21 (331 in T21; 210 shared) and down-regulated 233 genes (191 in T21, 112 shared; Padj 0.01). GSEA showed downregulation of MYC targets, consistent with prior studies in CDK8-depleted cells (Sooraj et al. 2022). Downregulation of the inflammatory "TNFα signaling via NFκB" pathway was also observed in CA-treated D21 and T21 cells (**Figure 2 – figure supplement 1C, D**); decreased expression of the NFKB1 and NFKB2 transcripts themselves likely contributed to this effect (**Figure 1 – source data 1**). Regulation of NFκB transcriptional programs has been previously linked to Mediator kinases (Chen et al. 2017) although direct control of NFκB transcription levels has not been reported. Activation of GSEA Hallmarks related to mTOR signaling, cholesterol and fatty acid metabolism were common in CA-treated D21 and T21 cells (**Figure 2 – figure supplement 1C, D**), in agreement with prior studies in other cell types (Poss et al. 2016; Audetat et al. 2017; Andrysik et al. 2021). To further characterize the CA-dependent transcriptional changes related to cholesterol and fatty acid metabolism, we highlighted the genes significantly up-regulated in these pathways in **Figure 2 – figure supplement 1E**; the data indicate that Mediator kinases broadly regulate cholesterol and fatty acid biosynthesis, and this is further confirmed by the metabolomics data (see below).

Based upon the RNA-seq data comparing CA-treated T21 or D21 cells vs. untreated controls, we completed an "upstream regulators" analysis (IPA; **Figure 2 – figure supplement 1F, Figure 1 – source data 5**). In agreement with the GSEA results, factors regulating cholesterol homeostasis (e.g. SCAP, SREBF1/2, INSIG1), fatty acid metabolism (e.g. ACSS2, FASN), and inflammation (e.g. NFKB1, MYD88, TLR3) were significantly altered by CA treatment in T21 cells (**Fig 2B; Figure 2 – figure supplement 1F**), with similar results in D21 (**Figure 2 – figure supplement 1F**). The IPA results also implicated CA-dependent repression of pro-inflammatory TFs specifically in T21 cells, including RELA (NFκB complex), FOXO3, and ILF3 (**Figure 2 – figure supplement 1F; Figure 1 – source data 5**), consistent with repression of chronic basal IFN

signaling upon Mediator kinase inhibition. This CA-dependent effect was further supported by the metabolomic and cytokine screen data, described below.

Metabolome: CA-dependent changes in the metabolomes of D21 and T21 cells were strikingly distinct. This is likely due to i) the longer time frame used for metabolomics analyses (24h vs. 4h for RNA-seq), and ii) the massive "pre-existing" basal metabolome differences in T21 vs. D21 cells (**Figure 2 – source data 1**). Direct comparison of T21 vs. D21 metabolomes (basal conditions) identified major differences in glycolysis, the pentose phosphate pathway, the citric acid cycle, various categories of lipids, and nucleotide biosynthesis pathways (**Figure 1 – figure supplement 2A, B**). Here, we will focus on the T21 vs. D21 metabolic differences that relate to pathways that were impacted by Mediator kinase inhibition.

In **Figure 2C**, we highlight examples of CA-dependent increases in anti-inflammatory lipid metabolites. In T21 cells selectively, CA treatment broadly increased levels of long chain mono- and poly-unsaturated FA (PUFA; **Fig 2C**), including oleic acid, eicosapentaenoate (EPA), docosapentaenoate (DPA), docosahexaenoate (DHA), and various other ω3 and ω6 PUFAs (**Figure 2 – source data 2**). These metabolites act as signaling molecules by binding to extracellular G-protein coupled receptors (GPCRs) to trigger anti-inflammatory signaling cascades (Basil and Levy 2016; Husted et al. 2017; Kimura et al. 2020; Jordan and Werz 2022), as shown in the simplified schematic in **Figure 2D**. Similar to LCFAs, endocannabinoids were broadly elevated in CA-treated T21 cells (vs D21; **Fig 2C**). Endocannabinoids mediate anti-inflammatory effects in part through PPAR TFs (Alhouayek and Muccioli 2014); for example, oleoylethanolamide (OEA) is a PPARα ligand (Fu et al. 2003) that can suppress pro-inflammatory NFκB signaling (Yang et al. 2016). Collectively, these results reveal new and unexpected ways in which Mediator kinase inhibition antagonizes pro-inflammatory IFN signaling in T21 (see Discussion).

Cytokine: CA treatment was also shown to be anti-inflammatory based upon the cytokine screen results. Under basal conditions, the levels of about two dozen cytokines changed in response to CA treatment (**Fig 2E**). In D21 cells, the anti-inflammatory cytokine IL10 increased upon Mediator kinase inhibition, in agreement with prior reports in other cell types (Johannessen et al. 2017), whereas the pro-inflammatory cytokines GMCSF, IL3, and MIP1α/β (a.k.a. CCL3/4) decreased; an exception was TNFα. Similar results were obtained in T21 cells; however, CA treatment reduced the levels of pro-inflammatory cytokines in T21 cells that were not reduced in D21 (e.g. IL32, IL33, IL34), although CXCL10 and GMCSF were exceptions. The selective CA-dependent decrease in IFNγ in T21 cells under basal conditions further supports the chronic high baseline IFN signaling in these cells (Sullivan et al. 2016). Note that some cytokines cannot be clearly defined as either pro- or anti-inflammatory and their roles may be cell type-specific (Dinarello 2007).

Because cytokine levels were measured 24h after CA treatment, we hypothesized that their altered levels would partially correspond to gene expression changes that were measured earlier, at 4h post-treatment. Indeed, this was observed for many cytokines impacted by Mediator kinase inhibition, as shown in **Figure 2F**. (Note that expression changes for cytokine receptors are also shown.) At basal conditions, several dozen cytokines and cytokine receptors were regulated by CA at the transcriptional level (**Fig 2F**), and most correlated with the cytokine screen data, including IL10, which was transcriptionally upregulated in D21 cells, and MIP1α/β (a.k.a. CCL3/4), which were transcriptionally downregulated in both D21 and T21 lines. We also observed that CA altered expression of two interferon receptors: IFNGR2 was downregulated and IFNAR2 was upregulated in both lines. Each of these IFN receptor genes is on chromosome 21 and associated with chronic inflammation in DS individuals (Sullivan et al. 2016).

Mediator kinase inhibition antagonizes IFNγ-induced transcriptional, metabolic, and cytokine responses

We next evaluated the transcriptomic, metabolomic, and cytokine data from IFNγ-treated cells. As shown in **Figure 3 – figure supplement 1A**, the transcriptional response to IFNγ was broadly similar in T21 and D21 cells; however, T21 cells showed higher basal expression of inflammatory genes. This can be seen upon comparison of gene expression in T21 vs. D21 cells under basal (**Figure 3 – figure supplement 1B**) or IFNγ-stimulated conditions (**Figure 3 – figure supplement 1C**; see also **Figure 3 – figure supplement 1D, E**). Only 10 genes showed greater induction in T21 cells in response to IFNγ, whereas 68 genes had reduced induction in T21 compared with D21 (**Figure 3 – figure supplement 1F**; **Figure 1 – source data 1**). Pathway analysis (GO Biological Processes) of these 68 genes showed predominant roles in IFN signaling and inflammatory responses (**Figure 3 – figure supplement 1G; Figure 1 – source data 6**). This result is consistent with hyperactive IFN signaling in T21 cells (Sullivan et al. 2016), such that induction upon exogenous IFNγ stimulation was reduced compared with D21 cells. Next, we focus on how Mediator kinase inhibition influenced transcriptional, metabolic, and cytokine responses in IFNγ-treated D21 and T21 cells.

Transcriptome: The regulatory roles of the Mediator kinases CDK8 and CDK19 are context- and cell type-specific, and their functions appear to be especially important for initiating changes in gene expression programs, such as during an acute stimulus (Luyties and Taatjes 2022). As shown in **Figure 3A**, many of the mRNAs responsive to IFNγ (activated or repressed) were impacted in the opposite way in CA + IFNγ-treated D21 or T21 cells (see also **Figure 3 – figure supplement 2A, B**). This CA-dependent antagonism of IFNγ-response genes is best illustrated with difference heatmaps that compared levels of IFN-dependent transcriptional changes with IFN +CA conditions (i.e. IFN vs. Ctrl – IFN+CA vs. Ctrl; **Figure 3 – figure supplement 2C, D**). An example at the CXCL9 gene locus is shown in **Figure 3B**; CXCL9 is a pro-inflammatory cytokine. GSEA also reflected CA-dependent suppression of the transcriptional response to IFNγ. In both D21 and T21 cell lines, IFNγ response and other inflammatory pathways were down-regulated with CA treatment compared with IFNγ alone (**Fig 3C, D; Figure 1 – source data 4**). Similar results were obtained with an assessment of inflammatory pathways using IPA (**Figure 3 – figure supplement 2E; Figure 1 – source data 5**). Thus, Mediator kinase inhibition blocked normal transcriptional responses to IFNγ, consistent with prior studies in MEFs and human cancer cells (Bancerek et al. 2013; Steinparzer et al. 2019). The up-regulated Hallmark pathways included cholesterol homeostasis and fatty acid metabolism (**Fig 3C, D; Figure 1 – source data 4**). A set of factors from the IPA upstream regulators analysis underscores this result, identifying TFs and enzymes involved in cholesterol and lipid metabolism in CA-treated cells T21 cells (**Fig 3E-G**), with similar results in D21 (**Figure 3 – figure supplement 2F, G; Figure 1 – source data 5**). These IPA results were consistent with CA-treated cells under basal conditions (**Fig 2B, Figure 2 – figure supplement 1C-F**) and were also supported by metabolomics data from IFN-treated cells (see below).

To probe further, we compared the GSEA "leading edge" IFN response gene set from a whole blood transcriptome dataset generated by the Human Trisome Project (Waugh et al. 2023) with the CA-responsive genes in our T21 cell line. As shown in **Figure 3 – figure supplement 2H**, 86% of these genes (69 out of 80) overlapped with those identified as elevated in the Human Trisome Project dataset, reflecting a common transcriptional response to IFNγ. Among these IFN-responsive genes were many whose expression decreased upon CA treatment, including STAT1, IRF1, GBP4, MX2, XCL1, and CXCL9. These results reveal that Mediator kinase inhibition suppresses transcriptional responses that are typically hyperactivated in DS individuals.

DNA-binding TFs are common targets for Mediator kinases (Poss et al. 2016) and TF phosphorylation contributes to CDK8 and/or CDK19-dependent effects on gene expression (Bancerek et al. 2013; Steinparzer et al. 2019). To test the notion that Mediator kinases suppress IFNγ transcriptional responses through TFs, we completed upstream regulators pathway analyses (IPA) based on the RNA-seq data. As shown in **Figure 3F, G** and **Figure 3 – figure supplement 2F, G**, an array of pro-inflammatory TFs were induced in IFNγ-treated T21 and D21 cells, as expected. Strikingly, however, activation of these same TFs was blocked in CA-treated cells (D21 and T21). These results suggest that inhibition of CDK8/CDK19 suppresses IFNγ responses, in part, through inhibition of pro-inflammatory TF activity, in agreement with prior experiments (Steinparzer et al. 2019).

Metabolism: Consistent with the RNA-seq results, metabolic changes in D21 and T21 cells reflected a CA-dependent suppression of IFNγ-induced inflammation. CA treatment reversed metabolic changes induced by IFNγ alone. For example, LCFAs, endocannabinoids, oleamide, desmosterol, and bile acids were reduced in D21 cells under IFNγ-stimulation conditions, consistent with the anti-inflammatory roles of these metabolites (Alhouayek and Muccioli 2014; Basil and Levy 2016; Husted et al. 2017; Kimura et al. 2020; Jordan and Werz 2022). By contrast, their levels were elevated in CA-treated D21 cells whereas the effects of Mediator kinase inhibition were reduced in scope in IFNγ-treated T21 cells, which may reflect the elevated basal IFN signaling in T21 cells (**Fig 4A–C; Figure 4 – source data 1**). As in D21 cells, however, CA-dependent changes in T21 were generally the opposite of those caused by IFNγ treatment alone, consistent with an anti-inflammatory effect (**Fig 4B, C; Figure 4 – source data 1**). For instance, quinolinic acid, a tryptophan derivative that is elevated in DS (Powers et al. 2019), was elevated in T21 cells following IFNγ stimulation but this was blocked by CA treatment (**Fig 4C**; **Figure 4 – source data 1**). Collectively, the data summarized in **Figure 4A–C** and **Figure 4 – source data 1** are consistent with CA-dependent anti-inflammatory effects.

Cytokines: Compared with basal conditions, many more cytokines showed CA-responsiveness in the context of IFNγ stimulation, and a majority of the changes countered IFNγ-induced inflammatory responses (**Figure 4 – figure supplement 1A**). This CA-dependent antagonism of IFNγ cytokine responses is illustrated in the difference heatmaps shown in **Figure 4D, E**. Levels of many pro-inflammatory cytokines were reduced in IFNγ + CA-treated D21 and T21 cells, including CXCL9, C5, IL33, IL1α, and others (**Figure 4 – figure supplement 1A**).

Because cytokine levels were measured 24h after treatment, we hypothesized that their altered levels would at least partially correspond to gene expression changes measured earlier, at 4h post-treatment. This was observed for many IFNγ-induced cytokines impacted by Mediator kinase inhibition, as shown in **Figure 4 – figure supplement 1B**. (Note that expression changes for cytokine receptors are also shown.) For example, gene expression changes for CXCL9, CXCL10, MIP1α/β (a.k.a. CCL3/4), FLT3 ligand, GCSF, MCSF, IL10, IL24, and TFR tracked with changes in their protein levels in CA-treated cells (**Figure 4 – figure supplement 1B**; compare with **Fig 2F**). Collectively, the cytokine screen demonstrated that inhibition of Mediator kinase function generally antagonized IFNγ signaling by downregulating pro-inflammatory cytokines while upregulating anti-inflammatory cytokines (e.g IL10, LIF, IL19). In the context of Down syndrome, this could potentially mitigate pathological immune system hyperactivation.

Mediator kinases regulate splicing in pathway- and cell type-specific ways

As Mediator-associated kinases, CDK8 and CDK19 are established regulators of RNAPII transcription (Luyties and Taatjes 2022); however, their potential impact on splicing has not been examined. Over 95% of human mRNAs are alternatively spliced and defects in this process contribute to errors in gene expression in human diseases (Scotti and Swanson 2016). We

compared alternative splicing events using rMATS (Shen et al. 2014), separately evaluating all RNA-seq replicates (biological triplicates; **Fig 1A**). The data revealed 741 differential exon skipping events in T21 cells (vs. D21) under basal conditions (**Fig 5A, B**; **Figure 5 – source data 1**), with approximately equal numbers of increased exon inclusion (n=382) and increased exon skipping events (n=359). A smaller number of other alternative splicing events (e.g. intron retention) were also detected (**Figure 5 – figure supplement 1A**). We observed similar trends in IFNγ-treated T21 and D21 cells, in which a greater number of exon skipping events (n=418 vs. n=296) occurred in IFNγ-stimulated T21 cells vs. D21 cells (**Figure 5 – figure supplement 1B, C; Figure 5 – source data 1**).

To determine whether Mediator kinase activity might influence splicing, we next evaluated CA-treated D21 and T21 cells. We found that CA treatment had a substantial impact on splicing, in both D21 and T21 cells (**Fig 5C**, **Figure 5 – figure supplement 1D**). We identified 432 or 444 sites with altered exon skipping events (D21 or T21, respectively) in CA-treated cells compared with controls (ΔPSI [percent spliced in]≥ 0.2, Padj < 0.05; **Fig 5C, Figure 5 – figure supplement 1E; Figure 5 – source data 1**), and CA treatment increased inclusion of alternative exons more frequently than it increased their skipping in both lines (n=239 vs. 193 in D21; n=256 vs. 188 in T21). Similarly in IFNγ-treated D21 and T21 cells (**Fig 5D, Figure 5 – figure supplement 1F; Figure 5 – source data 1**), Mediator kinase inhibition favored exon inclusion (n=178 in D21; 213 in T21) over exon skipping (n=130 in D21; 148 in T21).

To assess whether the exon inclusion events sensitive to Mediator kinase inhibition were selective for specific gene sets, we completed pathway analyses (IPA) across all conditions tested (**Fig 5E-G, Figure 5 – figure supplement 1H, I; Figure 1 – source data 5**). To increase statistical power, we grouped exon skipping events together (i.e. alternative exon inclusion events + alternative exon skipping events). Thus, the T21 vs. D21 comparison included 741 events, D21 +CA vs. Ctrl included 432 events, and so on. The results revealed that splicing changes triggered by Mediator kinase inhibition were cell type specific (T21 vs. D21) and selectively impacted inflammatory and metabolic pathway genes. As shown in **Figure 5E**, genes associated with inflammatory signaling (red highlighted pathways) showed evidence of differential splicing in CA-treated T21 cells (vs. D21) during IFNγ stimulation. In addition, pyrimidine salvage and biosynthesis genes were alternatively spliced in CA-treated T21 cells (vs. DMSO; **Fig 5G**). These pathway-specific effects in CA-treated cells were consistent with gene expression changes (e.g. GSEA Hallmark gene sets, **Figure 1 – source data 4**) and metabolic changes observed in CA-treated cells. For instance, the pyrimidine biosynthesis intermediates N-carbamoylaspartate, dihydro-orotate, and orotate were markedly depleted (1.4 – 3 fold) in CA-treated T21 cells (vs. DMSO; **Figure 1 – source data 2**). The data in **Figure 5G** implicate CA-dependent splicing changes as a contributing factor.

Altered TF function underlies metabolic and anti-inflammatory effects in Mediator kinase-inhibited T21 cells

Sequence-specific, DNA-binding TFs are a major target of Mediator kinases, based upon quantitative phosphoproteomics data (Poss et al. 2016). To broadly assess how TF activity changed in response to Mediator kinase inhibition, we completed PRO-seq experiments in control vs. CA-treated T21 cells. When sequenced at high depth (≥60M reads/replicate), PRO-seq allows reliable detection of short, bidirectional transcripts that center around consensus TF binding sites. Studies from numerous labs have shown that these bidirectional transcripts represent "signatures" of active TFs bound to these sites (Azofeifa et al. 2018; Wang et al. 2019; Kristjansdottir et al. 2020; Rubin et al. 2021). Similarly, loss of bidirectional transcription indicates reduced TF activity.

Because our primary interest was CA-dependent suppression of hyperactive IFN signaling in DS, we completed PRO-seq experiments in T21 cells under basal conditions, ±CA treatment. Based upon prior experiments (Steinparzer et al. 2019), PRO-seq was completed after 45 minutes CA treatment (or DMSO control). We then applied TFEA (Transcription Factor Enrichment Analysis), a well-tested method to globally assess TF activity from PRO-seq data (Rubin et al. 2021). As shown in **Figure 6A**, CA treatment broadly shut down pro-inflammatory TFs in T21 cells (e.g. IRF4, IRF8, SPIB, BACH1, RELA; examples shown in **Fig 6B**), consistent with the "downstream" RNA-seq (4h), cytokine (24h), and metabolomics data (24h). Furthermore, it was evident that CA treatment rapidly repressed the p53 family of TFs (**Fig 6A, B**; **Figure 6 – source data 1**). Although best known as a tumor suppressor, p53 actively represses cholesterol and lipid biosynthesis pathways, in part by blocking activation of SREBP (Moon et al. 2019). Rapid suppression of p53 by Mediator kinase inhibition is consistent with the metabolic changes observed in CA-treated T21 cells (**Fig 2C**). Moreover, the RNA-seq data implicated SREBP TF activation in CA-treated T21 cells (vs. DMSO controls), consistent with de-repression of SREBP following CA-dependent inhibition of p53 activity. From these data, we conclude that altered TF function is a major contributor to i) CA-dependent metabolic changes and ii) suppression of pro-inflammatory IFN signaling in T21 cells.

Discussion

Previous studies have shown that Mediator kinases regulate transcriptional responses to inflammatory stimuli (e.g. IFNγ or TNFα) in a variety of model systems (Chen et al. 2017; Steinparzer et al. 2019). Because individuals with Down syndrome have chronic, hyperactive IFN signaling (Sullivan et al. 2016), we hypothesized that Mediator kinase inhibition would antagonize inflammatory signaling cascades in T21 cells. This hypothesis was supported by the experimental results described here, which showed that Mediator kinase inhibition antagonizes inflammatory pathways at the transcriptional, metabolic, and cytokine protein levels. To maintain focus on Mediator kinase activity, we avoided knockdown or knockout experiments in this study, in part because CDK8/CDK19 proteins serve additional functions that are not kinase-dependent (Poss et al. 2016; Audetat et al. 2017; Sooraj et al. 2022; Chen et al. 2023). Collectively, our results expand upon established concepts for CDK8 and CDK19 (e.g. regulation of IFNγ signaling) and reveal new insights about their impact on TF activation, metabolic and cytokine responses, as well as their affect on pre-mRNA splicing. Our results also establish Mediator kinases as potential therapeutic targets to antagonize hyperactive IFN signaling in DS.

We acknowledge that individual-to-individual genetic variation could contribute to the differences we observe in our T21 vs. D21 comparisons (Sullivan et al. 2016; Hwang et al. 2021). We chose cell lines from sibling donors (both males of similar age) to minimize this possibility. Moreover, cross-referencing our results with data collected from large cohorts of individuals of varying age and sex (with/without T21) showed broadly similar trends in transcript (Waugh et al. 2023), metabolite (Powers et al. 2019), and cytokine levels (Malle et al. 2023) in the T21/D21 comparisons. Although our multi-omics T21/D21 comparisons yielded new insights, Mediator kinases were a focus of this study, and the T21/D21 comparisons primarily served as benchmarks to assess CDK8/CDK19 function in the context of DS and IFNγ signaling. A limitation of this study is that additional donor-derived cell lines were not evaluated; furthermore, the transcriptomic, metabolomic, and cytokine data were collected at select time points, such that some temporal trends in IFNγ responses can only be inferred (see below).

CDK8 and CDK19 kinase activity impacts core metabolic pathways

Metabolomics has been described as the most direct readout of cell state, yielding a “front line” assessment of the biochemicals that drive all cellular processes (Rinschen et al. 2019). The role of Mediator kinases in human cell metabolism is poorly understood. As a whole, our metabolomics data revealed the most striking CDK8/CDK19 effects on nucleotide biosynthesis and lipid homeostasis. Nucleotide levels (purines and pyrimidines) and their intermediates were broadly reduced in CA-treated cells whereas different classes of lipids were generally elevated (**Figure 2 – source data 2**).

The gene expression changes (RNA-seq, 4h) triggered by Mediator kinase inhibition tracked with these "downstream" metabolic changes (24h) (e.g. **Fig 2B, Figure 2 – figure supplement 1E**). For example, nucleotide biosynthesis mRNAs were down-regulated whereas lipid metabolism mRNAs were up-regulated in CA-treated cells; this was reflected in the GSEA Hallmarks, with positive enrichment for pathways such as cholesterol homeostasis, bile acid metabolism, and FA metabolism and negative enrichment for DNA repair pathways, which contains many nucleotide biosynthesis genes. Pyrimidine metabolism genes were also alternatively spliced in CA-treated cells. The differential timing of the transcriptomics and metabolomics experiments helps link CA-dependent metabolic changes to gene expression changes; however, we cannot exclude other factors and pathways. For example, reduced levels of nucleotides can trigger cellular compensation through up-regulation of FA levels (Schoors et al. 2015). Consequently, elevated levels of FA and other lipids may result, in part, from compensatory mechanisms triggered by CA-dependent reductions in nucleotide levels.

Pathway-specific regulation of splicing by Mediator kinases

Despite widespread study of Mediator kinases as regulators of RNAPII transcription, their potential function in splicing has not been addressed. We hypothesized that CDK8 and/or CDK19 may impact splicing based upon prior results that identified NAB2, SRRM2, and KDM3A as high-confidence CDK8/CDK19 substrates in human cells (Poss et al. 2016); each of these proteins has been linked to regulation of splicing (Gautam et al. 2015; Soucek et al. 2016; Baker et al. 2021). Our analysis revealed that exon skipping was dependent upon the kinase function of CDK8 and/or CDK19; increased inclusion or skipping of hundreds of alternative exons was observed in CA-treated cells (D21 or T21), and this was observed under both basal and IFNγ-stimulated conditions (**Fig 5C, D**).

Pathway analysis of the CA-sensitive alternative splicing events identified gene sets associated with signaling pathways known to be regulated by Mediator kinases, such as TGFβ and IFN signaling (**Fig 5E**). Interestingly, CA-dependent alternative splicing of these gene sets occurred specifically in IFNγ-stimulated T21 cells (not D21); moreover, other pathways were selectively affected by Mediator kinase inhibition in cell type and context-specific ways. For instance, mTOR pathway genes in CA-treated D21 cells and pyrimidine metabolism genes in T21 cells (**Fig 5F, G**).

Taken together, these results suggest that Mediator kinases regulate pre-mRNA splicing, which adds to the mechanisms by which CDK8 and CDK19 can influence gene expression. Our data further suggest that kinase-dependent splicing regulation occurs in cell type- and context-specific ways, similar to other known regulatory functions for CDK8 and CDK19 (Luyties and Taatjes 2022). However, the effects of CDK8/CDK19 on splicing appear to be modest, especially compared with CDK7, a different transcription-associated kinase (Rimel et al. 2020). The mechanisms by which CDK8 and/or CDK19 influence pre-mRNA splicing remain unclear, but are likely to involve both direct kinase-dependent regulation of splicing factors (e.g. through phosphorylation of NAB2 or SRRM2) and indirect regulation through modification of DNA-binding TFs (Rambout et al. 2018), which are common Mediator kinase substrates (Poss et al. 2016).

Differential expression of splicing regulatory proteins in CA-treated cells may also contribute, although the time frames of our analyses (RNA-seq at 4h post-treatment) minimize this possibility.

Mediator kinase inhibition broadly antagonizes IFN signaling

Independently and collectively, our transcriptomics, metabolomics, and cytokine screen data revealed that Mediator kinase inhibition suppressed pro-inflammatory signaling. This was most evident during IFNγ stimulation but was also apparent under basal conditions, especially in T21 cells due to their high "baseline" activation of IFN signaling pathways. We acknowledge that metabolites and cytokines can have variable, dynamic, and context-specific effects on inflammatory signaling. For example, sphingolipids (Maceyka and Spiegel 2014) and bile acids may serve pro- or anti-inflammatory roles depending upon cell type (Chiang and Ferrell 2019), and cytokine responses are complex, in part because cytokines regulate each other (e.g. MIP3α/CCL20 is down-regulated by IL10). For this study, a more straightforward metric for an anti-inflammatory change is whether CA treatment countered the effect of IFNγ stimulation alone. This was observed in an overwhelming number of cases across all experiments.

Among the IFNγ-responsive genes, the expression of many shifted in the opposite direction in IFNγ +CA conditions (**Fig 3A**). Based upon the RNA-seq data, IPA predicted CA-dependent inhibition of IFN-responsive TFs such as IRF1, IRF5, IRF7, NFKB1, and STAT1 (**Fig 3F, G; Figure 3 – figure supplement 2F, G**). Consistent with these findings, TFEA results showed rapid suppression of RELA (NFkB), IRF4, IRF8, and SPIB in CA-treated T21 cells (**Fig 6**), in agreement with prior results that showed that IFN-responsive TFs fail to activate when CDK8 and CDK19 are inhibited (Steinparzer et al. 2019). This CA-dependent suppression of IFN-responsive TFs is likely direct, given that DNA-binding TFs represent high-confidence Mediator kinase substrates (Poss et al. 2016).

Comparison of our T21 transcriptomics data with an RNA-seq analysis of whole blood from a large cohort of DS individuals (Waugh et al. 2023) identified a similar set of IFNγ-induced genes (**Figure 3 – figure supplement 2H**), linking our results to the general population. Importantly, expression of most of these so-called IFN "leading edge" genes was reduced upon Mediator kinase inhibition in T21 cells, suggesting that Mediator kinases help drive pro-inflammatory signaling in DS individuals.

To our knowledge, there has been no large-scale analysis of Mediator kinase-dependent effects on cytokine responses, despite much evidence that CDK8 and/or CDK19 help control inflammatory responses in a variety of mammalian cell types (Chen et al. 2017; Johannessen et al. 2017; Steinparzer et al. 2019; Freitas et al. 2022). Our screen of over 100 cytokines revealed that Mediator kinases control cytokine signaling under basal and IFNγ-stimulated conditions and generally had a pro-inflammatory effect (i.e. CDK8/CDK19 inhibition was anti-inflammatory). As with the transcriptional and metabolic changes, Mediator kinase inhibition reversed many IFNγ-induced changes in cytokine levels (**Fig 4D, E**). DS was recently characterized as a "cytokinopathy" and basal levels of as many as 22 cytokines were identified (among a screen of 29 cytokines) as elevated in the plasma of DS individuals (Malle et al. 2023). At least 6 of these 22 cytokines were reduced upon CA treatment in T21 cells (GMCSF, IL1α, IL3, IL13, IL15, GCSF), again suggesting that Mediator kinases drive inflammatory signaling in DS.

The CA-dependent metabolic changes also consistently reversed IFNγ-dependent effects; however, IFNγ treatment caused metabolic changes that were distinct in D21 vs. T21 cells. Interestingly, Mediator kinase inhibition increased levels of anti-inflammatory lipid signaling molecules (e.g. endocannabinoids, desmosterol, LCFA) in T21 cells under basal conditions (**Fig 2C**), whereas similar changes were observed in IFNγ stimulated D21 cells (**Fig 4A**). This may result from the elevated basal IFN signaling in T21 cells, such that CA treatment could measurably

suppress inflammatory pathways in the absence of exogenous IFNγ stimulation. In agreement, TFEA results showed rapid (45 min) and significant suppression of IFN-responsive TFs in CA-treated T21 cells (**Fig 6**). Furthermore, the CA-dependent suppression of p53 function (PRO-seq data t=45min, **Fig 6**) followed by evidence for CA-dependent activation of SREBP TFs (RNA-seq data t=4h, **Fig 2B**) provides a mechanistic basis for the elevated anti-inflammatory lipids in CA-treated cells. The ability of CA to antagonize the "basal" inflammatory signaling in T21 cells suggests a potential therapeutic strategy to mitigate the chronic immune system dysregulation in individuals with DS (Sullivan et al. 2017).

The anti-inflammatory lipids that were elevated in CA-treated cells included oleamide, desmosterol, endocannabinoids such as oleoylethanolamide, and PUFAs such as EPA, DPA, DHA and various other ω3 or ω6 PUFAs. These metabolites act as signaling molecules, at least in part, through binding nuclear receptors or GPCRs such as FFAR1, FFAR4, and GPR119, to initiate a cascade of anti-inflammatory responses (Fu et al. 2003; Basil and Levy 2016; Husted et al. 2017; Kimura et al. 2020; Jordan and Werz 2022). The wide-ranging impact of Mediator kinase inhibition on these lipid mediators of inflammation reveals new mechanisms by which CDK8 and/or CDK19 help control cellular responses to IFNγ; these results also mechanistically link Mediator kinase activity to nuclear receptor and GPCR function (**Fig 7**).

Working model: CDK8/19 inhibition enables persistent suppression of IFN signaling

Given the well-established roles for CDK8 and CDK19 as regulators of RNAPII transcription (Luyties and Taatjes 2022), the majority of direct CA-dependent effects likely result from gene expression changes. This hypothesis is also consistent with phosphoproteomics data that identified TFs and other RNAPII regulatory factors as high-confidence targets of CDK8/CDK19 in human cells (Poss et al. 2016). The staggered timing of our RNA-seq (4 hr), metabolomics (24 hr), and cytokine screen experiments (24 hr) allow inference of direct vs. indirect effects of Mediator kinase inhibition. Moreover, TFEA results in T21 cells (45 min) provided insights about CA-dependent changes in TF activity.

Taken as a whole, our results suggest that CDK8/CDK19 inhibition initiates a cascade of events that reinforce and propagate anti-inflammatory responses over time (**Fig 7**). At short timeframes (45 min), Mediator kinase inhibition suppressed activation of a cohort of IFN-responsive, pro-inflammatory TFs in T21 cells (**Fig 6**). Consistent with these results, mRNA levels of pro-inflammatory genes were suppressed in CA-treated T21 cells, as were protein levels of pro-inflammatory cytokines. TFEA also identified p53 TFs as suppressed in CA-treated T21 cells; p53 not only blocks cholesterol and lipid biosynthesis (e.g. to suppress growth), it also blocks SREBP activation (Moon et al. 2019). Suppression of p53 function in T21 cells could therefore elevate lipid biosynthesis and activate SREBP. In agreement, RNA-seq data identified SREBP (IPA upstream regulators), cholesterol homeostasis and fatty acid metabolism (GSEA Hallmarks) as activated upon Mediator kinase inhibition. SREBP TFs activate cholesterol and lipid biosynthesis pathways, and metabolomics data revealed elevated levels of anti-inflammatory lipids in CA-treated cells during IFNγ signaling (e.g. D21 cells +IFNγ; T21 cells at basal conditions). Among these elevated lipids, oleamide, desmosterol, and PUFAs are nuclear receptor agonists that activate PPAR and LXR (Yang et al. 2006; Sun and Bennett 2007; Spann et al. 2012; Roy et al. 2016); PPAR and LXR, in turn, broadly control fatty acid biosynthesis (Schultz et al. 2000), including PUFAs such as DPA, DHA, and EPA, whose levels were consistently elevated in CA-treated cells. These and other PUFAs bind GPCRs such as FFAR1/4 (Husted et al. 2017) to drive anti-inflammatory signaling cascades in an autocrine or paracrine manner. In this way, Mediator kinase inhibition may robustly and durably antagonize IFN signaling via metabolites that act downstream of initial gene expression changes (**Fig 7**).

Materials & Methods

Cell culture

Immortalized lymphoblastoid cell lines derived from age- and sex-matched sibling pairs (one D21: TIC0002294, one T21: TIC0001678) were obtained from the Nexus Biobank. Cells were cultured in RPMI medium (Gibco, 23400062) supplemented with 20% fetal bovine serum (Peak Serum Inc, PS-FB3), 1x GlutaminePlus (R&D Systems, R90210), and 1x Penicillin-Streptomycin (Gibco, 15140-163), on low-attachment flasks at 37C and 5% CO_2. Due to the lower proliferation rate of T21 cells compared to D21 cells, T21 cells were seeded at twice the density of D21 cells for all experiments unless otherwise noted. For all cortistatin A treatments, the compound remained in the media during IFNγ stimulation.

Western blotting

RIPA lysis buffer (Thermo Scientific, 89900) was used to generate cell lysates; all lysis was performed at 4ºC in the presence of Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100X) (Thermo Scientific, 78441) and Benzonase® endonuclease (Millipore Sigma, 101697). The BCA Protein Assay Kit (Pierce, 23227) was used to measure and normalize protein concentrations across samples. Protein samples were boiled for 5 minutes at 95ºC in 4x Laemmli Sample Buffer (Bio-Rad, 1610747) and run on SDS-acrylamide gels, followed by transfer to nitrocellulose membranes. Blocking was performed using either 5% milk in TBS-T or in 5% bovine serum albumin for phospho-antibodies.

Antibodies used are as follows: anti-STAT1 (RRID: AB_2198300; Cell Signaling Technology, 9172), anti-phospho STAT1 S727 (RRID: AB_2197983; Cell Signaling Techology, 9177), and anti-GAPDH (RRID: AB_1080976; GeneTex, GTX100118). HRP-conjugated anti-rabbit secondary antibody was used for detection (Invitrogen, 31480) and protein was visualized using Immunobilon Western Chemilumiescent HRP Substrate (Millipore, WBKLS0500) on an ImageQuant LAS 4000 (GE Healthcare). Intensity values were quantified and normalized using ImageJ.

RT-qPCR

Cells were seeded and allowed to recover for 24 hours prior to being treated with 10 ng/mL interferon gamma (Gibco PHC4031) or vehicle (40 mM Tris pH 7.4). Total RNA was isolated using TRizol (Invitrogen, 15596026) according to the manufacturer's instructions, quantified with a Qubit® 3.0 using the RNA High Sensitivity (HS) kit (Invitrogen™, Q32855), and 100 ng of RNA from each sample was converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368813) following the manufacturer's instructions. cDNA was diluted to 0.25 ng/µL for all samples, and amplification was performed using Sybr Select Master Mix (Thermo Fisher Scientific, 4472908) on a BioRad CFX384 Real Time PCR System. ΔΔCT values were calculated using GAPDH as a loading control. Treatments, RNA isolation, and RT-qPCR were performed in biological duplicate. The primers used for qPCR are as follows - GAPDH Forward: ACCACAGTCCATGCCATCAC, GAPDH Reverse: TCCACCACCCTGTTGCTGTA, GBP1 Forward: GTGCTAGAAGCCAGTGCTCGT, GBP1 Reverse: TGGGCCTGTCATGTGGATCTC, IRF1 Forward: GAGGAGGTGAAAGACCAGAGC, IRF1 Reverse: TAGCATCTCGGCTGGACTTCGA.

To probe the timing of the RNA-seq experiments, we completed RT-qPCR at various time points as shown in **Figure 1 – figure supplement 1B**. The 4h time point (post-IFNγ) was chosen because robust induction of GBP1 and IRF1 mRNA was observed. Whereas mRNA levels remained high at 6h, we selected the 4h time point because it would better represent the primary transcriptional response; moreover, the 4h time point was consistent with prior transcriptomics studies of IFNγ response in mammalian cells (Steinparzer et al. 2019).

Metabolomics

For each treatment condition, six flasks of cells were seeded and allowed to recover for 24 hours prior to treatment. Cells were pre-treated with 0.1% DMSO or 100 nM cortistatin A for one hour, then treated with 40 mM Tris, pH 7.4 or 10 ng/mL interferon gamma (Gibco, PHC4031). After 24 hours of interferon gamma treatment, cells were harvested and snap-frozen in liquid nitrogen. Sample preparation was carried out by Metabolon (Durham, North Carolina, USA) using a previously published workflow (Ford et al. 2020). Samples were prepared using the automated MicroLab STAR® system from Hamilton Company, and several

recovery standards were added prior to the first step in the extraction process for QC purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent.

All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 μm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion conditions, however it was chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same afore mentioned C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, however with 6.5mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 μm) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate, pH 10.8. The MS analysis alternated between MS and data-dependent MSn scans using dynamic exclusion. The scan range varied slighted between methods but covered 70-1000 m/z.

Identification of metabolites was performed through automated comparison of the ion features in experimental samples to a reference library of chemical standard entries (Dehaven et al. 2010), and are based on three criteria: retention index within a narrow RI window of the proposed identification, accurate mass match to the library +/- 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum. While there may be similarities between these molecules based on one of these factors, the use of all three data points can be utilized to distinguish and differentiate biochemicals.

Statistical and pathway analysis of metabolomics data

Two types of statistical analyses were performed: (1) significance tests and (2) classification analysis. Standard statistical analyses were performed in Array Studio on log-transformed data. For analyses not standard in Array Studio, the R program (http://cran.r-project.org/) was used. Following log transformation and imputation of missing values, if any, with the minimum observed value for each compound, Welch 2-sample *t* test was used as a significance test to identify biochemicals that differed significantly ($p < 0.05$) between experimental groups. An estimate of the false discovery rate (*q*-value) was calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies. Classification analyses included principal component analysis (PCA), hierarchical clustering, and random forest.

Pathway analysis of metabolomic data was performed using the Ingenuity Pathway Analysis software (Qiagen) (Kramer et al. 2014). For metabolomic analysis, a total of 566 out of 675 metabolites could be mapped using one of the following identifiers: CAS registry number, Human Metabolome Database (HMDB) identification, KEGG, PubChem CID, or RefSeq number; metabolites not used in IPA analysis can be found in **Figure 1 – source data 2**. Metabolomic pathway analysis used only mapped metabolites with a p-value of <0.1 when making pathway predictions.

RNA-seq
Cells were pre-treated with 0.1% DMSO or 100 nM cortistatin A for 30 minutes, then treated with 40 mM Tris, pH 7.4 or 10 ng/mL interferon gamma (Gibco, PHC4031). After four hours, total RNA was isolated from D21 or T21 cells using TRizol (Invitrogen, 15596026) as specified by the manufacturer and quantified with a Qubit® 3.0 using the RNA High Sensitivity (HS) kit (Invitrogen™, Q32855). 1 µg of total RNA with a RIN number of ≥8 was used for RNA-seq library prep. Libraries were constructed using Universal Plus™ mRNA-Seq library preparation kit with NuQuant® (Tecan, 0520). Library construction and sequencing were performed at the Genomics Shared Resource (CU Anschutz). Paired-end libraries (151 bp x 151 bp) were sequenced on the Illumina NextSeq 6000 platform (Firmware version 1.26.1, RTA version: v3.4.4, Instrument ID: A00405).

RNA-seq sequencing and computational analysis
For differential gene expression analysis, the workflow was as follows: adaptor sequences were trimmed from the RNA-seq raw fastq files using BBDuk (https://sourceforge.net/projects/bbmap/) and mapped using HISAT2(Kim et al. 2019). Gene counts were generated using featureCounts(Liao et al. 2014), and differential expression analysis was performed using DESeq2 (Anders and Huber 2010). Duplicate genes were filtered and those with the highest FPKM were kept for analysis. For inter-genotype (T21 vs D21) comparisons, expression of genes on chromosome 21 was normalized to ploidy. While ERCC RNA Spike-In Mix (Invitrogen™, 4456740) was added to isolated RNA samples prior to sequencing, ERCC gene counts were variable across vehicle treated samples even after accounting for read depth, and when using multiple normalization methods and a linear regression. As such, the median of ratios method native to DESeq2 was used to generate size factors and normalize samples. Qiagen ingenuity pathway analysis (IPA) version 90348151 (Kramer et al. 2014), GSEA 4.2.3 (Subramanian et al. 2005), and Gene Ontology analysis (Ashburner et al. 2000) were used for identification of activated and inhibited pathways. For IPA analysis, a total of 27,647 out of 28,265 genes could be mapped using Entrez Gene Symbols. For cross-genotype (i.e. T21 vs D21) pathway analysis, only genes with a fold-change > |1.25| and adjusted p-value <0.1 were used. For within-genotype (i.e. treatment effects) pathway analysis, only genes with an adjusted p-value <0.1 were used.

For splicing analysis, the workflow was as follows: duplicates were removed and adaptors trimmed using the bbTools function (v39.01), trimmed reads were mapped uniquely against the hg38 genome using HISAT2 (v2.1.0) (Kim et al. 2019), mapped reads were processed with rMATS (v4.0.1) (Shen et al. 2014), and results were filtered based on FDR < 0.05, absolute(IncLevelDifference) < 0.2, and ≥2 reads/replicate. Sashimi plots were generated from rMATS results using a modified script based on ggsashimi.py.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE220652 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE220652). Code used to process and visualize data can be found at https://github.com/kira-alia/Cozzolino2023.

Cytokine screen
Cells were pre-treated with 0.1% DMSO or 100 nM cortistatin A for 30 minutes, then treated with 40 mM Tris, pH 7.4 or 10 ng/mL interferon gamma (Gibco, PHC4031). After 24 hours, cells were lysed in RIPA buffer (Thermo Scientific, 89900) supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100X) (Thermo Scientific, 78441) and Benzonase® endonuclease (Millipore Sigma, 101697). Lysate concentrations were determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, 23225), and 250 µg of protein from each condition was incubated with a membrane array from the Proteome Profiler Human XL Cytokine Array Kit (R&D Systems, ARY022B). Membranes were processed according to the manufacturer's instructions and imaged using an ImageQuant LAS 4000 (GE Healthcare). Background-subtracted technical replicate values for each cytokine in each condition were quantified using ImageJ and averaged giving a normalized intensity value. The relative intensity was compared on a per cytokine basis and was statistically assessed using a one-way ANOVA test between the eight total conditions across two biological replicates.

PRO-seq
Cells were treated with 0.1% DMSO or 100 nM cortistatin A for 75 minutes before being transferred into a lysis buffer consisting of final concentrations of 10 mM Tris pH 7.4, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 0.5% NP-40, 10% glycerol, 1 mM DTT, 1x Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, 78441), and 2 U/µL SUPERase•In™ RNase Inhibitor (Invitrogen, AM2696). Nuclei were isolated and cleaned through repeated centrifugation and washes, and were counted, aliquoted, and snap-frozen in using liquid nitrogen in a freezing buffer consisting of 50 mM Tris pH 8.3, 5 mM $MgCl_2$, 40% glycerol. 0.1 mM EDTA, and 4 U/µL SUPERase•In™ RNase Inhibitor (Invitrogen, AM2696). Nuclear run-ons were performed for 3 minutes at 37ºC on a Groovin' Tubes thermoshaker (Boekel Scientific, 270500), on a mixture of 10 million human and 100,000 *Drosophila melanogaster* nuclei per replicate. PRO-seq library preparation was performed as described (Fant et al. 2020). Library sequencing was performed at the Cornell Genomics Facility (Cornell Institute of Biotechnology) on the Illumina NextSeq 2000 platform (Instrument ID VH00231).

PRO-seq data analysis
Raw fastq files from the sequencing pipeline described above were trimmed and mapped using the NascentFlow pipeline (DOI 10.17605/OSF.IO/NDHJ2). Bidirectional sites of transcription in the human genome (assembly hg38) were identified using the BidirectionalFlow pipeline (https://github.com/Dowell-Lab/Bidirectional-Flow), which runs TFIT (Azofeifa and Dowell 2017). Bidirectional calls across all replicates and conditions relevant to this study were merged using muMerge (https://github.com/Dowell-Lab/mumerge) and used as an annotation file for running Transcription Factor Enrichment Analysis (Rubin et al. 2021). Example traces of differentially active sites of bidirectional transcription were plotted using pyGenomeTracks (Lopez-Delisle et al. 2021).

The data in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE249549. Code used to process and visualize data can be found at https://github.com/kira-alia/Cozzolino2023.

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Competing interests: D.J.T. is a member of the SAB at Dewpoint Therapeutics; R.D.D. is a founder of Arpeggio Biosciences; J.M.E. has provided consulting services for Eli Lilly and Gilead, and serves on the advisory board of Perha Pharmaceuticals.

Figures

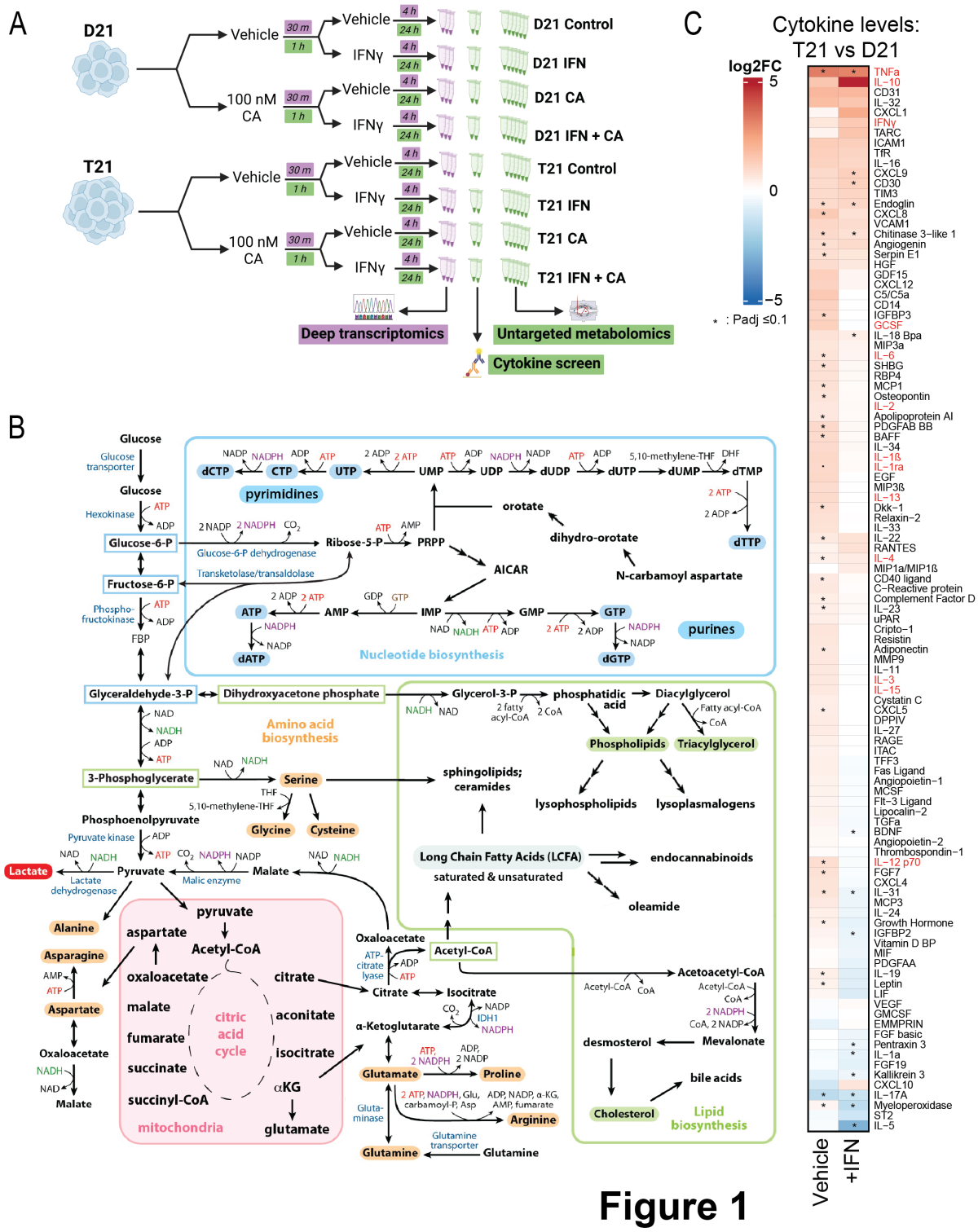


Fig 1. Experimental overview; elevated cytokines in T21 cells. (A) Schematic of cell treatment and data collection workflow for metabolomics, cytokine screen (green shading) and RNA-seq (purple shading). Created with Biorender.com. **(B)** Simplified diagram of human metabolic pathways, with an emphasis on those relevant to this study. Figure adapted from (Lunt and Vander Heiden 2011). **(C)** Heatmap of all cytokines measured (n = 105), comparing relative levels in vehicle-treated T21 cells vs. vehicle-treated D21 cells (left column) and relative levels in IFNγ-treated T21 cells vs. IFNγ-treated D21 cells (right column). Asterisk denotes Padj ≤ 0.1.

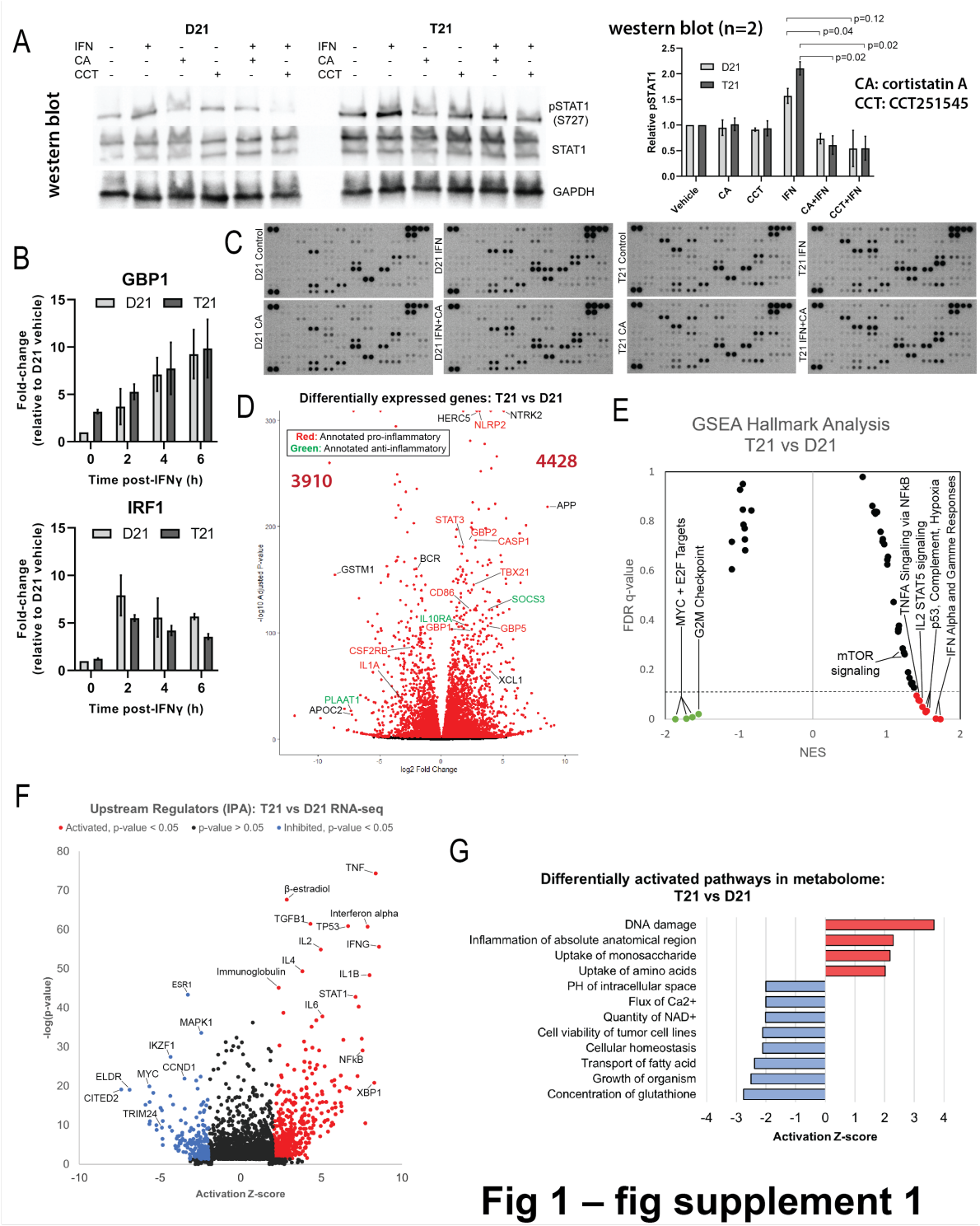
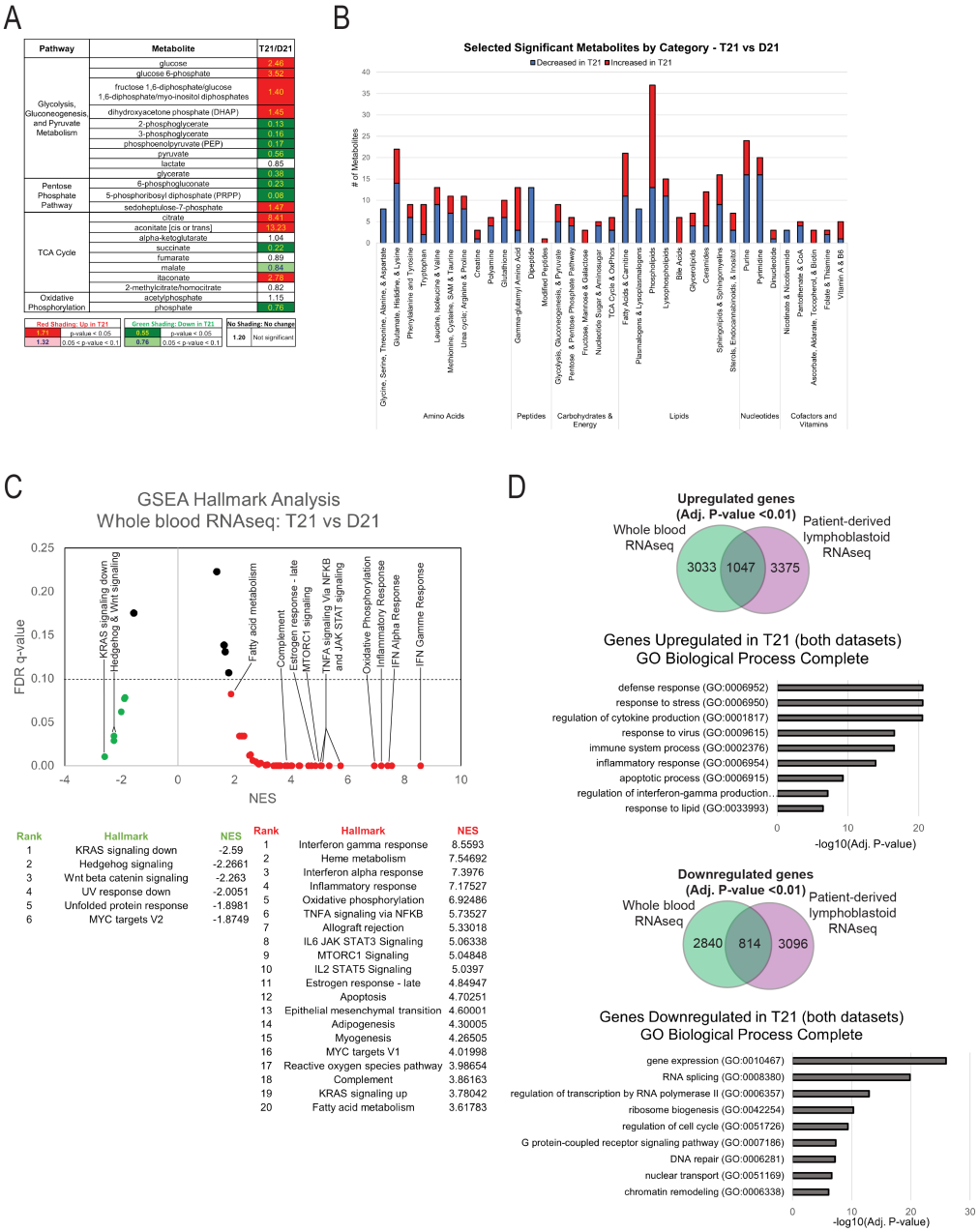


Figure 1–figure supplement 1. Comparisons between T21 and D21 cells. (A) Western blots of STAT1 S727 phosphorylation following 45min treatment with IFNγ with or without 30min pretreatment with cortistatin A (100nM) or CCT251545 (250nM), two structurally distinct inhibitors of CDK8/19. At right, data represented as mean ±SD from two biological replicate experiments. **(B)** Time course of relative gene expression levels measured by real-time quantitative PCR (RT-qPCR) of GBP1 and IRF1, two interferon stimulated genes, following treatment with 10 ng/mL IFNγ compared to untreated cells. Data normalized to expression of GAPDH and represented as mean ± SD from two independent experiments. Inset panel represents relative basal expression of each gene in untreated T21 cells compared to untreated D21 cells. **(C)** Representative data from

cytokine protein arrays. Arrays were incubated with cell lysates (250 μg total protein) treated as shown. Multiple exposures were taken to ensure that every spot was measured in the linear range; duplicate spots were averaged. Averaged intensity values from two biological replicates/condition were analyzed using ANOVA. **(D)** Volcano plot of differentially expressed genes in T21 cells compared to D21; selected genes are color-coded based on proinflammatory (red), anti-inflammatory (green), or other (black) roles in IFN signaling. Genes represented by red dots have an adjusted p-value of <0.01. **(E)** GSEA moustache plot of Hallmark pathways comparing T21 cells with D21. **(F)** Ingenuity Pathway Analysis of upstream regulators predicted to be responsible for differential transcription in T21 cells compared to D21 cells. Analysis used genes >1.25x up- or down-regulated, with an adjusted p-value <0.1. List of regulators was filtered to exclude drugs or other exogenous compounds. **(G)** Selected results of pathway analysis based on metabolite levels in untreated T21 compared with D21 cells, using the “Diseases & Functions” list from Ingenuity Pathway Analysis.

Fig 1 – fig supplement 2



Pathway	Metabolite	T21/D21
Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	glucose	2.46
	glucose 6-phosphate	3.52
	fructose 1,6-diphosphate/glucose 1,6-diphosphate/myo-inositol diphosphates	1.40
	dihydroxyacetone phosphate (DHAP)	1.45
	2-phosphoglycerate	0.13
	3-phosphoglycerate	0.16
	phosphoenolpyruvate (PEP)	0.17
	pyruvate	0.56
	lactate	0.85
	glycerate	0.38
Pentose Phosphate Pathway	6-phosphogluconate	0.25
	5-phosphoribosyl diphosphate (PRPP)	0.08
	sedoheptulose-7-phosphate	1.47
TCA Cycle	citrate	8.41
	aconitate [cis or trans]	11.94
	alpha-ketoglutarate	1.04
	succinate	0.22
	fumarate	0.89
	malate	0.84
	itaconate	4.76
	2-methylcitrate/homocitrate	0.82
Oxidative Phosphorylation	acetylphosphate	1.15
	phosphate	0.76

Red Shading: Up in T21	
1.73	p-value < 0.05
1.32	0.05 < p-value < 0.1

Green Shading: Down in T21	
0.35	p-value < 0.05
0.76	0.05 < p-value < 0.1

No Shading: No change	
1.20	Not significant

Rank	Hallmark	NES
1	KRAS signaling down	-2.59
2	Hedgehog signaling	-2.2661
3	Wnt beta catenin signaling	-2.263
4	UV response down	-2.0051
5	Unfolded protein response	-1.8981
6	MYC targets V2	-1.8749

Rank	Hallmark	NES
1	Interferon gamma response	8.5593
2	Heme metabolism	7.54692
3	Interferon alpha response	7.3976
4	Inflammatory response	7.17527
5	Oxidative phosphorylation	6.92486
6	TNFA signaling via NFKB	5.73527
7	Allograft rejection	5.33018
8	IL6 JAK STAT3 Signaling	5.06338
9	MTORC1 Signaling	5.04848
10	IL2 STAT5 Signaling	5.0397
11	Estrogen response - late	4.84947
12	Apoptosis	4.70251
13	Epithelial mesenchymal transition	4.60001
14	Adipogenesis	4.30005
15	Myogenesis	4.26505
16	MYC targets V1	4.01998
17	Reactive oxygen species pathway	3.98654
18	Complement	3.86163
19	KRAS signaling up	3.78042
20	Fatty acid metabolism	3.61783

Figure 1–figure supplement 2. Metabolic differences in T21 vs. D21; Data comparison with whole blood cohort clinical studies. (A) Relative levels of metabolites from core metabolic pathways in untreated T21 cells compared with untreated D21 cells. **(B)** Differential metabolites summary (ANOVA p-value <0.1) in untreated T21 cells relative to untreated D21 cells, broken down by category; excludes unknown and xenobiotic metabolites. **(C)** GSEA moustache plot of Hallmark pathway activation in whole blood samples from a cohort study of T21 individuals compared to euploid controls (Waugh et al. 2023). **(D)** Venn diagram of upregulated or downregulated genes (adjusted p-value <0.01) in Trisomy 21 from this study and in whole blood samples from the cohort study (NCT02864108). Genes either up- or down-regulated in both studies were analyzed using Gene Ontology Enrichment Analysis with the GO aspect "Biological Processes."

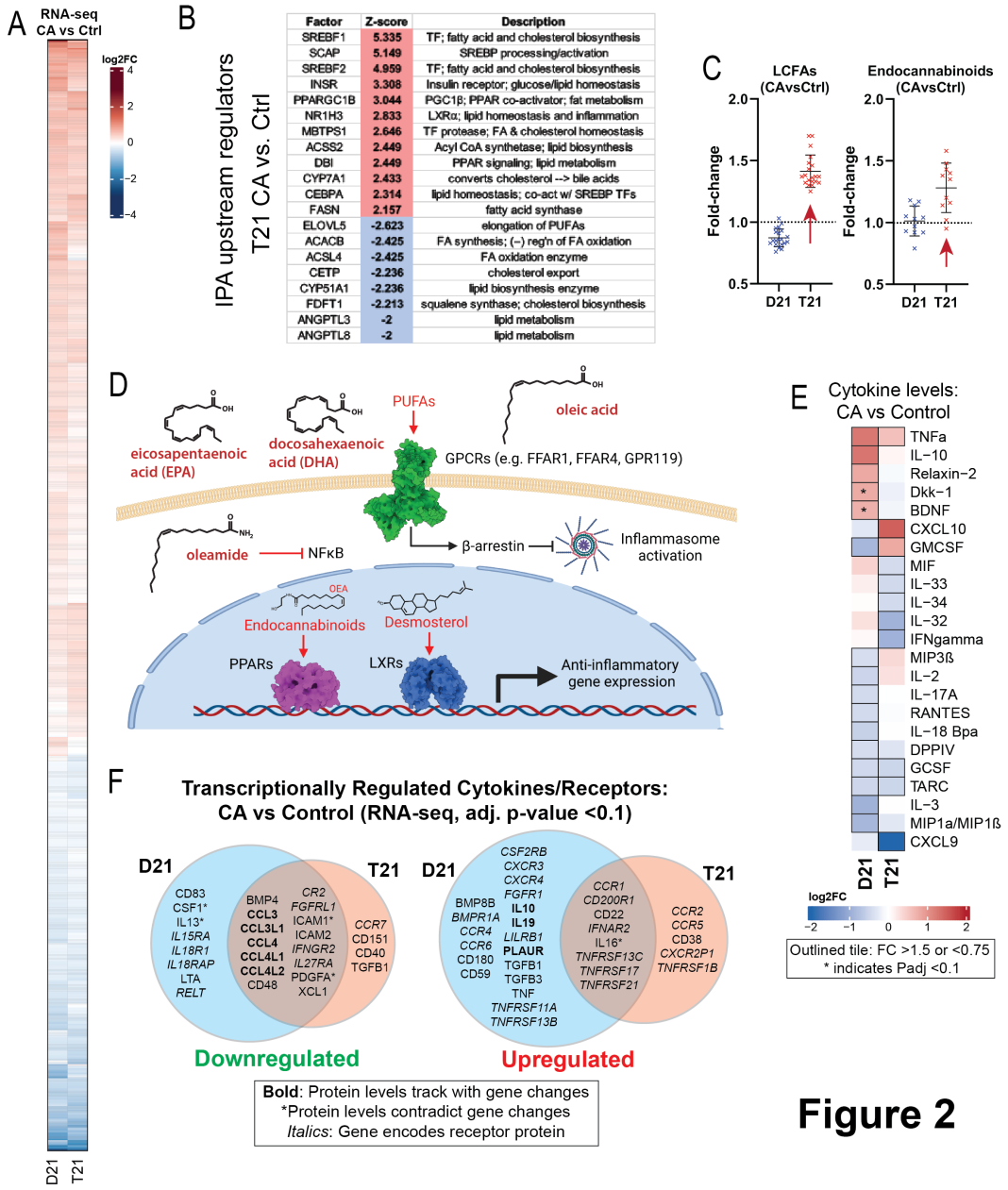
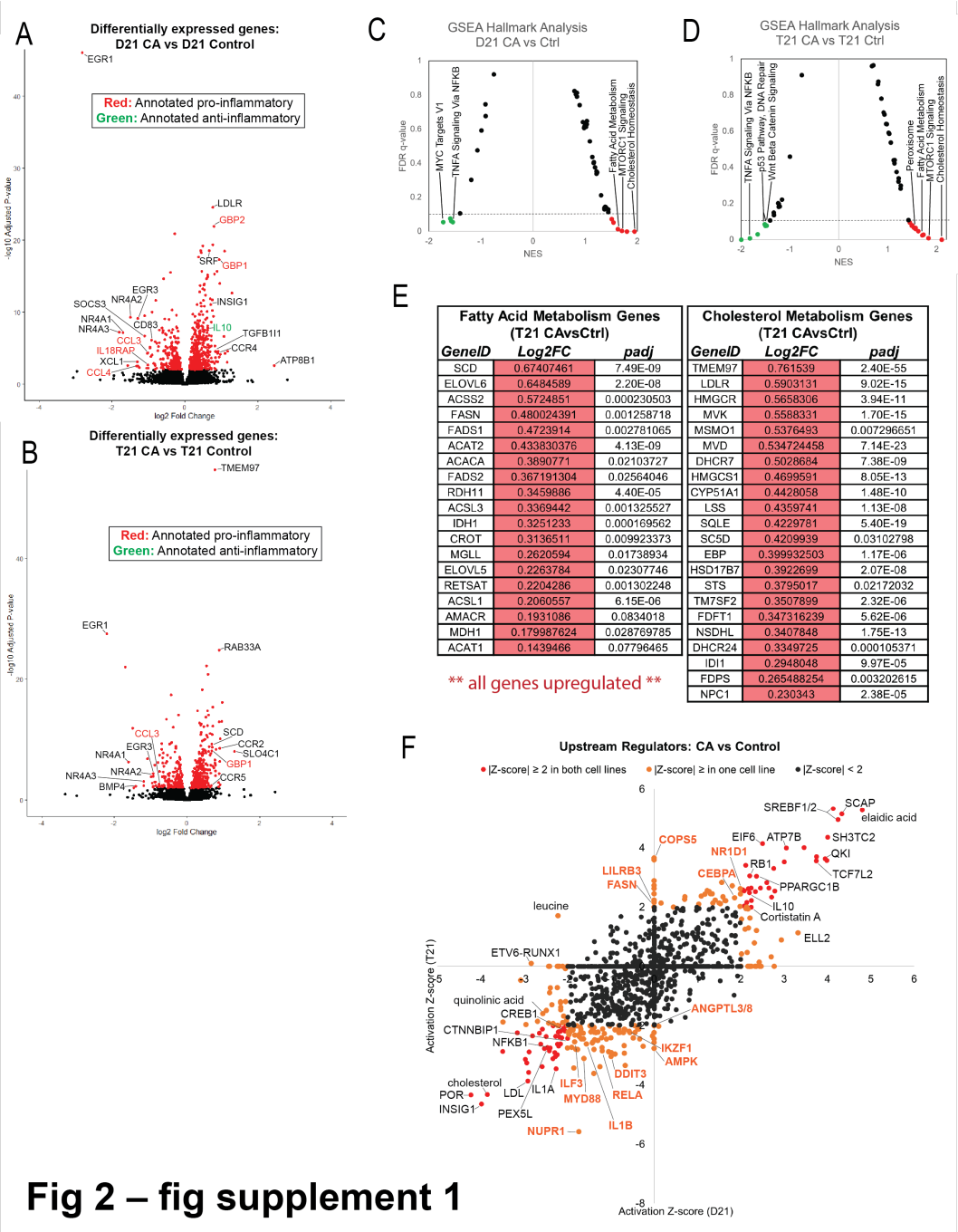


Fig 2. Mediator kinase inhibition tempers inflammatory pathways under basal conditions, with T21-specific effects. (A) Heatmap of genes with differential expression in D21 or T21 cells treated with CA (100 nM) compared to DMSO controls. Genes with adjusted p-value <0.01 in one or both cell lines are shown. **(B)** Table of activation Z-scores for selected upstream regulators predicted for gene expression changes in CA-treated T21 cells relative to DMSO controls. **(C)** Box plots showing relative levels of selected lipid metabolites in CA-treated D21 and T21 cells compared to DMSO controls. **(D)** Simplified diagram of pathways through which selected lipid metabolites regulate inflammation. **(E)** Heatmap of average relative cytokine levels in CA-treated cells compared to DMSO controls. Only cytokines with relative levels of ≥1.5 (log2FC; red shading) or ≤0.75 (blue shading) in one or both cell lines are shown; cytokines meeting this threshold are outlined in black. Asterisk (*) denotes adjusted p-value <0.1 using ANOVA. **(F)** Venn diagrams of cytokines and cytokine receptor genes (in *italics*) that were downregulated (FC <0.8, left diagram) or upregulated (FC >1.2, right diagram) in CA-treated D21 and T21 cells, compared to DMSO controls. Cytokines with matching trends from RNA-seq (4h) are listed in bold, whereas cytokines with inverse trends in at least one cell type are marked with an asterisk.



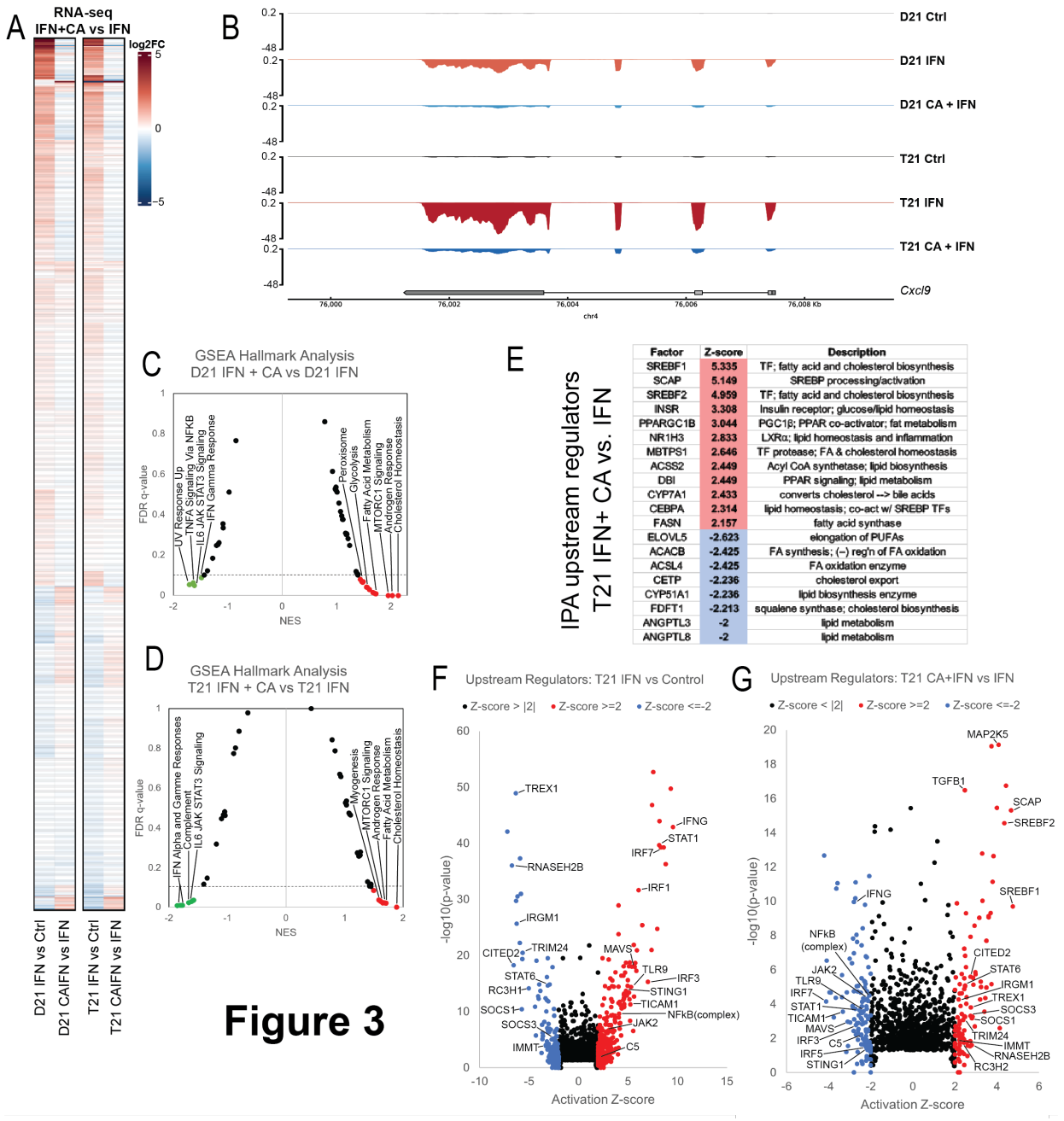
Fatty Acid Metabolism Genes (T21 CAvsCtrl)		
GeneID	*Log2FC*	*padj*
SCD	0.67407461	7.49E-09
ELOVL6	0.6484589	2.20E-08
ACSS2	0.5724851	0.000230503
FASN	0.480024391	0.001258718
FADS1	0.4723914	0.002781065
ACAT2	0.433830376	4.13E-09
ACACA	0.3890771	0.02103727
FADS2	0.367191304	0.02564046
RDH11	0.3459886	4.40E-05
ACSL3	0.3369442	0.001325527
IDH1	0.3251233	0.000169562
CROT	0.3136511	0.009923373
MGLL	0.2620594	0.01738934
ELOVL5	0.2263784	0.02307746
RETSAT	0.2204286	0.001302248
ACSL1	0.2060557	6.15E-06
AMACR	0.1931086	0.0834018
MDH1	0.179987624	0.028769785
ACAT1	0.1439466	0.07796465

** all genes upregulated **

Cholesterol Metabolism Genes (T21 CAvsCtrl)		
GeneID	*Log2FC*	*padj*
TMEM97	0.761539	2.40E-55
LDLR	0.5903131	9.02E-15
HMGCR	0.5658306	3.94E-11
MVK	0.5588331	1.70E-15
MSMO1	0.5376493	0.007296651
MVD	0.534724458	7.14E-23
DHCR7	0.5028684	7.38E-09
HMGCS1	0.4699591	8.05E-13
CYP51A1	0.4428058	1.48E-10
LSS	0.4359741	1.13E-08
SQLE	0.4229781	5.40E-19
SC5D	0.4209939	0.03102798
EBP	0.399932503	1.17E-06
HSD17B7	0.3922699	2.07E-08
STS	0.3795017	0.02172032
TM7SF2	0.3507899	2.32E-06
FDFT1	0.347316239	5.62E-06
NSDHL	0.3407848	1.75E-13
DHCR24	0.3349725	0.000105371
IDI1	0.2948048	9.97E-05
FDPS	0.265488254	0.003202615
NPC1	0.230343	2.38E-05

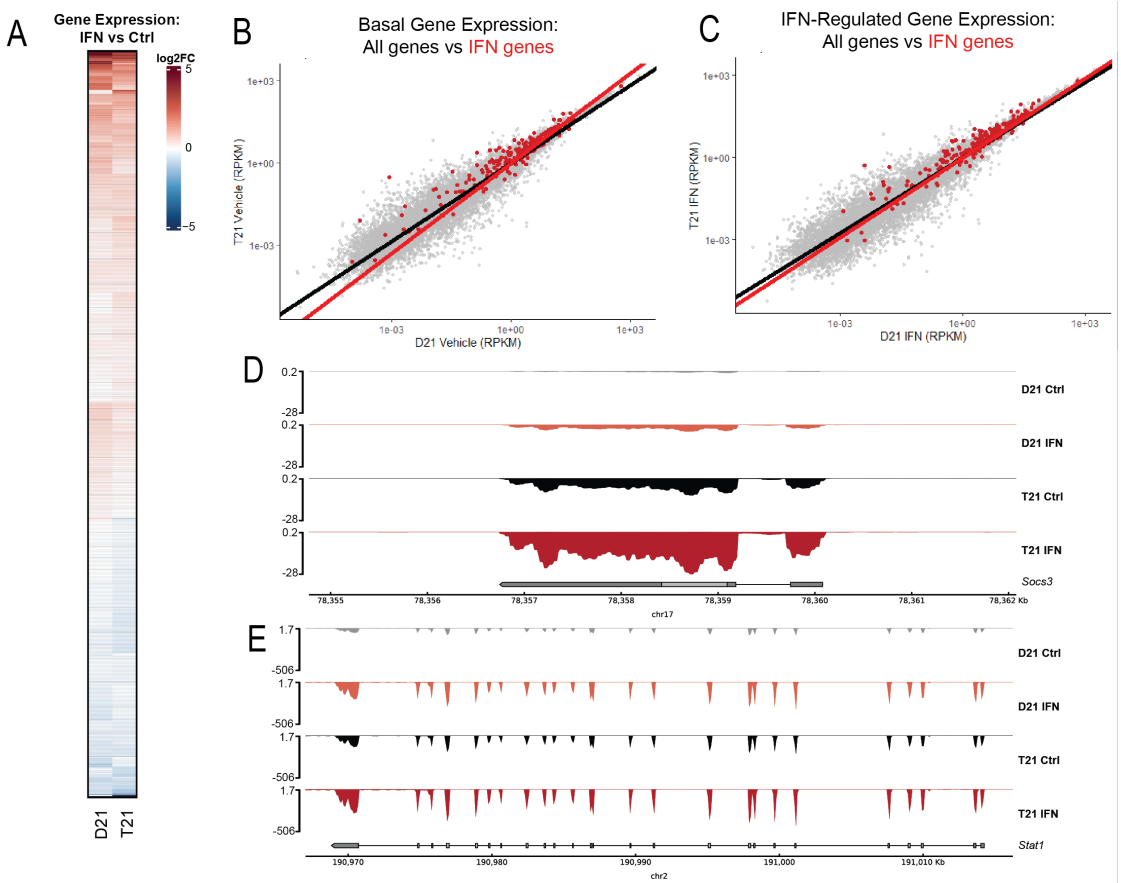
Fig 2 – fig supplement 1

Figure 2–figure supplement 1. CA suppresses T21 inflammatory pathways under basal conditions. (A, B) Volcano plots showing differentially expressed genes following CA treatment in D21 (A) or T21 (B) cells, with selected genes color-coded based on pro-inflammatory (red), anti-inflammatory (green) roles. Genes represented by red dots have an adjusted p-value of <0.01. **(C, D)** GSEA moustache plots of Hallmark pathway activation in CA-treated D21 (C) and T21 (D) cells compared to DMSO controls. **(E)** Tables of genes involved in fatty acid metabolism (left) or cholesterol metabolism (right) that are differentially expressed (adjusted p-value ≤ 0.1) in CA-treated T21 cells relative to vehicle controls, showing consistent upregulation in both pathways. **(F)** Ingenuity Pathway Analysis of upstream regulators predicted for differential gene expression (RNA-seq) in CA-treated D21 and T21 cells compared to DMSO controls. Predicted regulators specific for T21 cells are labeled in orange font. Analysis used genes with an adjusted p-value <0.1. List of regulators was filtered to exclude drugs or other exogenous compounds, with the exception of Cortistatin A.



Factor	Z-score	Description
SREBF1	5.335	TF; fatty acid and cholesterol biosynthesis
SCAP	5.149	SREBP processing/activation
SREBF2	4.959	TF; fatty acid and cholesterol biosynthesis
INSR	3.308	Insulin receptor; glucose/lipid homeostasis
PPARGC1B	3.044	PGC1β; PPAR co-activator; fat metabolism
NR1H3	2.833	LXRα; lipid homeostasis and inflammation
MBTPS1	2.646	TF protease; FA & cholesterol homeostasis
ACSS2	2.449	Acyl CoA synthetase; lipid biosynthesis
DBI	2.449	PPAR signaling; lipid metabolism
CYP7A1	2.433	converts cholesterol --> bile acids
CEBPA	2.314	lipid homeostasis; co-act w/ SREBP TFs
FASN	2.157	fatty acid synthase
ELOVL5	-2.623	elongation of PUFAs
ACACB	-2.425	FA synthesis; (−) reg'n of FA oxidation
ACSL4	-2.425	FA oxidation enzyme
CETP	-2.236	cholesterol export
CYP51A1	-2.236	lipid biosynthesis enzyme
FDFT1	-2.213	squalene synthase; cholesterol biosynthesis
ANGPTL3	-2	lipid metabolism
ANGPTL8	-2	lipid metabolism

Fig 3. Mediator kinase inhibition antagonizes IFNγ transcriptional responses in T21 and D21. (A) Heatmap comparing gene expression patterns (RNA-seq) in IFNγ-treated D21 or T21 cells ±CA. This comparison shows CA-specific effects during IFNγ stimulation, which broadly counter changes caused by IFNγ alone. Genes with statistically significant (Padj <0.01) levels in one or both cell lines in IFNγ vs. control comparisons are shown. **(B)** Representative genome browser tracks for CXCL9 locus in D21 and T21 cells treated with vehicle, IFNγ, or IFNγ +CA. **(C, D)** GSEA moustache plots of Hallmark pathways in D21 (C) and T21 (D) cells treated with IFNγ +CA compared to IFNγ alone. This shows CA-specific effects during IFNγ stimulation. **(E)** Table of activation Z-scores for selected upstream regulators in CA-treated T21 cells treated with IFNγ +CA relative to IFNγ alone. This curated set emphasizes lipid metabolite changes. **(F, G)** Ingenuity Pathway Analysis upstream regulators results from comparison of differential gene expression in T21 cells during IFNγ treatment (F) or in cells treated with IFNγ +CA compared to IFNγ alone (G). Selected TFs and other factors associated with inflammatory responses are labeled. Analysis used only genes with Padj <0.1.



F

Top Decreased IFN Responsiveness in T21		
GeneID	log2FC	padj
HAPLN3	-2.976	7.94E-41
GBP5	-2.192	5.92E-18
UBD	-1.876	2.23E-04
BCL6	-1.744	1.39E-04
MYH7	-1.618	3.03E-03
C4B	-1.589	4.67E-04
C4B_2	-1.589	4.67E-04
LOC110384692	-1.589	4.67E-04
C4A	-1.589	4.79E-04
CXCR5	-1.489	4.84E-03
SOCS3	-1.398	6.27E-06
TUSC1	-1.391	8.46E-02
SLFN12L	-1.300	1.23E-05
MUC1	-1.228	3.82E-03
ITK	-1.208	6.04E-04
BCL2L14	-1.154	9.35E-03
GBP1	-1.039	3.08E-10
GBP4	-0.964	4.83E-06
ETV7	-0.956	3.58E-06
ZNF697	-0.914	5.07E-05
GBP2	-0.894	2.98E-14
WARS	-0.868	8.12E-18
SIK1	-0.856	5.45E-03
SIK1B	-0.856	5.45E-03
GBP3	-0.839	1.19E-06
IRF1	-0.767	3.70E-17
FRMD6	-0.747	1.79E-02
RHOBTB3	-0.742	1.92E-03
AHR	-0.720	3.05E-05
STAT1	-0.684	4.79E-03
IRF5	-0.684	8.86E-06
CSF1	-0.665	4.79E-03
APOL6	-0.658	3.70E-04
FZD5	-0.650	6.50E-03
STAT3	-0.619	5.75E-10
TMEM140	-0.603	9.76E-03
CYTOR	-0.574	1.06E-02
TAP1	-0.560	4.49E-11
PIM2	-0.560	2.25E-02
UBE2L6	-0.558	2.54E-02
FBXO6	-0.554	8.06E-02
MDGA1	-0.550	5.53E-02
XKR8	-0.545	3.30E-06
SLC35F6	-0.539	7.82E-07
PRKXP1	-0.516	7.95E-02
AIM2	-0.507	8.33E-02

Increased IFN Responsiveness in T21		
GeneID	log2FC	padj
FAM156B	2.764	6.43E-02
KBTBD11	1.994	4.00E-03
CCNG2	0.786	5.55E-02
STAMBPL1	0.644	4.28E-03

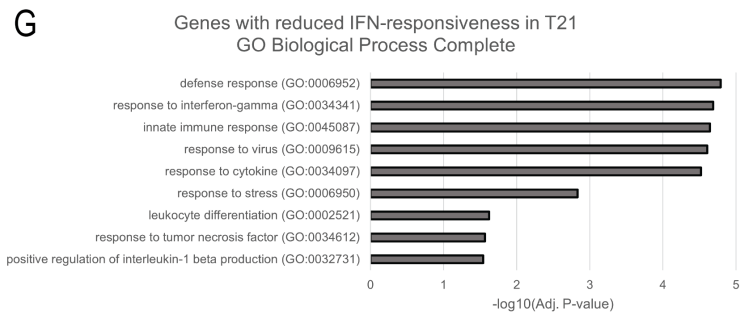
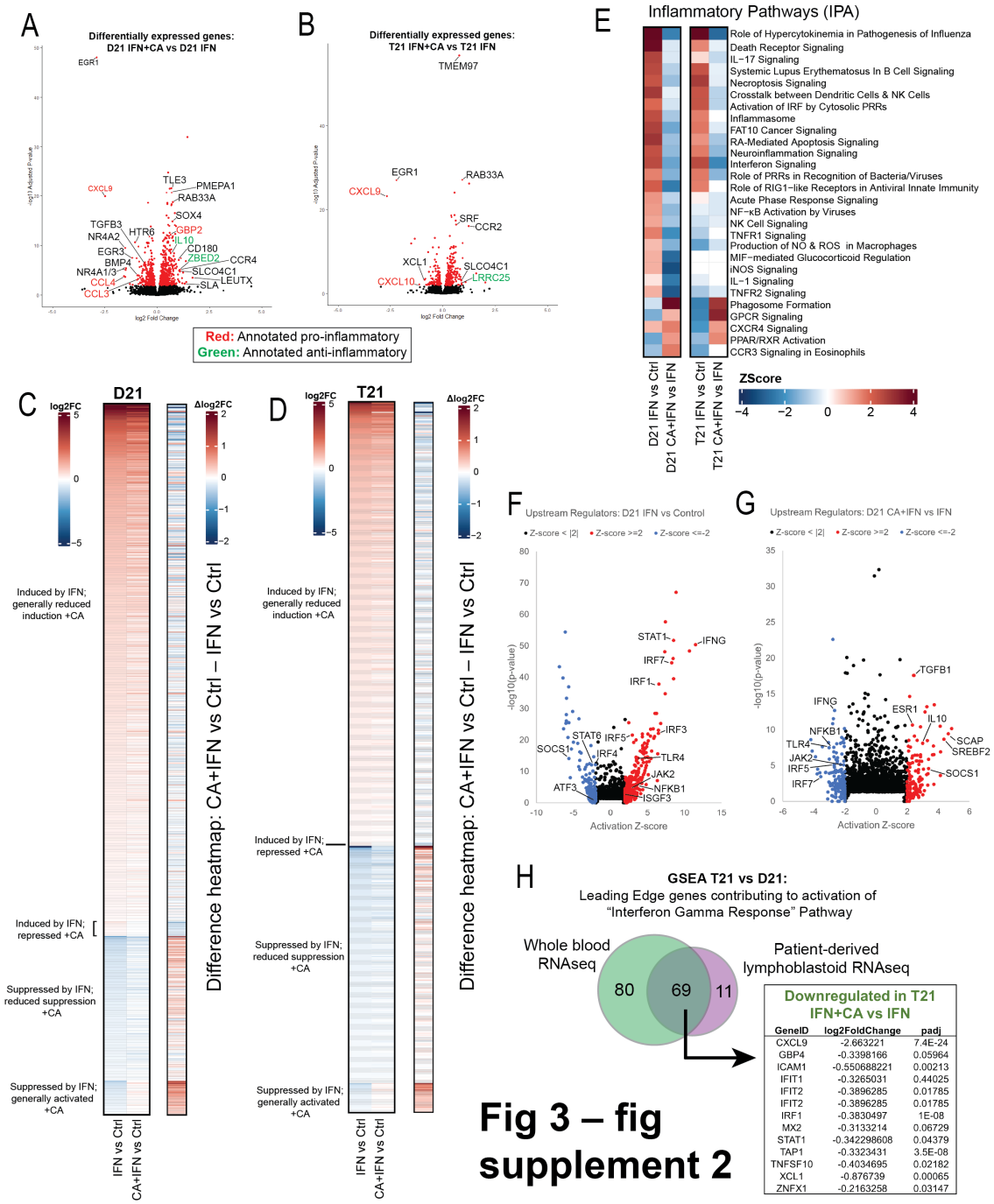


Fig 3 – fig supplement 1

Figure 3–figure supplement 1. The response to exogenous IFNγ is suppressed in T21 vs. D21 cells. (A) Heatmap of genes with differential expression in D21 or T21 cells treated with IFNγ compared to controls. Genes with adjusted p-value <0.01 in one or both cell lines are shown. **(B)** Average values across biological replicates for reads per kilobase of transcript, per million mapped reads (RPKM) in vehicle-treated D21 cells (x-axis) and vehicle-treated T21 cells (y-axis). Gray dots represent all genes with an RPKM value >0, and red dots represent genes from the GSEA Hallmarks IFNγ and IFNα signaling pathways. Linear regression trendlines for each set are depicted as follows; black: all genes; red: IFN response genes. The greater slope for IFN-responsive genes indicates higher basal expression of these genes in T21 cells. **(C)** Similar to panel B, except in IFNγ-treated D21 (x-axis) and IFNγ-treated T21 cells (y-axis). Note the linear

regression trendlines are more similar under IFNγ-stimulation conditions, compared with basal conditions (panel B), which provides evidence for dampened IFNγ transcriptional response in T21 cells, likely from higher basal level expression, such that gene expression ends up being similar in T21 and D21 cells treated with exogenous IFNγ. **(D, E)** Representative genome browser tracks (RNA-seq data) for SOCS3 (D) or STAT1 (E) in D21 and T21 cells ± IFNγ treatment. **(F)** Genes with lower (green) or higher (red) relative expression in IFNγ-treated T21 cells (vs IFNγ-treated D21; adjusted p-value <0.1). Only genes with a log2FC ≥ |0.5| are shown. **(G)** Gene Ontology Enrichment Analysis with the GO aspect "Biological Processes" for genes with reduced responsiveness to IFNγ stimulation in T21 cells. Many genes with reduced expression in T21 cells represent inflammatory pathways, consistent with a suppressed response to exogenous IFNγ in T21 cells, vs D21.



GeneID	log2FoldChange	padj
CXCL9	-2.663221	7.4E-24
GBP4	-0.3398166	0.05964
ICAM1	-0.550688221	0.00213
IFIT1	-0.3265031	0.44025
IFIT2	-0.3896285	0.01785
IFIT2	-0.3896285	0.01785
IRF1	-0.3830497	1E-08
MX2	-0.3133214	0.06729
STAT1	-0.342298608	0.04379
TAP1	-0.3323431	3.5E-08
TNFSF10	-0.4034695	0.02182
XCL1	-0.876739	0.00065
ZNFX1	-0.2163258	0.03147

Figure 3–figure supplement 2. Mediator kinase inhibition antagonizes IFNγ transcriptional responses. (A, B) Volcano plots of differentially expressed genes in IFNγ-treated cells ±CA in D21 (A) or T21 (B) cells. Names of selected genes are color-coded based on proinflammatory (red) or anti-inflammatory (green) roles. Genes represented by red dots have an adjusted p-value of <0.01. **(C, D)** Heatmaps and "difference" heatmaps based upon RNA-seq data under various conditions in D21 (C) or T21 (D) cells. At left (panel C, D), heatmaps represent all genes with an adjusted p-value <0.01 in IFNγ vs. Control comparisons for each cell line. Log2FC values were used to sort genes by expression trends in D21 and T21 cells. Data for IFNγ +CA vs. Ctrl are shown alongside IFNγ vs. Ctrl to better visualize CA-dependent effects. For difference heatmaps (at right in panel C, D), Log2FC values for each gene in the IFNγ vs. Control comparison were subtracted from Log2FC values for CA+IFNγ vs. Control comparisons to generate "difference" Log2FC values, which are shown in the difference heatmap on the right. **(E)** Heatmaps showing inflammatory

pathways in the Ingenuity Pathway Analysis “Diseases & Functions” list, generated from RNA-seq data. Heatmaps show pathway activation in IFNγ vs. Control compared to IFNγ+CA vs. IFNγ in both D21 and T21 cells, to focus on CA-dependent effects during IFNγ response. **(F, G)** Volcano plots from IPA upstream regulators results, derived from differential gene expression (RNA-seq) in D21 cells during IFNγ treatment (F) or in cells treated with IFNγ +CA compared to IFNγ alone (G). Selected TFs and other factors associated with inflammatory responses are labeled. Note prominent activation of pro-inflammatory TFs in +IFNγ cells (F), whereas these same TFs are predicted to be repressed in CA-treated cells (G). Analysis used only genes with an adjusted p-value <0.1. **(H)** Venn diagram showing overlap of leading edge genes in the GSEA “Interferon Gamma Response” pathway based upon RNA-seq data from a T21 vs. D21 whole blood cohort study (Waugh et al. 2023) and RNA-seq data from the sibling-matched T21 vs. D21 cells in this study. A substantial number of genes with the IFN “leading edge” designation in this study (i.e. increased expression in T21 vs. D21; 69 out of 80) were shared among the whole blood clinical cohort study; these 69 genes were down-regulated by CA treatment in T21 cells. A partial list of these genes is shown, with Log2FC and adjusted p-values.

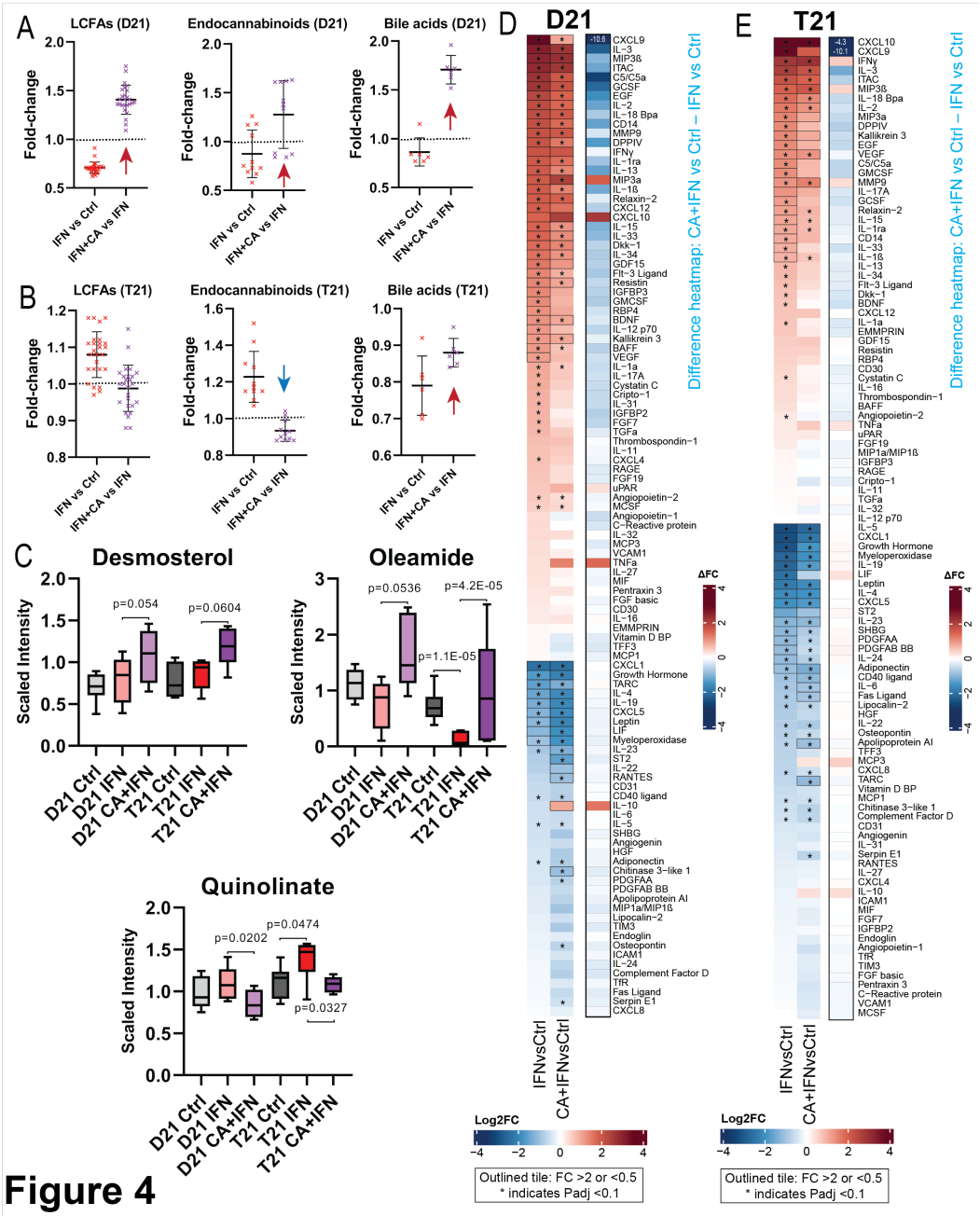


Figure 4

Fig 4. Mediator kinase inhibition reverses pro-inflammatory metabolic and cytokine changes triggered by IFNγ. (A, B) Effect of IFNγ treatment on select classes of lipid metabolites is shown (IFNγ vs. Ctrl) alongside the effect of CA treatment in IFNγ-treated cells, in D21 (B) and T21 (B) cells. The LCFAs represented here include saturated, mono- and poly-unsaturated FA, shown in rows 3-28 in **Figure 4 – source data 1**. Each point represents a different metabolite, with line and whiskers representing the mean and SD. Note CA treatment reverses IFNγ effects generally (arrows). **(C)** Box plots showing levels of anti-inflammatory metabolites desmosterol or oleamide, and pro-inflammatory metabolite quinolinate, in D21 or T21 cells treated with DMSO (Ctrl), IFNγ, or IFNγ +CA. **(D, E)** Heatmaps showing changes in cytokine levels in D21 (D) and T21 (E) cells, after the indicated treatments. Only cytokines with relative levels of ≥2.0 (log2FC; red shading) or ≤0.5 (blue shading) in one or both cell lines are shown; cytokines meeting this threshold are outlined in black. Asterisk (*) denotes adjusted Padj <0.1 using ANOVA. Alongside each heatmap set (D21 or T21) is a "difference" heatmap (IFN+CA vs. Ctrl – IFN vs. Ctrl levels) that highlights how Mediator kinase inhibition suppresses cytokine responses to IFNγ.

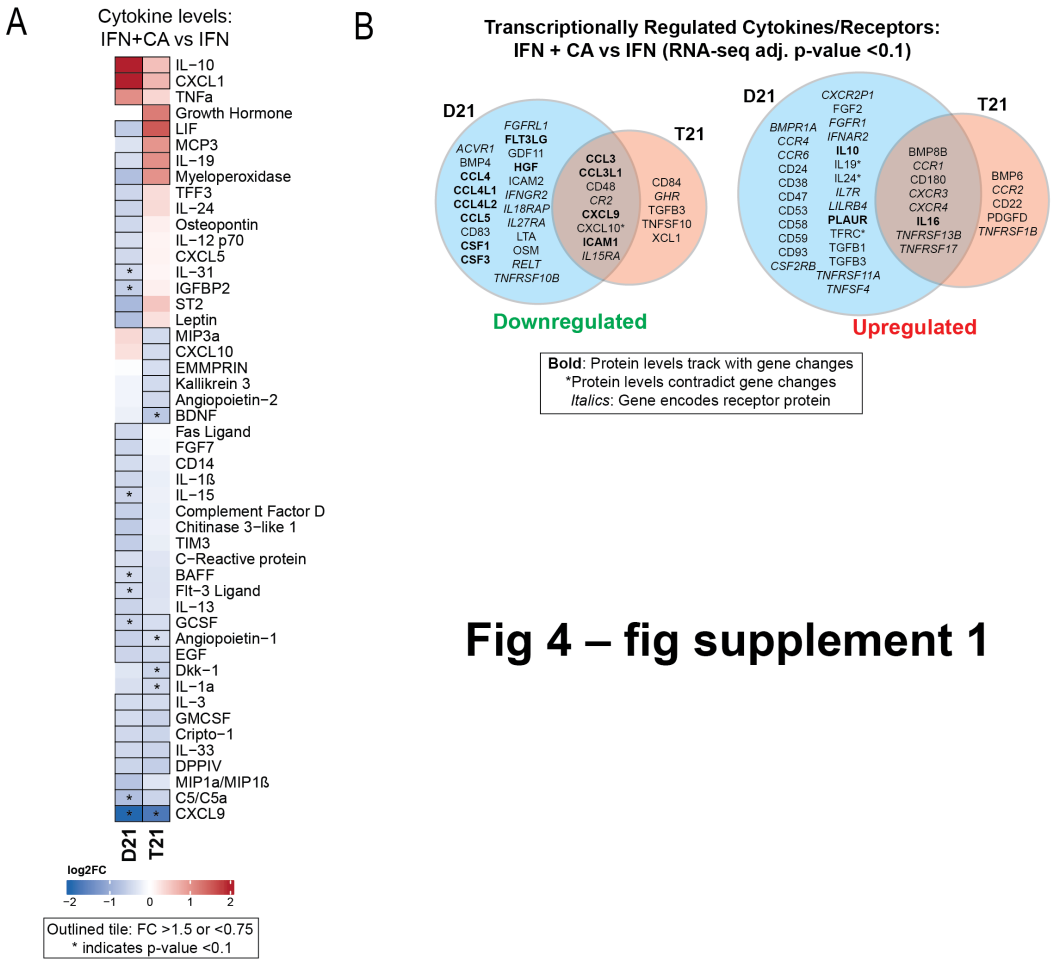


Fig 4 – fig supplement 1

Figure 4–figure supplement 1. Mediator kinases transcriptionally enable cytokine responses to IFNγ. (A) Heatmap of average relative cytokine levels in cells treated with IFNγ +CA compared to IFNγ alone. Only cytokines with relative levels of ≥1.5 (log2FC; red shading) or ≤0.75 (blue shading) in one or both cell lines are shown; cytokines meeting this threshold are outlined in black. Cytokines with an asterisk (*) had an adjusted p-value <0.1 using ANOVA. **(B)** Venn diagrams (RNA-seq data) of cytokines and cytokine receptor genes (in *italics*) that were downregulated (FC <0.8, left diagram) or upregulated (FC >1.2, right diagram) in IFNγ +CA-treated cells compared to IFNγ alone (D21 and T21). Cytokines with matching trends from RNA-seq (4h) and cytokine protein measurements (24h) are listed in bold, whereas cytokines with inverse trends in at least one cell type are marked with an asterisk.

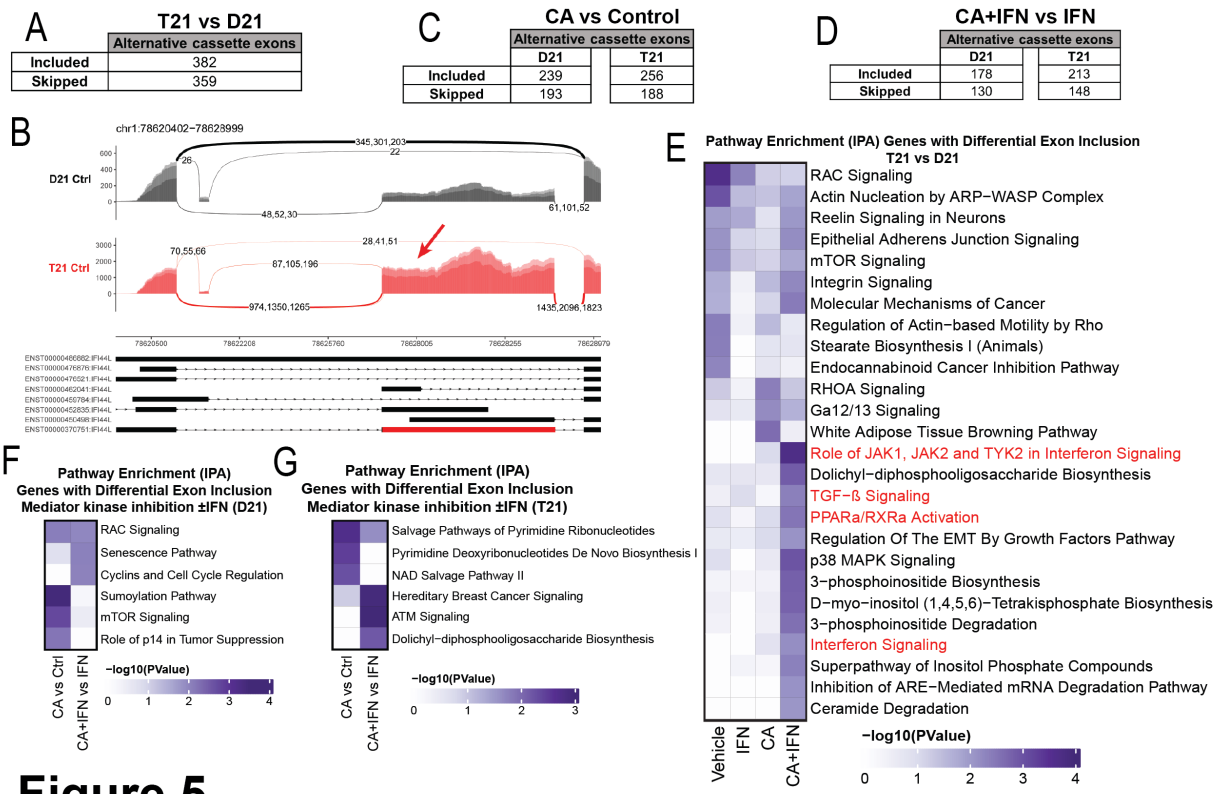


Figure 5

Fig 5. Mediator kinases regulate splicing in pathway-specific ways. (A) Table of alternative exon usage from untreated T21 cells compared to D21. Inclusion criteria were assessed at FDR <0.05, | InclusionLevelDifference | >0.2, and ≥2 reads/replicate. **(B)** Sashimi plots for the IFI44L gene, with normalized read numbers for D21 control (black) and T21 control (red) samples on the Y-axis, and splice junction read numbers (representing each of 3 replicate experiments). **(C)** Table of alternative exon usage from CA-treated D21 or T21 cells compared to controls. Inclusion criteria were same as for panel A. **(D)** Table of alternative exon usage from D21 or T21 cells treated with IFNγ and CA compared to IFNγ alone. Inclusion criteria were same as for panel A. **(E)** Ingenuity Pathway Analysis enrichment results of genes with alternative exon skipping events in T21 cells vs. D21; different treatment conditions indicated at bottom. Pathways relevant to IFNγ signaling are highlighted in red. Genes affected by alternative splicing in T21 vs. D21 cells could be grouped into different signaling pathways (e.g. RAC, mTOR, integrin) that are important for robust immune responses (Jones and Pearce 2017; Lee et al. 2021a), suggesting how alternative splicing may influence inflammatory signaling in T21 cells. **(F, G)** Ingenuity Pathway Analysis enrichment results of genes with alternative exon skipping events in D21 (F) or T21 (G) cells treated with CA (±IFNγ).

A Alternative Splicing Events: T21 vs D21

	Retained introns
Retained	31
Spliced	17

	Alt 5' splice sites
Downstream	34
Upstream	21

	Alt 3' splice sites
Downstream	50
Upstream	43

B Alternative Splicing Events: IFN vs Control

	Alternative cassette exons	
	D21	T21
Included	163	218
Skipped	133	200

	Retained introns	
	D21	T21
Retained	11	10
Spliced	11	9

	Alt 5' splice sites	
	D21	T21
Downstream	11	21
Upstream	19	20

	Alt 3' splice sites	
	D21	T21
Downstream	15	20
Upstream	19	27

D Alternative Splicing Events: CA vs Control

	Retained introns	
	D21	T21
Retained	13	19
Spliced	10	10

	Alt 5' splice sites	
	D21	T21
Downstream	21	29
Upstream	23	17

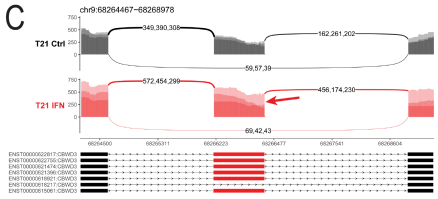
	Alt 3' splice sites	
	D21	T21
Downstream	27	20
Upstream	19	25

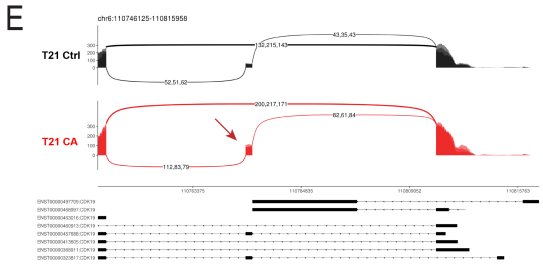
G Alternative Splicing Events: CA+IFN vs IFN

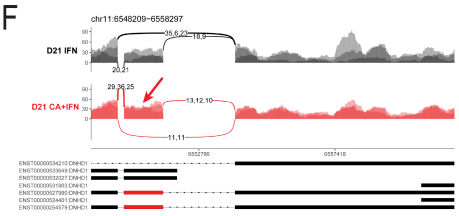
	Retained introns	
	D21	T21
Retained	14	7
Spliced	6	17

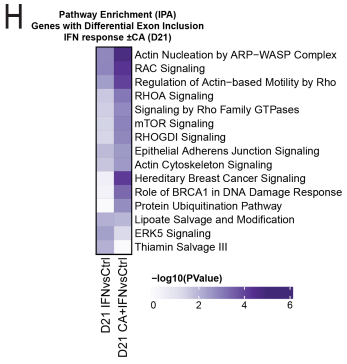
	Alt 5' splice sites	
	D21	T21
Downstream	9	19
Upstream	11	14

	Alt 3' splice sites	
	D21	T21
Downstream	23	31
Upstream	17	27









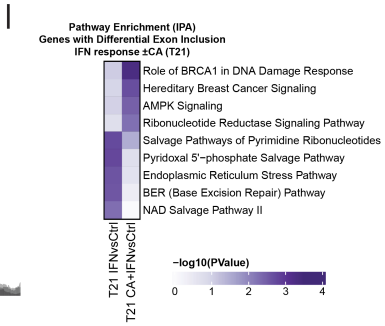


Fig 5 – fig supplement 1

Figure 5–figure supplement 1. Additional information about splicing changes. (A) Table of alternative splicing events (RNA-seq data) from untreated T21 cells compared to D21. Inclusion criteria were an FDR <0.05, | InclusionLevelDifference | >0.2, and ≥2 reads/replicate. **(B)** Table of alternative splicing events from D21 or T21 cells treated with IFNγ compared to control cells. Inclusion criteria were as indicated in A. **(C)** Sashimi plots of the CBWD3 gene, with normalized read numbers for T21 control (black) and T21 IFNγ (red) samples on the Y-axis and splice junction read numbers (representing each of 3 replicate experiments). **(D)** Table of alternative splicing events from D21 or T21 cells treated with CA compared to controls. Inclusion criteria were as indicated in A. **(E)** Sashimi plots for the CDK19 gene, with normalized read numbers for T21 Ctrl (black) and T21 +CA (red) samples on the Y-axis, and splice junction read numbers (representing each of 3 replicate experiments). Arrow denotes greater inclusion of alternative exon T21 +CA. **(F)** Sashimi plots for the DNHD1 gene, with normalized read numbers for D21 +IFNγ (black) and D21 +IFNγ +CA (red) samples on the Y-axis, and splice junction read numbers (representing each of 3 replicate experiments). Arrow denotes greater inclusion of alternative exon D21 +IFNγ +CA. **(G)** Table of alternative splicing events from D21 or T21 cells treated with IFNγ and CA compared to IFNγ alone. Inclusion criteria were as indicated in A. **(H, I)** Ingenuity Pathway Analysis enrichment results of genes with alternative exon skipping events in D21 (H) or T21 (I) cells treated with IFNγ (±CA).

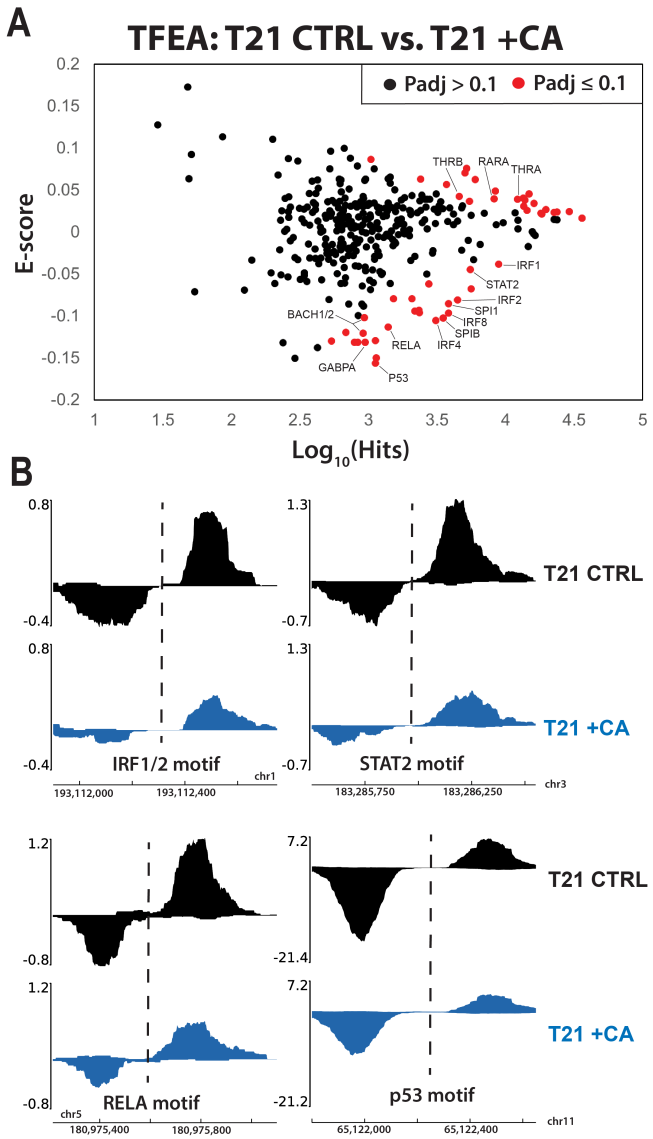


Fig 6. Mediator kinase inhibition rapidly suppresses pro-inflammatory TFs in T21 cells. (A) MA plot of TFEA results comparing control (DMSO) vs. CA-treated T21 cells under basal conditions, from PRO-seq experiments completed after 45min CA treatment. **(B)** Representative examples of bidirectional "eRNA" transcription from PRO-seq data, showing reduced levels in CA-treated cells. Location of TF consensus binding motifs indicated with dashed line.

Figure 7

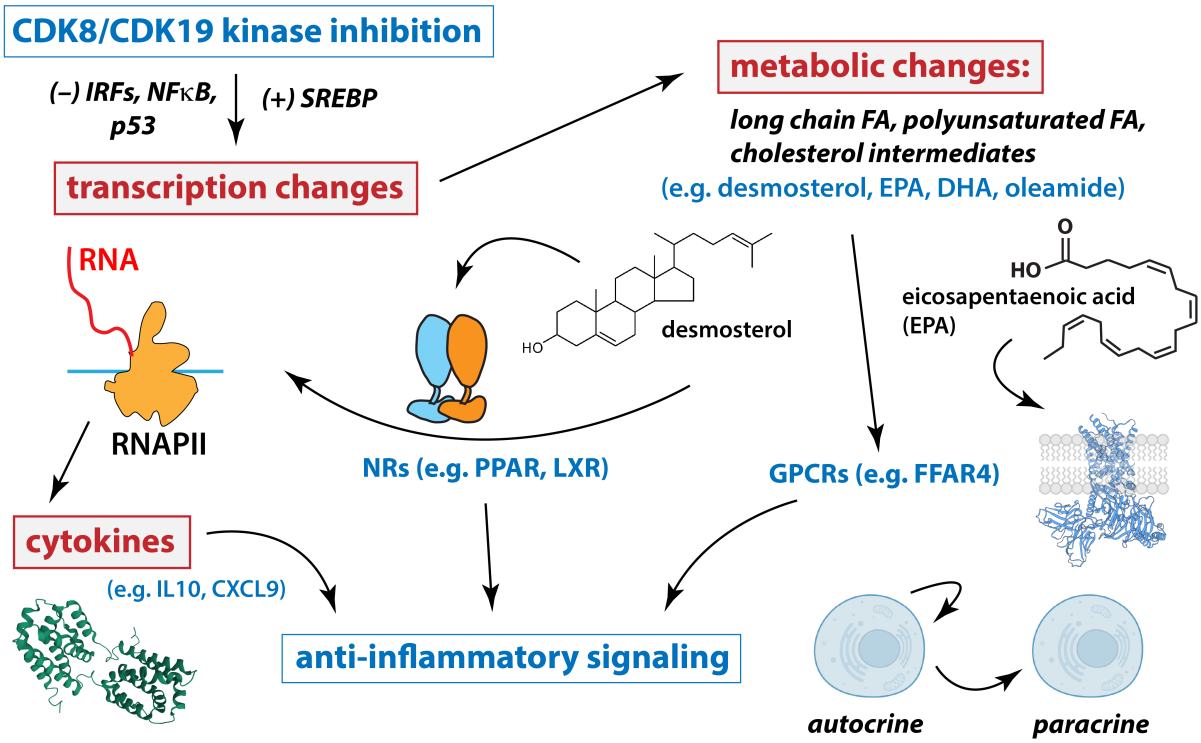


Fig 7. Working model for Mediator kinase-dependent suppression of IFNγ signaling. See text for details.

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