Understanding regulatory mechanisms of human T-helper 17 differentiation

A Thesis

Submitted in partial fulfilment of the requirements

Of the degree of

Doctor of Philosophy

By

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Under the guidance of

Prof. Sanjeev Galande and Prof. Riitta Lahesmaa

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Dedicated to

My late Grandfather

&

My mother……

DECLARATION

I declare that this written submission represents my ideas in my own words and where others' ideas have been included; I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources that have not been properly cited or from whom proper permission has not been taken when needed.

Ankitha Shetty

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Date: 27th July 2020

CERTIFICATE

Certified that the work incorporated in the thesis entitled, “Understanding regulatory mechanisms of human T-helper 17 differentiation” submitted by Ankitha Shetty was carried out by the candidate under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or Institution.

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Sincerely,
Ankitha

List of Contents

List of abbreviations

Th17	T-helper 17
HSC	Haematopoietic stem cells
CLP	Common Lymphoid Progenitors
TCR	T-cell receptor
Thconv	T-helper conventional
CD4	Cluster of differentiation 4
TF	Transcription factor
Thp	T-helper progenitor
Tfh	T-helper follicular
STAT	Signal transducer and activator of transcription
GATA3	GATA-binding protein 3
IL-	Interleukin-
FOXP3	Forkhead box P3
IFN	Interferon
EAE	Experimental encephalomyelitis
CIA	Collagen-induced arthritis
SLE	Systemic lupus Erythematosus
RA	Rheumatoid arthritis
CCL-	C-C Motif Chemokine Ligand
TNF	Tumour necrosis factor
MAPK	mitogen-activated protein kinase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
TGF- β	Tumour-growth factor β
T-BET	T-box expressed in T cells
TBX21	T-box transcription factor
JNK	Janus-kinases
SOCS3	Suppressor of cytokine signalling 3
RORγt	RAR-related orphan receptor gamma
IRF	Interferon regulatory factor
SATB1	Special AT-rich binding protein 1
RUNX1	Runt-related transcription factor 1
BASP1	Brain acid soluble protein 1

ATP1B1	ATPase Na+/K+ Transporting Subunit Beta 1
LMNA	Lamin A
KDSR	3-Ketodihydrosphingosine Reductase
COL6A3	Collagen Type VI Alpha 3 Chain
ITM2A	Integral membrane protein 2A
LC-MS	Liquid chromatography-Mass Spectrometry
FASP	Filter Aided Sample Preparation
LogFC	Logarithmic fold change
GO	Gene-ontology
IPA	Ingenuity pathway analysis
DE	Differentially-expressed
PALLD	Palladin, Cytoskeletal Associated Protein
ACSL4	Acyl-CoA Synthetase Long Chain Family Member 4
FHOD1	Formin Homology 2 Domain Containing 1
BATF	Basic Leucine Zipper ATF-Like Transcription Factor
CCR6	Chemokine receptor 6
OASL	2'-5'-Oligoadenylate Synthetase Like
ATF3	Activation of JNK and transcriptional repressor
PMA	Phorbol 12-myristate 13-acetate
RORC	RAR Related Orphan Receptor C
EF1a	Elongation factor 1-alpha.
FOSL	FOS-like
ETS1	ETS Proto-Oncogene 1, Transcription Factor
DOK1	Docking protein 1
VIM	Vimentin
IKZF-	Ikaros Zinc Finger
SIRT-1	Sirtuin 1
CTNNA1	Catenin alpha-1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
A488	Alexa Fluor 488
A647	Alexa Fluor 647
LFQ	Label free quantitative
RBPJ	Recombination Signal Binding Protein For Immunoglobulin Kappa J Region
AHR	Aryl Hydrocarbon Receptor
PARP	Poly (ADP-ribose) polymerase
DDX	DExD/H-Box Helicase 58

CASP1	Caspase-1 precursor
MX1	MX Dynamin Like GTPase 1
IFIT	Interferon-induced protein with tetratricopeptide repeats 1
VDR	Vitamin D receptor
Treg	T-regulatory
FACS	Fluorescence-activated cell sorting
FDR	False discovery rate
SEMA7A	Semaphorin 7A
BUR	Base-unpairing region
MAR	Matrix-attachment region
ULD	Ubiquitin-like domain
CD	Cut-domain
HD	Homedomain
CUTL	CUT-like
PML	Promyelocytic Leukemia
HDAC	Histone deacetylase
PCAF	P300/CBP-associated factor
CTBP	C-terminal binding protein
PD1	Programmed Cell Death 1
PTM	Post-translational modification
PLC	Phospholipase C
PKC	Phosphokinase C
Ub	Ubiquitinated
P	Phosphorylated
Ac	Acetylated
Me	Methylated
SILAC	Stable Isotope Labeling by/with Amino acids in Cell culture
ELISA	Enzyme-linked immunosorbent assay
RNAi	RNA-interference
SEM	Standard error of the mean
PRMT	Protein arginine methyltransferase
AP-1	Activator-protein 1
FRA	FOS-related antigen
FOSL	FOS-like
COL-	Collagen type-
NFAT	nuclear factor of activated T cells
PBMC	Peripheral blood mononuclear cells

SFMC	Synovial fluid mononuclear cells
UCB	Umbilical cord blood
NT5E	5'-Nucleotidase Ecto
IVT	In-vitro transcribed
KD	Knockdown
OE	Over-expression
SCR	Scramble
NT	Non-targeting
FASLG	Fas Ligand
PRDM1	PR/SET Domain 1
FGF2	Fibroblast Growth Factor 2
DUSP2	Dual specificity protein phosphatase 2
FUT7	Fucosyltransferase 7
DHRS9	Dehydrogenase/Reductase 9
HOPX	HOP Homeobox
GBP4	Guanylate Binding Protein 4
DMD	Dystrophin
AMP	Adenosine monophosphate
APOD	Apolipoprotein D
IDR	Irreproducible Discovery Rate
ChIP	Chromatin Immunoprecipitation
IP	Immunoprecipitation
WB	Western blotting
Ab	Antibody
DPP4	Dipeptidyl peptidase 4
MIAT	Myocardial infarction associated transcript
SGK1	Serine/threonine-protein kinase
ZAP70	Zeta Chain of T Cell Receptor Associated Protein Kinase 70
EIF4E	Eukaryotic Translation Initiation Factor 4E
SMARCE1	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily E, Member 1
MYO1D	Myosin ID
TRIM21	Tripartite motif containing-21
DHX	DEAH-box helicase
ID3	DNA-binding protein inhibitor
BCL3	BCL3 Transcription Coactivator

NR4A2	Nuclear Receptor Subfamily 4 Group A Member 2
ISG	Interferon-stimulated gene
NIPAL4	NIPA Like Domain Containing 4
CLEC17A	C-Type Lectin Domain Containing 17A
ATXN1	Ataxin 1
TSS	Transcription Start site
H3K27ac	Histone-3 Lysine-27 Acetylation

Synopsis

Introduction

Over the years, our understanding of T-cell-associated immunodeficiencies and pathologies has greatly advanced owing to an extensive investigation of cellular signaling networks. However, the origin of diseases caused due to dysregulation of the more recently discovered subsets such as Th17 and Th9 have only begun to be understood. Th17 cells are pro-inflammatory players of the adaptive immune system which not only contribute to bacterial and fungal immunity but are also causative of serious autoimmune disorders such rheumatoid arthritis and multiple sclerosis. This explains why investigating the molecular circuits governing Th17 responses is so crucial.

The privilege of ethical approvals and ease of genetic manipulation has resulted in an extensive use of murine models for conducting human disease research. Although the immune system appears largely similar between the two species, important cellular and molecular differences between them, are well reported in the field (reviewed in [1]). This includes significant discrepancies for expression of multiple T-cell associated genes involved in activation, differentiation and cytokine/chemokine function. Further, differences have also been reported for global transcriptional profiles of early-differentiating human and mouse Th17 cells, which could be suggestive of phenotypic dissimilarities [2]. Interestingly, factors such as AHR and BLIMP-1 have already been shown to oppositely regulate Th17-associated responses in the two species [3-5, 36]. This undisputedly conveys that though murine studies could largely be used to dissect functions of most Th17 regulators, their validation in the human counterpart is essential for them to hold relevance in disease therapy. Chapter 1 of the thesis elaborates the background of the study and introduces Th17 cells.

Aims and objectives

Although the past decade has witnessed an ever-increasing knowledge of novel regulators of Th17 circuits, only a limited number of these studies have been conducted using human CD4 T-cells. As a result, molecular information on human Th17 signaling is quite limited and this restricts our potential for progressing immunotherapy. Hence, there is a crucial requirement for expanding the number of studies investigating the human counterpart of the lineage. With this in view, our study focused on conducting a comprehensive analysis for profiling and characterizing novel regulators of human Th17 responses. Since the early stages of polarization involves a highly complex and stringently-finetuned transcriptional network, which allows lineage induction and alternate lineage inhibition in parallel, analysing the molecular details of this process holds great significance. We utilized human umbilical cord blood-derived naïve CD4 cells to obtain *in vitro* polarized Th17 cells and investigated key players of Th17 development.

In the past years, molecular networks defining T-helper cell lineages have been predominantly dissected using transcriptomic approaches. However, transcriptional profiles do not necessarily correspond to information at the protein level and this largely limits our ability to predict gene-function based on global RNA expression. Though human and mouse Th17 transcriptomes have been comparatively analysed, the corresponding proteomic profile has been investigated only in mouse [6]. Hence, we indulged in elucidating protein-level changes during early stages of human Th17 differentiation, using tools of mass spectrometry. We further exploited this resource in order to gather crucial hints on the potential regulators of the lineage, whose functions have not been previously determined in human systems. Furthermore, we also thoroughly characterised some of these candidates for their involvement in induction of Th17 responses.

During the course of this study, we focused on addressing the following aims -

1. To identify novel lineage regulators by studying protein-level dynamics of early-differentiating Th17 cells.

2. To determine the influence of SATB1 on human Th17 responses and identify its novel post-translational modifications in T-cells.

3. To comprehensively investigate role of FOSL1 and FOSL2 in controlling human Th17 differentiation

4. To study the functional antagonism between BATF and FOS-like proteins in orchestrating human Th17 fate.

Summary of the study

1. To identify novel lineage-regulators by studying protein-level dynamics of early differentiating Th17 cells

In the second chapter of this thesis, we investigated the differential proteome of human Th17 cells by utilizing tools of liquid chromatography combined with mass spectrometry. We used human umbilical cord blood (UCB)-derived naïve CD4 T-cells for this purpose. Dissimilarities between UCB and peripheral blood have been well-established in the field and the prior has been found to be a better source of 'naïve' T-cell populations [7-10]. Our MS-based proteomic analysis enabled us to identify a total of 148 and 175 proteins which were differentially upregulated or downregulated under the conditions of Th17 polarization at 24 and 72h respectively. The overlap between the DE proteins identified at the two time points was quite low, thereby indicating a time-distinguished regulatory profile. Among the differentially expressed (DE) candidates were many known (AHR, FOSL2, JUNB, SIRT1, RBPJ, SATB1, IRF8, SMAD3, STAT4, CCL20) and unknown regulators of the lineage (KDSR, ATF3, APOD, VIM, PALLD, IL16, UHRF1). Multiple proteins associated with anti-viral immunity and lipid metabolism were seen to be significantly altered, thereby potentially opening new avenues in the field of Th17 signaling. We also identified important changes in expression of proteins belonging to the AP-1/ATF superfamily (JUNB, FOSL2, ATF3), whose involvement in human Th17 responses is largely undetermined. Further strengthening our findings, we successfully validated the expression changes for more than 20 of the differentially expressed proteins using immunoblotting, immunostaining, flow cytometry or targeted mass spectrometry.

As a part of our analysis, we compared the differentiation-induced changes occurring at the transcript and protein level using RNA-seq and proteomic datasets. Although a high concordance (more than 90%) was observed between them, we found 21 candidates that appeared to be altered only in proteomics. More importantly, we identified 11 candidates which were differentially regulated in a contrasting manner at the RNA and protein level. These discrepancies highlighted the potential involvement of post-transcriptional and post-translational mechanisms and further underscored the importance for proteomic studies in investigating lineage dynamics.

Transcript-level dissimilarities have been well-reported for human and mouse Th17 cells [2]. To further investigate this divergence at the proteome level, we compared our DE candidates with the ones detected in a recently published murine study [6]. Strikingly, we discovered a very poor overlap between the datasets, thereby underlining species-specific regulatory profiles. More importantly, we identified a total of 18 proteins which showed an opposing fashion of differential expression in human and mouse, including SATB1 and CD44. Such discrepancies could be indicative of a species-specific role.

Briefly, our study delivers an important resource to the Th17 field where we have identified potentially novel regulators of human Th17 differentiation. This is the first report to conduct a detailed comparison between transcriptomics and proteomics of human Th17 cells and also elucidate the species-specific protein level changes in early differentiating populations. All findings from this chapter are a part of the published study by - *Tripathi, Valikangas, Shetty et al. iScience, 2019* (https://doi.org/10.1016/j.isci.2018.12.020).

2. To determine the influence of SATB1 on human Th17 responses and identify its novel post-translational modifications in T-cells

Characterization of protein profiles has been found to deliver useful hints on molecular function of regulatory proteins. As an important highlight of our MS analysis, SATB1 emerged as a key candidate showing a species-specific expression profile. SATB1 is a global chromatin regulator whose role in T-cell development, activation and differentiation has been extensively studied [11-16]. Results from Chapter 2 demonstrated that SATB1 was differentially upregulated in mouse but downregulated

in human Th17 cells. Such disparity could indicate opposing functions in the two species. Upregulated levels of SATB1 in mouse Th17 cells have been previously attributed to a positively influence on the lineage [17]. Since its human specific role had not been addressed, we focused on investigating the same, as a part of the third chapter of this thesis.

First, the opposing expression profile of SATB1 was successfully validated at both RNA and protein level, using human and mouse Th0/Th17 cells. Further, SATB1-silencing showed a significant upregulation in expression of the lineage markers - CCR6, IL17A and IL17F. This evidently demonstrated its inhibitory effect on Th17 signaling and further confirmed a species-specific role. Although SATB1 protein in human and mouse shows 98% sequence homology, regulatory circuits differentially controlling their expression levels could largely contribute to such functional dissimilarities. Thus, our study provides an important line of investigation for inter-species comparison and their importance in validation of gene function.

Previous reports have shown that SATB1 function is highly context specific and can be fine-tuned by its post-translational modifications [18-21]. A landmark study in this field was the discovery of phosphorylated and acetylated forms of SATB1, which were found to differentially dictate T-cell activation [19]. Since PTM profiling of this chromatin regulator has been limitedly investigated over the years, we focused on employing a MS-based approach for screening novel post-translational modifications of SATB1 in human T-cells. Our analysis discovered seven phosphorylations and an acetylation (K11-Ac, T298-P, S309-P, T310-P, S313-P, T630-P, S633-P and S637-P), spanning various inter-domain regions of SATB1 in both Jurkat and activated primary T-cells. Interestingly, the detected phosphorylations appeared to occur on closely placed serine and threonine residues around the CUT, CUTL and homeo-domains, which determine DNA-binding features. Notably, we found that most of the identified PTMs (except for K11-Ac), have been reported in other studies using cancer cell lines [22]. Our analysis is the first instance to report them in activated primary T-cells. Given the wide occurrence of these modifications across cell types, they could be involved in governing more fundamental features such as protein stability.

3. To comprehensively investigate role of FOSL1 and FOSL2 in controlling human Th17 differentiation

As a part of our MS-based proteomic analysis, we discovered FOSL2 and a few associated AP-1 proteins to be differentially upregulated under Th17-polarizing conditions. Functionally, FOSL2 has been shown to restrain murine Th17 responses and also act as a crucial modulator of T-helper cell plasticity [17]. Interestingly, its known paralog FOSL1, has been shown to operate antagonistically and support the differentiation process. Given their undetermined roles in human Th17 cells, we focused on holistically dissecting their function by using high-throughput approaches. We initially characterized their expression profiles and discovered that activation leads to induction of both these proteins. However, IL-6/STAT3 signaling further upregulates their expression in Th17 polarizing cells.

As a part of our initial experiments, we individually silenced FOSL1 and FOSL2 and discovered a significant upregulation in IL-17 secretion, thereby suggesting an inhibitory effect on the lineage. This contradicts the previously established scenario in mouse where FOSL1 was shown to act as a positive regulator [23]. AP-1 proteins are well known for having synergistic or compensatory equations with similarly-operating members (reviewed in [24]). Knowing this, we adopted a simultaneous perturbation approach and investigated whether a functional coordination exists between FOSL1 and FOSL2. Interestingly, co-depletion of FOSL1 and FOSL2 additively enhanced IL-17 expression whereas their dual-overexpression resulted in a pronounced inhibition (when compared to the Single perturbation controls). Further strengthening these findings, a comparative RNA-seq analysis for the single- and co-perturbed samples depicted a FOSL-mediated synergistic regulation profile for multiple Th17 markers and associated molecules. Focusing on the DE targets identified in the simultaneously perturbed datasets, we meticulously analysed expression changes for genes with known roles in Th17 function (IL23R, IL21, JUNB, CD70, IL12RB1, CD52, CXCR3, PRDM1, DUSP2, NT5E), and found confirmative evidence to establish that FOS-like proteins indeed cooperatively inhibit human Th17 differentiation. Interestingly, pathway analysis for these targets revealed multiple autoimmune related processes to be significantly enriched, thereby suggesting their involvement in development of inflammatory phenotypes.

To mechanistically dissect this molecular synergy, we determined the global occupancy profile of these proteins using ChIP-seq experiments. Highlighting their coordinated action, an extensive overlap was detected for genome-binding sites of FOSL1 and FOSL2. These included many of their cooperatively regulated, Th17-asociated RNA-seq targets, thereby underscoring a direct transcriptional control over lineage-defining molecules. Furthermore, motif analysis of FOSL1 and FOSL2 ChIP peaks revealed nearly identical binding sequences for the two proteins. Additionally, it also indicated similar motifs for other AP-1 family proteins such as FOS, ATF3 and BATF. Overlapping occupancy of AP-1 members is a characteristic feature of this family and could potentiate both synergy and competition between them. We propose a similar kind of intersection to mediate the transcriptional cooperativity between FOS-like proteins.

AP-1 proteins are known to exhibit functional versatility by utilizing their highly dynamic protein interacting abilities. Moreover, proteins within the same regulatory complex are known to exhibit functional synergy by sharing interacting partners [25]. With this in view, we used tools of mass spectrometry to elucidate the interactors of FOS-like proteins. Our analysis yielded a total of 36 proteins (including RUNX1, SIRT-1, JUN, JUNB) to commonly bind to FOSL1 and FOSL2. Intriguingly, except for JUN, all the other factors have been shown to positively regulate murine Th17 responses [26-28]. Assuming their murine roles to be conserved, a shared interaction with lineage inhibitors such as FOSL1 and FOSL2 could easily indicate functional sequestration or interactome-dependent contextual roles for these identified binding partners. Our analysis is the first study of its kind to holistically compare the networks of AP-1 proteins in human T-cells and might serve as an important resource for understanding AP-1- coordinated Th17 signaling circuits.

Summing the above findings, our study provides a detailed report on function and mechanism of FOSL1 and FOSL2 in instrumenting human Th17 responses.

4. To study the functional antagonism between BATF and FOS-like proteins in orchestrating human Th17 fate

The motif searches for FOSL1/FOSL2 ChIP peaks (Chapter 4) illustrated that BATF could bind to similar genomic sequences as FOS-like proteins. Interestingly, many of

the earlier studies have found BATF to largely inhibit transcriptional activity of FOS proteins [29]. This was further underscored by a murine study in 2012 which showed that BATF and FOSL2 oppositely regulate Th17 responses by competing for genomic occupancy on common lineage-associated loci [17]. Since such a regulatory interplay between these AP-1/ATF factors had not been explored in the human counterpart, we focused on studying their functional inter-relatedness during early stages of human Th17 differentiation. We initiated the study by assessing effects of BATF silencing on human Th17 cells. In agreement with its function in mouse [30], BATF appeared to positively regulate important Th17 markers (IL-17A, IL-17F, RORC and CCR6). Furthermore, using RNA-seq analysis we discovered many key Th17 genes to be altered in a fashion that supported its role as an inducer of the lineage. This evidently emphasized that BATF and FOSL antagonistically regulate human Th17 fate.

To further dissect this functional contrast at the molecular level, we compared the RNA-seq targets of BATF and FOS-like proteins and focused on the common but oppositely regulated genes. As a result of this analysis, we discovered multiple Th17 lineage-characterizing molecules (IL17A, IL17F, CCR6, FUT7, IL21, RORA, IL23R, HOPX) to be positively regulated by BATF while being synergistically suppressed by FOS-like proteins. Likewise, known Th17-inhibitors (PRDM1, ID3) were repressed by BATF but cooperatively driven by FOSL1 and FOSL2.

Overlapping genomic occupancy of murine BATF and FOSL2 has been previously reported and could be proposed as a potential mechanism for molecular competition. To explore further on this front in the human counterpart, we determined the correspondence in DNA-binding sites of human BATF, FOSL1 and FOSL2 using ChIP-seq analysis. More than 70% of FOSL and BATF ChIP peaks were found to occur within intergenic and intronic regions, complying with the earlier trend established for AP-1/ATF occupancy. Strikingly, upon comparing the three ChIP-seq datasets, we found a total of 2,624 sites to be commonly bound by these TFs. Among the shared sites were multiple of their antagonistically regulated genes, which have been known to influence Th17-lineage establishment. This indicated a direct genomic control of these proteins over factors determining the Th17 fate. More interestingly, the overlapping AP-1 peaks over many of the shared direct targets, were seen to be flanked by H3K27ac marks (obtained using a published human Th17 enhancer dataset [31]). This is consistent with previous findings in the field and might indicate a

predominant enhancer-based regulation of the lineage, by BATF and FOS-like proteins [32-34].

BATF has been previously shown to form transcriptionally inert dimers with JUN, which allows repression of regulatory functions by FOS:JUN complexes [35]. On similar lines, we investigated whether the BATF/FOSL antagonism detected in human Th17 cells is based on a contest for common interacting partners. Interestingly, we discovered RUNX1, JUNB and JUN as the successfully validated shared interactors of FOSL and BATF. These common binding partners could contextually regulate expression of lineage-associated genes, depending on who they interact with.

AP-1 activity has been known to be highly contextual, owing to a constant interplay between its family members (reviewed in [24]). Our study makes an attempt at investigating this front by holistically deciphering the inter-relatedness between BATF and FOS-like proteins during initiation of human Th17 differentiation.

Conclusions

The primary aim of this thesis was to investigate the molecular signaling networks of human Th17 cells. We initiated this aim by employing an LC/MS-MS strategy to analyse human Th17 cell proteome and discovered a highly dynamic and time-distinguished proteomic profile for early-differentiating populations. Based on the DE candidates identified, we propose a significant number of potentially novel regulators involved in induction of human Th17 responses. Our study importantly highlights multiple genes which exhibit polarization-induced changes at the protein level but not at the transcript level. Intriguingly, our inter-species proteomic comparison revealed a total of 18 proteins which showed opposite expression profiles in the two species. SATB1 being one them, we further investigated its function in human Th17 cells and found it to act as a negative regulator, which contradicts its role in mouse and underscores the existence of species-specific molecular functions. Among the other detected DE targets in our proteomic analysis was FOSL2, which was found to synergize with its paralog protein FOSL1 and inhibit human Th17 responses. This was another finding from our study which presented a scenario different from the one reported in mouse. As the final part of this thesis, we focused on investigating the

functional correspondence between FOS-like proteins and BATF. We found an evident antagonism between their transcriptional activities and identified their largely overlapping occupancy over their contrastingly regulated targets. We further discovered multiple binding partners which were shared between BATF and FOS-like proteins. This is indicative of a potential competition and also highly contextual roles of signaling proteins. Our study is the first instance of meticulous analysis of such inter-relatedness between AP-1/ATF family members in human Th17 cells.

Based on the multiple findings from this thesis that emphasize human versus mouse differences, we contribute an importance resource to the field that highlights the significance of gene function validation in human systems. An attempt to acknowledge the molecular differences between the two species might help in increasing the translatability of murine results for advancement of human disease therapy.

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Chapter 1

A comprehensive review on Th17 cells

1.1 Introduction

Lymphocyte function constitutes a critical branch of the adaptive immune system in vertebrates. Antigen specificity and an ability to exhibit immunological memory are important characteristics of lymphocyte function. B and T cells are two types of lymphocyte populations which concomitantly orchestrate distinct immune responses to confer protection against a spectrum of pathogens. Each of these populations are characterized by unique antigen-specific receptors on their surface, owing to which adaptive immune responses acquire specificity. B lymphocytes are majorly involved in mediating humoral immune responses whereas T lymphocytes govern cell-mediated immunity. B cells develop in the bone marrow from haematopoietic stem cells (HSCs) and further migrate to secondary lymphoid organs where they differentiate into antigen-specific, antibody-producing plasma cells. Development of T-cells however occurs in parts, at two distinct immunological sites. Initially, T-cell precursors developing from common lymphoid progenitors (CLPs), arise from the fetal liver and bone marrow. They further migrate to the thymus where they are educated by the micro-environment to develop into immunocompetent T-cells that finally enter peripheral circulation. Thymic maturation includes an important step of TCR rearrangement, owing to which developing T-cells express either γδ or αβ TCR receptor chains. αβ T-cells form the majority of mature T lymphocytes and their effector functions significantly contribute to adaptive immune responses. Mature αβ-T-cells enter the periphery as CD4 or CD8 positive cells, each of which perform discrete functions. CD8 T-cells or cytotoxic T-cells combat malignant or virus-infected cells by releasing cytolytic enzymes. On the other hand, CD4 T-cells are divided into two

lineages – conventional T-helper cells (Thconv) and regulatory T-cells (Tregs). Helper T-cells when stimulated, produce a repertoire of cytokines/effector molecules that help B cell-mediated antibody production as well as cytotoxic T-cell and macrophage function (reviewed in [1]). However, regulatory T-cells are surveillance-players that suppress immune-reactivity to self-antigens and control exaggerated responses that are deleterious to the host (reviewed in [2]) (Graphical representation in Figure 1).

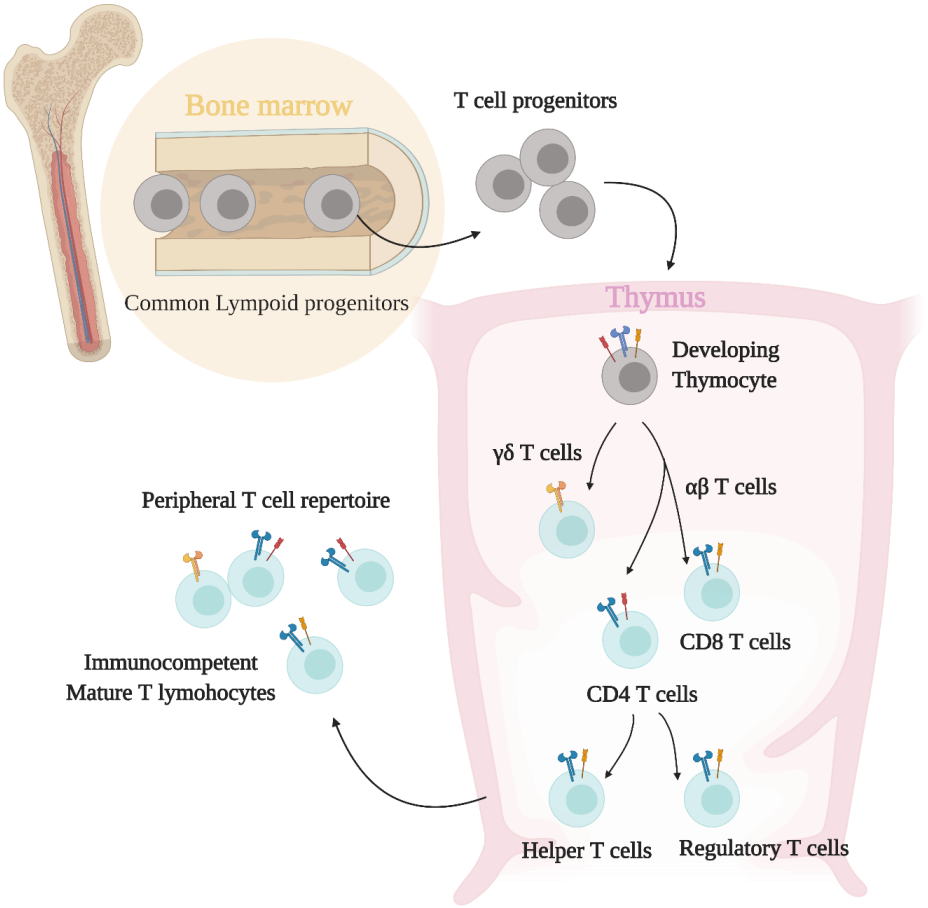

Figure 1. Overview of development and function of T-cell subsets. T-cell progenitors arising from the bone marrow migrate to the thymus where the micro-environment educates them further to form mature αβ- or γδ- T-cells, which enter peripheral circulation. Immunocompetent αβ-T-cells govern adaptive immune responses through functionally distinct T-Cytotoxic (CD8), T-Helper (CD4) or T-Regulatory cells (CD4). Illustration is modified and recreated from Skapenko et al, 2005 [3].

Since T lymphocytes are involved in crucial operations of vertebrate host immunity, investigating the mechanistic details of their development and effector functions is imperative for enhancing our understanding of immunological disorders. Genetic and epigenetic mechanisms establish the ground work for transcriptional regulation of all cellular responses. It has long been established that mammalian immune cell

modulation is highly complex and thus cannot be pioneered by just a handful of transcription factors (TFs). These recent years of research have thus focussed on dissecting signaling networks of different T-helper lineages, using an extensive application of high-throughput methods.

1.2 T-helper cell function

T-helper cells or Th cells, are functionally intriguing populations that possess the striking ability to fine-tune their responses based on the surrounding cytokine milieu [4]. Th1, Th2, Th9, Th17, Th22, Tfh and Tregs are the multiple T-helper subpopulations that a Th precursor (Thp) or naïve T-cell can polarize into, in presence of different cytokines [5-7] (See Figure 2). Amongst these, the Th1, Th2 and Treg cells have been extensively studied over the past few decades. Each of these subsets are regulated by specific transcription factors that act as master regulators and define lineage-commitment for the polarizing T-cell. For example, STAT4-induced T-BET expression primes Th1 differentiation, STAT6-induced GATA-3 expression primes Th2, whereas STAT5-induced FOXP3 expression stimulates Treg differentiation [8]. Once committed to a particular lineage, each of these subsets secrete specific cytokines that specialize in combating a defined spectrum of immunological-encounters. Th1 cells are known to secrete IFN-γ, which eliminates intracellular bacteria/viruses whereas Th2 cells secrete IL-4, which is detrimental to helminthic infections [5]. Regulatory T-cells on the other hand, have been shown to dampen immune responses and execute immunological surveillance [9].

Lineage-commitment stabilizes cytokine expression in the polarized populations by means of epigenetic mechanisms [10]. For example - In case of Th1 cells, Th1 lineage-specific gene loci show permissive histone marks, whereas alternate-lineage gene loci like *Il4* are associated with repressive histone modifications [11-16]. Interestingly, molecular mechanisms which allow these subsets to defy lineage commitment and differentiate into other T-helper fates have also been well-studied. This enables CD4 populations to accommodate rapid changes in requirements of host immunity [6, 7].

In early 2000's, researchers studying Th1 and Th2 responses, encountered a novel CD4 subset, with distinct function and developmental origin. These cells were named

'Th17 cells' based on their characteristic secretion of IL-17, a pro-inflammatory cytokine. Role of IL-17 secreted by innate immune cells, in governing vertebrate immune responses has been well-studied over the years. However, identification of an 'IL-17-secreting CD4 subset' has been acknowledged in the field only recently. Comprehensive investigation of Th17 function has since then been an intriguing area of research.

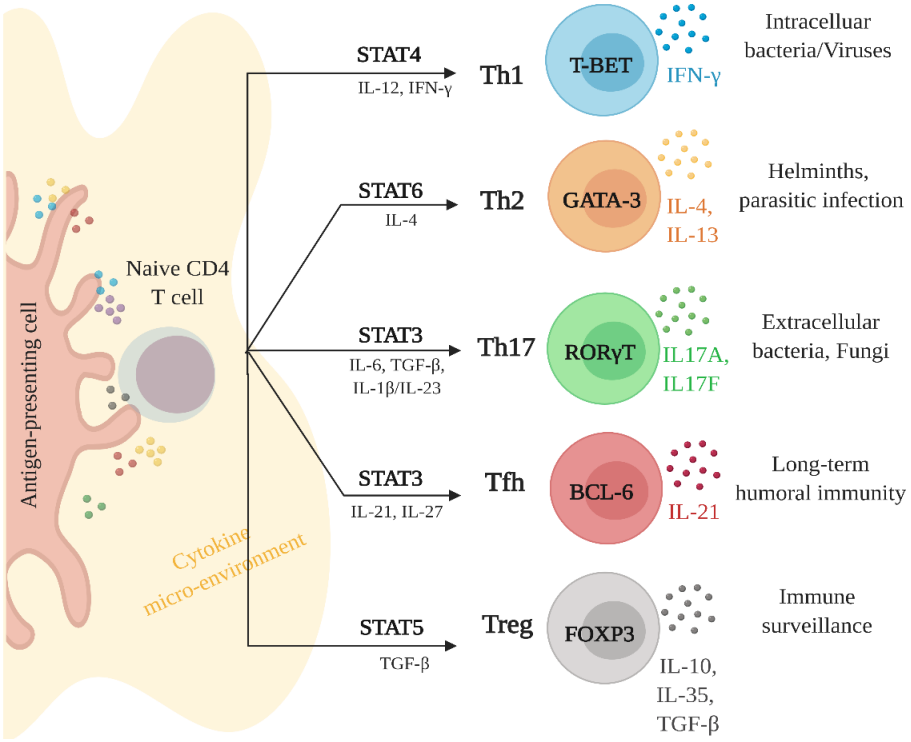

Figure 2. Naive T-cells and differentiation into functional T-helper subsets. Based on the cytokine milieu, naïve CD4 cells are subjected to differential transcriptional reprogramming that facilitates production of specific effector-cytokines, distinguishing T-helper subsets with specialized functions. Th1, Th2, Th17, Th9, Tfh and Tregs are the different lineages that a naïve cell can polarize into. This illustration has been recreated from McKee et al, 2010 and O'shea et al, 2010 [8, 17].

1.3 Introduction to Th17 cells – Protective or pathogenic?

Over the past two decades, multiple research groups have demonstrated Th17 cells to play a dual role in host immunity. Depending on the cytokine environment, these cells can perform either immunoprotective or immunopathogenic functions. Murine Th17 cells are classified as pathogenic or non-pathogenic (protective), based on their ability to induce autoimmunity in the host. In a broader sense, protective or non-pathogenic Th17 cells induce stringently regulated, anti-microbial responses with

restoration of tissue homeostasis upon pathogen-clearance. Conversely, pathogenic Th17 cells lack the ability to control self-induced, pro-inflammatory responses, thereby causing host tissue damage and autoimmunity. Extensive research over the past decade has now established that these responses are highly dynamic and cannot be defined with uniformity, owing to their contextual nature.

1.3.1 Immunoprotective functions of Th17 cells

One of the earlier reports identifying an IL-17 secreting CD4 population, distinct from Th1 and Th2, was published by Infante-Duarte and others [18]. The authors reported that naïve T-cells challenged with *B. burgdorferi* or mycobacterial lysates, secrete more IL-17 as compared to primed Th1 and Th2 cells. Further, a supporting study found IL-17 secreting T-cells to be indispensable in clearance of *Klebsiella pneumoniae*, an extracellular bacteria uncontrolled by Th1 or Th2 responses [19]. CD4-mediated IL-17 signaling was also shown to reduce the pathogenic burden of *Pneumocystis carinii,* a fungus causing life-threatening lung infections [20]. A number of reports that followed later, additionally established the requirement of Th17 cells in combating infectious agents such as *S. aureus* and *C. rodentium* [21, 22].

Th17 cells have been widely-known to dominate epithelial and mucosal barriers where they mediate clearance of many extracellular bacteria and fungi. They primarily secrete two major cytokines - IL-17A and IL-17F, both of which are known to similarly regulate host immune responses. Reports suggesting redundancy in their function have been previously published, where eliminating both cytokines was found to be essential for animals to gain susceptibility to *S. aureus* infections [21]. Nevertheless, their exclusive roles in other bacterial and fungal infections have also been reported [23, 24]. In support of the above murine studies, an immunoprotective role of Th17 cells has also been demonstrated in humans. Patients with defective IL-17 signaling are found to be highly susceptible to *S. pneumoniae* and *C. albicans infections* (reviewed in [25]). More importantly, individuals with S. aureus infections show the existence of IL-17 and IL-10 co-producing CD4 populations, which represent the classic non-pathogenic Th17 phenotype [26].

1.3.2 Th17 cells in Immunopathology

Our understanding of autoimmune disorders has advanced revolutionarily owing to use of murine models of Experimental encephalomyelitis (EAE) and Collagen-induced

Arthritis (CIA). EAE mouse models are excellent systems to study multiple sclerosis in humans whereas CIA models help in understanding responses related to rheumatoid arthritis. Initial study reports by multiple research groups emphasized that autoimmune-related inflammation results from IL-12 induced Th1 responses [27-29]. These findings were further supported by murine studies by Becher et al. which reported ablation of EAE inflammatory responses in mice deficient for the p40 subunit of IL-12 [30]. However, in 2003, the sole involvement of IL-12 in autoimmune responses was critically questioned, since the p40 subunit of the cytokine was found to be shared with another member of the same family, IL-23. IL-12 is a heterodimer consisting of p40 and p35 subunits, whereas IL-23 is composed of p40 and p19 subunits [31]. A classic series of murine experiments using mice models deficient for the exclusive subunits of *Il12(p35-/-)* and *Il23(p19-/-)* revealed that only loss of IL-23 function abolished EAE- and CIA-associated inflammation [32-34]. This strongly opposed previously interpreted roles of IL-12 in murine autoimmunity.

Strikingly, EAE resistance of *Il23p19-/-* mice was accompanied by a significant reduction in IL-17 producing T lymphocytes, with unchanged proportions of Th1 cells [32]. Besides, IL-23 induced T-effector cells were seen to produce pro-inflammatory cytokines such as IL-17A/F, with the ability to initiate EAE upon adoptive transfer in mice. More importantly, these cells showed absence of IFN-γ secretion and their gene expression profiles significantly differed from Th1 and Th2 lineages [34]. Interestingly, mice models deficient for Th1-associated factors like STAT4 and IFN-γ, depicted enhanced disease scores instead of abrogating EAE development. This certainly ruled out any other potential links between Th1 responses and Th17-associated autoimmune development [35]. These findings clearly marked the discovery of “Immunopathological Th17 cells” - a new, IL-23 induced CD4 population that is functionally distinct from other T-helper lineages.

Few of the early indications for existence of Th17 responses in human autoimmune disorders came from a study which demonstrated the detection of IL-17 transcripts in blood and CSF of patients suffering from multiple sclerosis [36]. Soon after, other studies reported expression of IL-17 in sera and diseased tissue fluids of patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [37-39]. The inflamed joints/synovial fluid of RA patients were also shown to have significant

expression of IL-23p19 subunit and CCL20, a chemokine ligand required for Th17 migration [37, 40]. More importantly, IL-17A/F producing $CD4^+$ memory T-cells have been detected under conditions of polyclonal stimulation in peripheral blood/gut of healthy individuals as well as Crohn's disease patients. Interestingly, IL-17F function has been shown to be more crucial in immunopathology of Crohn's disease [41]. It is however important to highlight that since most the previous reports in humans were simply based on detection of IL-17 RNA/protein in tissues or body fluids, the idea of Th17 responses to be solely causative of inflammation can be challenged for some of the findings [42].

In recent years, researchers in the field have focused on generating and accumulating crucial information on molecular mechanisms of Th17 function in order to gain new insights into therapy of associated immunodeficiencies and autoimmune disorders. The next sections are focused on elaborating this further.

1.4 'A tale of Th17' – Effector cytokines, molecular inducers and transcriptional regulators

1.4.1 Effector cytokines governing Th17 responses

IL-17A and IL-17F, the two major IL-17 subtypes secreted by Th17 cells, are known to share a 55% homology and are produced during differentiation in a co-ordinated fashion [43]. Importance of their function in immunoprotection has already been highlighted in Section 1.3.1. Secreted IL-17 binds to its receptors on a wide range of immune and non-immune cells and induces expression of anti-microbial peptides, proinflammatory cytokines (IL-6, TNF-α, GM-CSF) and chemokines (IL-18, CCL20 and CCL1), by stimulating the (NFκB)/MAPK pathway [44, 45]. The prime role of these inflammatory mediators is to chemo-attract important components of the innate immune system to the response site. Both IL-17A and IL-17F have been found to be elementary in recruitment and activation of neutrophils during an immunological encounter [46]. Further, importance of IL-17 signaling has been highlighted by studies using IL-17RA gene-deficient mice, which exhibit impaired host defence responses against *Klebsiella* and *Candida* infections [19, 47, 48]. However, it is important to note

that IL-17 secretion from other innate immune counterparts might also contribute to this phenotype.

Multiple reports in the past decade have demonstrated that Th17 function involves participation from many other cytokines apart from IL-17 (Summarized in Figure 3). Lineage expansion by autocrine signaling is well studied for Th1 and Th2 subsets where their secreted cytokines are further capable of inducing their own production [4]. IL-17, however, is incapable of inducing such self-activation in Th17 cells as they lack receptors for its responsiveness. Interestingly, these cells are known to secrete an important cytokine - IL-21, which is a member of the IL-2 family and a known B cell survival and differentiation factor (reviewed in [49]). Th17-polarized cells are known to express receptors for IL-21, which allows the cytokine to stimulate autocrine signaling and promote lineage establishment (reviewed in [50]). IL-21 is also known to inhibit development of Tregs, which further favours Th17 differentiation [49].

Another Th17-secreted cytokine important for its function is IL-22, which belongs to the IL-10 family of proteins. IL-22 acts primarily on epithelial cells/keratinocytes and stimulates them to produce anti-microbial peptides [24, 51, 52]. During Th17 responses, it synergizes with IL-17 to help clear bacterial infections caused by *K. pneumoniae* and others [51]. Further, *Il-22* deficient mice show no resistance to EAE development, suggesting that the cytokine assumes a pathogenic function only in combination with other Th17 cytokines, and not on its own [52]. As a matter of fact, it has been found to assume both pro-inflammatory and anti-inflammatory roles, in a context-dependent manner [53, 54]. Additionally, since its receptors are present mainly on non-immune cells, Th17 cells use IL-22 signaling to communicate with tissue environments. Eminently, studies have reported that most of the Th17-effector cytokines including IL-17A, IL-17F, IL-21 and IL-22 are found to be commonly secreted by human and mouse Th17 cells [55]. Furthermore, apart from inflammation-regulating cytokines, chemokines such as CXCL8 and CCL20, that aid in neutrophil infiltration are also produced during the course of Th17 polarization [56].

Given the nature of responses induced by Th17 effector molecules, it is crucial to develop a mechanism that tightly regulates them and prevents detrimental effects of inflammation to host cells. To achieve this, protective Th17 cells secrete an important

cytokine, IL-10, which is known to curb inflammatory responses [57]. Its anti-inflammatory potential is highlighted by studies demonstrating restoration of pro-inflammatory cytokine levels (IL-17 and IL-21), upon neutralization of IL-10 [58]. Cytokines like IL-22 or conditions of hypoxia (in absence of pathogenic exposure), are also shown to induce IL-10 production in Th17 cells [54, 59]. Further, in cases of murine EAE, adoptive transfer of Th17-primed cells along with blocking antibodies against IL-10, causes severe and more rapid disease progression [57]. Its functional implications are also established in human patients with early RA, where IL-10$^+$ Th17 cells are seen to expand upon receiving effective drug therapy [60]. Hence, IL-10 function is crucial for restoring tissue homeostasis and its differential expression is one of the factors that distinguishes protective and pathogenic functions of Th17 cells. Since pathogenic Th17 cells lack IL-10 and cause host tissue destruction, discovery of this cytokine has opened new horizons for autoimmune therapy.

Multiple reports also suggest Th17-specific secretion of alternative-lineage cytokines like IFN-γ [55], IL-4 [61], IL-9 [62], thereby highlighting a dynamic nature. IFN-γ is the major Th1-defining cytokine and its influence on Th17 responses appears to be complicated. Presence of IFN-γ has been shown to induce STAT1 phosphorylation [63] and T-BET expression [64], both of which significantly inhibit Th17 responses. Intriguingly, both mouse and human studies have reported presence of IL-17$^+$ IFN-γ$^+$ co-expressing T-cells, under conditions of chronic inflammation, thereby underscoring plasticity along the Th17-Th1 lineage. Especially in CIA models and human RA patients with prevalence of these co-expressing populations, whether IL-17 or IFN-γ drives synovial inflammation, has been a topic of debate. An interesting report on synovial T-cells of RA patients, demonstrated that inflammation in early stages of RA is driven by Th17 cells, which gradually transition into Th1/Th17 cells and finally assume Th1 fate [65]. Thus, Th17-mediated IFN-γ secretion and its role in further influencing Th17 responses is largely governed by cues from other co-expressing cytokines and cellular interactions.

Peculiarly, Th17 cells have been found to be poor secretors of IL-2, the conventional cytokine which promotes T-cell survival and proliferation for most other T helper subsets [66]. Presence of IL-2, in fact suppresses differentiation of Th17 cells [67]. Further, Th17 pathogenicity has been positively linked to GM-CSF (granulocyte

macrophage colony stimulating factor) expression in both mouse and human [68, 69]. Chemokines such as CXCL3, CCL4 and cytokines like IL-3 have also been functionally implicated in Th17-pathogenicity (reviewed in [70]).

1.4.2 Factors inducing Th17 responses

Development of protective or pathogenic Th17 cells is largely governed by the surrounding cytokine milieu. Responsiveness to tissue-micro environments is conferred by vital receptors expressed on the surface of T-cells, which enable them to modify expression of their effector cytokines (See figure 3). Initial reports on Th17 discovery suggested that IL-23 could stimulate IL-17 secretion in activated CD4 populations. However, since naïve CD4 cells lack the receptors for its responsiveness, IL-23 was found to be incapable of inducing Th17 differentiation in them [71]. Ever since, researchers in the field have invariably invested in dissecting cytokine requirements and culture conditions for induction of mouse and human Th17 cells from naïve CD4 lymphocytes. It is well established that a combination of TGF-β, IL-6 and IL-1β, successfully induces murine Th17 differentiation [71-73]. TGF-β has been well studied for its ability to induce Treg cells, but when used in combination with IL-6, it is known to block Treg development and promote Th17 responses [71, 73-75]. TGF-β/IL-6 stimulated Th17 responses are generally non-pathogenic in nature. However, further exposure to IL-23 for prolonged periods can induce a pathogenic phenotype in these cells. Thus, the dual nature of Th17 fate is primarily distinguished by the availability of IL-23. Besides, this cytokine also plays a critical role in maintenance and expansion of the Th17 lineage, with the ability to induce its own receptor in both human and mouse [34, 72, 75, 76].

Human autoimmune disorders including Inflammatory bowel disease (IBD) and ankylosing spondylitis have been strongly linked to polymorphisms in IL-23R, thereby underscoring the importance of IL-23 signaling in human diseases [77, 78]. Factors such as IL-6 and IL-21 have already been shown to induce expression of IL-23R [79]. Reports also suggest that IL-21 can substitute IL-6 in some cases and induce de-novo Th17 differentiation, in combination with TGF-β. Thus, IL-21 not only causes Th17 expansion, but also participates in development of the lineage. Further, factors like IL-

1β and TNF-α are seen to amplify Th17 responses, however, they are incapable of initiating differentiation on their own [71-73].

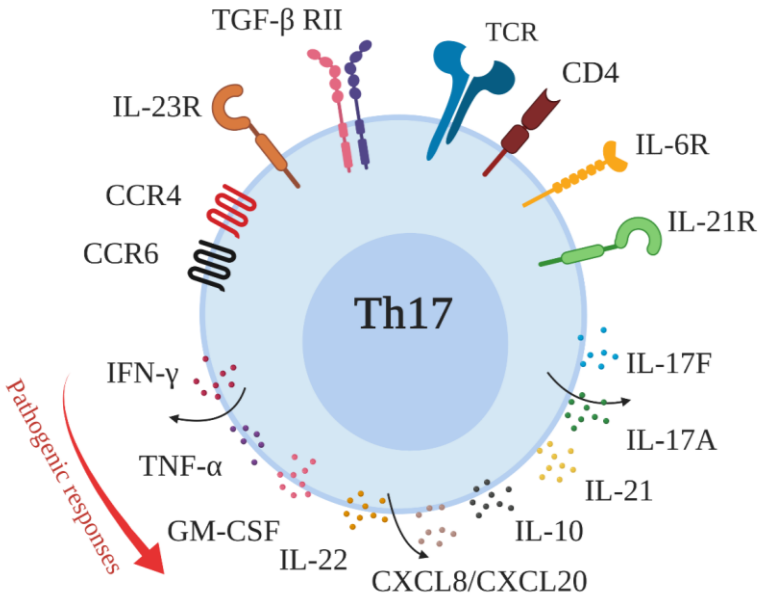

Figure 3. Molecular markers expressed on mouse and human Th17 cells. Th17 lineage-defining transcriptional networks induce the expression of various chemokines and cytokines which regulate inflammatory responses. The surface of Th17 cells expresses multiple receptors for growth factors, cytokines and chemokines, enabling these cells to communicate with the tissue-microenvironment to further modulate their effector responses.

An essential role of TGF-β in promoting Th17 differentiation is well-proven from studies that have reported a significant reduction in IL-17 producing cells upon deletion of the *Tgfb1* gene in activated T-cells [80]. Intriguingly, different classes of TGF-β have been found to induce differential Th17 responses, in combination with IL-6. TGF-β1 induces protective Th17 signatures, whereas TGF-β3 induces pathogenic ones (similar to IL-23 induction). Though both populations are seen to express similar levels of IL-17, TGF-β1 promotes IL-10 expression whereas TGF-β3 actively downregulates it [81]. Similar downregulation of IL-10 has been observed upon IL-23 exposure to Th17 cells [57]. Though IL-23 is found to be dispensable for TGF-β3 induced pathogenicity, it appears to be essential in stabilizing the induced-responses [81].

Besides this study, multiple other researchers in parallel have reported confused findings for TGF-β. One such study by *Ghoreschi et al*. reported about 2000 differentially expressed genes between TGF-β-dependent and -independent Th17-induction programs. Presence of this growth factor was shown to induce non-pathogenic Th17 cells, whereas its absence induced pathogenic ones [82]. The pathogenic populations selectively expressed T-BET and Th1-like features, which confirmed an essential role of TGF-β in inhibiting Th1 responses. However, correlation of TGF-β with non-pathogenic Th17 signatures has largely been disproved by reports on EAE and IBD, where Th17 cells grown in its presence were seen to exhibit pathogenic features [83-85]. Thus, elucidation of the precise role of this growth factor seems to require a more comprehensive investigation in order to decipher whether or not it has a contextual function.

Over a decade of relevant-research has now established that determining Th17 culture conditions in humans is more complex than in mouse. Remarkably, the role of TGF-β has been controversial even in the human counterpart. Initial attempts at in-vitro development of Th17 cells using peripheral-blood naive CD4 T-cells suggested significant discrepancies in cytokine requirements. Studies by Acosta Rodriguez and colleagues reported that IL-1β and IL-6, but not TGF-β, is essential for Th17 specification whereas Wilson and colleagues proved that IL-23 or IL-1β alone is sufficient for induction of IL-17 producing cells. Several other groups suggested similar differences in regard to Th17 culture conditions [76, 86-88] Most discrepancies from the above findings and other associated studies were later concluded to be a probable effect of the method by which $CD45RA^{+}$ (naïve) cells were isolated from peripheral blood, leading to contamination of the naïve CD4 pool by memory T-cells. To resolve this, some researchers later resorted to the use of umbilical cord blood (UCB) as a source of naive CD4 T-cells for human studies. In UCB naïve CD4 cells, a combination of IL-23 and IL-1β has been shown to induce a mixture of Th17 and Th1/Th17 clones. This was found to be corrected by exogenous addition of TGF-β, which shifted the stoichiometry of these clones predominantly to Th17 fate. This further confirms TGF-β mediated suppression of Th1 lineage during human Th17 differentiation as well [46]. Since physiological levels of TGF-β1 in human plasma is >2ng/ml, with a wide range of immune cells expressing it, tissue micro-environments which serve as niches for polarization are quite unlikely to be devoid of this growth factor [89]. More recently, a

combination of IL-6, TGF-β and IL-1β has been shown to successfully establish Th17 responses in UCB naïve CD4 cells. Presence of neutralizing antibodies against IFN-γ and IL-4 in Th17 priming cultures is seen to further promote differentiation [90]. Furthermore, lipid compounds such as prostaglandins, known to have hormone-like functions, have also been found to enhance Th17 responses in both human and mouse [91]. A few well-known induction factors for mouse and human Th17 differentiation have been depicted in Figure 4.

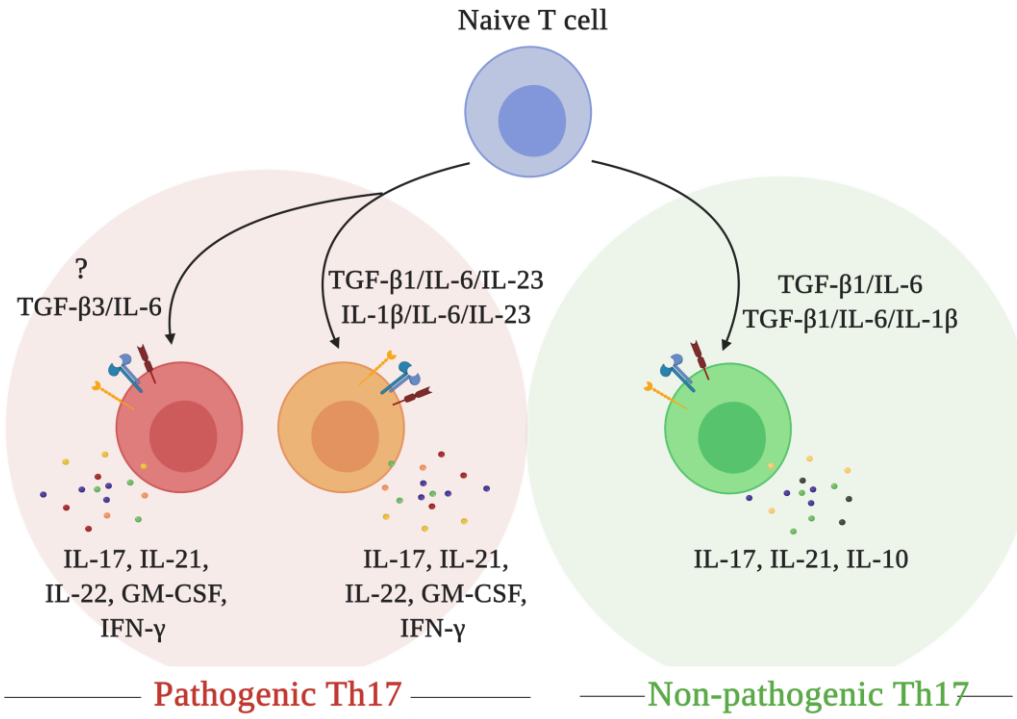

Figure 4. Factors inducing differential responses of Th17 cells. Figure depicts various cytokines and growth factors that have been demonstrated to distinguish the immunoprotective and immunopathogenic signaling cascades in mouse and human Th17 cells. This Illustration is modified and recreated from Wu et al, 2018 and Korn et al, 2009 [70, 85]. Question marks indicate uncharacterized role of the factor in induction of human Th17 differentiation.

Higher-order of plasticity is an important feature of Th17 cells, wherein their ability to inter-switch with alternative lineages like Th1 and Treg, has been constantly reviewed in the field. Studies have indicated existence of freshly-derived Th1/Th17-like cells (IL-17$^+$ IFN-γ$^+$) in both human and mouse, indicative of lineage plasticity between the subsets. These clones can eventually bias their phenotype completely towards Th1 or Th17 in the presence of suitable inducing factors (reviewed in [46]). Interestingly, context-dependent plasticity is also known to exist between protective and pathogenic

Th17 cells, where changing cytokine niches could promote the interconversion between these transient fates. Phenotypic switching from a non-pathogenic to pathogenic phenotype can be detrimental to the host. Hence, exploring molecular circuits that regulate these functions independently and in association with each other is crucial.

1.4.3 The 'protagonists' - Classical regulators of Th17 differentiation

Trafficking of Th17 cells to specific lymphoid and non-lymphoid organs is vital for its tissue-specific migration and function. This is largely dictated by heterogeneously expressed chemokine receptors like CCR4, CCR5, CXCR5, CCR6 and CCR7 on the surface of Th17 cells [92]. Notably, expression of most of these receptors is shared by Th1 and Th2 cells. Among these, CCR6 is the only receptor to be uniformly expressed on most Th17 cells, regardless of tissue-specific chemotaxis, in both human and mouse [93, 94]. CCR6 is induced by TGF-β and its expression allows Th17 cells to migrate to inflamed sites of the gut, CNS and other tissues where epithelial/stromal cells produce CCL20. CCL20 is a ligand for CCR6 and possesses both chemotactic and anti-microbial potential [95]. Deficiency of CCR6-CCL20 axis leads to aberrant migration of Th17 cells and disrupts the effector T-helper cell balance in the inflamed tissue. CCR6 expression is also used as a marker to distinguish in-vitro polarized Th17 cells in both human and mouse [96].

Distinguished molecular signatures for most T-helper subsets are governed by lineage-specific STAT signaling proteins. Th1 and Th2 responses are dictated by STAT4-induced T-BET expression and STAT6-induced GATA-3 expression respectively (reviewed in [6]). Intriguingly, responses induced by these STATs are shown to be inhibited during Th17 differentiation by polarizing factors like TGF-β [97, 98]. It is well-established that early events of Th17 differentiation are defined by STAT3 (Signal transducer and activator of transcription 3), a master-regulator of the lineage in both human and mouse [99-101]. Cellular Janus kinases are known to phosphorylate STAT3 at specific sites, which promotes its homo- or hetero-dimerization, followed by its nuclear translocation, where its acts as a transcriptional activator (reviewed in [102]). In Th17 cells, polarizing factors such as IL-6 or IL-23 induce the phosphorylation of STAT3 and stimulate its transcriptional function [103].

Significance of STAT3 function has been highlighted by studies employing T-cell specific STAT3-deficient mice, which exhibit reduced Th17 numbers and show EAE resistance [99, 104]. Further, over-expression of active STAT3 or deletion of a known negative-regulator of STAT3 (Suppressor of cytokine signaling 3/SOCS3), both have been shown to enhance Th17 responses; thereby strengthening its role as an important positive regulator of the lineage [105, 106]. With respect to human T-cell differentiation, the significance of STAT3 function was highlighted when patients with a severe immunodeficiency condition called Job's syndrome (Hyperimmunoglobulin E syndrome), were found to harbour mutations in the *STAT3* gene. These patients show altered DNA binding and impaired SH2 domain function by STAT3 and suffer from recurrent infections and enhanced innate immune responses [105].

Role of STAT3 as an orchestrator of Th17 fate has been well-emphasized by multiple reports that show STAT3-dependent induction of key marker genes (*IL-17A/Il-17a, IL-17F/Il-17f, IL-23R/Il-23r & IL-21/Il-21)*, in accordance with genomic occupancy of STAT3 on regulatory regions of many Th17-associated loci [85, 100, 101, 107]. Another Th17-relevant factor found to be a direct target of STAT3 is RORγT (RAR-related orphan receptor gamma T), which is encoded by the *RORC* gene and specifically induced under conditions of Th17 differentiation. Though its function is found to be required for IL-17 secretion, its involvement is known to be partial. This is because mice deficient for *Rorγt* show reduced numbers but not complete abolishment of IL-17 secreting cells [108]. Regardless of a potential binding site on Il-17 promoters, regulation of IL-17 expression via direct binding of RORγT has not been definitively addressed. Another member of the same family, RORα, is also induced by STAT3 and has been proposed to synergistically regulate Th17 responses along with RORγT [109].

IRF4 is another transcriptional regulator which belongs to the interferon regulatory factor family and whose role in Th17 lineage commitment has been well-established. IRF-4 is already known to be important in regulating Th2, Th9 and Treg lineages [110-113]. Its role as a positive regulator of Th17 responses is demonstrated by *Irf4*$^{-/-}$ mice which show EAE resistance and drastically reduced Th17 numbers [114]. However, its effect has been found to be independent of STAT3. These findings are further supported by studies on human Th17 cells, wherein STAT3 occupies the *IRF4* locus,

but does not transcriptionally regulate it [101]. Further, IRF4 deficiency is seen to elevate IFN-γ and FOXP3 levels under conditions of Th17 polarization, thereby underlining its function in suppressing alternative lineages [115].

Another transcriptional modulator, BATF which belongs to the AP-1 family of proteins has been reported to be essential for induction of Th17 responses [116]. Th1 and Th2 lineages also express significant levels of BATF, but its deficiency is not known to affect their function. EAE resistance of *Batf*$^{-/-}$ mice with reduced levels of IL-17, RORγT and IL21 demonstrate its instrumental role in the induction of Th17 responses. Further, AP-1 proteins like JUNB are known to dimerize with BATF and occupy promoter/intergenic regions of Il17a, Il17f, Il21 and Il22 [116]. Strikingly, under Th17 conditions, BATF signaling and not STAT3, is found to be crucial for IL-6 mediated suppression of the iTreg fate.

Eminently, IRF-4 and BATF have been highlighted as the 'pioneer factors' or 'initiator proteins' for T-helper cell differentiation. TCR signaling promotes co-operative assembly of IRF4 and BATF on Th17-relevant gene loci, thereby enhancing chromatin accessibility for lineage-defining factors like STAT3 and RORγT [117]. Since this occurs independently of polarizing cytokines, it is believed that these regulatory proteins prime the chromatin for subsequent binding of most lineage-specific TFs. Under conditions of Th17 polarization, IL-6 signaling phosphorylates STAT3 and translocates it to the nucleus, where BATF/IRF4/STAT3 co-operatively induce expression of the lineage-defining genes (*Il17a, Il17f, IL23r)* [117].

Thus, multiple studies have now highlighted that Th17 differentiation occurs in three transcriptional waves – The first wave involves expression of the classical regulators STAT3, IRF4 and BATF, which induce genes such as *Il-21* and *Il-23a*. The second wave involves expression of RORγT, whereas the final wave witnesses production of Th17-specific cytokines and suppression of other T-helper effector molecules [118-120].

Over the years, the conventional transcriptional regulators governing Th17 responses have been extensively characterized for their functional mechanisms. However, an intriguing study comparing gene expression profiles of protective and pathogenic Th17 populations in mouse, revealed as many as 233 differentially-expressed transcripts

[81]. This suggests that many other regulatory molecules might coordinate with the classical Th17 players to distinguish the two signaling phenotypes. Interestingly, immunologically-significant factors such as STAT4 and TBX21 have been found to be enriched in pathogenic Th17 cells, whereas the non-pathogenic counterparts exhibit a higher expression of regulators such as IKZF3, which is known to promote IL-10 production (reviewed in [70]). Such differences in expression profiles of TFs need to be further investigated in detail for their possible significance in governing the dual-nature of Th17 responses.

1.4.4 Molecular players with newly-emerged Th17-associated roles

Apart from the conventional orchestrators of Th17 cell identity, recent research has assigned novel Th17-specific function to many other immunoregulatory proteins. Murine Th17 studies highlighting functions for some of these candidates and the corresponding human-specific reports (for the ones available), have been elaborated in the next section (Outlined in Figure 5).

1.4.4.1 CD73/(NT5E)

CD73/NT5E is a 5' ectonucleotidase that catalyses consecutive steps of ATP hydrolysis along with CD39. CD39 breaks ATP to produce AMP, which is further converted to adenosine by CD73. Cellular ATP induces inflammatory responses whereas free adenosine inhibits them, by differentially binding to purinergic receptors [121-123]. Since CD73 function limits availability of free ATP and generates free adenosine, it majorly functions as a suppressor of inflammation [124, 125]. Independent studies have reported a high expression of CD73 on regulatory T-cells, where it dampens immune responses in both human and mouse [126-128]. However, correlation of CD73 expression with Th17 disease-development has been controversial. A 2008 EAE study observed increased T-cell mediated inflammation upon CD73 deficiency, thereby underscoring its anti-inflammatory function. However, disease progression in this case was still found to be impeded owing to impaired migration of inflammatory T-cells to the CNS [129]. Contradictory to this, another study found CD73 expression to be positively correlated with EAE disease progression. Nevertheless, lack of its function was not seen to affect disease severity [130]. More

recent reports though, have indicated its importance in resolving murine autoimmune conditions like colitis [131].

Interestingly, human autoimmune disorders show a disease-specific correlation with CD73. A study on patients with active IBD reported high CD73 expression on cells of the lamina propria with a positive correlation to RORC and IL-17 expression. Also, resolution of inflammation upon anti-TNF treatment reduced CD73 expression, thereby portraying it as an inflammatory marker in IBD patients [132]. However, most reports on other patient cohorts suggest the opposite. Especially in cases of Juvenile Idiopathic Arthritis (JIA), CD73 expression negatively correlates with disease severity. Also recently, non-membrane bound forms of this protein have been shown to mediate anti-inflammatory effects [133, 134].

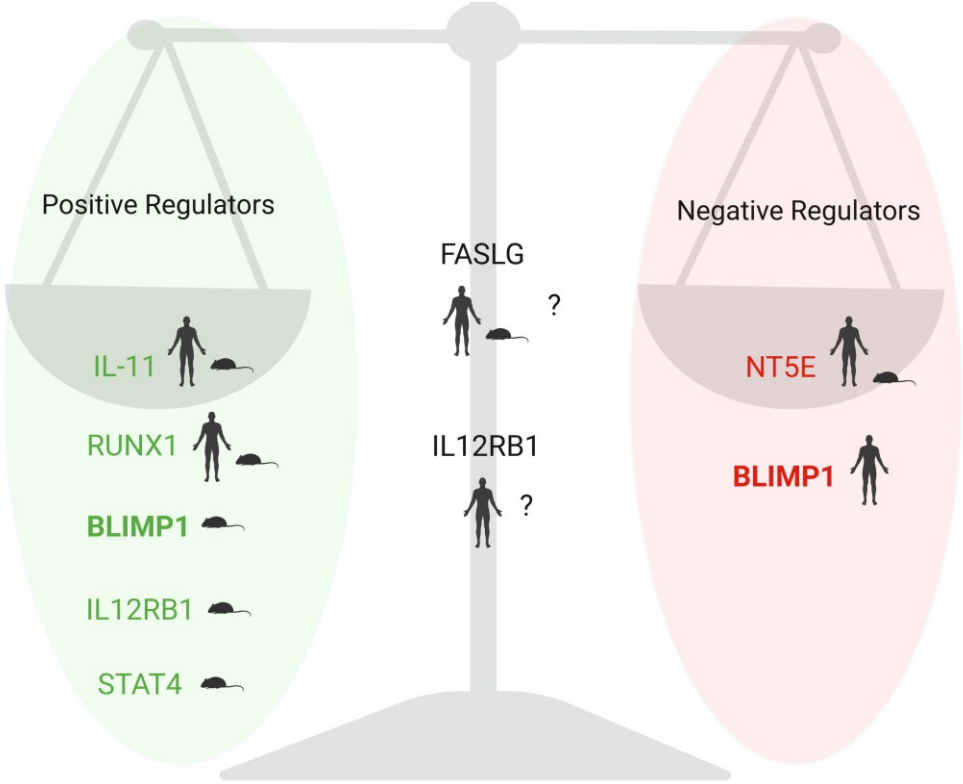

Figure 5. Recently discovered transcriptional regulators influencing Th17 differentiation. The figure shows the more recently-characterized positive and negative regulators of Th17 differentiation. The corresponding findings in human and mouse have been highlighted using the respective icons. Genes at the centre of the balance represent the candidates known to contextually regulate the lineage. BLIMP1 has been highlighted owing to its opposing function in the two species.

1.4.4.2 PRDM1/BLIMP-1

PRDM1 or BLIMP-1 (B lymphocyte-induced maturation protein-1) is an important regulator of T effector and Treg function. A 2014 study reported severe intestinal inflammation in mice with thymic T-cell deficiency of *Blimp1* gene. These mice showed impaired production of IL-10 and increased expression of pro-inflammatory cytokines [113, 135]. However, contradictory to these thymic-deletion studies, peripheral T-cell-specific depletion of *Blimp1* was found to reduce EAE symptoms, indicating it as a positive regulator of murine Th17 responses. This also suggests a tissue-specific function for BLIMP-1 in T-cells. Additionally, its levels are found to be much higher in mouse pathogenic Th17 cells as compared to non-pathogenic ones [136]. Counter-intuitively, under Treg conditions, it appears to restrain Th17 signaling. BLIMP-1 function is known to be critical for the overall suppressive ability of FOXP3+ RORγT+ murine Tregs, where it binds and suppresses Th17 cytokine-gene loci [137]. Also, in Tregs of inflamed sites, BLIMP-1 sustains FOXP3 expression by inhibiting IL-6/STAT3 induced methylation of its genomic loci, thereby preventing a Treg-to-Th17 fate-switching [138].

Strikingly, its role in human Th17 cells is found to be opposite to that of mouse. Studies using *in vitro* cultured Th17 cells from peripheral blood, show enhanced IL-17 secretion upon BLIMP-1 deficiency, thus highlighting a negative role [90].

1.4.4.3 RUNX1

RUNX1 function in Th17 differentiation has been found to be quite intriguing, since it has the potential of promoting and suppressing T-cell mediated IL-17 expression, in a context-specific manner. In both human and mouse Th17 cells, RUNX1 induces RORγT, followed by their association in a complex that binds IL-17 locus, and induces its transcription. However, under Treg conditions, FOXP3 co-operatively binds to RUNX1 and prevents RORγT-RUNX1 assembly, thereby impeding IL-17 expression [139-142]. A similar mechanism is also seen in case of the Th1-defining factor T-BET, which physically interacts with RUNX1 and inhibits its RORγT-associated Th17-promoting function [64]. As a result, role of RUNX1 appears to be crucial in regulating the balance between inflammatory and anti-inflammatory responses, majorly by exploiting its contextually tripartite interaction with RORγT, FOXP3 and T-BET. Further

highlighting this idea, its function has also been found to be required for the IL-17 producing Treg cells (Tr17) and the IFN-γ-producing Th17 cells [143, 144]. Since these findings easily suggest that differential regulation at the level of RUNX1 could contribute to changes in lineage-development, studying its molecular mechanisms could provide useful insights into dynamics of T helper cell plasticity.

1.4.4.4 FAS-FASLG

FASLG (ligand) is a TNF (tumour necrosis factor) family protein that binds to FAS (receptors) on target cells. Ligation of FAS-FASLG initiates the caspase cascade for apoptosis [145]. FAS signaling-induced apoptosis is critical for central and peripheral tolerance, as well as to regulate lymphocyte numbers during a pathogenic encounter. Previous studies report that mice defective in FAS signaling, develop lymphadenopathy and splenomegaly, but are relatively resistant to EAE development [146, 147]. In support of this, a more recent study found FAS to be a target of STAT3 and BATF in murine Th17 cells, where its genetic deficiency was seen to abrogate EAE development. Further, in-vitro differentiation of *Fas*$^{-/-}$ T-cells under Th17 conditions, represses IL-17A and enhances expression of IFN-γ, thereby suggesting its role in balancing Th1/Th17 responses [120]. However, none of these reports comment on its T-cell specific function during inflammation.

A study in 1999 interestingly suggested that a balance between the expression of FASLG on inflammatory T-cells and expression of FAS-receptor in target tissue cells, decides the clinical manifestations of EAE [148]. Additionally, differential susceptibilities of inflammatory and anti-inflammatory cells to FAS signaling-mediated apoptosis, has been found to dictate the final outcome of the disease. For example, human patients with Acute Coronary Syndrome depict an enhanced expression of FAS/FASL. However, in this study, Tregs were seen to have a higher susceptibility to FAS-mediated apoptosis than Th17 cells. This was observed to create an imbalance in Treg and Th17 populations, leading to pronounced inflammation [149]. Interestingly, human patients suffering from multiple sclerosis, show defects in the FAS/FASL pathway. This is shown to reduce apoptosis and increase survival of pathogenic T-cell populations, thereby promoting inflammatory conditions [150-152].

1.4.4.5 IL12Rβ1

IL-12R is the primary receptor for IL-12 signaling, which majorly regulates Th1 development in both mouse and human. It consists of 2 subunits – IL12Rβ1 and IL12Rβ2, which are induced upon antigenic stimulation. IL12Rβ1 has binding specificity to the p40 subunit shared between IL-12 and IL-23 cytokines. Hence, defects in its function, affects both IL-12 and IL-23 signaling. *Il12rβ1*$^{-/-}$ mice are completely resistant to EAE, owing to defects in both APC and CD4 T-cell function. This emphasizes its requirement in development of autoimmunity [153]. Also, these mice were shown to have severely compromised IL-23 and IL-18 responses [154, 155].

Role of this receptor in human Th17 cells however, seems contextual and quite unclear. IL-17 treatment of human PBMCs promotes IL12Rβ1 and IL-23 levels, suggesting its expression to be positively correlated with Th17 function. However, researchers have found a patient with mendelian susceptibility to Crohn's disease to be genetically deficient for IL12Rβ1 function. This indicates a negative correlation between IL12Rβ1 expression and incidence of IBD [156].

1.4.4.6 IL-11

IL-11 is a member of the IL-6 cytokine family, known to promote Th17 differentiation in both human and mouse [86]. Injection of IL-11 in RREAE mice, a model for relapsing-remitting MS disease (RRMS), showed increased disease scores and thus a supporting role for this cytokine. Further confirming this, IL-11 antagonists were seen to reduce disease severity in both acute and RREAE cases [157]. Besides, IL-11 has been shown to induce encephalitogenic T-cells, in a manner similar to IL-23 [158].

In humans, patients with early as well as relapsed stages of MS disease show elevated serum IL-11 levels and increased numbers of IL-11-secreting CD4 cells. A distinctive cross-talk exists between IL-11 and Th17 cytokine signaling, where they both can induce each other's expression in $CD4^{+}$ T-cells and can potentially re-enforce inflammatory signaling in cases of RRMS. Also, IL-11 is solely capable of inducing Th17 differentiation in naïve CD4 cells of early stage MS patients, in absence of other polarizing cytokines [157, 159].

1.4.4.7 STAT4

Signal transducer and activator of transcription 4 (STAT4) is Th-1 lineage transcriptional regulator known to induce expression of IFN-γ and other Th1-effector molecules, in response to IL-12 [160-162]. Regulation of Th17 responses by STAT4 signaling has been a point of debate. Studies by Harrington et al. and Park et al. initially reported IL-23 primed IL-17 secretion to be independent of STAT4 signaling. However, this was later proposed to be an effect of phenotype-obscuration on account of PMA/Ionomycin re-stimulation [63, 98]. It is well established that STAT4 KO mice are EAE resistant [163]. Supporting studies have also reported the requirement of STAT4 in achieving maximal expression of IL-17 expression in IL-23 primed cells; thereby emphasizing its role in Th17 pathogenicity [100]. Regardless, involvement of STAT4 has been found to be dispensable for TGF-β/IL-6 induced protective Th17 responses in mouse (reviewed in [164]). More importantly, the differential role of STAT4 in regulating pathogenic and non-pathogenic Th17 signaling in humans, is yet to be deciphered.

Apart from the candidates discussed in this section, many other proteins with well-documented roles in key signaling processes are yet to be reviewed for their influence on Th17 effector function. The above-mentioned findings evidently depict some regulatory proteins with species-species differences in their Th17-specific roles. An important drawback in the field is that most reports on discovery of novel regulators are based on murine phenotypes and hence molecular networks governing human Th17 differentiation are still far from being understood.

1.5 Conclusion and future perspectives

Discovery of Th17 cells in both mouse and human has opened new horizons in the field of immunoprotection and autoimmunity. In the past decades, immunological research has been dominated by murine model systems. However, their usage is not always optimal for research involving development of novel therapeutic targets for humans. The prime reason being that multiple regulatory proteins have shown significant functional discrepancies in mouse and human systems [90, 136, 165, 166].

Such observations underscore the necessity to validate murine gene findings using cells of human origin. This approach is imperative for us to be able to harness information from mouse experiments for applications in human immunotherapy. An important concern on this front is that although there has been a steady surge in Th17-related articles, very few of them have focused on addressing species-specific differences. This is primarily owing to poor-characterization of lineage-defining transcriptional networks in human systems. Developmental origin of Th17 cells have already been shown to differ in mouse and human (reviewed in [46]). Further, the interplay between Th17 and other T-helper cell subsets also appear discordant in the two species. Thus, there exists an increasing need for molecular investigation of human Th17 responses.

The basal regulatory machinery defining Th17 cell identity involves participation of multiple transcription factor families that function in a synchronized manner. The AP-1/ATF superfamily is one such group of proteins which has been recently studied for their ability to influence murine Th17 differentiation. These proteins have already been well-reviewed for their ability to modulate crucial signaling events during T-helper cell activation and differentiation. Interestingly, exhibiting context-specific regulatory potential is an important feature of AP-1/ATF proteins. Many members of this family namely - JUNB, JUND, FOSL1, FOSL2 and BATF have been well-investigated for their Th17-associated roles using genetically-deficient mouse models. While JUNB, FOSL1 and BATF positively regulate the lineage, JUND and FOSL2 have been found to inhibit Th17 differentiation [117, 167-169]. It is noteworthy that FOSL1 and FOSL2, despite being paralogs, have similar functions in most cell-types, but show conflicting roles in regulation of murine Th17 responses [117, 169]. More interestingly, none of these AP-1 proteins have been explored for their ability to alter human Th17 phenotypes. Whether or not, there are species-specific differences for functions of these proteins, is something that stands undiscovered. In addition to the AP-1 family, other immune signaling molecules such as SATB1, IKZF1, RBPJ and C-MAF, with recently discovered roles in murine Th17 establishment, need to be reviewed for their human-specific function.

Plasticity of Th17 cells, especially across the Th1 or Treg axis appears to be critical in regulating the balance between pro-inflammatory and anti-inflammatory responses within the host. Though there are murine reports elucidating molecular mechanisms

of T-helper cell plasticity, such phenotypic transitions in human T-cells have not been thoroughly examined. Further, transcriptional networks distinguishing the protective and pathogenic signaling cascades of human Th17 cells are still unexplored. Detailed investigation into such blurred areas of human Th17 research is a crucial requirement for us to comprehensively understand the developmental origins of human autoimmune disorders. Achieving this could help in significant advancement of Th17-associated immunotherapy.

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Chapter 2

Mass spectrometry-based proteomic analysis reveals novel changes in protein signatures of early differentiating human Th17 cells

2.1 Introduction

Functional implication of Th17 cells in protective-inflammation and autoimmune disorders has been well-established in both human and mouse. Being one of the more recently discovered T-helper subsets, knowledge regarding mechanistic regulation of Th17 molecular players is quite limited. Permissive histone marks and DNA demethylation at promoters of *IL17a*, *IL17f and Rorc,* have been shown to sustain cytokine expression and lineage commitment in Th17 primed cells [1-3]. However, unlike Th1 and Th2 cells, epigenetic modifications repressing alternate lineage-cytokines are relatively more dynamic in Th17 cells, thereby suggesting their pronounced plasticity as compared to other T-helper subsets [4]. This makes it obligatory to possess a strong, minimally error-prone circuit that maintains the balance between Th17 and other T-helper cell responses. A tightly-regulated, well-orchestrated network of lineage-specific transcription factors is required to accomplish this. Until recently, most of our understanding on molecular players of Th17 differentiation has emerged from studies involving high-throughput transcriptional analysis [5-8].

2.1.1 Employing transcriptomics to explore differentiation-specific networks

Temporal profiling of transcripts in both mouse and human Th17 cells has been useful in indicating time-resolved roles of key transcription factors in initiation and maintenance of the lineage. Transcriptional analysis of murine Th17 cells at different

stages of polarization indicates that classical regulators like STAT3/BATF/IRF4 are expressed during early phases, followed by RORγT expression at intermediate time points; whereas cytokine genes are subsequently induced only at later stages. Noteworthily, expression of these key Th17 genes in a time-point-dependent manner is known to be critical for lineage-establishment. For example, it has been shown that early expression of BATF and IRF4 enables them to act as initiation factors, which bind to chromatin and make it accessible to other lineage-defining proteins like STAT3 and RORγT. More importantly, apart from marker genes, global RNA profiling across the differentiation window has revealed many other regulatory proteins and chromatin-remodellers to exhibit a Th17-specific expression profile. Interestingly, a significant number of these proteins, namely TSC22D3, POU2AF1, FAS, BCL11B, ETV6, JMJD3, FOSL2 and SATB1 have been successfully validated for their ability to alter expression of Th17 effector cytokines in mouse [5, 6]. Thus, global transcriptional studies have not only embellished our knowledge regarding core Th17 factors but have also helped us predict lineage-associated roles for proteins with previously unidentified functions.

Time-resolved transcriptome analysis has also been performed for in-vitro polarized human Th17 populations using umbilical cord blood-isolated naive CD4 cells [7]. More importantly, upon comparing this study with previously reported murine findings, transcripts corresponding to a significant number of Th17-associated genes (including IL23R, NOTCH1, RORA, RUNX1 and VDR) were found to be similarly altered in the two species, upon induction of polarization [5, 7, 9-12]. However, some key discrepancies were also witnessed. Additionally, many other genes with previously unidentified roles in the process, such as BASP1, ATP1B1, LMNA, KDSR, COL6A3 and ITM2A were found to be differentially expressed at the RNA level between human Th0 and Th17 conditions. Lineage-specific expression of these functionally novel molecules could be indicative of their ability to influence human Th17 responses.

2.1.2 Need for a proteomics approach to study potential players of T-helper cell differentiation

Over the years, high-throughput transcriptomics has shown immense potential for enhancing our knowledge regarding regulatory networks of T cells. However, it does

have important limitations. It is well established that transcriptional signatures represent only half of the story and do not necessarily translate into protein profiles [13]. In fact, about 60% of the protein-level variance cannot be explained by transcript information alone. Further, cellular protein concentrations have a wider range of dynamicity as compared to RNA molecules [14, 15]. This is a consequence of multiple post-transcriptional, post-translational and protein-degradation mechanisms, which are known to alter the expression and stability of cellular proteins. This mandates that transcript levels alone cannot be used to predict protein abundances and hence deciphering gene expression profiles at the protein level is equally essential for understanding biological processes and cellular pathways [16].

Earlier methods for global proteomic analysis posed many challenges majorly owing to poor-reproducibility and limited detection-sensitivity. This made it difficult to characterize low-abundance proteins. Since many transcription factors and cytokine receptors are known to express at lower concentration ranges, developing better techniques for proteomics-based analysis of functional gene-networks is imperative. Recently, significant technological advances have greatly improved our approach for studying protein-level changes in biological cells. Notably, employing mass-spectrometry in combination with liquid-chromatography (LC-MS) has proven to be a robust technique capable of determining both qualitative and quantitative changes in proteins with much improved detection sensitivity. MS-based analysis has been widely used to study proteomes of many immune cell-types including B cells [17], macrophages, dendritic cells [18], cytotoxic T-cells [19] and T-helper cells (Th1, Th2 and Treg) [20-25]. Besides, it has enabled identification of differential protein signatures in natural and induced Tregs in both human and mouse, which is an important resource to the field [21, 24, 25]. MS tools have also been used to characterize the proteomic profile of human disease conditions like Crohn's disease, wherein Th1/Th17 mixed clones from gut biopsies were analysed [26].

Interestingly, the proteome of in-vitro differentiated murine Th17 cells was reported only recently [27]. However, none of the existing studies have characterized the cellular proteome for the human counterpart. Striking dissimilarities have already been reported for the transcriptome of early-differentiating mouse and human Th17 cells [28]. Since multiple other reports have also established prominent discrepancies in gene-expression profiles of the two species, it is essential to characterize the protein

level changes in human Th17 cells and determine its concordance with mouse [14, 29-32].

Extrapolation of murine findings and its translation to human research has shown limited success and stands as a major challenge in the field [33, 34]. Thus, validating murine gene-function results, using cells of human origin is crucial for advancement of immune therapy and drug development, targeting human diseases.

2.2 Materials and Methods

2.2.1 Human $CD4^+$ T-cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from umbilical cord blood of healthy neonates (Turku University Central Hospital, Turku, Finland) using density gradient method (Ficoll-Paque PLUS; GE Healthcare). Naive $CD4^+$ cells were further purified from the PBMC pool using Dynal $CD4^+$ positive isolation kit (Invitrogen). Purified $CD4^+$ cells from individual donors were either directly cultured or pooled before culturing (in case of experimental validations).

2.2.2 In-vitro Th17 culture

Isolated $CD4^+$ cells were activated using plate bound α-CD3 (3750 ng/6-well culture plate well; Immunotech) and soluble α-CD28 (1 µg/mL; Immunotech), in a maximum density of 2.5×10^6 cells/mL of X-vivo 20 serum-free medium (Lonza). Media was supplemented with L-glutamine (2 mM, Sigma-Aldrich), and antibiotics (50 U/mL penicillin and 50 µg/mL streptomycin; Sigma-Aldrich). For Th17 priming, a cytokine cocktail of IL-6 (20 ng/mL; Roche), IL-1β (10 ng/mL, R&D Systems) and TGF-β (10 ng/mL, R&D Systems), in the presence of neutralizing anti-IFNγ (1 µg/mL, R&D Systems) and anti-IL-4 (1 µg/mL, R&D Systems) antibodies was used. For control cells (Th0), $CD4^+$ T-cells were plainly activated with similar amounts of α-CD3 and α-CD28 in the presence of neutralizing antibodies without any cytokines and cultured in parallel. All cell cultures were maintained at 37°C in a humidified atmosphere of 5% (v/v) CO_2/a.

2.2.3 Isolation of mouse cells and *in vitro* cell culture

BALB/c mice purchased from the University of Turku animal facility were housed in accordance with the University of Turku animal welfare guidelines. Spleens of 8- to 10-week-old mice were first macerated using a cell strainer and syringe plunger to make a single cell suspension and red blood cells were lysed using ACK lysis buffer (Gibco by Life Technology, cat# A10492-01). Cells were then isolated by positive selection using $CD4^+CD62L^+$ coupled magnetic beads (MACS Miltenyi Biotec; cat# 130-106-643), using a MACS LS/MS column (MACS Miltenyi Biotec). Cells were cultured in IMDM (Gibco) media supplemented with 5% fetal calf serum, 2 mM L-glutamine (Sigma-Aldrich), and 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich) and 50 µM β-mercaptoethanol (Gibco). Cells were further stimulated using plate-bound α-CD3 (1 µg/mL; BD PharMingen, cat# 553238) and soluble α-CD28 (2 µg/mL; BD PharMingen, cat# 557393) for 72h (unless otherwise indicated) and cultured either under Th0/TCR control conditions or Th17 differentiation conditions. Differentiation was primed using TGFβ (1 ng/ml; R&D, cat# 240-B), IL-6 (20 ng/ ml; R&D, cat# 406-ML), and IL-1β (10 ng/ml; R&D, cat# 201-LB). Neutralising antibodies - anti-IFN-γ (cat# 557530), and anti-IL-4 (cat# 559062) (both at 10 µg/mL, BD PharMingen) were added to both control and Th17 conditions, to inhibit Th1 and Th2 differentiation.

2.2.4 Mass-spectrometry sample preparation, pre-processing and preliminary analysis

2.2.4.1 Cell lysis

Proteins were extracted from the cell pellet using a lysis buffer (4% SDS, 0.1 M DTT, 0.1 M Tris-HCl, pH 7.6), heated at 95ºC for 5 min. The lysate was then sonicated at high voltage with a setting of 5 cycles for 30 seconds and 30 seconds rest between cycles. The cell debris were cleared by centrifugation at 16000x g for 20 min, and a DC Protein Assay (#5000116, BioRad) was used to estimate protein amounts.

2.2.4.2 Filter Aided Sample Preparation (FASP) Method

Briefly, an aliquot corresponding to 50 µg of protein from each biological replicate corresponding to different time points (i.e., 24h and 72h) (n=5 for both Th17 and corresponding Th0 controls) were mixed with FASP urea buffer 8 M urea in 0.1 M Tris-

HCl, pH 8.5) in a 30 kDa filter tube (Millipore) to eliminate the SDS. The proteins were reduced with dithiothretiol (DTT) and alkylated with iodoacetamide in the dark for 20 min. Finally, they were digested with sequencing grade modified trypsin in 1:30 (protein:protease) ratio overnight at 37ºC. The digested peptides were then acidified and desalted using Sep-Pak C18 cartridges (WAT054955 Vac 1 cc 50 mg, Waters). The desalted samples were dried using a centrifugal evaporator (Thermo Scientific) and stored at -80ºC until further LC-MS/MS analysis.

2.2.4.3 Mass spectrometry analysis

The dried peptides were reconstituted in formic acid/acetonitrile mixture, and an amount corresponding to 400 ng was analysed using EasynLC 1200 coupled to Q Exactive HF mass spectrometer (Thermo Scientific). The peptides were separated on a 75 µm ID X 40-cm HPLC column, packed in-house with 1.9 µm Reprosil C18 particles (Dr Maisch GmbH). The peptides were eluted with a gradient from 7 to 25% B phase in 75 min, then to 90% B in 15 min, at flow rate of 300 nL/min. The mobile phase compositions were, water with 0.1 % formic acid (A) and 80% acetonitrile 0.1% formic acid (B). The temperature of the column was maintained at 60ºC using a column oven. The tandem mass spectra were acquired with higher-energy C-trap dissociation (HCD) of the 10 most intense ions (m/z 300–2000, charge states > 1+). The MS1 resolution was set to 120,000, with 3 x 10^6 AGC target value and a maximal injection time of 100 ms. MS/MS spectra were acquired in the Orbitrap with a resolution of 15,000 (at m/z 400), a target value of 50,000 ions, a maximum injection time of 250 ms. Dynamic exclusion was set to 30 s. Triplicate analysis was performed for all samples in randomized batches.

2.2.5 Peptide and protein identification and quantification

The mass spectrometry raw files were processed using MaxQuant software version 1.5.5.1 (Cox and Mann, 2008). Uniprot human database (May 2017) was used to search the peptide data using Andromeda (Cox et al., 2011) as a search algorithm. The search parameters specified trypsin digestion with a maximum of two missed cleavages, carbamidomethylation of cysteine as fixed term modification and N-terminal acetylation and methionine oxidation as variable modifications. The peptide and protein level false discovery rates (FDR) were set to 0.01. The match between the

runs option was enabled to transform the identifications across the mass spectrometric measurements. The label free quantification method (MaxLFQ) was used to determine the relative intensity values of proteins and to normalize the protein intensities between the samples (Cox et al., 2014). Prior to the downstream data analysis, data was filtered to remove proteins with less than two unique peptides. Contaminants and reverse hits were also removed (Table S1). The proteomic mass spectrometry data presented in this paper were submitted to PRIDE (Vizcaíno et al., 2016) and have the accession number PXD008973.

2.2.6 Proteomics data analysis

All data analyses were performed using the R statistical programming software environment version 3.4.3 (R Core Team, 2015).

2.2.6.1 Exploratory data analysis

To explore the similarity of the samples and the grouping of the biological replicates in the LFQ-normalized data, the R-package pheatmap (Kolde, 2015) was used. Pearson correlation coefficient was used as a similarity measure and hierarchical clustering with complete linkage for clustering the samples.

2.2.6.2 Differential expression analysis

The Reproducibility Optimized Test Statistic (ROTS) (Elo et al., 2008; Suomi et al., 2017) was used to detect the DE proteins between the conditions. Differential expression was examined separately for each comparison and time point. The examined comparisons were Th0 – Thp at 24h, Th0 – Thp at 72h, Th17 – Thp at 24h, Th17 – Thp at 72h, Th17 – Th0 at 24h and Th17 – Th0 at 72h. Technical replicates for a biological replicate were averaged and the data was log2-transformed prior to the differential expression analysis. FDR of 0.05 was used as a threshold to define the DE proteins. Differentially expressed proteins whose logarithmic fold change (LogFC) was > 0, were considered as up-regulated and proteins whose LogFC was < 0 were considered as down-regulated. Z-score standardization of the DE proteins in the compared samples was used for visualizing the changes in expression with heatmaps.

2.2.7 Enrichment analysis

The enriched gene ontology (GO) biological processes were identified using the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8

(Huang et al., 2009a, 2009b). The GO FAT terms, which filter out the broadest terms, were considered. The enrichment analysis was performed using the DE proteins over both time points as the input and the whole detected and filtered proteome as the background reference. A biological process was considered enriched if it had FDR ≤ 0.05. The enrichment of molecular types and cellular locations was further examined using the Ingenuity Pathway Analysis (IPA) (QIAGEN Inc.) (Krämer et al., 2014). The enrichment of cellular locations and protein types between Th17 and Th0 was examined at 24h and 72h using the time point specific DE proteins as input for IPA and the whole detected and filtered proteome as a background reference. All the resulting IPA location and type information was collected.

2.2.8 Targeted Proteomics Validation

Selected reaction monitoring (SRM) mass spectrometry was used to validate the relative abundance of ATP1B1, PALLD, ACSL4, FHOD1, SMTN and RDX in the Th17 cells at 72 h. Heavy-labelled synthetic peptides (lysine $^{13}C_6$ $^{15}N_2$ and arginine $^{13}C_6$ $^{15}N_4$) were obtained for the targets of interest (Thermo Fischer Scientific) and were selected on the basis of their stability, consistency and intensity in the discovery data. For these validations, four additional cultures were prepared from the cord blood of four donors. Skyline software (MacLean et al., 2010) was used to evaluate the top five most intense transitions from the MS/MS spectra of the heavy labelled standard peptides and assess the relative performance of the native peptides in the spiked validation samples. The samples were prepared using the same FASP digestion and desalting protocols used for discovery. These were then spiked with synthetic heavy labelled analogues of the peptide targets and a retention time standard (MSRT1, Sigma) for scheduled selected reaction monitoring. The LC-MS/MS analyses were conducted using Easy-nLC 1000 liquid chromatograph (Thermo Scientific) coupled to a TSQ Vantage Triple Quadrupole Mass Spectrometer (Thermo Scientific). The column configuration included a 20 x 0.1 mm i.d. pre-column in conjunction with a 150 mm x 75 µm i.d. analytical column, both packed with 5 µm Reprosil C18-bonded silica (Dr Maisch GmbH). A separation gradient from 8% to 43% B in 27 min, then to 100% B in 3 min, was used at a flow rate of 300 nl/min (the mobile phase compositions are as indicated above). The raw SRM data are available through PASSEL (Farrah et al., 2012) with the dataset identifier PASS01204 Skyline was used to select the transition used for the assays and subsequently processed to generate the data. The MSStats

(3.8.4) plugin included in the Skyline software was used for the group comparison between cases and controls. The summed intensities of GAPDH peptides were used as a global standard to normalize the data from each analysis and Tukey's median polish method was used as the summary method.

2.2.9 Transcriptomics data analysis

2.2.9.1 RNA-seq sample preparation

RNA samples from five biological replicates derived from Th0 and Th17 cultures of five individual donors were collected at 72h time point. RNA was isolated (RNeasy Mini Kit, QIAGEN) and DNase treated (RNase-Free DNase Set; QIAGEN). Library preparation was performed according to Illumina TruSeq® Stranded mRNA Sample Preparation Guide (part # 15031047). RNA-seq with 50 nucleotide read length was performed at the Finnish Functional Genomics Centre (FFGC) with HiSeq 3000 instrument using TruSeq chemistry and base calling was performed with CASAVA1.8.

2.2.9.2 Pre-processing of the raw data

The RNA-seq raw reads were mapped to the Ensembl human reference genome GRCh38 (Genome Reference Consortium Human Build 38) (Zerbino et al., 2018) using the STAR aligner (Dobin et al., 2013) version 2.5.2. The read counts were generated using the featureCounts tool (Liao et al., 2014) in the Subread software package (Liao et al., 2013) version 1.5.1. Uniquely mapped reads were used for further analysis. The uniquely mapped reads were filtered for lowly expressed genes (genes with counts per million (cpm) >1 in at least 5 replicate samples were retained) and used for further analysis. The filtered gene counts were normalized using the trimmed mean of M-values (TMM) normalization from the Bioconductor package edgeR (McCarthy et al., 2012; Robinson et al., 2010) after which the data was transformed to counts per million (cpm), offsetted by 1 and log2-transformed. Differential expression analysis was performed similarly to proteomics using ROTS (Elo et al., 2008; Suomi et al., 2017). An FDR of 0.05 was used as a threshold to define the DE genes. The RNAseq data presented in this study was submitted to the Gene Expression Omnibus (GEO) and has the Series record GSE118974.

2.2.10 Comparison between human and mouse proteomics data

To compare differentially regulated proteins (differentially expressed proteins and proteins detected in only one condition) between Th17 and Th0 in human and mouse

at 72h during Th17 polarization, we used the published mouse proteomics raw data (Mohammad et al., 2018) and pre-processed it similarly to the human data using MaxQuant (version 1.5.5.1) with LFQ-normalization, filtering out proteins with less than two unique peptides, and removing contaminants and reverse hits. Similarly, as with the human proteomics data, the differential expression analysis was performed using ROTS. To make the comparison of the differentially regulated proteins more comprehensive, we used a threshold of FDR 0.1 in both datasets to define the DE proteins. Mouse genes related to proteins were mapped to orthologous human genes for the comparison using Ensembl BioMart (Zerbino et al., 2018). All the orthologous mouse genes from Ensembl 92 database to the human reference genome GRCh38 were considered. If multiple orthologous human genes existed for a given mouse gene, the most similar human orthologous gene according to the Ensembl database was selected.

2.2.11 Immunoblot Analysis

Cells were harvested and lysed in either RIPA (Pierce, #89901) or Triton-X buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.5% Triton-X-100; 5% glycerol; 1% SDS), supplemented with proteinase (Roche) and phosphate inhibitors (Roche). Lysed samples were sonicated for 7 min under ice cold conditions (Bioruptor UCD-200; Diagenode), followed by centrifugation at 14,000 rpm for 20 min at 4°C. Supernatants were collected and quantified using the DC Protein Assay (Bio-Rad). Samples were boiled with 6x sample loading dye (330 mM Tris-HCl, pH 6.8; 330 mM SDS; 6% β-ME; 170 mM bromophenol blue; 30% glycerol) and loaded on 4–20% precast gradient SDS-polyacrylamide gels (Biorad). Gel proteins were transferred to nitrocellulose membranes (Bio-Rad), and probed with the antibodies listed in Table 1 in 5% BSA. In some cases, blots were striped with striping buffer (25 mM Glycine and 1%SDS; pH 2.5) and re-probed successively with different antibodies recognizing proteins with different molecular mass.

2.2.12 IL-17A secretion

IL-17A levels were analysed in supernatants of 72h cultured cells. Milliplex MAP human IL-17A kit (Merck Millipore; HCYTOMAG-60K-01), Bioplex Human IL-17A Cytokine/Chemokine 96-Well Plate Assay (Bio Rad; Cat. no. 171B5014M, 171304090M) or Human IL-17A Duoset ELISA kit (R&D Biosystems DY317-05,

DY008) was used for detection. The amount of IL-17A secreted by Th17 cells was normalized with the number of living cells determined based on forward and side scattering in flow cytometric analysis (LSRII flow cytometer; BD Biosciences).

2.2.13 IFN-γ secretion

Culture supernatants from $CD4^+$ T-cells polarized under Th17 conditions for 72h were assayed by ELISA for IFN-γ secretion (Milliplex MAP human IL-17A+IFN-γ kit; Cat.no. HCYTOMAG-60K-02), according to the manufacturer's protocols. For appropriate analysis, all values below the detectable range were considered zero.

2.2.14 Flow cytometry

Flow cytometry analysis of cell-surface receptor CCR6 detection was performed at 72h post Th17 cell priming. Cells were washed twice with PBS, and staining was performed in FACS staining buffer (0.5% FBS/0.1% Na-azide/PBS) for 20 min at 4°C followed by two rounds of washes with staining buffer. Data was either acquired using LSRII flow cytometer (BD Biosciences) on the same day or cells were fixed with 1% formalin and analysed on the following day. Live cells were gated for analysis based on forward and side scattering. Detection of IFN-γ producing cells was determined by intracellular cytokine staining with anti-IFN-γ-FITC (BD Biosciences). Cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin initially for 2h, GolgiStop (BD Biosciences) was added at 2h and activation was continued to go on for another 3h. Cells were then fixed in 4% paraformaldehyde solution, followed by staining with fluorescent antibodies in 0.1% saponin permeabilization buffer and analysed on LSRII (BD Biosciences). For OASL and ATF3 staining, cells were first fixed with 4% paraformaldehyde and permeabilized with Perm III buffer (BD Biosciences). Cells were incubated with primary antibodies (1:25) for 30 min in Perm buffer, followed by washes and secondary antibody treatment (1:500, Perm buffer). Suitable isotype controls/Only secondary antibody controls were maintained. Information for the used antibodies has been provided in Table 1.

2.2.15 Quantitative Real-time PCR

Total RNA was isolated using RNeasy kit (Qiagen, Valencia, CA). cDNA was synthesized with Reverse Transcription kit (Applied Biosystems, Foster City, CA) using oligo dT primers according to the manufacturer's instruction. TaqMan primers and probes for IL-17A, IL-17F, RORC and SATB1 were designed with Universal Probe

Library Assay Design Centre (Roche), in Absolute QPCR ROX Mix (Thermo Scientific). EF1a gene was used as endogenous control. The qPCRs were run using the 7900HT Fast Real-Time PCR System (Applied Biosystems). All primer sequences have been provided in Appendix I.

2.2.16 Immunostaining

$CD4^+$ T-cells were cultured for 72hrs under Th0 and Th17 differentiation conditions and then spun down on Poly-L-Lysine coated coverslips at 800rpm. Cells were washed, fixed and permeabilized using Ebioscience Intracellular Staining kit (Invitrogen; Cat no.00-5223-56 and Cat no.00-5123-43; Invitrogen; Cat no.00-8333-56). Permeabilized cells were further incubated overnight with primary antibody against Lamin A/C (Santacruz Biotechnology, Cat no. sc-7292). Cells were washed with Permeabilization buffer and further incubated for 60 mins with anti-mouse Alexa flour 488 secondary antibody (Life Tech Cat no. A11001). Atto-Phalloidin A647 (Sigma; Cat no.65906) was used to stain cytoplasmic actin. Stained cells were finally mounted in Prolong Gold Antifade mountant with DAPI (Cat no. P36941) and imaged on Zeiss 780 Confocal microscope.

2.2.17 Statistical analysis

A two-tailed student's t-test was used for determining the statistical significance of IL-17A and IFN-γ secretion, % CCR6-expressing cells, IL-17A and IL-17F transcription and protein expression of OASL and ATF3 at 72h of culture from three to five independent cultures. Statistical analysis of the mass spectrometry data is described in the respective methods section.

2.2.18 Antibodies and other reagents

The following are the antibodies used in the study – SATB1 (Abcam, Cat no. ab109122), GAPDH (Hytest, Cat no. 5G4 MAB 6C5), β-Actin (Sigma, Cat no. A5441), STAT3 (Cell Signaling, Cat no. D3Z2G), BASP1 (Santa Cruz, sc-66994), JUNB (Santa Cruz, Cat no. sc-8051), LMNA (Santa Cruz, Cat no. sc-7292), IRF7 (Abcam, Cat no. ab70069), SMAD3 (Cell Signaling, Cat no. C67H9), STAT4 (Cell Signaling, Cat no. C46B10), ETS1 (Santa Cruz, Cat no. sc-112X), OASL (Abcam, Cat no. ab38325), DOK1 (Santa Cruz, Cat no. sc-6934), ATF3 (Santa Cruz, Cat no. sc-22798), CTNNA1 (Santa Cruz, Cat no. sc-47753), SIRT1 (Cell Signaling (D1D7), Cat no. 2496), FAS (Santacruz Biotech., Cat no. sc-715), VIM (Abcam, Cat no. ab71144), RORC

(eBioscience, Cat no.14-6988-82), BATF (Cell signaling Tech., Cat no. 8638S), CD44 (Cell Signaling, Cat no.37259) and PE-CCR6 (BD biosciences, Cat no. 559562). DOK1 and OASL antibodies were as primary antibodies for FACS staining. Respective A647 labelled antibodies from invitro were used for secondary antibody incubations (A647 goat anti-mouse Life Tech, Cat no. A21235; A647 goat anti-rabbit Life Tech, Cat no. A21245).

2.3 Results

2.3.1 Quantitative proteomic analysis of Th17 cells during early stages of polarization

We used Shotgun label-free (LFQ) proteomics to investigate quantitative changes in protein signatures of human Th17 cells in comparison with activated T-cells. Fig 2.3.1A illustrates the detailed workflow for the proteomics study. Naïve CD4 cells were freshly isolated from umbilical cord blood samples of five different donors. Isolated cells were either TCR-activated using CD3/CD28 antibodies (Th0) or cultured in presence of the Th17-polarizing cytokines (IL-6, IL-1β, TGF-β) in combination with TCR crosslinking. Polarization was confirmed by analysing expression of important Th17-lineage markers including CCR6 and IL-17 cytokine at 72h of differentiation (Fig 2.3.1B&C). In order to disregard any possibility of the differentiated cells bearing an IFN-γ-expressing pathogenic Th17 phenotype, we estimated levels of secreted IFN-γ in 72h polarized cells and found it to be comparable to Th0 (Figure 2.3.1D). For proteomic characterization, cells were harvested at 24 and 72h of culture and samples were prepared using FASP (Filter-assisted sample preparation), following which they were analysed by Liquid Chromatography-Tandem MS (LC-MS/MS) in triplicates.

Altogether, our study identified more than 5,600 proteins using Label-free mass-spectrometry. Samples within the normalized data, clustered well on the basis of biological replicates or cell-lineages, thereby indicating successful normalization, good quality and good reproducibility. Importantly, the proportion of missing values across samples was found to be quite low (<7%).

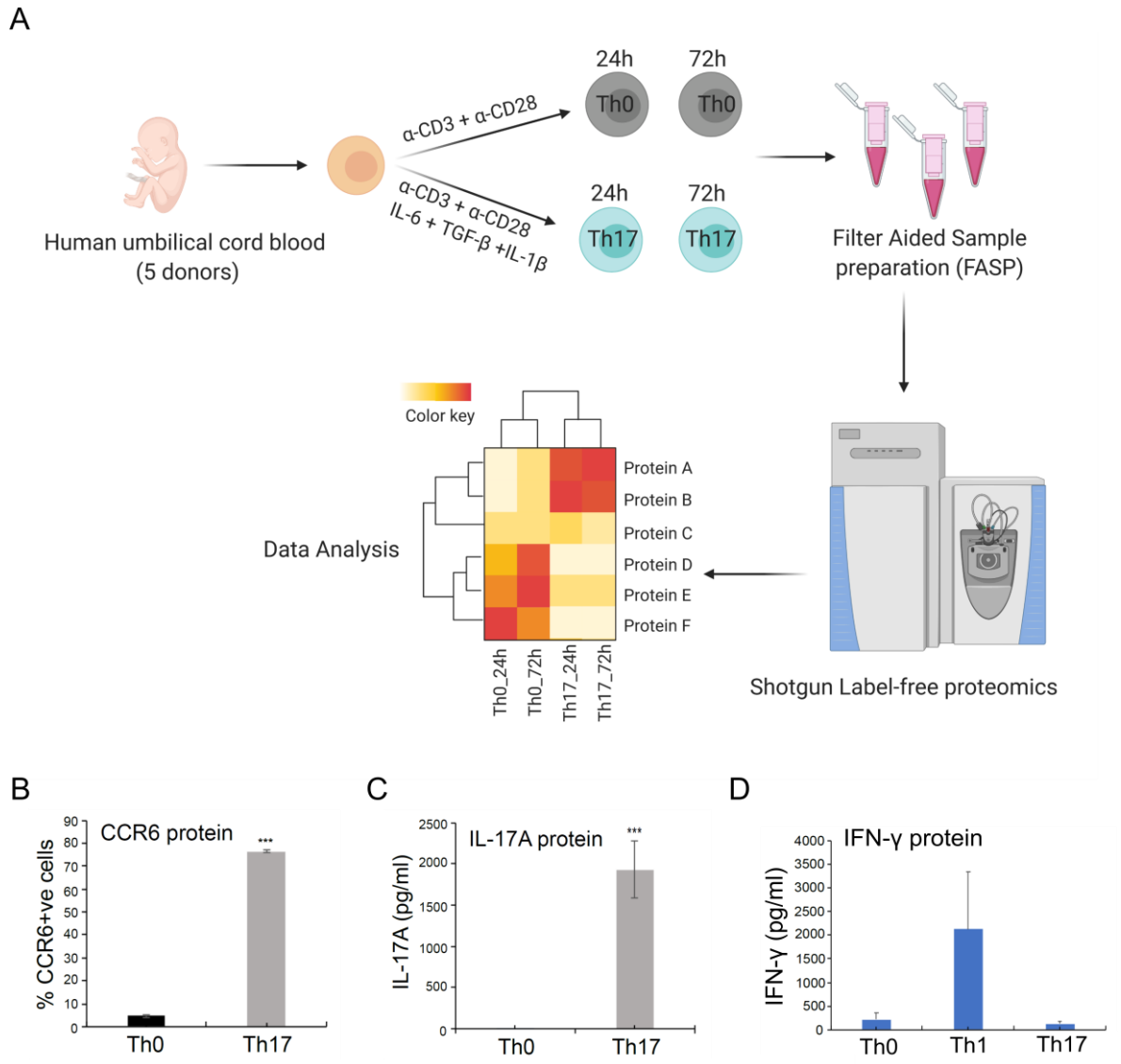

Figure 2.3.1. Workflow of the study and analysis of Th17 specific markers. **A.** Graphical representation of the workflow for the proteomics study **B.** Percentage of CCR6 positive cells was estimated in 72h Th0 and Th17 cultures using Flow cytometry. Data represents values for five individual donors **C&D.** Luminex analysis was used to measure secreted IL-17A and IFN-γ levels in Th0 and Th17 cells at 72h post activation. Data is representative of five individual donors for IL-17A and three individual donors for IFN-γ estimation. Error bars across the mean represent SEM values. Significance was calculated using paired-end Students T-test (***p<0.001).

2.3.2 Identification of Differentially Expressed (DE) proteins between Th0 and Th17 cells having known and unknown lineage-associated function

A quantitative comparison between proteomic identifications of Th0 and Th17 samples at both time points was performed in order to identify the differentially expressed (DE) protein targets. We found 148 and 175 proteins to be differentially regulated between the two lineages at 24h and 72h respectively (FDR of 0.05 was used to define the DE proteins). Among the DE proteins that were detected at only one time point, 60 and 92 proteins were upregulated whereas 56 and 52 proteins were downregulated at 24h and 72h respectively (Fig 2.3.2A). Intriguingly, the overlap between DE proteins detected at the two time points was quite low, thereby suggesting a stage-specific role of these proteins in Th17 lineage-induction (Figure 2.3.2B). Included within the DE list were many proteins with previously-established Th17-associated function including AHR [35], FOSL2 [5], JUNB [36], REL [37], SIRT1 [38], RBPJ [39], CCL20 [40] and TNFSF8 [41]. Additionally, many targets with unexplored roles in Th17 lineage-specification such as ICAM1, KDSR, ATF3, APOD, VIM, PALLD, IL16, LAIR1, KDM6A and UHRF1 also depicted a differential expression profile. We found poly (ADP-ribose) polymerase (PARP) superfamily proteins (PARP9, PARP10, PARP14) and RNA-helicase Dead-box proteins (such as DDX58 and DDX60) to be upregulated in Th17-polarizing conditions. Earlier reports have already established the ability of PARP-14 to modulate phosphorylation of the Th17 master regulator STAT3 and positively drive differentiation in mouse [42]. Besides, many known negative regulators of Th17 lineage (IRF8, ETS1, SMAD3, CASP1, STAT4) were seen to be significantly downregulated in our study [5, 6]. Also, Th17-specific enhanced expression was detected for multiple proteins with known functions in antiviral immunity (IRF7, OAS, OASL, MX1), thereby indicating a potential role of interferon signaling in driving the differentiation process.

For functional annotation of the differentially expressed proteins at both time points, we conducted Gene-ontology (GO) analysis and found significant enrichment of approximately 300 biological processes (FDR<0.05). These included a number of immunologically-relevant processes such as regulation of immune system development, cytokine production, cytokine-mediated signaling pathways, leucocyte cell-cell adhesion, cellular response to type 1 interferon, immune system processes, T-cell activation, and lymphocyte differentiation (Proportions for chosen GO-annotated

processes have been shown in Fig 2.3.2C). As anticipated, a number of immune-related proteins were common to the GO terms ‘response to cytokine’ and ‘immune system process’, including candidates such as CCL20, IRF7, ETS1 and IFIT. Also, most of the targets linked to antiviral immunity or known to be interferon-induced were seen to be included under the ‘response to cytokine’ group. Recent reports have indicated a significant role of lipid signaling and fatty acid biosynthesis in driving Th17 differentiation [43, 44]. Interestingly, our analysis identified many proteins with functional implications in lipid-metabolism to be highly upregulated in Th17 cells (VDR, MSMO1 and CYP51A1). Involvement of VDR has been previously shown to enhance Th17 polarization and MSMO1 and CYP51A1 are known to modulate RORC expression and Th17-pathogenicity [10, 43]. Our study additionally encountered many other lipid-signaling associated molecules whose role in Th17 biology has not been determined. This highlights an important area of investigation in the field.

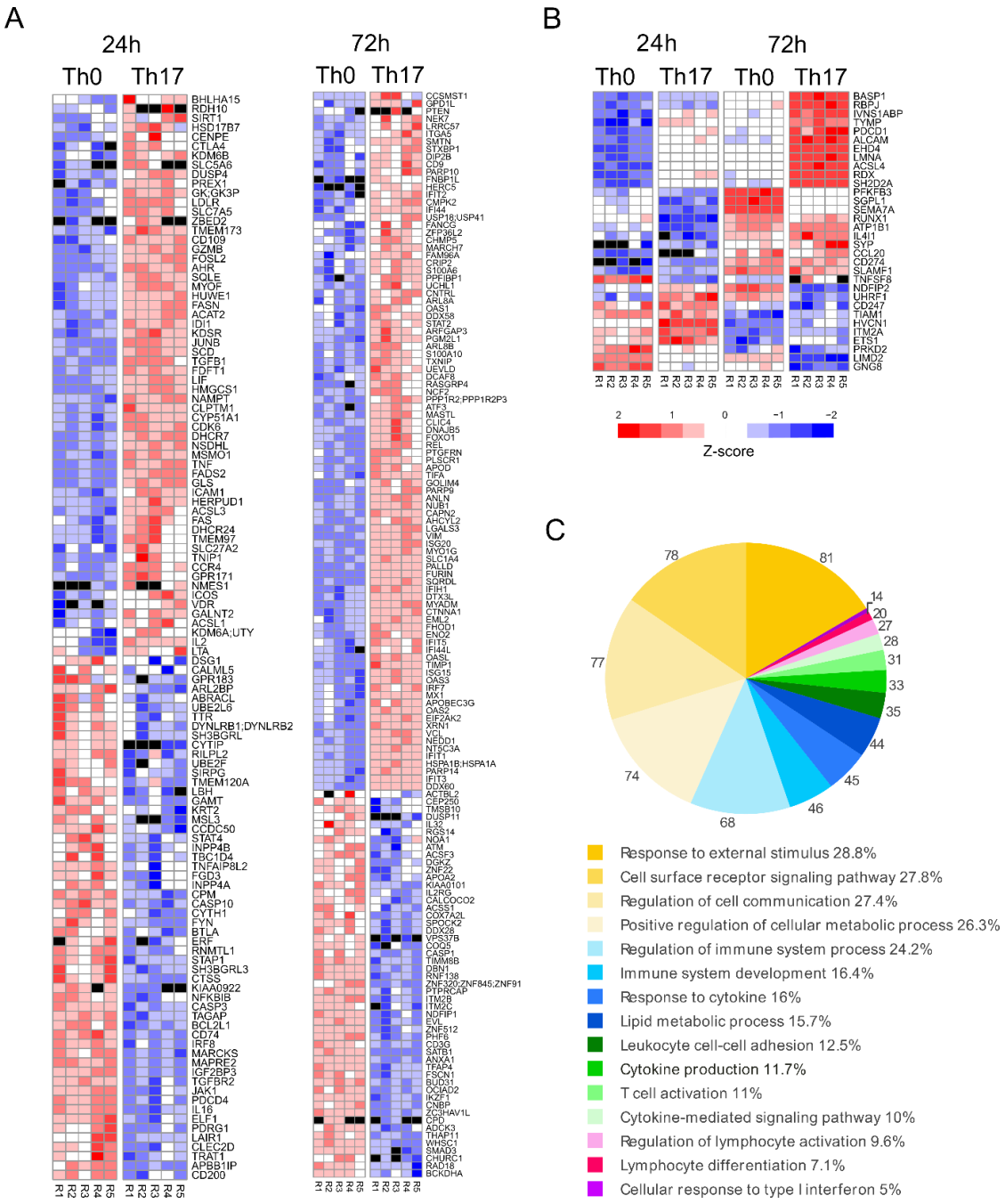

Figure 2.3.2. The MS-identified Differentially Expressed (DE) proteins and associated enrichment of biological processes. A. Heatmaps depict Z-score standardized expression of DE proteins between Th17 and Th0 cells at 24h and 72h post activation. An FDR of 0.05 was used to define the differentially expressed proteins. DE proteins with Log (FC)>0 were considered as upregulated and those with Log (FC)<0 were considered as downregulated. **B.** Common proteins differentially expressed at both times have been plotted in the heatmap for their standardized Z-score values. R1, R2, R3, R4 and R5 represent the scores for the individual biological replicates in panels A and B. The colour scale for the heatmaps represents direction of differential expression. Red indicates upregulated proteins and blue indicates downregulated proteins in Th17 cells (relative to Th0). Black colour represents missing values for the corresponding proteins in the given condition **C.** Gene ontology analysis was performed for enrichment of biological processes for DE proteins detected between Th17 and Th0 at both time points (24h and 72h). Proportions for fifteen of

the chosen GO processes are shown. GO analysis was performed using DAVID and the detected proteome was used as a reference background.

2.3.3 Significant concordance between differentially expressed targets detected in proteomics and transcriptomics

Discrepancies in transcript and protein information at any given time is well established across cell types. In order to evaluate this as a part of our study, we determined the degree of concordance for differential regulation seen between proteomics and RNA-seq data for the same set of samples at 72h. We altogether detected 12,400 transcripts in our RNA-seq analysis. Importantly, transcripts were found corresponding to 95.5% (5,661) of the total proteomic identifications (5,923) in LC-MS. Conversely, we detected only about half (54.3%) of the RNA-seq transcripts in our proteomics study, which is in agreement with some of the recently published reports [24, 25].

Next, we focused on the proteins having corresponding transcripts detected in RNA-seq and searched for DE proteins among them. Of the identifications showing correspondence, we found 172 proteins to be differentially expressed between Th0 and Th17 at 72h. On the other hand, out of the proteins exclusively identified in proteomics, only 3 were seen to be differentially expressed between Th0 and Th17 conditions. A good correlation for all the common identifications between proteomics and transcriptomics was shown based on the Pearson correlation coefficient analysis of the logarithmic fold changes (0.519, p value < 0.001, n = 5,661) (Figure 2.3.3A). An improved correlation was observed for the targets commonly DE at protein and transcript level (0.825, $p < 0.001$, n = 154) whereas the identifications detected as DE exclusively in proteomics displayed a poor correlation, as expected (0.233, $p = 0.35$, n = 18).

Further, to comprehensively determine the overlap of Th17-induced changes between our proteomics and transcriptomics data, we compared the differentially regulated proteins with the corresponding DE transcripts between Th0 and Th17 at 72h. [The term “differentially regulated” refers to proteins detected as DE or those detected only in one condition in MS analysis (9 proteins; detected in either Th0 or Th17)]. Interestingly, more than 90% of the differentially regulated proteins (having associated transcripts) were seen to be altered in a similar fashion at the RNA and protein level. Out of these, 107 proteins and their corresponding mRNAs exhibited consistent

upregulation in Th17 cells which included RBPJ, VIM, FOXO1, ATP1B1, LMNA, RUNX1 and FURIN. Likewise, 45 proteins such as SMAD3, SATB1, ETS1 and IL2RG showed Th17-specific downregulation at both RNA and protein level (chosen targets shown in Fig 2.3.3B). An interesting highlight of this comparison was the discovery of 11 candidates that displayed an opposing profile of differential expression between proteomics and transcriptomics (chosen candidates depicted in Fig 2.3.3C). Such an anti-correlation profile between mRNA and protein levels has also been reported earlier in iTreg cells [25]. Moreover, we found 21 candidates to be differentially expressed exclusively in the proteome and not in RNA-seq analyses (Fig 2.3.3D). This could either be a result of the temporal lag between RNA and protein expression or could be suggestive of post-transcriptional regulation. Hence, our comparative analysis indicated a highly similar profile of differential expression between proteomics and transcriptomics, while suggesting important dissimilarities for some candidates.

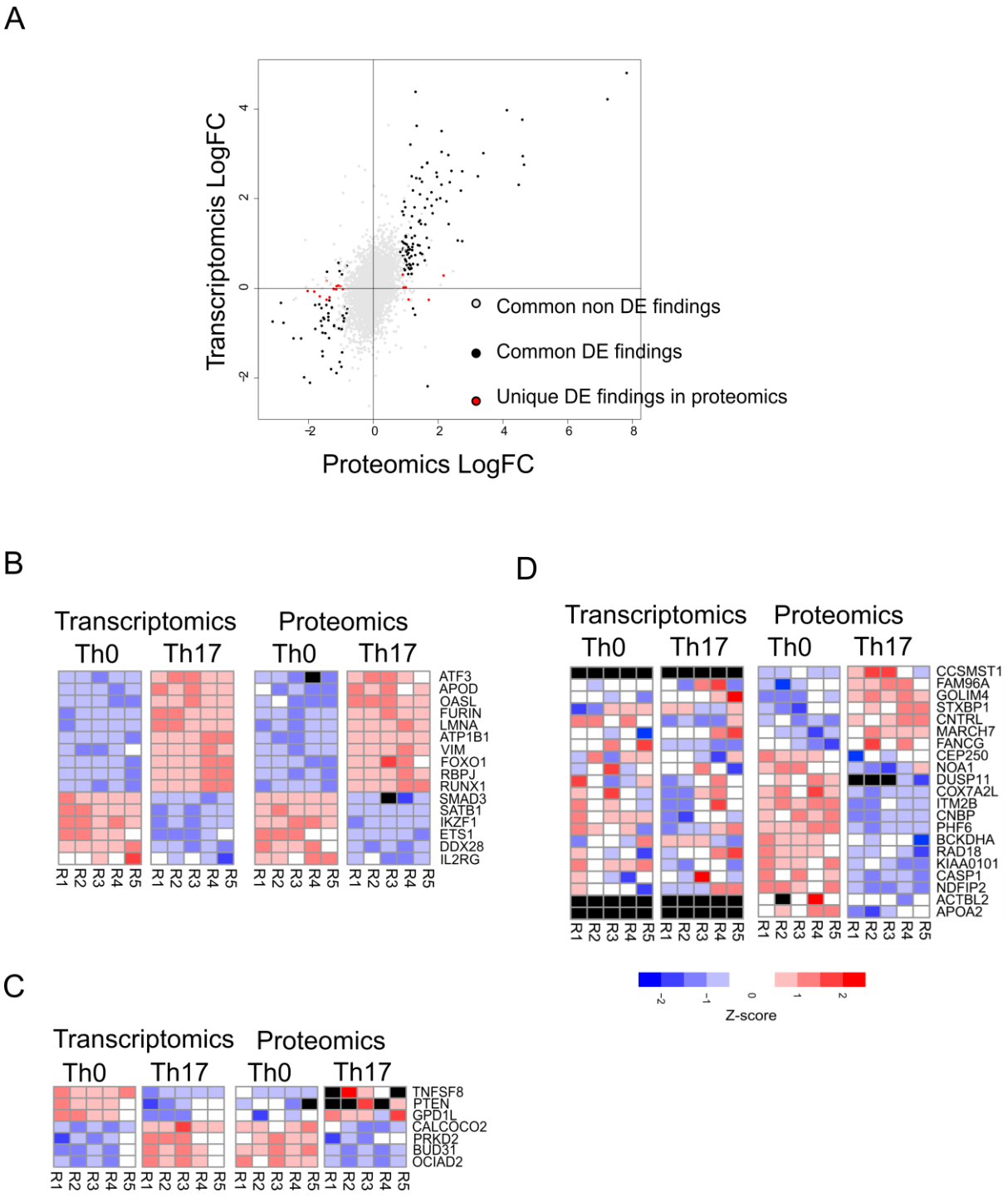

Figure 2.3.3. Comparison of differentially expressed targets between Th0 and Th17 cells in proteomics and transcriptomics data. Proteomics and transcriptomics datasets were compared for their corresponding differentially expressed candidates between Th0 and Th17 cells at 72h of polarization (FDR<0.05). **A.** The logarithmic fold changes (logFC) of all the common detections in proteomic and transcriptomic analyses have been plotted. The targets detected as DE in both proteome and transcriptome are marked in black, whereas those uniquely DE in proteome are marked in red. The Pearson correlation coefficient for the logFCs of the common detections was 0.519 (p-value <0.001, n=5661), for the common DE findings was 0.825 (p<0.001, n=154) and for the DE findings unique to the proteome was 0.233 (p=0.35, n=18) **B.** Standardized z-scores are plotted for some chosen targets showing similar fashion of differential expression at RNA and protein level. **C.**

Chosen DE targets depicting antagonistic fashion of differential expression between proteome and transcriptome are plotted for their standardized z-scores. **D.** Heatmap for the 21 candidates showing differential expression exclusively in the proteome dataset (not DE in transcriptome dataset). R1, R2, R3, R4 and R5 represent the scores for the individual biological replicates in panels B, C and D. The colour scale for the heatmaps represents direction of differential expression. Red indicates upregulated proteins and blue indicates downregulated proteins in Th17 cells (relative to Th0). Black colour represents missing values for the corresponding proteins in the given condition.

2.3.4 Validation of MS-identified DE proteins using western blotting, immunostaining and flow cytometry methods

Mass-spectrometry analysis depends on probability algorithms and hence targets identified by MS-based approaches require experimental validation. To validate the differential expression of chosen DE targets from our study, we employed western blotting, flow cytometry and immunostaining methods. Immunoblots for some of the successful validations are depicted in Fig 2.3.4(I)A&B. Based on these results, BASP1, VIM, CTNNA1, LMNA, ATP1B1, FAS, SIRT1, JUNB and IRF7 showed upregulation in Th17 cells whereas SMAD3, SATB1, STAT4 and ETS1 showed Th17-specific downregulation, which is in agreement with our proteomic findings. We additionally validated upregulated expression of LMNA or Lamin A/C by performing immunostaining in 72h cultured Th0 and Th17 cells (Figure 2.3.4(II)C). We observed that some of the validated targets such as ATP1B1, LMNA and BASP1 have previously been documented for their differential expression at protein level in human Th17 cells [7].

Flow cytometry is a robust technique widely used for quantitation of surface and intracellular protein levels. We utilized a FACS-based approach for successful validation of two additional proteins ATF3 and OASL. Both these targets showed enhanced expression in 72h Th17 cells, consistent with our proteomics analysis (See Fig 2.3.4(II)A&B). Notably, many of the successfully validated DE targets from our study have been previously examined for their ability to influence Th17 differentiation in mouse. The upregulated proteins JUNB, SIRT1, ATF3 and IRF7 have been shown to positively regulate murine Th17 differentiation [45-48] whereas the downregulated targets ETS1 and SMAD3 are well-documented for their ability to inhibit murine Th17 responses [49, 50]. However, functional profiling of these candidates in human Th17 cells has not been performed yet. Likewise, the role of other targets validated for differential expression (SATB1, FAS, ATP1B1, BASP1, VIM, STAT4, LMNA and

CTNNA1) have not been reported in dictating human Th17 differentiation. Addressing the function of these proteins in lineage-specification would be important to understand Th17-specific functional circuits.

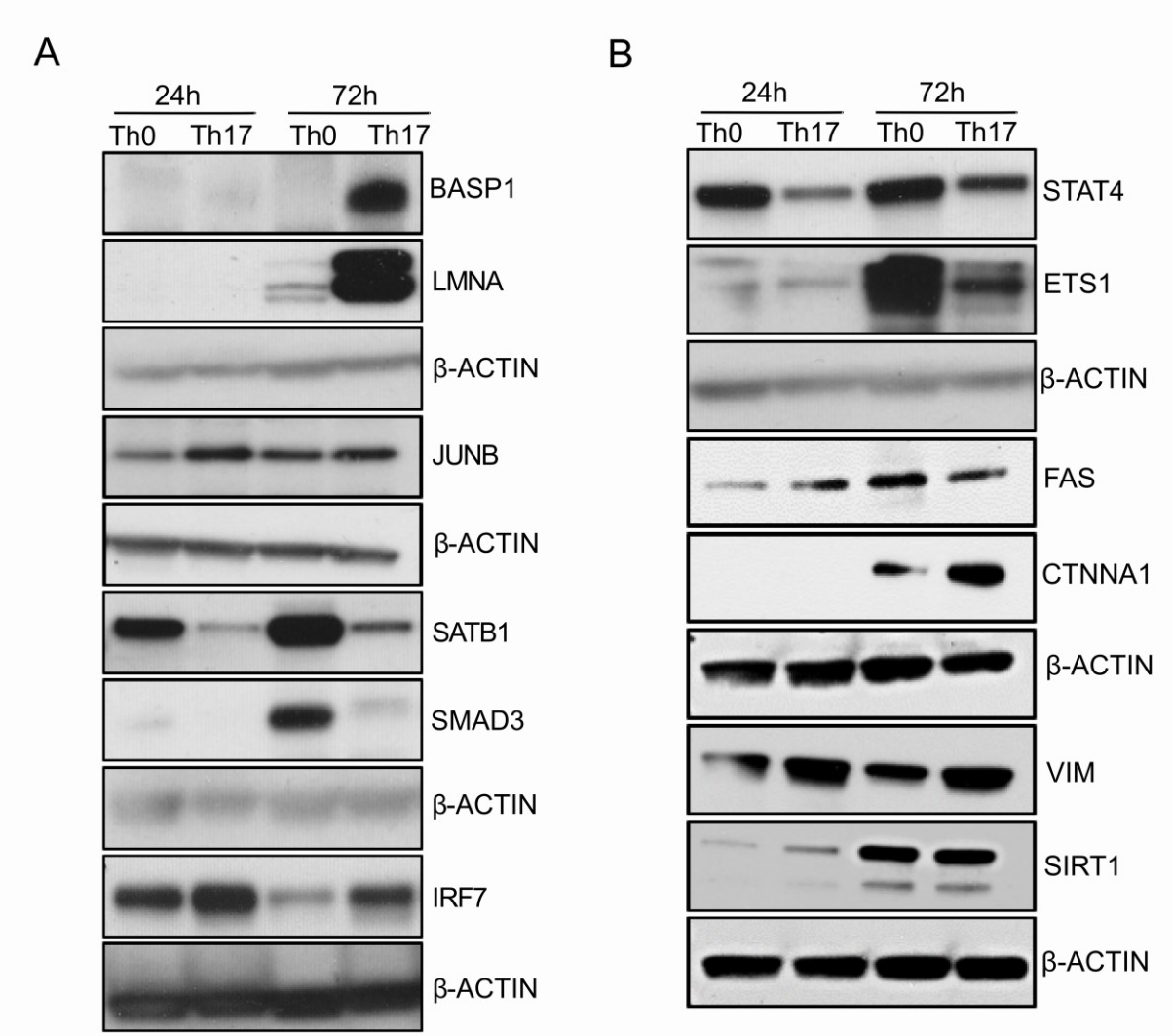

Figure 2.3.4(I). Immunoblotting-based validation of proteins identified as differentially expressed in proteomic analysis. A&B. Western blotting was used to validate differential expression of identified targets with known and unknown Th17-associated function. Protein extracts of Th0 and Th17 cells at 24h and 72h post activation, were used for immunoblotting. Representative blots from three biological replicates are shown.

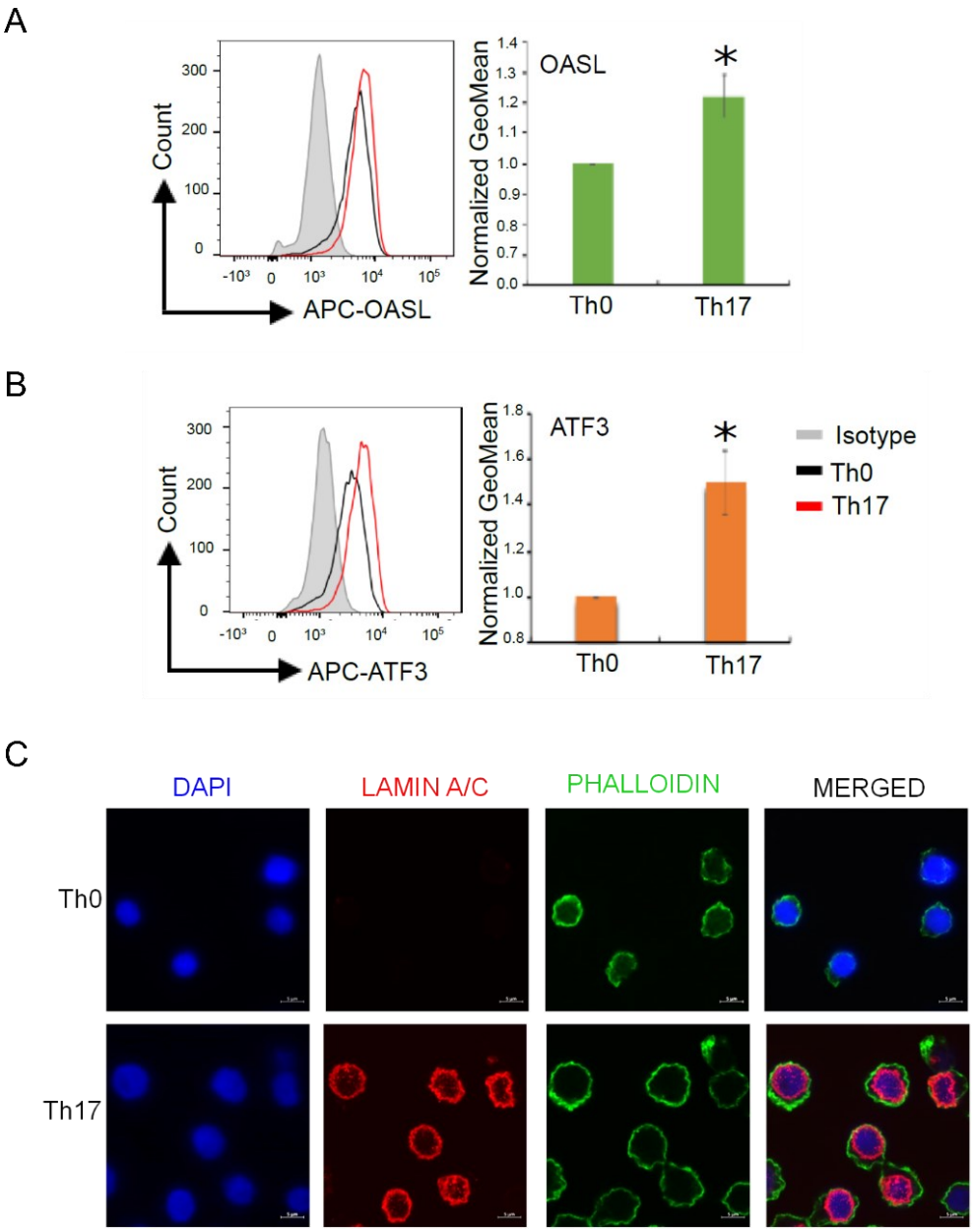

Figure 2.3.4(II). Validation of MS-identified DE proteins using flow-cytometry and immunostaining. A&B. Flow-cytometry was used to validate differential upregulation of OASL and ATF3 in Th17 cells, as identified by proteomics. Representative histogram images show isotype in grey, Th0 expression in black and Th17 expression in red. Normalized Geo Mean values for OASL and ATF3 are plotted for three biological replicates in the adjoining bar graph. Error bars across mean represent SEM values. Significance was calculated using paired-end Students T-test (*p<0.05). **C.** Immunostaining images show differential expression of Lamin A/C in Th0 and Th17 cells at 72h post activation. Nuclear and cytoplasmic regions have been marked with DAPI and Phalloidin respectively. Images were acquired using a confocal microscope (Zeiss 780) and the acquisition settings were maintained constant for Th0 and Th17 samples.

2.3.5 Human and mouse proteomes show significant dissimilarities between their DE protein signatures

Over the years, murine models have been extensively used for in-vivo gene-function studies. However, their ability to recapitulate human conditions has been questioned by multiple reports and hence extrapolating their findings to human disease biology has found limited success. The field of immunological research has repeatedly witnessed a significant number of discrepancies between the two species with respect to gene expression and function [32, 34]. Importantly, the convergent and divergent transcript signatures between early stages of human and mouse Th17 differentiation have already been reported [28]. To explore more into species-specific differences, we wanted to determine the concordance between Th17-specific protein signatures of human and mouse. Recently, the proteome of murine Th17 cells at 72h of polarization was published by Mohammad *et al.* [27]. We performed a detailed proteomic comparison between the published mouse study and our human study using Th0 and Th17 conditions at 72h. Among the 3,731 and 5,917 proteins identified in mouse and human respectively, we were able to detect homologs for almost 85% of the mouse proteins in our human proteome dataset. Also, we used a common FDR threshold of 0.1 (used in Mohammad *et al*., 2018) to uniformly define the differentially expressed proteins for both the datasets.

Interestingly, our comparative analysis discovered a very limited overlap between the DE proteins of the two species, which is in accord with the dissimilarities reported for their corresponding transcriptomes [28]. Among the 758 and 397 differentially regulated candidates detected at 72h in mouse and human respectively, only 51 appeared to be common between the two proteomes (Figure 2.3.5A). More importantly, only 33 of those were found to be regulated in a similar fashion (See Fig 2.3.5B) whereas 18 proteins appeared to show an opposing manner of differential regulation in human and mouse (See Fig 2.3.5C). Within the target group showing concurrent regulation between the two species, we observed 15 proteins being upregulated (including BACH2, VIM, RBPJ, FOXO1) and 18 proteins being downregulated in Th17 cells (including ITM2A, IL2RG, AGK, COX5B, ANXA1) (See Fig 2.3.5B). We then focused on the oppositely regulated target group in order to investigate the species-specific differences. We discovered 8 proteins showing downregulation in mouse but upregulation in human Th17 cells such REL, PRDX4,

CD44 and DDX58. Likewise, an additional 10 candidates were seen depicting downregulation in human but upregulation in mouse Th17 conditions including SATB1, POLR2J, IL16 and LPXN (Chosen candidates shown in Fig 2.3.5C). We used western blotting to successfully validate the antagonistic expression profile shown by SATB1 and CD44 in the two species, at 72h of culture (See figure 2.3.5D). BATF and RORγT expression was used to confirm polarization of murine Th17 cells (Figure 2.3.5E).

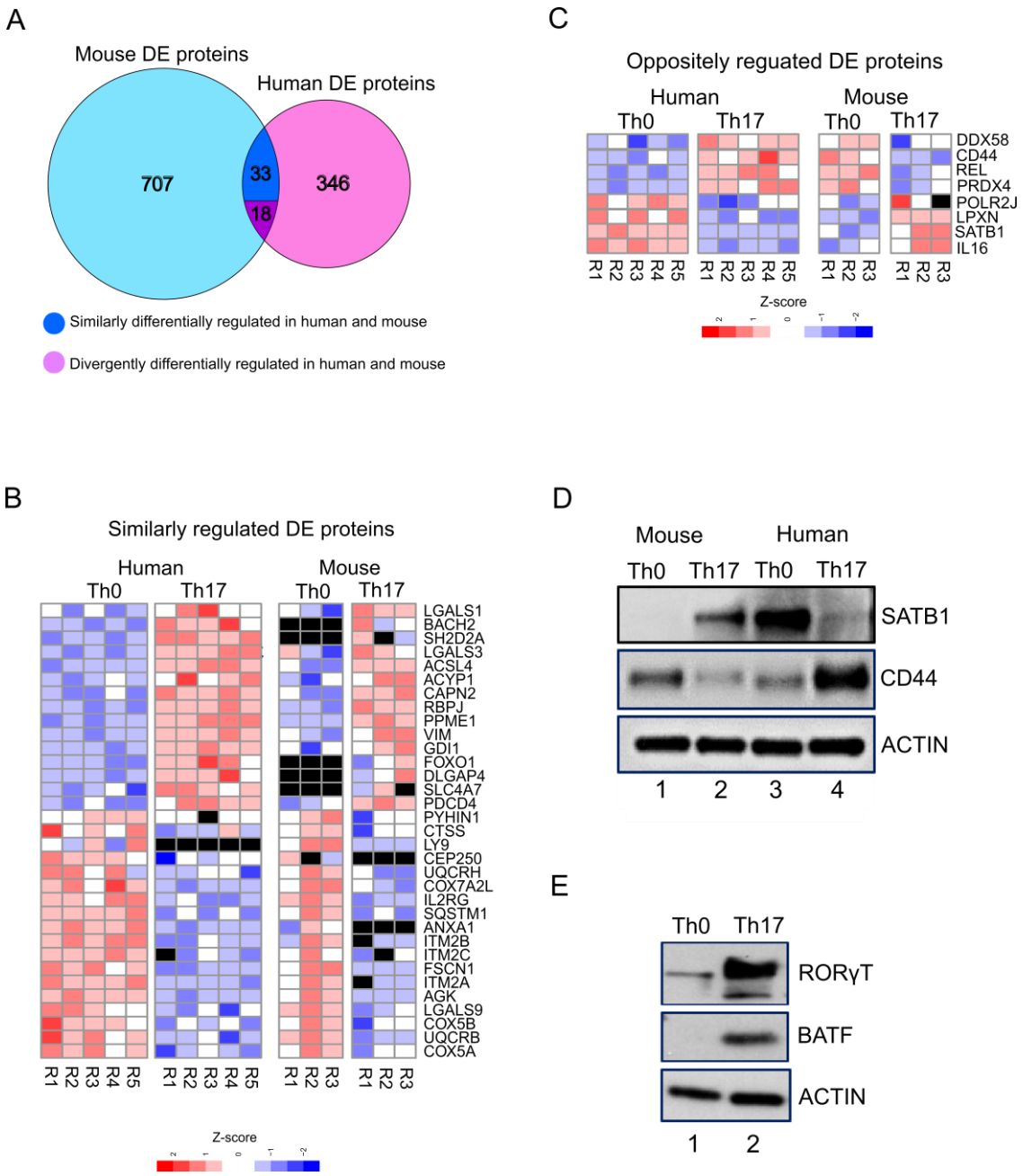

Figure 2.3.5. Comparison of differentially regulated proteins in human and mouse Th17 proteomes. A. Venn diagram shows intersection between differentially regulated targets in human and mouse 72h Th17 proteomes. Differentially regulated proteins are defined as differentially expressed targets between Th17 and Th0 OR proteins detected in only one of these

conditions (lineage-specific). Numbers for common targets which are differentially regulated in similar and opposite fashion in human and mouse have been indicated. **B.** Heatmap depicts standardized z-score expression values for proteins differentially regulated in a similar fashion in mouse and human proteomics data. **C.** Proteins showing differential regulation in an opposing manner in mouse and human proteomics. Heatmap in panel C depicts standardized Z-score expression for some chosen oppositely regulated targets between human and mouse. **D.** Immunoblotting results for validation of the opposing DE pattern of SATB1 and CD44 in human and mouse. Protein lysates of Th0 and Th17 cells at 72h of differentiation were used for immunoblotting. **E.** Immunoblot depicting RORγT and BATF levels in mouse Th0 and Th17 cells at 72h post activation. The immunoblot is representative of three biological replicates.

SATB1 is a chromatin organizer known to be involved in transcriptional regulation of a large number of genes involved in T-cell development, activation and differentiation, in both mouse and human systems [51-66]. FOXP3-mediated repression of SATB1 has been shown to be required for the suppressive ability of human Tregs [60]. SATB1 is also known to be an important regulator of mouse and human Th2 differentiation where it forms transcriptionally active chromatin loops at the Th2 cytokine locus and orchestrates expression of differentiation-specific genes [56, 62, 63]. Notably, recent findings indicate that SATB1 positively regulates murine Th17 differentiation. However, its involvement in human Th17 responses has not been investigated.

CD44 is a glycoprotein known to have significant roles in diverse signaling processes in multiple cell types [67-69]. Its function in various T-helper differentiation subsets has been well-studied. Lack of CD44 in Th1 cells impairs cell survival and impedes generation of cellular memory which is required for anti-viral immunity [68]. CD44 also plays an important role in Treg-mediated immune-suppression by enhancing expression of FOXP3 and suppressive cytokines like TGF-α and IL-10 [70]. Importantly, absence of CD44 function is known to enhance IL-17 secretion and EAE progression, thereby suggesting an inhibitory role in murine Th17 responses [71]. However, a contrasting report shows that CD44 deletion downregulates Th1/Th17 differentiation, enhances Th2 polarization and abrogates clinical scores for EAE [72]. Most of the reports dissecting the function of CD44 and SATB1 in T-cells are based on murine studies. Given their contrasting expression profiles in mouse and human Th0 and Th17 conditions, our study indicates that these proteins might function in a species-specific manner during differentiation.

In the current study, we observed that a majority of the DE proteins in human Th17 cells did not exhibit differential expression in mouse and vice versa. Furthermore, there

were many candidates which were detected in both Th0 and Th17 conditions of one species but were detected only in one of the conditions in the other species. Moreover, analysis of the Top 25 differentially regulated proteins in human and mouse identified only IL2RG as a common candidate. Even among the common DE targets, a significant number of proteins showed opposing patterns of differential regulation between the two species. In a nutshell, our study highlights substantial dissimilarities in the DE protein profiles of human and mouse during initiation of Th17 lineage. Additionally, the comparative analysis reported here presumably underscores the limited success in the field, for modelling human diseases using murine findings.

2.4 Summary of the study

All findings in the above study are a part of the published report by Tripathi, Valikangas, Shetty *et al.* (iScience, 2019) [73]. Our study has utilized the tools of label-free quantitative proteomics to explore the dynamic changes in protein signatures during early stages of Th17 differentiation using umbilical cord blood-derived naïve $CD4^+$ T-cells. The statistically significant list of proteins differentially expressed between Th0 and Th17 cells at 24h and 72h, suggest a time-distinguished, lineage-specific proteome for these conditions. To further confirm the MS-based findings, selected DE proteins with known and unknown Th17-associated functions were successfully validated using flow cytometry, immunostaining and immunoblotting techniques. Moreover, the DE targets identified by Mass-spectrometry were found to be in high concordance with those detected in transcriptomics data. However, our study also discovered some proteomic-specific targets. Interestingly, a systematic comparison of the human Th17 proteome with previously published mouse Th17 proteomic dataset revealed a poor overlap for the DE proteins identified in the two species. Species-specific differences were further highlighted by proteins that portrayed an opposing fashion of differential expression in human and mouse. Experimental validation of some chosen targets (SATB1 and CD44) depicting such an antagonistic profile between the two species further strengthen these findings.

2.5 Discussion

Th17 cells have important implications in development of pro-inflammatory conditions involved in immunoprotection and autoimmunity. Our understanding of Th17-biology and associated immunopathology largely stems from murine studies characterizing expression and function of important regulatory proteins. However, studies using human cells for dissecting differentiation-specific mechanisms are quite limited. So far, our holistic view on human Th17 regulation is based on findings from high-throughput transcriptomic studies [7, 8]. However, molecular changes at the transcript level do not necessarily translate into phenotypic profiles owing to post- transcriptional and post-translational events. Hence, studying protein dynamics is essential.

Our study focused on analysing proteomic profiles of Th0 and Th17 cells at 24 and 72h post-activation, in order to determine the differentially expressed protein signatures during initiation of Th17 polarization. Among our DE findings, we found a large number of known Th17-associated proteins (including CCR4, CTLA-4, ICOS, CCL20, RUNX1, SIRT-1, JUNB and FOSL2). Notably, we also discovered significant differential expression for candidates that might have a potential role in regulating Th17 responses. These included SEMA7A (CD108) and CD109, both of which are GPI-anchored surface glycoproteins. SEMA7A (Semaphorin 7A) is a well-known inhibitor of T-cell function and plays an important role in autoimmune disorders like rheumatoid arthritis, colitis and multiple sclerosis [74-77]. However, its exact influence on Th17 lineage has not been determined. The other candidate CD109 is a reported co-receptor for TGF-β and a negative regulator of signaling associated with it. CD109 couples with Caveolin-1 which triggers receptor-mediated endocytosis of TGF-β receptors and degrades them [78]. It is also known to induce degradation of TGFBR1 by SMAD7/SMURF-dependent mechanisms [79]. Role of TGF-β signaling is known to be pivotal for development of Th17 cells [80, 81]. While it promotes Th17-lineage at lower concentrations, it enhances Treg lineage at higher concentrations. CD109 has also been reported to increase activation of STAT3 in human keratinocytes, which is a master transcription factor for Th17 development [82]. Another related DE protein identified in our study is FURIN, which is a known regulator of CD109-associated functions. Murine studies indicate that lack of FURIN in T lymphocytes causes impaired production of TGF-β1, induces T-cell activation/expansion and also disturbs peripheral tolerance [83]. Given that both FURIN and CD109 showed significant

upregulation in Th17 cells in our study, their potential effect on Th17 development might utilize fine-tuning of the overlapping TGF-β signaling pathway.

Multiple proteins involved in the maintenance of nuclear architecture and chromatin structure were seen to be differentially upregulated (LMNA, BASP1, PARP9, PARP10, PARP14) or downregulated (including SATB1, IRF8, IKZF1, ELF1, CNBP) in Th17 cells from our study. We also noticed that many of these targets have been previously examined for their Th17-specific role in mouse. For instance, IRF-8 has been shown to physically interact with RORγT and suppress IL-17 transcription, thereby functioning as an inhibitor of murine Th17 responses [84]. Additionally, IKZF1 has been shown to positively regulate development and maintenance of Th17 cells in mouse [85]. So far, these proteins have not been reviewed for their role in dictating human Th17 fate. Their differential expression detected in our proteomic study is an important indication for their potential involvement in human Th17 differentiation.

Our experimental validations for the chosen DE targets included multiple proteins (JUNB, SATB1, SMAD3, ETS1, IRF7) with previously characterized roles in murine Th17 regulation. However, their involvement in human Th17 responses is yet to be studied. More importantly, we detected significant differential expression for many proteins with unidentified roles in the field (BASP1, LMNA, IRF7, ATF3, ACSL4, ATP1B1, FHOD1, RDX, PALLD, OASL), which makes them good candidates for follow-up studies. Gene ontology analysis found lipid-metabolic processes to be one of the top biological pathways enriched for our DE findings. Few of the DE targets associated with lipid metabolism (AHR, FASN, ACC1, HSD17B7, FASD2) have been recently reported for their importance in murine Th17 responses [43, 44, 86-88]. It would be interesting to determine the significance of these and other lipid signaling molecules like ACSL4 for their influence on induction of human Th17 lineage.

Multiple members of the AP-1/ATF family (JUNB, FOSL2, ATF3) were included among the differentially-upregulated proteomic targets. One of the most significant ones was FOSL2, which is a known regulator of murine T-cell plasticity and a repressor of Th17 differentiation [5]. Strikingly, its paralog protein FOSL1, has been found to assume an antagonistic role and support the murine Th17 lineage [89]. Since these proteins have been poorly characterized for their human-specific function, it would be exciting to decipher their inter-relatedness in human Th17 cells.

Upon comparing the DE targets detected in our proteomics and transcriptomics data, a significant overlap was seen, which is consistent with other findings in the field. However, we discovered 21 candidates showing a differential expression profile exclusively in proteomics. This indicates the involvement of post-transcriptional and post-translational mechanisms in regulation of these targets. Amongst these, only two proteins ITM2B and COX7A2L were found to be similarly differentially expressed in the published mouse proteomics dataset as well [27]. We noticed that none of the previous reports have explored the proteome-specific DE targets detected in our study for their role in Th17 lineage induction. This could be an important area of investigation.

Transcriptional signatures conserved between human and mouse have enabled follow-up studies on potential therapeutic targets using in-vivo mouse models [28, 31]. However, divergent molecular and functional profiles between the two species have also been widely acknowledged. Important dissimilarities have already been previously highlighted for RNA-level changes in Th17 cells of human and mouse [28]. To explore further into the interspecies differences, we compared the human Th17 proteome from our study with previously published mouse Th17 proteome for their respective DE targets and found remarkable differences. The highlight of this comparison was the discovery of 18 proteins that showed an opposing fashion of differential regulation in human and mouse. While we successfully validated this expression trend for some candidates (SATB1 and CD44), reports on these proteins for their potential involvement in human Th17 differentiation are still lacking. It is important to address if the proteins showing species-specific expression profiles also depict specific-specific roles in development of Th17 lineage. This could prove useful in explaining the molecular basis for why mouse models poorly recapitulate human disease conditions. Our comparative analysis also discovered 33 proteins to be similarly regulated between the two species, which hints at the potential targets for which functional studies can be followed up in mouse.

SATB1 was found to be differentially upregulated in mouse Th17 cells and downregulated in the human counterpart. Previous reports have significantly underscored the importance of SATB1 as a chromatin-regulator in mediating T-helper cell function [56, 58-60, 62, 64, 66, 90]. While it has been found to enhance Th2 cytokine responses [56, 62], its repression has been shown to be necessary for the suppressive ability of regulatory T-cells [60, 64]. However, its role in Treg precursor

populations have been found to be supportive [91]. This suggests that the cytokine signaling events governing lineage establishment in T-cells presumably involve a contextual participation of SATB1. While recent murine studies have found it to be a positive modulator of Th17 fate [5], its involvement in the human counterpart is still unexplored. It would be interesting to determine whether the relative inhibition of SATB1 levels seen in human Th17 cells, translates into a lineage-suppressing role and conveys a species-specific influence. Such findings are quite limited in the field and have been shown only for a handful of factors such as AHR [92,96] and BLIMP1 [93, 94]. More of such inter-species functional studies could certainly help us in defining the exact scope of utilizing mouse models for advancing human immunotherapy.

Summing up our study findings, our analysis reveals the protein level-dynamics during initiation of human Th17 differentiation. With differential expression shown for multiple candidates having unknown roles in Th17 biology, our study serves as an important resource for targets with potential function in this T-helper subset. Our comparative analysis also highlights changes specific to protein signatures in Th17 cells and the important discrepancies in the Th17 molecular profile of human and mouse.

2.6 Limitations of the study

Our inter-species comparison is based on the changes shown at 72h of polarization since it was the only time point available in the published mouse report [27]. This analysis was hence performed assuming that differentiation kinetics for human and mouse is similar, which may not be true. It is equally important to explore other differentiation time points in order to have a comprehensive understanding regarding the differences between the two species.

2.7 Data and software availability

The PRIDE accession number [95] for the mass spectrometry proteomic profiling data presented in this chapter is PXD008973. The RNA-seq data from this study is submitted to the Gene Expression Omnibus (GEO) with identifier GSE118974.

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Chapter 3

Exploring SATB1 as a regulator of human Th17 responses and studying its PTM profile in T-cells

3.1 Introduction

SATB1 (Special AT rich sequence binding protein 1) is a T-cell lineage enriched transcription factor, known for its role in maintaining global chromatin structure and function. It is well-established that SATB1 mediates regulation of a wide variety of genes involved in T-cell responses [1-4] [5-10]. With a propensity to largely bind base unpairing regions (BURs) in the genome, it acts as an adaptor for recruitment of chromatin remodelling proteins on its target gene loci. The genome is tethered to the nuclear matrix via matrix attachment regions or MARs [11]. SATB1 binds to AT-rich sequences within these MAR's and rearranges transcriptionally poised chromatin into distinct loops, thereby enabling regulation of its distal targets [2, 3, 12, 13]. In mouse thymocytes, SATB1 exhibits a peculiar euchromatin-rich 'cage-like structure' which appears to demarcate the active and inactive domains of the chromatin [2, 3]. Interestingly, SATB1 function is known to be highly context-specific where the signaling milieu in the cell dictates if SATB1 acts an activator or repressor for a given set of gene targets [5, 14]. Apart from T-cell responses, SATB1 has been found to be crucial for regulation of haematopoietic stem cell differentiation, embryonic development, dendritic cell maturation and neuronal responses (review, [15]). SATB1 expression has also been found to be dysregulated in many cancers; however its ability to promote or suppress cancerous phenotypes appears to be largely contextual (review, [15-17]).

3.1.2 SATB1 domain structure

Human and mouse SATB1 are highly similar with about 98% homology in their protein sequence [18]. Human SATB1 consists of 763 amino acids and its domain structure comprises of an N-terminal ubiquitin like domain (ULD), a middle Cut domain (CD) and a C-terminal homeo-domain (HD) (Figure 3.1.2). The ULD (70-170 a.a) mediates protein-protein interactions; CD (346-495 a.a) enables DNA binding and the HD (641-702 a.a) dictates specificity of DNA binding [12, 19, 20]. Earlier, region 90-204 a.a was proposed to be a PDZ-like domain that mediates SATB1 homo- and hetero-dimerization[20]. However, Wang et al. in 2011 uncovered the crystal structure for SATB1 N-terminal region and demonstrated that it folds like a Ubiquitin-like domain (ULD) instead [19]. The ULD is known to mediate SATB1 oligomerization which is a critical feature for its DNA binding function. SATB1 is one of the very few nuclear-localizing proteins that possess a ULD for mediating protein-protein interactions. A number of proteins like PML, β-catenin, HDAC1, PCAF, CTBP, etc. have been established as interactors of SATB1 (review, [15]. These interacting partners further recruit additional proteins and form a transcriptional hub around SATB1-regulated genes. Notably, based on its interactome, SATB1 is capable of significantly altering its regulatory potential for underlying gene-targets. For instance, the histone deacetylase HDAC1 and PCAF acetyltransferase enzymes are known to exclusively interact with SATB1 under different signaling conditions and antagonistically regulate the expression of the *IL-2* gene [14].

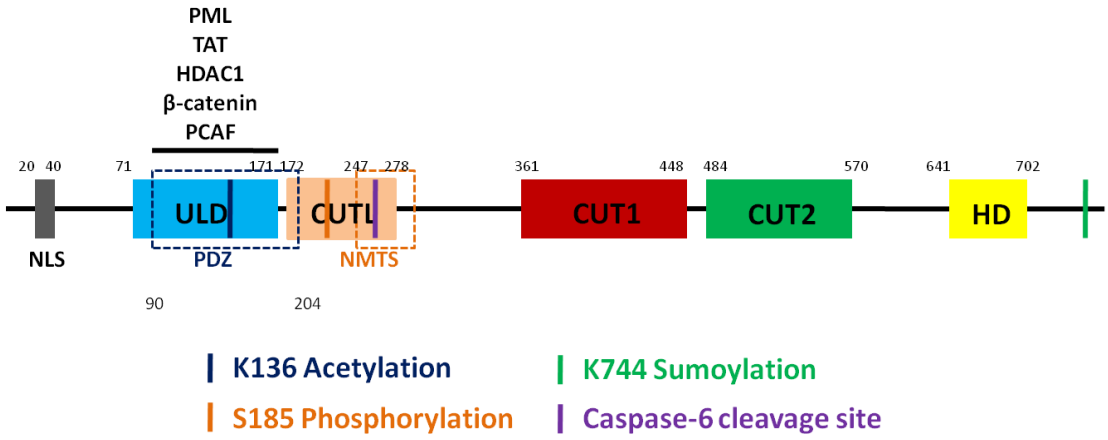


Figure 3.1.2. Detailed domain structure of human SATB1. Human SATB1 bears a N-terminal ULD (70-170) involved in protein-protein interactions, followed by two Cut domains (361-570) involved in DNA binding and a C-terminal homeo-domain that dictates the specificity for DNA binding.

The CUT-like domain (CUTL) harbours the matrix attachment sequence that tethers SATB1 to the nuclear matrix. Indicated with different coloured bars are the various PTMs known for human SATB1.

3.1.3 SATB1 as a regulator of T-cell development and differentiation

SATB1 function is largely attributed to its ULD-mediated protein interaction and Cut domain-mediated DNA binding features. As a T-cell lineage-enriched chromatin regulator, its role in development and effector-functions of T lymphocytes has been well established. T-cells confer immunoprotection against a wide variety of pathogens and also show a pronounced involvement in anti-tumour immunity, allergy, autoimmune inflammation and other immunopathological disorders. Immunocompetency of T-cells develops majorly in the thymus where progenitor populations arriving from the bone marrow undergo a systematic process of maturation to form CD4 and CD8 mature T-cells, each of which play exclusive roles in adaptive immunity. Both these populations have dedicated receptors on their surface which enables them to sense external stimuli and initiate intracellular signaling cascades for induction of effector responses. These receptors include the TCR (T-cell receptor) and other cell-surface proteins which bind to different cytokines and growth factors. It is well-established that CD4 cells or T-helper cells, under the influence of TCR activation and differential cytokine signaling, polarize into Th1, Th2, Th17 and Treg lineage populations. The cytokine profiles characterizing these lineages are quite distinct from each other and perform discrete immunological roles. For e.g. Th2 cells functionally deal with helminthic infections, Th17 cells confer protection against extracellular bacteria and fungi whereas Tregs participate in dampening exaggerated immune responses. Such specialized effector functions are governed by a complex network of lineage-specific transcriptional proteins. SATB1 is one of the leading players regulating such networks in T-cells.

SATB1 has been shown to dictate the expression of a wide-variety of T-cell-relevant genes including BCL-2, IL-10, IL-13, IL-2R, IL-2, and IL-4 [1, 5, 6, 8, 14]. Physiological significance of its function in T lymphocyte responses has been established in cell types as early as hematopoietic precursors, where progenitors with elevated levels of SATB1 are seen to differentiate into lymphoid lineage cells [21]. Also, its gene regulatory roles in thymic T-cell development and peripheral T-cell differentiation have been well studied. During thymopoiesis in mouse, SATB1 expression is majorly

regulated by TCR signaling. Interestingly, murine thymocytes show a bimodal expression of SATB1, which corresponds to its differential regulation in immature and mature CD4 populations [7]. Importantly, SATB1 null mice have defective thymocyte development with a block at the DP stage of thymopoiesis and die within 3 weeks. Role of SATB1 is particularly crucial at the DP stage, since it regulates the RAG1/RAG2 hub for TCR rearrangement [22]. Even mice with conditional deletion of SATB1 show reduced T-cell numbers, impaired T-cell differentiation and susceptibility to autoimmune conditions [1, 21, 23]. Additionally, its function has also been implicated in apoptotic signaling of early T-cell populations. Considering its role in holding the genome architecture together, caspase 6-dependent cleavage and removal of SATB1 has been found to be essential in enabling chromatin fragmentation during apoptosis [24]. Recently, SATB1 has also been shown to contribute to anti-tumour immunity where it inhibits PD1 signaling upon TCR activation and prevents premature exhaustion of T-cells [25].

Apart from T-cell development and survival, role of SATB1 in T-cell differentiation has been found to be quite intriguing. Whether it acts as a positive or negative regulator of differentiation appears to depend on the T-helper cell subset. For instance, under Th2-polarizing conditions, SATB1 coordinates with WNT signaling to promote expression of GATA3, which primes Th2 lineage commitment [5]. Additionally, it induces a synchronized expression profile of Th2 cytokine genes like IL-4, IL-5 and IL-13 [8]. A recent study also indicated promoter-switching mediated fine-tuning of SATB1 expression in activated versus Th2 differentiated cells, suggesting some functional implications [26]. It is well-known that regulatory T-cells play a crucial role in immune-surveillance and tissue homeostasis, with transcription factor FOXP3 acting as a master regulator of the lineage. Inhibition of SATB1 by FOXP3 or FOXP3-regulated miRNAs has been found to be essential for Treg function (review, [27]). It is also known that de-repression of SATB1 makes regulatory T-cells lose their suppressive ability and assume effector T-cell functions, thereby confirming its role as an inhibitor of Treg responses [9]. Counter-intuitively, in Treg precursor cells, SATB1 has been found to positively regulate lineage-development by activation of Treg-specific super enhancers [28]. These reports thus demonstrate a widely contextual role of SATB1 in regulating T-helper cell phenotypes.

Apart from transcriptional mechanisms, SATB1 function in T-helper cells is also known to be modulated by some key post-translational modifications (PTMs). On this front, cellular milieu has been found to play an important role, where distinguished signaling pathways confer different PTMs on SATB1 protein and alter its regulatory abilities [5, 14, 29]. PTMs like phosphorylation, acetylation and SUMOylation are known to influence the functional diversity of transcriptional regulators in many T-helper cell subsets (review, [30]). Although SATB1 function is extensively studied in developing and differentiated T-cells, its PTM profile has been characterized only in activated T lymphocytes [14, 31]. The next section is focussed on how PTMs influence the role of SATB1 in activation-associated responses.

3.1.4 T-cell activation and role of SATB1 post-translational modifications

T-cell activation plays an important role in survival, proliferation and effector function of T lymphocytes. Activation occurs when TCR/CD3 complexes and other co-stimulatory molecules on the surface of T-cells bind to MHC/peptide complexes on antigen presenting cells (review, [32]). The first events of activation involve stimulation of membrane bound kinases which phosphorylate cytoplasmic tails of different T-cell receptors and create docking sites for important signaling proteins (review, [33, 34]). This is followed by phosphorylation of Phospholipase C (PLC-γ), which catalyses the production of secondary messengers and activates key signaling molecules like Raf and PKC θ (Phosphokinase C). Finally, these proteins initiate a MAPK/NFκB signaling cascade that stimulates nuclear transcription events and induces expression of genes like IL-2 (review, [35, 36]). IL-2 is the primary cytokine secreted by activated T-cells and is known to be crucial for maturation, survival and differentiation of T lymphocytes. Extensive research on TCR signaling has emphasized on how post-translational modifications alter function of cytoplasmic signaling proteins and nuclear transcription factors during activation responses (reviewed in [37], [33, 38]). SATB1 is one such chromatin regulator, whose functional status is fine-tuned by this mechanism.

SATB1 protein sequence contains multiple serine, threonine, lysine and arginine residues, which makes it a great target for many PTMs. Particularly, phosphorylation and acetylation are two modifications which have been proficiently characterized for their ability to influence SATB1 function in T-helper cell activation. In Jurkat T-cells,

human SATB1 function as an IL-2 regulator is shown to be modulated by acetylation at its lysine 136 or phosphorylation at its serine 185 residue [14]. Depending on the status of cellular signaling, the two PTMs occur in a mutually exclusive fashion and have contrasting effects on SATB1 function. Upon activation of T-cells, phosphokinase C (PKC) levels rise and SATB1 is phosphorylated. Phospho-SATB1 shows increased DNA-binding and represses IL-2 expression by recruiting histone deacetylase-1 (HDAC1) to its gene locus. Conversely, when PKC levels fall, SATB1 is dephosphorylated and loses its DNA-binding ability. This further allows an acetyl-transferase PCAF to acetylate SATB1 and recruit transcriptional co-activators to the IL-2 locus, thereby inducing its expression [14]. Acetylation also governs interaction of SATB1 with CtBP1, which in turn-modulates SATB1-mediated regulation of IL-2 under Wnt signaling conditions in mouse T-cells [29].

Apart from the above mentioned PTMs, SATB1 function has also been shown to be regulated by SUMOylation. SUMOylated SATB1 is directed to PML bodies where the protein undergoes caspase-6 dependent cleavage, thereby limiting its transcriptional ability [39]. These reports thus emphasize that a single residue post-translational modification can act as a molecular switch for altering its function. Hence, a comprehensive analysis of its PTM profile would largely help in dissecting contextual roles of SATB1. Recently, a mass-spectrometry-based study using a combination of isomethionine methyl-SILAC and antibody-mediated peptide enrichment reported the global profile of protein arginine-methylation in Jurkat and primary human T-cells [31]. Interestingly, SATB1 was found to be one of the proteins with a novel methylation status, being arginine methylated at the R42 residue. Apart from this report, many studies conducted in the past decade have revealed that MS based analysis could serve as an efficient tool for comprehensive identification of protein post-translational modifications. Since PTMs have been shown to significantly modulate SATB1 function as a T-cell regulator, it would be worthwhile to employ this approach for studying some of its novel modifications in activation and differentiation subsets.

On a different note, although the role of SATB1 in T-cell activation and Th2/Treg lineage establishment has been well-studied, its involvement in Th17 responses has only been merely indicated. In the next section, I will discuss the unexplored area of SATB1 function in mouse and human Th17 lineage development.

3.1.5 SATB1 as a regulator of T-helper 17 responses

Th17 cells are pro-inflammatory players that combat extracellular bacteria and fungi by inducing tissue inflammation. However, under certain cytokine conditions, they portray a pathogenic phenotype and launch exacerbated inflammatory responses, causing host-tissue damage and autoimmunity [40, 41]. Transcriptional networks steering Th17 responses have been quite well-investigated using mouse models [42, 43]. In 2012, an integrative network analysis of murine Th17 cells demonstrated a significant involvement of SATB1-mediated transcription. The study indicated that loss of SATB1 impairs expression of key Th17 genes including *Il-22, Il-17a, Il-17f, Il21, Il1R1* and *Ccl20* [42]. Besides, SATB1 has also been found to promote expression of Th17-associated genes while inhibiting Th1-lineage markers in cutaneous human T-cell lymphomas [44]. Noteworthily, a recent study demonstrated its differential involvement in pathogenic and non-pathogenic Th17 signaling in mouse. Their findings indicate that SATB1 is required for pathogenicity of encephalitogenic T-cells but has no significant role in the non-pathogenic counterpart [10].

Regardless of multiple murine studies, involvement of SATB1 in Th17 responses of humans has still not been investigated. MS-based analysis of mouse and human Th17 proteome interestingly depicts an opposing profile of SATB1 regulation in the two species ([45], Results 2.3.5). SATB1 levels are elevated in mouse but downregulated in human Th17 cells, as compared to their respective activation controls. In spite of extensive homology in mouse and human SATB1 protein sequence, such discrepancies in the regulation of its expression could be indicative of a species-specific role in Th17 responses. Since the two systems have already witnessed multiple contradictions for gene expression and function, determining human specific role of SATB1 in Th17 signaling is essential for us to utilize these findings in immunotherapeutics [46-49].

3.2 Materials and Methods

3.2.1 Human CD4⁺ T-cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from umbilical cord blood of healthy neonates (Turku University Central Hospital, Turku, Finland) using density gradient method (Ficoll-Paque PLUS; GE Healthcare). Naive CD4 cells were further purified from the PBMC pool using Dynal CD4 positive isolation kit (Invitrogen). Purified CD4 cells from individual donors pooled before culturing.

3.2.2 *In vitro* Th17 culture

Isolated $CD4^+$ cells were activated using plate bound α-CD3 (3750 ng/6-well culture plate well; Immunotech) and soluble α-CD28 (1 µg/mL; Immunotech), in a maximum density of 1×10^6 cells/mL of X-vivo 20 serum-free medium (Lonza). Media was supplemented with L-glutamine (2 mM, Sigma-Aldrich), and antibiotics (50 U/mL penicillin and 50 µg/mL streptomycin; Sigma-Aldrich). For Th17 priming, a cytokine cocktail of IL-6 (20 ng/mL; Roche), IL-1β (10 ng/mL, R&D Systems) and TGF-β (10 ng/mL, R&D Systems), in the presence of neutralizing anti-IFNγ (1 µg/mL, R&D Systems) and anti-IL-4 (1 µg/mL, R&D Systems) antibodies was used. For control cells (Th0), CD4+ T-cells were plainly activated with similar amounts of α-CD3 and α-CD28 in the presence of neutralizing antibodies without any cytokines and cultured in parallel. All cell cultures were maintained at 37°C in a humidified atmosphere of 5% (v/v) CO_2/a.

3.2.3 Immunoblot analysis

Cells were harvested and lysed in either RIPA (Pierce, #89901) or Triton-X buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.5% Triton-X-100; 5% glycerol; 1% SDS), supplemented with proteinase (Roche) and phosphate inhibitors (Roche). Lysed samples were sonicated for 7 min under ice cold conditions (Bioruptor UCD-200; Diagenode), followed by centrifugation at 14,000 rpm for 20 min at 4°C. Supernatants were collected and quantified using the DC Protein Assay (Bio-Rad). Samples were boiled with 6x sample loading dye (330 mM Tris-HCl, pH 6.8; 330 mM SDS; 6% β-ME; 170 mM bromophenol blue; 30% glycerol) and loaded on 4–20% gradient SDS-PAGE gels (Biorad). Gel proteins were transferred to nitrocellulose membranes (Bio-Rad), and probed with the antibodies listed in Table 1 in 5% BSA. In some cases, blots were

striped with striping buffer (25 mM Glycine and 1%SDS; pH 2.5) and re-probed successively with different antibodies recognizing proteins with different molecular mass.

3.2.4 IL-17A secretion

IL-17A levels were analysed in supernatants of 72h cultured cells. Milliplex MAP human IL-17A kit (Merck Millipore; HCYTOMAG-60K-01), Bioplex Human IL-17A Cytokine/Chemokine 96-Well Plate Assay (Bio Rad; Cat. no. 171B5014M, 171304090M) or Human IL-17A Duoset ELISA kit (R&D Biosystems DY317-05, DY008) was used for detection. The amount of IL-17A secreted by Th17 cells was normalized with the number of living cells determined based on forward and side scattering in flow cytometric analysis (LSRII flow cytometer; BD Biosciences).

3.2.5 Flow cytometry

Flow cytometry analysis of cell-surface receptor CCR6 detection was performed at 72h post Th17 cell priming. Cells were washed twice with PBS, and staining was performed in FACS staining buffer (0.5% FBS/0.1% Na-azide/PBS) for 20 min at 4°C followed by two rounds of washes with staining buffer. Data was either acquired using LSRII flow cytometer (BD Biosciences) on the same day or cells were fixed with 1% formalin and analysed on the following day. Live cells were gated for analysis based on forward and side scattering.

3.2.6 Quantitative Real-time PCR

Total RNA was isolated using RNeasy kit (Qiagen, Valencia, CA). cDNA was synthesized with Reverse Transcription kit (Applied Biosystems, Foster City, CA) using oligo dT primers according to the manufacturer's instruction. TaqMan primers and probes for IL-17A, IL-17F and SATB1 were designed with Universal Probe Library Assay Design Centre (Roche), in Absolute QPCR ROX Mix (Thermo Scientific). EF1a gene was used as endogenous control. The qPCR runs were analysed using the 7900HT Fast Real-Time PCR System (Applied Biosystems). (Primer sequences have been provided in Appendix I)

3.2.7 siRNA-based silencing of SATB1

Isolated $CD4^+$ T-cells from umbilical cord blood were re-suspended in OptiMEM I cell culture medium (Invitrogen) and nucleofected with SATB1-targeting siRNA (Sigma)

using Amaxa nucleofector II (Lonza). Four million cells were transfected with 5ug of siRNA after which the cells were rested at 37°C for 24h in RPMI 1640 medium (Sigma-Aldrich) supplemented with Pen/Strep, 2 mM L-glutamine and 10% FCS ($2x10^6$ cells/ml). Post resting, cells were activated and cultured under Th17 conditions as described above. (siRNA sequences have been provided in Appendix II)

3.2.8 Statistical analysis

A two-tailed student's t-test was used for determining the statistical significance of IL-17A secretion, % CCR6-expressing cells, IL-17A and IL-17F transcription and protein expression of SATB1 at from three to five independent cultures. Statistical analysis of the mass spectrometry data is described in the respective methods section.

3.2.9 Antibodies used in the study

The following antibodies were used in the study – SATB1 (Abcam, Cat no. ab109122), LSD1 (Diagenode, C15410067), GAPDH (Hytest, Cat no. 5G4 MAB 6C5), β-Actin (Sigma, Cat no. A5441), PE-CCR6 (BD biosciences, Cat no. 559562).

3.2.10 Methods for SATB1 PTM analysis

3.2.10.1 Jurkat T-cell and Primary T-cell culturing for PTM analysis

Jurkat T-cells or freshly isolated naïve CD4 cells from human umbilical cord blood were activated using plate bound α-CD3 (3750 ng/6-well culture plate well; Immunotech) and soluble α-CD28 (1 µg/mL; Immunotech) for 72h. Cells were harvested and lysed according to manufacturer's protocol from Pierce MS-Compatible Magnetic IP Kit (Thermo Fischer, Cat no. 90409).

3.2.10.2 Immunoprecipitation of SATB1 for MS analysis

SATB1 was immunoprecipitated using Pierce MS-Compatible Magnetic IP Kit (Thermo Fischer, Cat no.90409). 72h activated Jurkat or Primary T-cell culture pellets were lysed in appropriate volumes of kit provided Cell-lysis buffer. SATB1 antibody (Abcam, Cat no. ab109122) was pre-incubated with Protein A/G beads to form bead-Ab complexes. Lysates were precleared with beads pre-bound to control IgG (Rb). Pre-cleared lysates were then incubated overnight with beads pre-bound to SATB1 Ab (Abcam, Cat no. ab109122). Immunoprecipitated protein complexes were washed (following manufacturer's protocol) and further eluted with appropriate volume of elution buffer.

3.2.10.3 Proteomics sample preparation

The eluents from SATB1 pull down experiments were denatured with the 8 M urea solution. The denatured proteins were further treated with dithiothreitol (10 mM) at 37ºC for 1 h to reduce the disulfide bridges followed by alkylation using iodoacetamide in dark for 30 mins. The IP samples were then diluted to reduce the urea concentration (less than 1M) and subsequently digested with sequencing grade modified trypsin at 37ºC overnight. The tryptic digested peptides were then desalted using in-house C_{18} stage tips by utilizing Empore C_{18} disks (3M, Cat No 2215). The desalted samples were dried in a SpeedVac and stored at -80ºC prior to LC-MS/MS analysis.

3.2.10.4 LC-MS/MS analysis

The dried peptides were reconstituted in formic acid/acetonitrile mixture and the NanoDrop-1000 UV spectrophotometer was used to measure the peptide amounts. The peptide samples were then analysed by using Easy-nLC 1000 coupled to Q Exactive HF (Thermo Fisher Scientific) mass spectrometer. Briefly, peptides were loaded on pre-column (20 x 0.1 mm i.d) and separated with a 75 µm x 150 mm analytical column. Both columns were packed in house with 5 µm Reprosil C_{18} (DrMaisch GmbH). The peptide mixture was separated with a gradient from 5 to 35% solvent B in 78 mins (Solvent A: 2% ACN in MiliQ and Solvent B: 95% ACN in MiliQ) at a flow rate of 300 nl/min. Tandem mass spectra were acquired in a positive ion mode using high energy collisional dissociation (HCD) setting for top 10 most intense ions (300 -2000 mz). MS1 spectra were acquired in a profile mode at 120,000 resolution with AGC value of 3 X 10^6. The MS2 spectra were acquired at 15,000 resolution in centroid mode with 50,000 AGC values.

3.2.10.5 Data analysis

The MS/MS raw files were searched in Proteome Discoverer (v 2.1) using MASCOT search engine against Swissprot homo sapiens database. The search criteria included specificity for trypsin with two missed cleavages, fixed modification for carbamidomethylation of cysteine and variable modification of methionine oxidation and N-terminal acetylation. Furthermore, acetylation for lysine, methylation for arginine and phosphorylation for serine, threonine and tyrosine were included as variable modifications. A false discovery rate of strict (0.01) and relaxed (0.05) setting for peptide were applied using Percolator node.

3.2.11 Generation of anti-K51me SATB1 antibody

3.2.11.1. Raising the antibody

All conducted procedures were as per the approved guidelines from the ethical committee at the National Toxicology Centre (NTC), Pune. To generate the methylation-specific antibody, K51-me SATB1 peptide (GRGRLGSTGGK^{me}MQGVPLKHSG) was synthesized commercially (Apeptide, China). Antibodies were produced in New Zealand white Rabbits, as per the protocol from Tony Hyman's laboratory, MPI-CBG with modification as below (https://hymanlab.mpi-cbg.de/hyman_lab/general/). A suitable amount of the peptide was conjugated with KLH using glutaraldehyde followed by subsequent dialysis (to remove glutaraldehyde). Conjugated peptides were mixed with Freund's complete adjuvant (Sigma Aldrich) for the first immunization. Rabbits were intradermally immunized. Further, after every 21 days, rabbits were immunized using peptides mixed with Freund's incomplete adjuvant until sufficient titre for the antibody was obtained.

3.2.11.2. Purifying the antibody

The antisera obtained was purified by employing a dual round of 'affinity column purification'. To begin with, two separate purification columns were prepared by conjugating 'SulfoLink-coupling resin' to the unmethylated or K51-methylated SATB1 peptides, as per manufacturer's instructions (Thermo). Antiserum was first passed through the unmethylated column and the flow-through devoid of peptide-backbone antibody was further introduced onto the methylated column. The bound methylation-specific antibody was finally eluted and stored in 50% glycerol solution at -20^{o}C.

3.2.12 Dot-blot analysis

200ng of synthetic peptides for unmethylated and K51-methylated SATB1 were spotted, next to each other on nitrocellulose membranes. 2μg BSA was spotted in parallel to be used as negative control. The membrane was allowed to air-dry and non-specific sites were blocked using 5% BSA (in TBST) for 1h at RT. 1:500 dilution of the purified methylation-specific Ab was pre-incubated with unmethylated peptide in order to eliminate any residual backbone specificity. This fraction was then used to probe the membrane (overnight at 4^{o}C). Blots were washed with TBST and further incubated with HRP-conjugated anti-rabbit secondary antibody (Star124P Goat anti Rabbit IgG

(H/L), Cat no. 38220090). Blots were finally developed using Pierce™ ECL Western Blotting Substrate (Thermo Scientific, Cat no. 32106, 32209, 32109)

3.3 Results

3.3.1 Nuclear protein SATB1 is differentially expressed in human Th17 cells

Umbilical cord blood-derived naïve CD4 cells were cultured under activation (Th0) or Th17-polarizing conditions and SATB1 levels were assessed in these cells using qPCR and immunoblotting methods. We discovered significant downregulation of SATB1 RNA and protein levels in Th17 cells (relative to Th0) at both 48 and 72h of culture (Fig 3.3.1A&B). Reduction in SATB1 transcription upon induction of Th17-lineage has also been shown by another study [50], which confirms our findings. Across cell types, SATB1 has been reported to localize within nuclear regions. However, none of the studies on human Th17 cells have reviewed its cellular compartmentalization. In order to address the same, we performed cellular fractionation followed by immunoblotting, using Th0 and Th17 lysates at 24 and 72h post activation. SATB1 was predominantly detected in nuclear fractions irrespective of lineage or time point (Fig 3.3.1C). LSD1 and GAPDH were used as nuclear and cytoplasmic markers respectively, for confirming successful fractionation.

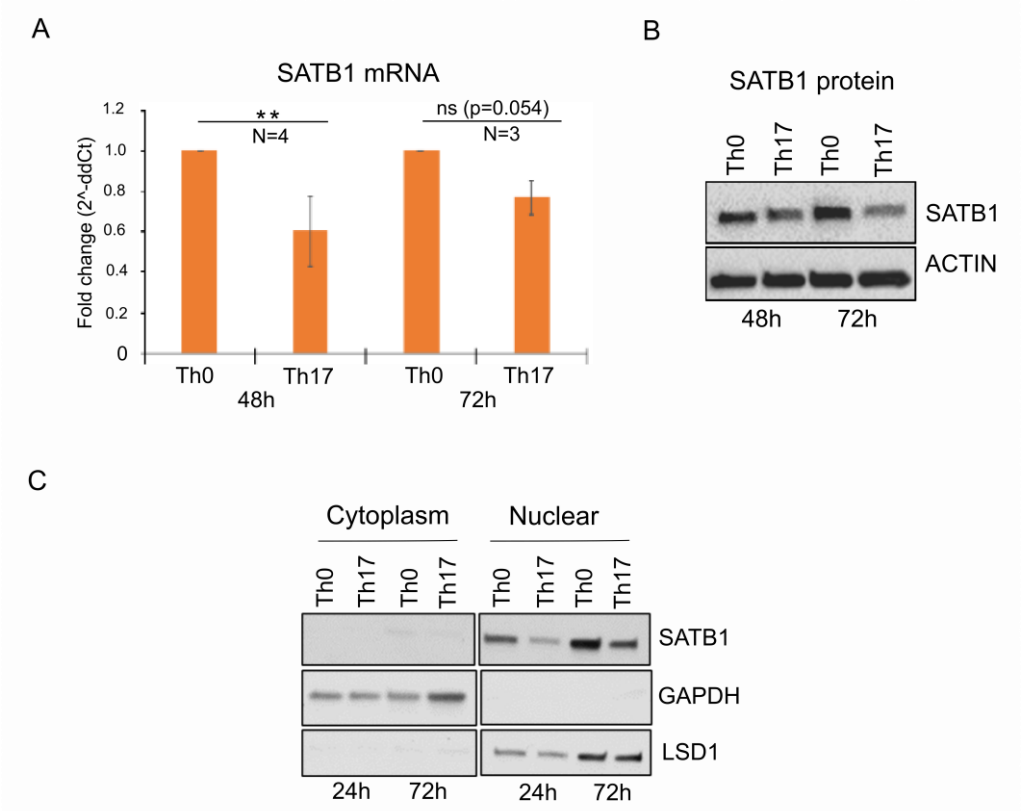


Figure 3.3.1. Differential expression and localization of human SATB1 in Th0 and Th17 cells. A. qRT-PCR analysis was performed to estimate SATB1 mRNA levels in Th0 and Th17 cells at 48h and 72h post activation. Error bars across the mean depict standard error values for the biological replicates (N=4 for 48h and N=3 for 72h). Significance was calculated using paired-end Students T-test (**p<0.01). **B.** Immunoblot shows differential expression of SATB1 protein in Th0 and Th17 cells at 48h and 72h post activation. Blot is representative of three biological replicates **C.** Cellular fractionation was performed for Th0 and Th17 protein lysates at 24h and 72h post activation. Immunoblot depicts expression of SATB1 in nuclear and cytoplasmic fractions of Th0 and Th17 cells at the mentioned time points. GAPDH and LSD1 serve as markers for cytoplasmic and nuclear fractions respectively.

3.3.2 Effect of SATB1 silencing on early differentiating human Th17 cells

Interspecies comparison of proteomic datasets indicated an antagonistic expression profile for SATB1 during initiation of Th17 differentiation. Such discrepancies could indicate a species-specific role. SATB1 has previously been shown to function as a positive regulator of murine Th17 differentiation [42]. However, its involvement in human Th17 signaling has not been reviewed. In order to investigate the same, we used RNAi mediated silencing of SATB1. We treated naïve CD4 cells with SATB1-targeting siRNA and further cultured them under Th17 conditions for 24 and 72h (Workflow in Figure 3.3.2A). qPCR and immunoblotting methods were used to confirm knockdown of SATB1 at transcript (Fig 3.3.2B) and protein level (Fig 3.3.2C&D)

respectively, at 24h of culture. Additionally, percentage of SATB1 protein was estimated for non-targeting and siSATB1 conditions to determine extent of knockdown.

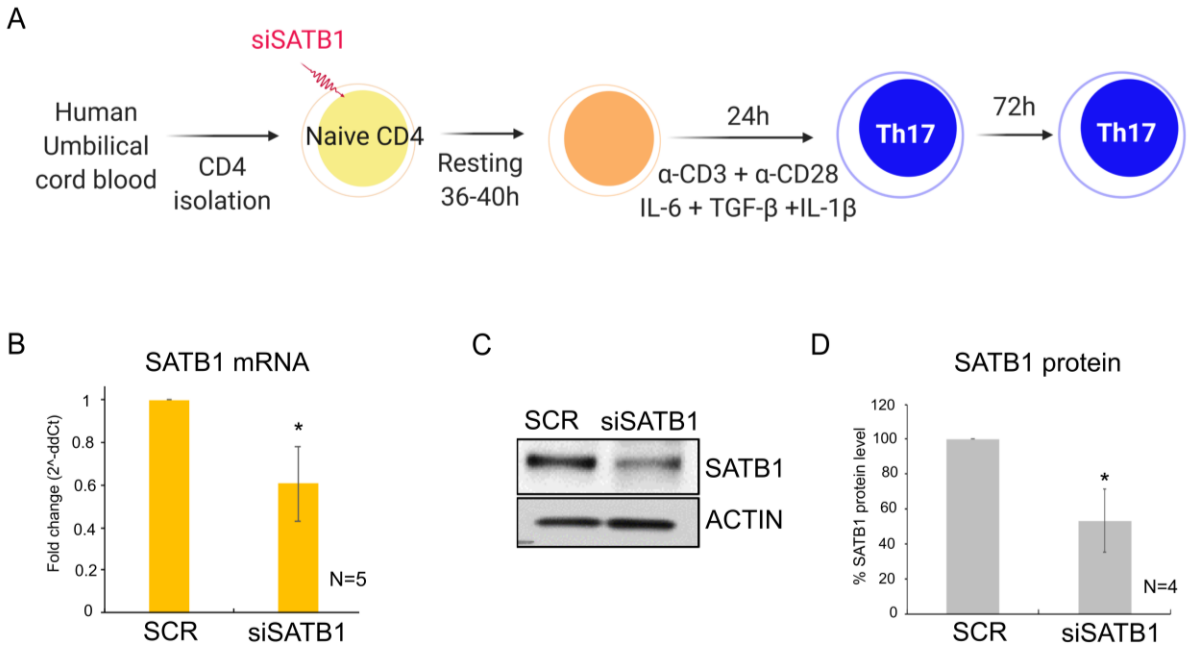


Figure 3.3.2. RNAi-mediated knockdown of SATB1 in human Th17 cells. A. Workflow for siRNA mediated knockdown of SATB1 in Th17 cells **B.** Bar plot shows qRT-PCR-based analysis of SATB1 mRNA levels at 24h of Th17 polarization. SCR or non-targeting siRNA was used as negative control. Fold changes were calculated and normalized to SCR values before plotting. Data represents mean ± SEM for five biological replicates. **C&D.** Immunoblot (Panel B) shows protein levels of SATB1 in siRNA treated Th17 cells at 24h of polarization. SCR or non-targeting siRNA was used as negative control. Immunoblots were quantitated using ImageJ and percentage of SATB1 expression relative to non-targeting control was plotted (Panel C). Data shows mean ± SEM for four biological replicates. Statistical significance for the bar plots in panels A&C was calculated using paired-end Student's t-test (*p<0.05).

3.3.3 SATB1 portrays a species-specific role by negatively regulating Th17 differentiation in human

After optimizing the knockdown, we analysed the effect of SATB1 silencing on Th17 lineage-associated markers at 72h of polarization. Naïve CD4 cells were treated with SATB1 targeting siRNA and further cultured under Th17-polarizing conditions for 72h. Chemokine receptor 6 (CCR6) is a cell-surface protein which shows pronounced upregulation upon induction of Th17 lineage. Upon knockdown of SATB1, we observed a significant increase in percentage of CCR6 expressing cells using flow cytometry analysis (Fig 3.3.3A). Geometric mean values for CCR6 expression were

also seen to be prominently enhanced (Fig 3.3.3B). Additionally, we performed qPCR analysis on SATB1-depleted cells and noticed a substantial upregulation in transcript levels of IL-17A and IL-17F (Fig 3.3.3C). To confirm these findings, IL-17A protein levels were estimated in supernatants of SATB1-silenced Th17 cultures. In agreement with the transcript data, we saw a significant increase in IL-17A secretion upon knockdown of SATB1 (Fig 3.3.3D). Overall, our results suggest that SATB1 negatively regulates human Th17 differentiation, which conflicts its previously reported function in mouse. This proves that in context of Th17 polarization, SATB1 portrays species-specific expression as well as function.

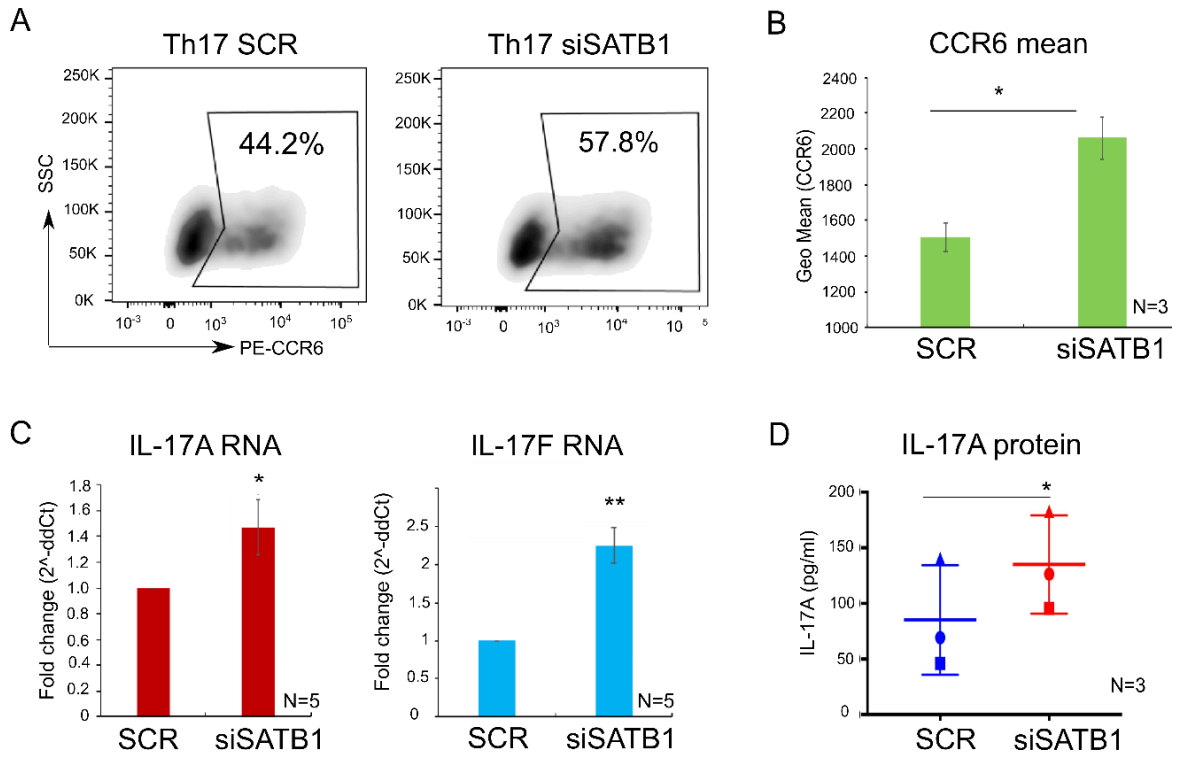


Figure 3.3.3. Loss of SATB1 enhances expression of Th17 specific markers. **A.** SATB1 was silenced in Th17 cells and percentage of CCR6 positive cells were determined at 72h of polarization using flow cytometry. SCR siRNA was used as non-targeting control. Representative Contour plots for three biological replicates have been shown. **B.** Bar plot depicts geometric mean expression values for CCR6 in non-targeting versus SATB1 siRNA treated Th17 cells at 72h of polarization. Data represents mean ± SEM for three biological replicates. Statistical significance was calculated using paired-end Students T-test (*p<0.05) **C.** IL-17A (red) and IL-17F (blue) mRNA levels were estimated using qRT-PCR analysis and fold change values normalized to SCR have been plotted. Data represents mean ± SEM for five biological replicates. Statistical significance was calculated using paired-end Students T-test (*p<0.05, **p<0.01) **D.** Secreted IL-17A levels were estimated using Luminex analysis. Values were normalized to cell count before plotting. Error bars represent standard error values across three biological replicates Significance is calculated using Students T-test (*p<0.05).

3.3.4 Identification of novel post-translational modifications of SATB1 in activated human T-cells

Role of post-translational modifications in governing the regulatory ability of transcription factors has been well-documented [51]. Extensive research for over 20 years in Galande lab has focused on characterizing the molecular function of SATB1 using multiple model systems. These have included multiple experiments pertaining to elucidation of its interacting partners as well as PTMs. In fact, a previously published report from the lab and a landmark discovery in the SATB1 field demonstrated that contextual signaling dictates whether SATB1 is phosphorylated or acetylated, and this in turn significantly alters its transcriptional potential in activated T-cells [6]. Nevertheless, studies characterising other modifications of this chromatin regulator have been quite limited so far.

3.3.4.(I) Methylation of human SATB1

During the course of exploring the influence of SATB1 on chromatin structure and function, immunostaining analysis revealed that SATB1 co-localizes with specific histone marks such as H3K4me3, in mouse thymocytes (Data courtesy: PhD thesis_Ranveer Jayani). This prompted us to hypothesize a potential interaction between SATB1 and protein methyltransferases. In order to investigate this further, a series of co-immunoprecipitation experiments were performed for screening some of the known histone methyltransferases that could bind to SATB1 (Data by Jangid & Jayani, Galande Laboratory). Among the positive interactions were two important enzymes - PRMT7 and SET9, which are known to deliver methyl groups on arginine and lysine residues respectively, of their corresponding substrates. Follow-up experiments further suggested that apart from regulating histone methylation, a close association with these enzymes could possibly result in methylation of SATB1 itself (Data courtesy: PhD thesis by Ranveer Jayani). For preliminarily assessing this, *in vitro* methylation assays were performed and it was observed that the N-terminal PDZ-like domain of SATB1 was indeed methylated by SET9. The *in vitro* methylated product was then analysed by mass-spectrometry, revealing Lysine 51 (K51) as the target residue, which is located amidst the NLS (nuclear-localization signal) and the ULD domains of SATB1.

Methylation as a post-translational modification is known to significantly affect processes such as ribosome biogenesis, mRNA stability, DNA repair and proteasome degradation (reviewed in [52-54]). Although it does not drastically influence the substrate's electrostatic nature, it does alter the local hydrophobicity. Methylation-dependent regulation is also known to hold significance in immune signaling events such as T-cell development, activation, differentiation and cytokine secretion [55, 56]. Immunological relevance of this modification is further underlined by human studies where patients with mutations in methylated residues of STAT proteins and p-65 show impaired IFN signaling and effector responses [57]. Given this scenario, it was suggestive that exploring the methylation status of SATB1 could provide key insights on SATB1-mediated regulation of T-cell function.

A crucial pre-requisite for such characterization was to confirm the presence of the modification *in vivo*. This was particularly important since the *in vitro* results were obtained using only the PDZ-like domain, which cannot mimic the native conformation of the protein. Moreover, the assay conditions used may not really exist in the cellular context. For the purpose of validating the *in vivo* occurrence, an anti-K51 methylated SATB1 Ab was raised by intradermally injecting rabbits with commercially synthesized peptides (Sequence in Fig 3.3.4(I)A). The peptide sequence was first tested for its uniqueness to SATB1 (Since a related homolog SATB2 shows a high sequence similarity with the protein). The anti-serum obtained was further purified using a very stringent protocol (Workflow in Fig 3.3.4(I)B), which ensured that the resulting antibody does not detect any unmethylated protein.

In order to confirm the desired specificity for the antibody, we performed dot-blot analysis using the methylated and unmethylated peptides (utilized for the immunization) (Fig 3.3.4(I)C). We observed that the initial eluted antibody fraction showed a strong signal for K51-methyl SATB1 but also depicted detectable, residual specificity for the unmethylated form. However, pre-blocking the Ab with the unmethylated peptide before usage appeared to completely resolve this issue. Having confirmed this, we next used the antibody for examining methyl-SATB1 levels in whole cell lysates. We performed the initial immunoblot experiments using control and activated mouse thymocytes, since the peptide sequence was conserved in mouse (Fig 3.3.4(I)D). Though the pre-blocked antibody efficiently detected only methylated SATB1 peptide in dot-blot analysis, it appeared to non-specifically bind to multiple

other proteins in the thymocyte lysates. This indicates that the antibody-specificity needs to be significantly improved by trimming the peptide-length for booster injections, for it to be usable in case of immunoblot applications.

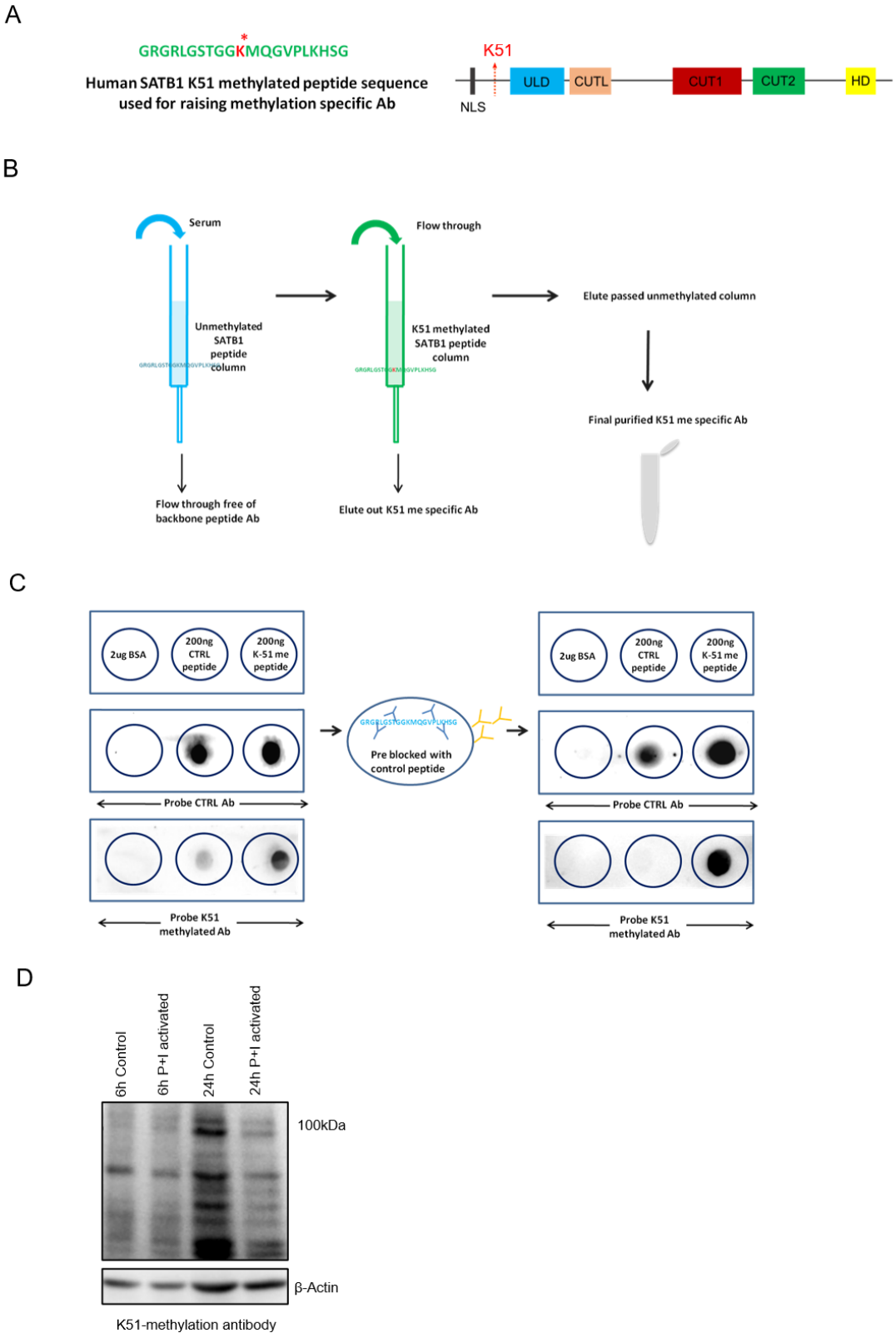


Figure 3.3.4(I). Purification and specificity of anti-K51 methyl SATB1 antibody. **A.** Figure in the left shows the peptide sequence for the commercial K51 methyl SATB1 peptide. Figure on the right demonstrates the domain structure highlighting the K51 target residue **B.** Work flow for 'Double round of antibody purification' used for obtaining anti-K51methyl SATB1. Details in methods section 3.2.11. **C.** Dot blots show specificity of the initially purified anti-K51 Ab and the pre-blocked anti-K51 Ab for unmethylated versus K51-methylated SATB1 peptides. BSA was used as negative control.

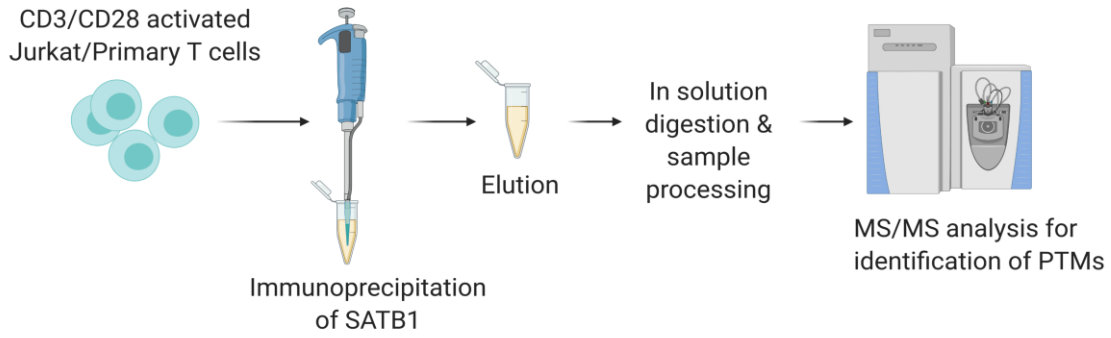
Data is representative of three biological replicates **D.** Immunoblot analysis showing reactivity of anti-K51methyl SATB1 Ab for mouse thymocyte samples activated using PMA+Ionomycin (6h and 24h). Data represents one biological replicate.

3.3.4.(II) LC/MS-MS analysis for PTM profiling of human SATB1

A much elegant and comprehensive strategy for studying *in vivo* protein level identifications like PTMs, is by employing tools of mass-spectrometry. In parallel to an antibody-based approach, we adopted LC/MS-MS methods in order to confirm K51 methylation as well as to determine the other SATB1 PTMs in activated human T-cells. For this study, Jurkat T-cells and naïve CD4 cells derived from umbilical cord blood (UCB) were TCR crosslinked using CD3/CD28 antibodies and cultured for 72h. SATB1 was immunoprecipitated from lysates of the cultured cells, samples were processed and MS analysis was performed in duplicates (Work flow described in Figure 3.3.4(II)A). Upon analysing the individual Mass-spec datasets for Jurkat and Primary T-cells, we discovered a significant number of modifications including methylation, acetylation and phosphorylation. Information regarding position, residue, abundance and sequence-motif, specific to each of the detected PTMs has been shown in figures 3.3.4(II)B and 3.3.4(II)C.

Among the detected PTMs was R42 methylation, which has previously been reported for SATB1 in peripheral blood (PB) primary T-cells, under both naïve and activated conditions [31]. However, our analysis detected this modification only in activation conditions of Jurkat and not cord blood T-cells. This could be a valid discrepancy since dissimilarities between UCB and PB isolated CD4 cells are well-known in the field [58-61]. Interestingly, the previously characterized SATB1 modifications, namely S185-P and K136-Ac were not detected in this study. This could be on account of the mentioned PTMs occurring at a low abundance in our samples. Moreover, the previously published study for these PTMs used PMA-ionomycin treatments to induce T-cell activation as opposed to CD3/CD28 crosslinking (used in our analysis), which might explain the disparity. Likewise, as a key observation of this analysis, we noticed that the *in vitro* discovered K51-methylation was absent among the significantly-detected PTMs. This could indicate a purely *in vitro* or cell-type specific occurrence of this modification. However, follow-up experiments involving analysis of other cell-types like cancer cells would help in further elucidating this contextual incidence.

A



B

Activated Jurkat_Technical Replicate 1

Position	Target	Modification	Classification	Highest Peptide Confidence	Sequence Motif
11	K	Acetyl	Multiple	Low	NEATQGkEHSEMS
38	S	Phospho	Post-translational	High	RLEQNGsPLGRGR
42	R	Methyl	Post-translational	Low	NGSPLGrGRLGST
298	T	Phospho	Post-translational	High	SQPSVRtPLPNLH
309	S	Phospho	Post-translational	High	LHPGLVsTPISPQ
310	T	Phospho	Post-translational	High	HPGLVStPISPQL
313	S	Phospho	Post-translational	High	LVSTPIsPQLVNQ
630	T	Phospho	Post-translational	Low	LPPRQPtVASPAE
633	S	Phospho	Post-translational	Low	RQPTVAsPAESDE
637	S	Phospho	Post-translational	Low	VASPAEsDEENRQ

Activated Jurkat_Technical Replicate 2

Position	Target	Modification	Classification	Highest Peptide Confidence	Sequence Motif
11	K	Acetyl	Multiple	Low	NEATQGkEHSEMS
38	S	Phospho	Post-translational	High	RLEQNGsPLGRGR
42	R	Methyl	Post-translational	Low	NGSPLGrGRLGST
44	R *	Methyl	Post-translational	Low	SPLGRGrLGSTGA
298	T	Phospho	Post-translational	High	SQPSVRtPLPNLH
309	S	Phospho	Post-translational	High	LHPGLVsTPISPQ
310	T	Phospho	Post-translational	High	HPGLVStPISPQL
313	S	Phospho	Post-translational	High	LVSTPIsPQLVNQ
630	T	Phospho	Post-translational	Low	LPPRQPtVASPAE
633	S	Phospho	Post-translational	Middle	RQPTVAsPAESDE
637	S	Phospho	Post-translational	Middle	VASPAEsDEENRQ

C

Activated Primary T cell_Technical Replicate 1

Position	Target	Modification	Classification	Highest Peptide Confidence	Sequence Motif
11	K	Acetyl	Multiple	Low	NEATQGkEHSEMS
298	T	Phospho	Post-translational	High	SQPSVRtPLPNLH
309	S	Phospho	Post-translational	High	LHPGLVsTPISPQ
310	T	Phospho	Post-translational	High	HPGLVStPISPQL
313	S	Phospho	Post-translational	High	LVSTPIsPQLVNQ
630	T	Phospho	Post-translational	Low	LPPRQPtVASPAE
633	S	Phospho	Post-translational	Middle	RQPTVAsPAESDE
637	S	Phospho	Post-translational	High	VASPAEsDEENRQ

Activated Primary T cell_Technical Replicate 2

Position	Target	Modification	Classification	Highest Peptide Confidence	Sequence Motif
11	K	Acetyl	Multiple	Low	NEATQGkEHSEMS
298	T	Phospho	Post-translational	High	SQPSVRtPLPNLH
309	S	Phospho	Post-translational	High	LHPGLVsTPISPQ
310	T	Phospho	Post-translational	High	HPGLVStPISPQL
313	S	Phospho	Post-translational	High	LVSTPIsPQLVNQ
465	S *	Phospho	Post-translational	Low	GPAPLIsTPPSRP
466	T *	Phospho	Post-translational	Low	PAPLIStPPSRPP
630	T	Phospho	Post-translational	Low	LPPRQPtVASPAE
633	S	Phospho	Post-translational	Low	RQPTVAsPAESDE
637	S	Phospho	Post-translational	High	VASPAEsDEENRQ

Figure 3.3.4(II). Novel post-translational modifications of SATB1 identified in activated human T-cells. A. Work flow for MS-based PTM analysis of human SATB1. **B&C.** List of novel post-translational modifications of SATB1 in activated Jurkat T-cells (Panel B) and activated primary T-cells (Panel C) at 72h post initiation of culture. The tabular data provides information on the position, the target residue, the type of modification, the detection abundance and the sequence motif

for each of the detected PTMs. Data is shown for two technical replicates of MS/MS analysis for both Jurkat and Primary T-cells (* in blue indicate PTMs identified only in one of the technical replicates).

We noticed that phosphorylation was the most dominant modification identified in our study. As a part of the downstream analysis, we defined the high-confidence detections as those PTMs which were consistently seen in both technical replicates of either samples. We found 8 and 10 of such modifications respectively, for primary T-cells and Jurkat T-cells. Notably, upon comparing the two groups, we discovered a significant overlap (Figure 3.3.5). Except for R44 methylation and S38 phosphorylation which were exclusively identified in Jurkat cells, all of the other scored PTMs were found to be shared between the two samples (including K11-Ac, T298-P, S309-P, T310-P, S313-P, T630-P, S633-P and S637-P). Strikingly, we discovered two prime regions of SATB1, to depict sequential phosphorylations. The first one between the Cut-like and CUT1 domain which includes T298, S309, T310 & S313 residues and the other between the CUT2 and homeodomains which includes T630, S633 & S637 (Representation in Fig 3.3.5). Additionally, we also discovered K11 acetylation which lies quite close to the NLS.

We observed that most of these PTMs (except for K11-Ac) have been previously reported in cancer cells as well [22]. However, ours is the first study to identify them in activated primary T-cells. Considering their wide-occurrence across cell-types, these modifications might govern more fundamental features such as protein stability.

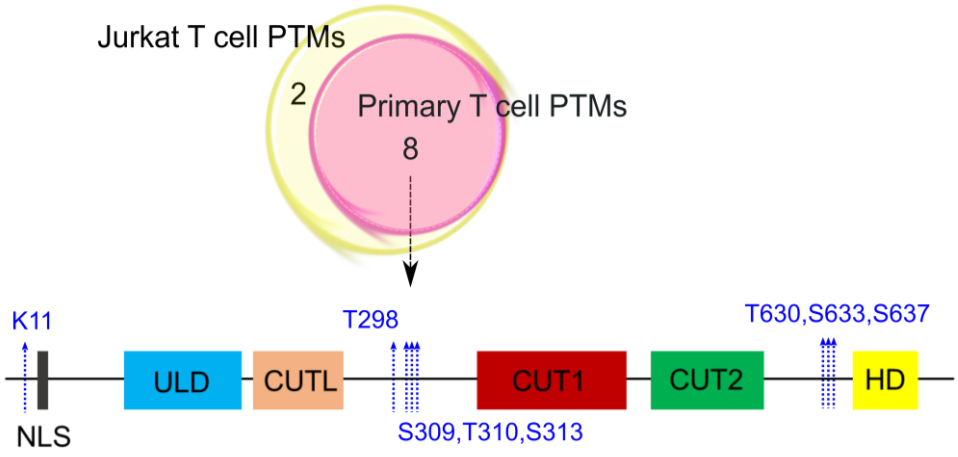


Figure 3.3.5 Commonly occurring post-translational modifications of SATB1 in activated Jurkat and Primary T-cells. Venn diagram shows overlap between SATB1 PTMs identified in 72h activated Jurkat and Primary T-cells. Positions of the shared PTMs are pictorially represented on the adjoining domain structure of human SATB1.

3.4 Summary

We have aimed at studying expression and function of SATB1 during early stages of human Th17 polarization using umbilical cord blood-derived CD4 cells. Complementing its opposing expression pattern in human and mouse Th17 conditions, our study has demonstrated a species-specific role for SATB1 in regulating Th17 responses. Our findings confirmatively highlight SATB1 as a negative regulator of Th17 lineage where we discovered its inhibitory effects on multiple Th17-associated markers including CCR6, IL-17A and IL-17F. Additionally, we employed tools of mass spectrometry to identify novel post-translational modifications of SATB1 in activated T-cells. We found 8 PTMs to be common between activated Jurkat and primary T-cells including K11-Ac, T298-P, S309-P, T310-P, S313-P, T630-P, S633-P and S637-P.

3.5 Discussion

Th17 differentiation in human and mouse has been recently studied for largely divergent proteomic signatures, with multiple candidates showing opposing expression profiles in the two species (Chapter 2, [45]). SATB1 is one such transcription factor and gene regulator which is upregulated in mouse and downregulated in human Th17 cells. Enhanced SATB1 expression in murine Th17 cells has been functionally implicated in promoting the differentiation process [42]. However, its downregulation in the human counterpart had not been functionally reviewed. Our study shows SATB1 as a negative regulator of early human Th17 responses and underscores the antagonistic roles that it assumes in human and mouse. This is an important addition to the field since such species-specific roles have previously been reported only for a handful of factors [62] [63, 64]. On a parallel note, given that SATB1 levels are already downregulated in human Th17 cells, it might be important to authenticate its effects on the lineage using over-expression strategies. Noteworthily, molecular mechanisms regulating SATB1 function have not been studied in either human or mouse. Being a chromatin-organizer that regulates its targets by directly binding to gene-loci [1-4, 8, 22, 24, 29], it would be imperative to

determine its transcriptional targets and ChIP-seq binding sites, in order to holistically investigate its function in Th17 regulation.

Different T-helper subsets are known to be capable of inter-switching lineages, under contextual signaling conditions (reviewed in [65]). Since SATB1 has been shown to modulate Th2 [5] and Treg [9] responses, it would be interesting to examine if altering its levels in Th17 cells affects the expression of genes defining these alternate lineages (IL-4, GATA3, FOXP3). This would elucidate its potential involvement in controlling T-helper cell plasticity. So far, our understanding of molecular networks driving pathogenic roles of Th17 cells largely originates from mouse model experiments, whereas gene-circuits dictating human Th17 pathogenicity are far from being understood. A recent study in mouse showed that conditional deletion of SATB1 abrogates EAE development by compromising IL-17 and IFN-γ production in inflammatory Th17 cells [66]. On similar lines, it would be important to determine if T-cells in human disease conditions such as rheumatoid arthritis, multiple sclerosis and colitis analogously portray SATB1 as a modulator of human Th17 pathogenicity.

Our PTM analysis detected multiple post-translational modifications (methylation, phosphorylation and acetylation), spanning different regions of the SATB1 primary structure, in both Jurkat and Primary T-cells. Interestingly, we identified two prominent sections, where groups of closely situated serine and threonine residues were seen to be phosphorylated. We discovered these to be in the vicinity of the CUT, CUTL and homeodomain, which determine the DNA binding and specificity of the chromatin regulator. This suggests that the phosphorylations in the neighbouring regions could potentially influence the genomic occupancy of SATB1 and alter its regulatory functions.

Modifications existing outside of a protein's functional domains might impact domain-specific roles, on account of protein folding. It would hence be equally essential to review if SATB1 tetramerization or its ULD-mediated protein interactions are influenced by these PTMs [19]. Additionally, previous literature reveals that a distinct cross-talk might exist among modifications which are in close proximity to each other [67]. On these lines, it would be crucial to determine if the phosphorylations identified in this study, control each other's ability to be post-translationally modified.

Our MS-based analysis also identified acetylation of the K11 residue at the N-terminus of SATB1. Considering its proximity to the nuclear localization signal, it would be intuitive to primarily assess its effect on cellular compartmentalization of SATB1. It is well established that PTMs such as acetylation and phosphorylation show a high level of dynamicity and reversibly control protein function (reviewed in [68]). Even on single residues, these modifications could drastically alter cellular processes such as apoptosis, protein translation and gene-transcription (reviewed in [68]). Intriguingly, in many cases, these effects are known to occur in a context-specific manner. For example, the eIF4E-binding protein, an inhibitor of eIF4E elongation factor, is known to be dephosphorylated under conditions of nutrient deprivation or infection. This results in enhanced binding between the two proteins, which further inhibits translation [69]. Another example is of microtube proteins, which are known to be acetylated in response to reactive-oxygen species, which eventually causes perinuclear distribution of the mitochondria and copes with cellular stress [70]. Similar reports also exist for a number of signaling proteins whose activities have been shown to be contextually dictated by a plethora of post-translational modifications [71]. Given these findings, it would be important to decipher if such dynamicity occurs for any of our detected PTMs. Different cytokine and growth-factor signaling conditions would have to be assessed for their influence on the PTM status of SATB1. With this in view, it might also be important to reconsider our *in vitro* characterized K51 methylation, which failed to be detected in activated T-cells. It is plausible that this modification occurs only circumstantially within specific cell types. Therefore, it is necessary to systematically profile multiple cell-types and culturing conditions for the existence of K51 methylation in SATB1.

Residue-specific mutants could be one of the ways to address the many queries regarding the novel PTMs detected in our study and their influence on SATB1 function. Over the years, role of SATB1 in differentiated T-helper cell subsets (Th2, Treg and Th17) has been well-investigated [5, 9, 42, 45, 72]. However, information regarding its post-translational modification status in these subsets, is lacking in the field. Whether SATB1 possesses a lineage-specific PTM profile, where these modifications alter its ability to instruct T-helper cell polarization, requires further investigation.

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Chapter 4

Role of FOSL1 and FOSL2 in human Th17 differentiation

4.1 Introduction

Transcription factor (TF) families are majorly classified on the basis of similarity in sequence and DNA-binding domain structure. Many closely resembling TF families further form a superfamily. It is interesting to note that individual members of these families portray great functional diversity in regulating the same set of biological processes. AP-1 is one such interesting superfamily which consists of multiple 'basic leucine zipper' protein families with DNA binding function. Homo- and hetero-dimers formed between members of the JUN and FOS families majorly constitute for AP-1 transcriptional activity. The JUN family comprises of c-JUN, JUNB and JUND, whereas the FOS family includes c-FOS, FOSB, FOSL1 (FRA-1) and FOSL2 (FRA-2) proteins (review, [1]). Dimerization between AP-1 members occurs via leucine-rich sequences that form α-helical interaction domains [2, 3]. Additionally, different combinations of AP-1 dimers show differential stability as regulatory complexes. Reports have suggested that FOS proteins lack the ability to dimerize with other FOS members. Conversely, JUN can homo- or hetero-dimerize with FOS and JUN proteins and a JUN-FOS heterodimer forms the most stable association [4, 5]. Furthermore, structural studies have demonstrated that during dimerization, the α-helical DNA binding regions of AP-1 proteins juxtapose, which is essential for its genome-binding function [6-8]. (Illustration in Fig 4.1).

It has been well-acknowledged that genes containing consensus AP-1 binding sequences (also known as TPA-responsive elements), act as classical targets of AP-1 proteins (reviews [9-11], [12]). Interestingly, transactivation potential for individual

AP-1 members is quite different. JUNB, JUND, FOSL1 and FOSL2 act as weak transactivators whereas JUN, FOS and FOSB demonstrate a strong potential. In some cases, members with weaker transcriptional potential could act as repressors of AP-1 activity; either by competing for common DNA-binding sequences or by forming inactive dimers with the strong regulators [13, 14]. Thus, AP-1 function as a transcriptional modulator, is also influenced by the nature of the dimer pair (reviews [1, 15, 16]). Recently, members of gene families like ATF/CREB and MAF, which include well-known regulatory proteins like BATF, c-MAF and ATF1 have been found to bear a close resemblance to AP-1 structure and function [17, 18]. Such groups of proteins with highly basic α-helical domains for dimerization and DNA-binding have been termed as BZIP proteins (reviewed in [8],[19]). Hetero-dimerization of these proteins with AP-1 members have been shown to regulate various transcriptional signaling events.

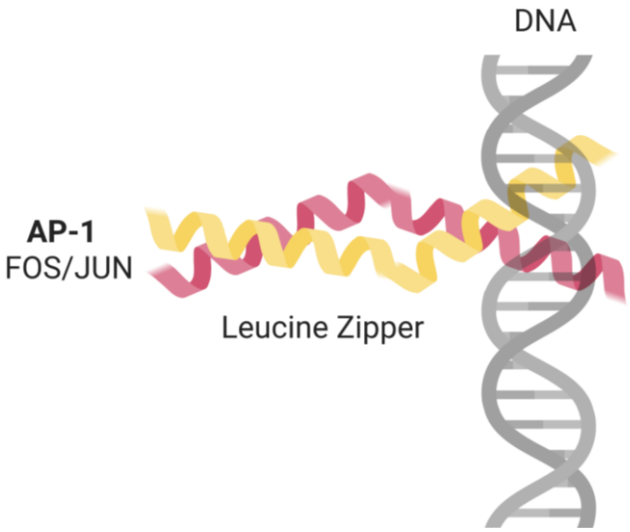


Figure 4.1 Binding of AP-1 dimers to DNA regions. The bZIP domain of AP-1 proteins consists of a 'leucine-zipper' and a 'basic-region'. Leucine-rich stretches mediate dimerization whereas the residues from the basic region interact with the DNA.

4.1.2 Overview of AP-1 function

A plethora of cellular processes including cytokine signaling, oncogenic responses, cell cycle progression, stem cell differentiation, embryonic development, growth factor signaling, apoptotic responses and immune reactions, are known to be influenced by AP-1 function (reviews [9-11]), [20, 21]. Using genetic knockout mice models, various members of the AP-1 family have been assigned essential roles in early development. While functions of C-JUN, JUNB, FOSL1 and FOSL2 are required for normal

embryogenesis, roles of C-FOS, FOSB and JUND are reported to be dispensable (review, [22]). Since absence of different AP-1 members targets development of different tissues, functional diversity within the family is evidently seen. Apart from embryonic development, many cancers report a contextual involvement of AP-1, where it assumes oncogenic or suppressive roles, based on cell-type and genetic background of the tumour [1, 23]. Further, since AP-1 members influence expression of cell-cycle regulators like Cyclins, they play an important role in cellular proliferation [24, 25]. In the recent years, investigation of AP-1 function in immune cell responses has demonstrated an important role in activation, differentiation and apoptosis of leucocyte populations (reviews [9, 10, 12, 22]). However, given the dynamicity of AP-1 function, a thorough understanding of its involvement in immune-regulation has not been achieved yet.

4.1.3 FOSL1 and FOSL2: A general introduction

A co-ordinated network of JUN and FOS proteins is known to influence a wide-range of biological processes. Interestingly, proteins of the FOS family are known paralogs of each other, with FOSL1 and FOSL2 (also known as FOS-like proteins) being the most recently discovered members [26]. FOSL1 protein consists of 271 amino acids whereas FOSL2 has 326 residues. Although these proteins exhibit strong homology in specific regions, the overall sequence only shows about 45% identity between them. The most critical feature distinguishing FOSL1 and FOSL2 from other members is the lack of a transactivation domain, which explains the utmost need of dimerization with a JUN protein to mediate its transcriptional function [27]. Studies on expression kinetics of FOS proteins under conditions of serum treatment demonstrate delayed and prolonged expression of FOSL1 and FOSL2 as compared to c-FOS and FOSB, thus indicating differential induction and stability profiles for these proteins [28]. Apart from being regulated transcriptionally, FOSL1 and FOSL2 function is also known to be altered by post-translational modifications. MAP kinases have been shown to phosphorylate these proteins under conditions of growth-factor and cytokine signaling, which enhances their DNA binding ability and transactivation potential [29-33]. Remarkably, phospho-forms of these proteins are also known block ubiquitin-independent proteasomal degradation events, thereby enhancing their stability [32, 34].

The function of FOS-like proteins has important implications in the regulation of tumour responses, apoptotic signaling, embryonic development, cell cycle progression, cell motility, adipocyte differentiation, immune signaling and associated inflammatory responses (reviews [35-37], [38]). During early stages of development, the deficiency of *Fosl1* or *Fosl2* causes embryonic lethality, thereby underlining its crucial involvement in embryogenesis. Lack of *Fosl1* or *Junb* causes severe defects in placental vascularization [39, 40], whereas *Fosl2* deficiency results in skeletal abnormalities and growth retardation [41]. Further, many cancers like squamous carcinomas, colon cancers, breast cancers and adenocarcinomas, have reported elevated levels of these proteins with a positive correlation with tumour development, progression and metastasis. Specific cancer types have also detected hyperphosphorylated forms of FOSL1 and FOSL2, which appear to have enhanced stability, resulting in protein accumulation and increased invasiveness (review, [42]).

FOSL2 in particular, has been found to play a distinguished role in ECM production and deposition where it induces collagen-synthesis genes like COL1A1, COL1A2, COL5A1 and COL6, in fibroblasts and immune cell populations [43, 44]. Additionally, FOS-like proteins significantly influence inflammatory responses by targeting multiple immune and non-immune cell-types. FOSL2 is seen to promote systemic and tissue-specific inflammation [45-47], whereas FOSL1 appears to contextually regulate these responses [48-51]. An essential role of FOS-like proteins has also been established in immunoprotection and autoimmune signaling reactions. Multiple studies on myeloid and lymphoid cells have demonstrated significant roles of these proteins in macrophages, B cells, NK-T cells and T-cells [9]. Particularly, T-helper cell responses portray distinguished functions of FOSL1 and FOSL2, in coordination with JUN and ATF family members.

4.1.4 FOSL1 and FOSL2 in T cell activation and differentiation

T-helper cell populations have been widely acknowledged for their versatility, based on their ability to mediate a repertoire of immunological functions. Activation and differentiation are two prime events that program a naïve CD4 cell to develop into a functionally specialized effector T cell. T-cell activation is induced via engagement of

the T cell receptor and stimulates expression of the IL-2 cytokine. T cell differentiation, on the other hand, occurs in response to specific cues from the surrounding micro-environment and results in generation of either Th1, Th2, Th17 or Treg subsets; each of which have specialized functions.

Multiple studies in the field have elaborated on the activation and differentiation-specific roles of FOSL1 and FOSL2. T-cell activation responses like IL-2 production have previously been shown to positively correlate with expression of FOS-like proteins, in both human and mouse [52, 53]. Further, studies on primary human T-cells have found FOSL1 to positively regulate IL-2 expression, in association with JUNB. These reports also indicate that TCR signaling in fact, induces FOSL1 and JUNB, which then bind within NFAT-1 complexes on the *IL-2* enhancer and stimulate its transcription [53]. Besides, FOS-like proteins have been reported to regulate migration and cell-cycle phenotypes in T lymphocytes. For e.g., aberrant expression of FOSL2 in association with JUND in adult T cell leukaemia, promotes cell proliferation and expression of the chemokine receptor - CCR4 [54].

The above findings and most other reports in previous years have majorly studied the involvement of FOS-like proteins in T lymphocyte activation and T-cell associated cancers. T cell differentiation subsets however, have been poorly investigated for their function. Until now, only Th2 and Th17 cells have been characterized for regulatory roles of FOS-like proteins. In case of murine Th2 clones, both FOSL1 and FOSL2, in association with JUN and NFAT-1 are known to induce expression of the lineage-specific cytokine IL-4 [55]. As for human CD4 cells under Th2 conditions, though significant levels of FOSL2 have been detected during early stages of polarization, its role in induction and maintenance of the lineage has not been explored [56]. Further, immune-related inflammation caused by macrophages, T lymphocytes and other non-immune cells, have also reported significant involvement of FOS-like proteins (as described in Section 4.3) [45-47] [48-51]. It is well-known that amongst the T cell subsets, Th17 cells lead in orchestrating such inflammatory responses [57]. Using T-cell specific gene-deficient mice, both FOSL1 and FOSL2 have been investigated for their ability to influence Th17 lineage. The next section elaborates on the Th17-specific role of these proteins.

4.1.5 Regulation of Th17 lineage by FOS-like proteins

Exposure of naïve CD4 cells to micro-environments consisting of cytokines like IL-6 and TGF-β, is known to induce Th17 development. Extensive research has demonstrated Th17 cells to be dual-natured. While non-pathogenic Th17 cells confer protection at mucosal barriers, aberrant expression of 'inflammatory mediators' by pathogenic Th17 cells, results in autoimmune-development. It is well-studied that pro-inflammatory cytokines such as IL-17A, IL-17F, IL-21, IL-22, GM-CSF act as molecular effectors of these cells. Furthermore, a dynamic interplay between Th17 and Treg responses dictates the outcome of inflammatory phenotypes.

4.1.5.1 FOSL2 negatively regulates murine Th17 differentiation

A couple of seminal studies in 2012 used integration of transcriptomics and genome-wide occupancy analysis to sketch a functional network of transcriptional regulators governing murine Th17 responses [58, 59]. One of the key findings of these studies was discovery of FOSL2, as an important modulator of T-helper cell plasticity. It was found that *Fosl2*-deficient CD4 cells, primed for Th1, Th2, Th17 or Treg fates, showed dysregulated expression of lineage-specific cytokines. For e.g. - Th1 and Th2 cells atypically expressed IL-17 cytokine, whereas IFN-γ expression was unusually promoted in cells primed for Th17 and Th2 fate. These findings thus demonstrate an essential role of FOSL2 in suppressing expression of alternate-lineage cytokines in differentiated cells. More importantly, FOSL2-deficient Th17 cultures showed enhanced IL-17 levels along with generation of atypical FOXP3+IL17+ cells, thereby indicating it as a negative regulator of IL-17. FOSL2 was also found to inhibit other genes involved in Th17-induction and cytokine expression, such as - *Batf, Ccr6, Il17f* and *Ccl20*. However, it was intriguing to note that factors required for maintenance and survival of Th17 cells were positively regulated by this AP-1 protein (*Il23r, Il12rb1, Il7r, Il21*). Thus, FOSL2 function seems to be more complicated than merely being a repressor of Th17 cytokines. Furthermore, although pro-inflammatory molecules were elevated in absence of FOSL2, development of EAE was observed to be severely compromised. This could be a probable effect of some Treg-like features exhibited by FOXP3-expressing inflammatory T-cells seen in *Fosl2*-deficient mice.

As another interesting finding from these studies, FOSL2 was seen to co-occupy many Th17-specific loci, along with initiation factors such as BATF and IRF4. Since BATF/IRF4 complexes positively regulate murine Th17 responses and contradict

FOSL2 function, the authors imply that a shared genomic occupancy could hint at potential competition between them. However, since these studies have been performed on in-vitro polarized cells which are well-reported to create asynchronous populations, commonly occupied sites may not necessarily indicate 'competitive regulation'. Mechanisms by which these functionally antagonistic proteins co-regulate Th17 responses, regardless of parallel expression profiles, needs to be thoroughly investigated. Moreover, none of the existing reports elaborate on the function of FOSL2 in human Th17 cells.

4.1.5.2 FOSL1 as a positive regulator of murine Th17 lineage

A study reported in 2017 utilized viral-vector based strategies for perturbation of FOSL1 in mouse models. Findings from this study demonstrated a supportive role of FOSL1 in Th17 responses, in a JUNB-STAT3 dependent manner [60]. Interestingly, both FOSL1 and JUNB were shown to be induced by STAT3, following which these proteins associate with each other and co-operatively transcribe Th17 cytokine-gene loci. Further, in regard to development of autoimmunity, overexpression of FOSL1 and JUNB was seen to aggravate collagen-induced arthritis in mice, in a Th17-dependent manner. Strikingly, though embryonic development has found FOSL1 and c-FOS to have overlapping functions [61], their roles in Th17 development have been shown to be conflicting. Aberrant expression of c-FOS and c-JUN appeared to repress IL-17 levels, emphasizing on the contextual role of AP-1 proteins in Th17 responses. An important highlight of this study was the parallel investigation of FOSL1 in human Th17 differentiation. FOSL1 levels were found to be elevated in mononuclear cells isolated either from peripheral blood (PBMCs) or synovial tissue (SFMCs) of RA patients. Notably, in support of the murine findings, perturbation of FOSL1 in CD4 T-cells extracted from healthy PBMCs or diseased SFMCs, indicated a positive role in IL-17 expression. However, human-specific responses reported in this study need to be reviewed on specific grounds.

The authors report the phenotype for FOSL1 perturbation in human T-cells, but do not indulge in exploring molecular mechanisms associated with its function. Transcriptional targets, DNA-binding sites or interacting partners of human FOSL1 have not been investigated in this study. Given the widely reported context-dependent roles of AP-1 proteins, examining the mechanism of action for FOSL1 function is

crucial. Further, this study has employed the use of retroviral and lentiviral strategies for performing gene-silencing and over-expression on human $CD4^+$ T-cells. Such strategies have been previously known to have undesirable effects on immune phenotypes (review, [62, 63]). Virus-mediated transduction requires pre-activation of $CD4^+$ T-cells, which could potentially affect differentiation and associated kinetics for in-vitro cultures. Although it is true that lentiviral vectors illicit minimal inflammation as compared to retroviral ones, reports stating their influence on in-vivo T-cell responses have been well-acknowledged (review, [64]).

Another aspect of this study that needs reconsideration is the use of PBMCs as a source of $CD4^+$ T-cells. Past research in the field has witnessed discrepancies related to Th17-phenotypes owing to the use of peripheral blood as a source of naïve cells. Resultantly, some studies resorted to the use of umbilical cord blood (UCB) [57], which has been found to possess a relatively higher fraction of 'truly-naïve' cell populations [57, 65]. Other differences in cord blood and peripheral blood immune cells have also been highlighted before [66-68]. Hence, validating these studies using non-viral methods of gene perturbation in cord blood-isolated CD4 cells is required for a comprehensive investigation of FOSL1 function in human Th17 differentiation.

Murine reports on FOSL1 and FOSL2 portray opposing roles of these paralog proteins in regulation of Th17 lineage. Mouse versus human discrepancies in gene function have been well-established and hence investigating human-specific role of FOS-like proteins is crucial. Use of high-throughput analysis for determining transcriptional targets, genome-wide binding sites and interacting partners could help in deciphering the mechanism of action of these proteins in human Th17 responses. Additionally, the molecular landscape differentially dictating non-pathogenic and pathogenic Th17 fates is largely unexplored in humans, and a holistic analysis of AP-1 function could provide useful hints in understanding this paradigm.

4.2 Materials and Methods

4.2.1 Primary Human $CD4^+$ T-cell isolation and Th17 culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from the umbilical cord blood of healthy neonates (Turku University Central Hospital, Turku, Finland) by the Ficoll- Paque density gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare).

Naïve $CD4^+$ cells were further purified using $CD4^+$ Dynal positive selection beads (Dynal CD4 Positive Isolation Kit; Invitrogen). $CD4^+$ T-cells were stimulated with plate-bound α-CD3 (3.75 µg/ml; Immunotech) and soluble α-CD28 (1 µg/mL; Immunotech) in X-vivo 20 serum-free medium (Lonza). X-vivo 20 medium was supplemented with L-glutamine (2 mM, Sigma-Aldrich), and antibiotics (50 U/mL penicillin and 50 µg/mL streptomycin; Sigma-Aldrich). Th17 cell polarization was induced using a cytokine cocktail of IL-6 (20 ng/mL; Roche), IL-1β (10 ng/mL) and TGF-β (10 ng/mL) in the presence of neutralizing anti-IFN-γ (1 µg/mL) and anti-IL-4 (1 µg/mL) to block Th1 and Th2 differentiation, respectively. For the control cells (Th0), $CD4^+$ T-cells were TCR stimulated with α-CD3 and α-CD28 in the presence of neutralizing antibodies without differentiating cytokines and cultured in parallel. All cytokines and neutralizing antibodies used in the study were purchased from R&D Systems unless otherwise stated. All cultures were maintained at 37°C in a humidified atmosphere of 5% (v/v) CO_2/air.

4.2.2 Flow Cytometry

The following antibodies were used for Flow cytometry: anti-CCR6 PE (BD Cat no. 559562); anti-FOSL1 (Santacruz Biotechnology, sc-28310); anti-FOSL2 (Cell Signaling Tech., Cat no.19967); anti-STAT3 (Cell Signaling Tech., Cat no. 9139); anti-BATF (Cell Signaling Tech., Cat no. 8638); Alexa 647 anti-mouse (LifeTech, Cat no.A21235); Alexa 647 anti-rabbit (LifeTech, Cat no. A21245), APC-CD73 Monoclonal Antibody (AD2), APC (Thermo Fischer, Cat no.17-0739-42), PE anti-human CD70 Antibody (Biolegend, Cat no. 355103).

CCR6 surface staining was performed 72h after initiation of Th17 culture, for which cells were washed twice with FACS buffer (0.5% FBS/0.1% Na-azide/PBS) and incubated with antibody for 20 min at 4ºC. For intracellular staining, cells were fixed and permeabilized as per manufacturer's instructions by Invitrogen IC staining buffers (Cat nos. 00-5223-56; 00-5123-43; 00-8333-56). Cells were incubated with primary antibodies for 2 hours and subsequently washed using Perm Buffer. This was followed by 30 min incubations with labelled secondary antibodies. This step was excluded for anti-CD70 and anti-CD73, which were pre-labelled. Suitable isotype or secondary antibody controls were maintained. Samples were acquired on LSRII (BD Biosciences,

Franklin Lakes, NJ); live cells were gated based on forward and side scattering. Acquired data was analysed with FlowJo (FLOWJO, LLC).

4.2.3 FOSL1 or FOSL2 single and double knockdown by RNAi

For individual silencing, $CD4^+$ T-cells from umbilical cord blood were suspended in Optimem I (Invitrogen) and transfected with two different FOSL1 or FOSL2-targeting siRNAs (Sigma, Sequences in Appendix II) using the nucleofection technique by Lonza. Scrambled non-targeting siRNA was used as control (Sigma). Four million cells were transfected with 5 µg of siRNA after which the cells were rested at 37ºC for 40-42h in RPMI 1640 medium (Sigma-Aldrich) supplemented with pen/strep, 2 mM L-glutamine and 10% FCS (2 million cells/ml) and subsequently activated and cultured under Th17 conditions as described above.

For simultaneous silencing, 4 million cells were nucleofected with 10 µg of FOSL-targeting siRNA [5 µg FOSL1 + 5 µg of FOSL2 (Thermo Scientific, Cat no. 115633)] or 10 µg of Scramble siRNA. FOSL1 or FOSL2 single knockdown controls were also maintained (5 µg FOSL1/FOSL2 siRNA + 5 µg of control siRNA). Cells were rested for 36-40 hrs post nucleofection before culturing under Th17 conditions. For identification of global targets - SCR, Single KD and Double KD Th17 cells were harvested at 24 and 72h of polarization. Three such biological replicates were subjected to sample preparation, as described in 3.2.11. All siRNA sequences have been provided in Appendix II.

4.2.4 FOSL1 and FOSL2 double over-expression

I. Generating in-vitro transcribed RNA

In order to generate linearized vectors for the IVT reaction, T7 promoter containing plasmids - Empty pGEM-GFP64A, GFP-FOSL1 (Origene, Cat no. RG202104) and GFP-FOSL2 (Origene, Cat no. RG204146), were in-invitro digested using the restriction enzymes Spe1 (NEB, Cat no. R0133), Xma1 (NEB, Cat no. R0180) and Ssp1 (NEB, Cat no. R3132) respectively. Digestion was performed for 1h using Cut Smart Buffer (NEB, Cat no. B7204S). Next, using the generated templates, in-vitro transcribed (IVT) RNA was produced using Cell Script MessageMAXTM T7 ARCA-Capped Message Transcription Kit (Cat. No. C-MMA60710) as per manufacturer's instructions. 10M Lithium chloride precipitation was used to precipitate the product (-20°C, O/N), followed by 70% Ethanol washes and resuspension in nuclease-free

water. The size of the RNA was confirmed using BioRad Experion or Agilent Bioanalyzer at this step. The RNA was further Poly-adenylated using Cell script A-Plus™ Poly(A) Polymerase Tailing Kit (Cat no.C-PAP5104H). LiCl precipitation was repeated and the final pellet was resuspended in nuclease-free water. RNA concentration was determined using Nanodrop and the IVT RNA was stored at -80°C till further use.

II. Nucleofection

For dual over-expression, 4 million cells were nucleofected with either FOSL1+FOSL2 IVT RNA (56 pmoles FOSL1 + 56 pmoles FOSL2) or control GFP RNA (112 pmoles). Single over-expression controls (OE) for FOSL1/FOSL2 were also maintained (56 pmoles of FOSL1/FOSL2 + 56 pmoles of control siRNA). We ensured equimolar RNA amounts across the different nucleofection conditions. Cells were rested for 16-20 hrs post nucleofection and further cultured under Th17 conditions. For identification of global targets - GFP, Single OE and Double OE Th17 cells were harvested at 24 and 72h of polarization. Three such biological replicates were subjected to sample preparation, as described in 3.2.11.

4.2.5 siRNA mediated silencing of STAT3

$CD4^+$ T-cells from umbilical cord blood were suspended in Opti-mem I (Invitrogen) and transfected with STAT3 targeting siRNA or non-targeting control siRNA (Sigma) (siRNA Sequences in Appendix II) using the nucleofection technique (Lonza). Four million cells were transfected with 6 µg of siRNA after which the cells were rested at 37º C for 40-42h in RPMI 1640 medium (Sigma-Aldrich) supplemented with pen/strep, 2 mM L-glutamine and 10% FCS (2 million cells/ml) and subsequently activated and cultured under Th17 culturing condition as described above.

4.2.6 Immunoprecipitation

Immunoprecipitation for FOSL1 & FOSL2 was performed using Pierce MS-Compatible Magnetic IP Kit (Thermo Fischer, Cat no.90409). 72h Th17 cell culture pellets were lysed in appropriate volumes of kit provided Cell-lysis buffer. All antibodies used, were pre-incubated with Protein A/G beads for 4-5h to form bead-Ab complexes. Lysates were first pre-cleared with control IgG-bead complexes for 3h. Pre-cleared lysates

were then incubated overnight with FOSL1 (Santacruz Biotechnology, Cat no.sc-28310) or FOSL2 (Cell Signaling Technology, Cat no.19967) Ab-bead complexes. Species-specific control IgG antibodies were used as negative-IP control. Immunoprecipitated protein complexes were washed (following manufacturer's protocol) and further eluted with appropriate volume of elution buffer. Eluted protein was subjected either to vacuum drying for MS analysis or run for western blotting. Antibodies used for IP-immunoblotting have been described in 3.2.9.

4.2.7 Immunofluorescence analysis

$CD4^+$ T-cells were cultured for 72hrs under Th17 differentiation conditions and then spun down on Poly-L-Lysine coated coverslips at 800rpm. Cells were washed, fixed and permeabilized using Ebioscience Intracellular Staining kit (Invitrogen; Cat no.00-5223-56 and Cat no.00-5123-43; Invitrogen; Cat no.00-8333-56). Permeabilized cells were further incubated overnight with primary antibodies against FOSL1 (Santacruz Biotechnology, Cat no. sc-28310) / FOSL2 (Cell Signaling Tech; Cat no.19967) and Lamin A/C (Santacruz Biotechnology, Cat no. sc-7292). Cells were washed with Permeabilization buffer and further incubated for 60 mins with respective anti-mouse or anti-rabbit Alexa flour secondary antibodies (Invitrogen Cat nos. A11031; A31572; A21202). Atto-Phalloidin A647 (Sigma; Cat no.65906) was used to stain cytoplasmic actin. Stained cells were finally mounted in Prolong Gold Antifade Mountant with DAPI (Cat no. P36941) and imaged on Zeiss 780 Confocal microscope.

4.2.8 Quantitative Real-time PCR

Total RNA was isolated using RNeasy kit (Qiagen Cat No. 74104) which included on-column DNAse treatment. Genomic DNA removal was further ensured with an additional treatment with DNAseI (Invitrogen). cDNA was synthesized with Reverse Transcription kit (Applied Biosystems) using oligo dT primers as per manufacturer's instructions. TaqMan primers and probes were designed with Universal Probe Library Assay Design Centre (Roche). All Taqman reactions were performed using Absolute QPCR Mix, ROX (Thermo scientific, Cat no. AB1139A). EF1α was used as endogenous control. The qPCR runs were analysed using the 7900HT Fast Real-Time PCR System (Applied Biosystems). All taqman primers and probes are listed in (Appendix I).

4.2.9 Western Blotting

Cell culture pellets were lysed using RIPA buffer (Pierce, Cat no. 89901), supplemented with protease and phosphatase inhibitors (Roche) and sonicated using Bioruptor UCD-200 (Diagenode). Sonicated lysates were centrifuged at 14,000 rpm for 20 min at 4°C and supernatants were collected. Samples were estimated for protein concentration (DC Protein Assay; Bio-Rad) and boiled with 6x Laemmli buffer (330 mM Tris-HCl, pH 6.8; 330 mM SDS; 6% β-ME; 170 µM bromophenol blue; 30% glycerol). Samples were loaded on gradient Mini-PROTEAN TGX Precast Protein Gels (BioRad, Helsinki, Finland) and transferred to PVDF membranes (Trans-Blot Turbo Transfer Packs, BioRad).

The following antibodies were used – anti-FOSL1 (Cell Signaling Tech, Cat no. 5281), anti-FOSL2 (Cell Signaling Tech., Cat no.19967); anti-STAT3 (Cell Signaling Tech., Cat no. 9139); anti-BATF (Cell Signaling Tech., Cat no. 8638), anti-STAT4 (Cell Signaling, 2653); anti-NT5E/CD73; anti-APOD; anti-JUNB; anti-GAPDH (Hytest, Cat no. 5G4), anti-β-actin (SIGMA, Cat no. A5441) and anti-LSD1 (Diagenode, Cat no. C15410067). HRP conjugated anti-mouse IgG (SantaCruz, Cat no. sc-2005) and anti-rabbit IgG (BD Pharmingen, Cat no. 554021) were used as secondary antibodies.

List of antibodies used for IP-immunoblotting are as follows. Anti-FOSL1 (Santacruz, Cat no. sc-28310); anti-FOSL2 (Cell Signaling, Cat no. 19967); anti-RUNX1 A-2 (Santa Cruz, Cat no. sc-365644); JUNB C-11 (Santa Cruz, Cat no.sc-8051); anti-SIRT1 (Cell signaling, Cat no.2496); anti-JUN (BD Biosciences, Cat no.610326). Conformation specific Rabbit HRP (Cell Signaling, Cat no.5127) and Mouse HRP (Cell signaling, Cat no. 58802)

4.2.10 Cellular Fractionation

24 and 72h cultured Th0 and Th17 cell lysates were fractionated into their Cytoplasmic and nuclear components using the NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo Fischer Scientific, Cat no. 78833), by following manufacturer's instructions. Extracts were analysed by western blotting as described in section 3.2.9. Localization of FOSL1 and FOSL2 was detected using primary antibodies specific for the respective proteins. GAPDH and LSD1 were used as cytoplasmic and nuclear markers, respectively. (Antibody details in section 3.2.9).

4.2.11 RNA-seq analysis

I. RNA Isolation and RNA-Seq Sample Preparation

RNA was isolated (RNeasy Mini Kit; QIAGEN, Hilden, Germany) and given on-column DNase treatment (RNase-Free DNase Set; QIAGEN) for 15 min. The removal of genomic DNA was ascertained by an additional treatment of the samples with DNase I (Invitrogen). After RNA quantification (using Nanodrop 2000) and quality control (using BioRad Experion or Agilent Bioanalyzer), libraries for RNA-Seq were prepared. The high quality of the libraries was confirmed with Advanced Analytical Fragment Analyzer (Advanced Analytical Technologies, Heidelberg, Germany) or with Agilent Bioanalyzer, and the concentrations of the libraries were quantified with Qubit® Fluorometric Quantitation (Life Technologies, ThermoFisher). Sequencing was performed at the Finnish Functional Genomics Centre (FFGC) using HiSeq3000 Next-Generation Sequencing platform.

II. Alignment and Differential Expression Analysis

Obtained sequencing reads were checked for quality using FastQC (v.0.11.14) [69] and MultiQC (v.1.5)[70]. High quality reads were aligned to the human reference genome (hg38) using R (v.3.6.1) [71]/ Bioconductor(v.3.9) [72] package-Rsubread (v.1.34.6)/method-align [73]. Gene-wise read counts were obtained using the parameters ‘strand specificity: reversely stranded’ and ‘paired end reads: NO’. Statistical testing and differential expression analysis was performed using Bioconductor package ROTS (v.1.12.0) [74]. Note that for each comparison, the expressed genes (CPM expression value >1) in at least 50% of the replicates in one of the compared sample groups were included in the statistical testing. Further DE genes were detected with cut offs FDR<0.1, Fold change<1.8 (unless otherwise specified).

III. Data representation

Heatmaps showing z scores or Log2FC values for the differentially expressed genes were generated using gplots R package.

4.2.12 ChIP-seq analysis

I. Sample preparation

$CD4^+$ T-cells were cultured under Th17 cell polarizing conditions for 72 hrs. Chromatin was prepared using Diagenode Chromatin shearing optimization kit (Cat no. C01010055) and further subjected to sonication using Bioruptor sonicator (Diagenode) to obtain chromatin fragments of 100–500 bps. Fragmented chromatin was incubated with 10-12µg of FOSL1 (Santacruz Biotechnology, Cat no.sc-28310) or FOSL2 (Cell Signaling Tech, Cat no.19967) antibody and incubated with magnetic beads for crosslinking (Cat no. 112.04 Dynal Biotech, Invitrogen). The crosslinks were further reversed (65°C for 12–16 h, mixer conditions), treated with Proteinase K and RNase A and then purified using QIAquick PCR purification kit, QIAGEN. DNA libraries were prepared (Fasteris Life Sciences) and sequenced on Illumina HiSeq 4000.

II. Analysis

Raw read quality control was performed with FastQC (v. 0.11.4) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The adapter sequences present in the raw reads were trimmed using TrimGalore (v. 0.4.5) (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and the trimmed reads were mapped to the hg38 reference genome using Bowtie2 [75] (v. 2.4.3.1). Duplicate reads were marked with Picard tools' (v. 2.20.2) MarkDuplicates function and reads with mapping quality < 30 were filtered out using samtools (v. 1.9). Sample quality was controlled by calculating cross-correlation scores and the non-redundant fraction with phantompeakqualtools (v. 1.2) and preseq (v. 2.0), respectively. Peaks were called using MACS2 (v. 2.1.0) and reproducible peaks were identified using IDR with FDR cut-off of 0.01. [76-81]

4.2.13 ChIP-peak Anno

R package ChIPpeakAnno was used to annotate the peaks and identify regions common to the two transcription factors with a minimum overlap of 200 bases. [82]

4.2.14 Motif analysis

Enriched transcription factor binding site motifs within the peaks were identified by Homer (v. 4.11) using both de novo and known motifs. A 200 bp window was used for motif finding.

4.2.15 Ingenuity Pathway Analysis

Pathway analysis was performed using Ingenuity Pathway Analysis (IPA, www.qiagen.com/ingenuity; Qiagen; March 2019) tool. IPA pathways with p-value <0.01 were considered as significantly enriched.

4.2.16 Volcano plots (using online Galaxy Europe Tools)

I. Volcano plot for Double KD and Double OE RNA-seq targets

List of DE targets was acquired from RNA-seq analysis of Double KD (24 and 72h) and Double OE Th17 (72h) cells. Volcano plots were generated using the 'Volcano Plot' function of Galaxy Europe under 'Graph/Display Data' [83]. Targets with FDR<0.1 and fold change>1.8 were highlighted using respective colors. Chosen Th17-relevant genes were represented with labelled boxes.

II. Volcano Plot for Shared direct targets of FOSL1 and FOSL2

FOSL1 and FOSL2 common sites obtained from ChIP-peak Anno analysis, were annotated to the nearest TSS using Homer. Of these, the genes differentially regulated in Double KD or Double OE (FDR<0.1 and fold change>1.5) were considered. The corresponding RNA-seq expression changes for the listed targets were acquired using 'Joint two files' operation under 'Text manipulation' on Galaxy Europe. Subsequent volcano plots were created as described in 3.2.16 I. [83]

4.2.17 Mass Spectrometry for interactome analysis of FOSL1 and FOSL2

I. Sample preparation

The IP eluates for IgG, FOSL1 and FOSL2 were denatured with urea buffer (8 M urea, 50mM Tris-HCl pH 8.0), followed by reduction using dithiothreitol (10 mM) at 37^0C for 1 h. The reduced cysteine residues were subsequently alkylated using iodoacetamide (14 mM, in darkness) at room temperature for 30 min. The samples were diluted to reduce the urea concentration (<1 molar), followed by digestion with sequencing grade modified trypsin at 37°C overnight (16-18 hours). The digested peptides were acidified and then desalted using C18 Stage Tips, prepared in house using Empore C18 disks (3M, Cat No 2215). The desalted samples were dried in a SpeedVac (SAVANT SPD1010, Thermo Scientific) and then stored at -80°C until further analysis.

For validation measurements, synthetic isotopic analogues (lysine 13C6 15N2 and arginine 13C6 15N4) were obtained for unique peptides from selected protein targets

identified in the AP-MS discovery data (Thermo Fischer Scientific). The same sample preparation procedure was used for the validation experiments, with the exception that the samples were spiked with isotope-labeled peptides and MSRT retention time peptides standards (Sigma), prior to MS analysis.

II. LC-MS/MS Analysis

A. Data-Dependent Analysis

The dried peptides were reconstituted in formic acid/acetonitrile (both 2% in water) and a NanoDrop-1000 UV spectrophotometer (Thermo Scientific) was used to measure the peptide amounts. Equivalent aliquots of the digested peptides were analyzed by LC-MS/MS using an Easy-nLC 1200 coupled to Q Exactive HF mass spectrometer (Thermo Fisher Scientific). The peptides were loaded onto a 20 x 0.1 mm i.d. pre-column and separated with a 75 µm x 150 mm analytical column, both packed with 5 µm Reprosil C18 (Dr Maisch GmbH). A separation gradient from 5 to 36% B in 50 min was used at a flow rate of 300 nl/min (Solvent A: 0.1% formic acid in MiliQ H_2O and Solvent B: 80% acetonitrile, 0.1% formic acid in MiliQ H_2O). The tandem MS spectra were acquired in positive ion mode with a data dependent Top 15 acquisition method from 300-1750 m/z using HCD fragmentation. The singly and unassigned charged species were excluded from the fragmentation. The MS1 and MS/MS spectra were acquired in the Orbitrap, at a resolution set to 120,000 and 15,000 (at m/z 200), respectively. The AGC target values for MS1 and MS/MS were set to 3,000,000 and 50,000 ions, with maximal injection times of 100 and 150 ms, respectively, and the lowest mass was fixed at m/z 120. Dynamic exclusion was set to 20 s. Triplicate analysis were performed for all samples in randomized batches.

B. Parallel reaction monitoring (PRM)

Synthetic peptide analogues for validation targets were analyzed together with MSRT retention time peptides standards (Sigma) by LC-MS/MS using an Orbitrap Fusion Lumos mass spectrometer, coupled to Easy-nanoLC (Thermo Fisher Scientific) with the same column configuration as above. On the basis of these data a PRM method was developed for the analysis of these targets and their endogenous counterparts in AP validation samples. For the targeted analysis, the peptides were separated with a 30 min gradient from 8% to 39% solvent B. The data was acquired in a PRM mode with an isolation window setting of 1.6 m/z at a resolution of 15,000 for the Orbitrap, using a target AGC value of 50,000 and maximum injection time of 22ms.

III. Data Analysis

A. AP-MS Data

The mass spectrometry raw files were searched against a UniProt FASTA sequence database of the human proteome (downloaded, May 2019, 20415 entries:) using the Andromeda search engine, incorporated with the MaxQuant software (Version 1.6.0.16) [84, 85]. Trypsin digestion, with a maximum of two missed cleavages, carbamidomethylation of cysteine as a fixed modification, and variable modification of methionine oxidation and N-terminal acetylation were specified in the searches. A false discovery rate (FDR) of 1% was applied at the peptide and protein level. MaxQuant's label-free quantification (LFQ) algorithm [86] was used to calculate the relative protein intensity profiles across the samples. The "match between run" option was enabled to perform matching across the mass spectrometric measurements.

The proteinGroup.txt file from the MaxQuant output was further processed using Perseus (Version 1.6.2.3) [87]. The output was filtered to remove contaminants, reverse hits and proteins only identified by site. Protein LFQ values were log2 transformed and the medians of the technical replicates calculated. The data was filtered to retain proteins with three valid values in at least one group (IgG, FOSL1 and FOSL2 pulldown). The resulting data matrix was then analyzed using the mass spectrometry interaction statistics (MiST) algorithm. The algorithm calculates a MiST score for each of the potential interactors on the basis of their intensity, consistency and specificity to the bait [88]. A MiST score criteria of ≥0.75 for FOSL1 and FOSL2-prey interaction and ≤0.75 for interaction with IgG was applied. Further, to eliminate proteins frequently detected as contaminants in IP experiments, comparison was made with a list of proteins frequently detected with IgG-mock baits derived elsewhere in our research (This was based on 126 other IP experiments). Proteins detected with a frequency of 40% were retained. The subsequent list of proteins was mapped against STRING database, and the assigned protein-protein interaction (PPI) network was further visualized using Cytoscape [89].

B. Validation Data

The data from analysis of the synthetic peptides was analyzed using Proteome Discoverer (Version 2.2, Thermo Fisher Scientific) using a Fasta file containing the sequences the peptide targets. The MSF file from Proteome Discoverer was then used to construct spectral library in Skyline (v4.2) software [90] and define their retention

time indices. Skyline was then used to create scheduled isolation lists for PRM analysis [90]. Skyline was used to process the PRM-MS raw files and review the transitions and integration of the peptide peaks. The transition signals of endogenous peptides were normalized to their heavy counterparts and the statistical analysis was performed using in built MSStat plugin [91] on the basis of sum of transition areas.

4.2.18 Graphical representation and Venn diagrams

All graphs were plotted using GraphPad Prism software (V8.3.0). Two-tailed students T-test was used to calculate statistical significance, unless otherwise mentioned. All Venn diagrams were generated using Biovenn [92] or Venny [93].

4.3 Results

4.3.1 FOSL1 and FOSL2 are upregulated in human Th17 cells

For evaluating the expression kinetics of FOS-like proteins during human Th17 differentiation, we used data from a previously published study (from Lahesmaa group), that compared mouse and human Th17 transcriptomes [94]. We plotted FOSL1 and FOSL2 transcript levels for human umbilical cord blood (UCB)-derived naïve CD4 cells which were cultured either under activation (Th0) or Th17-polarizing conditions (TGF-β, IL-6 and IL-1β) for 0.5, 1, 2, 4, 6, 12, 24, 48 and 72h (Fig 4.3.1A). We observed that induction of Th17 differentiation showed significant upregulation of both FOSL1 and FOSL2 RNA at all of the assessed time points. We further validated this trend at the protein level by performing immunoblot analysis (Fig 4.3.1B). We observed that both proteins were the most differentially upregulated at 24h of polarization, with FOSL2 depicting a more pronounced profile.

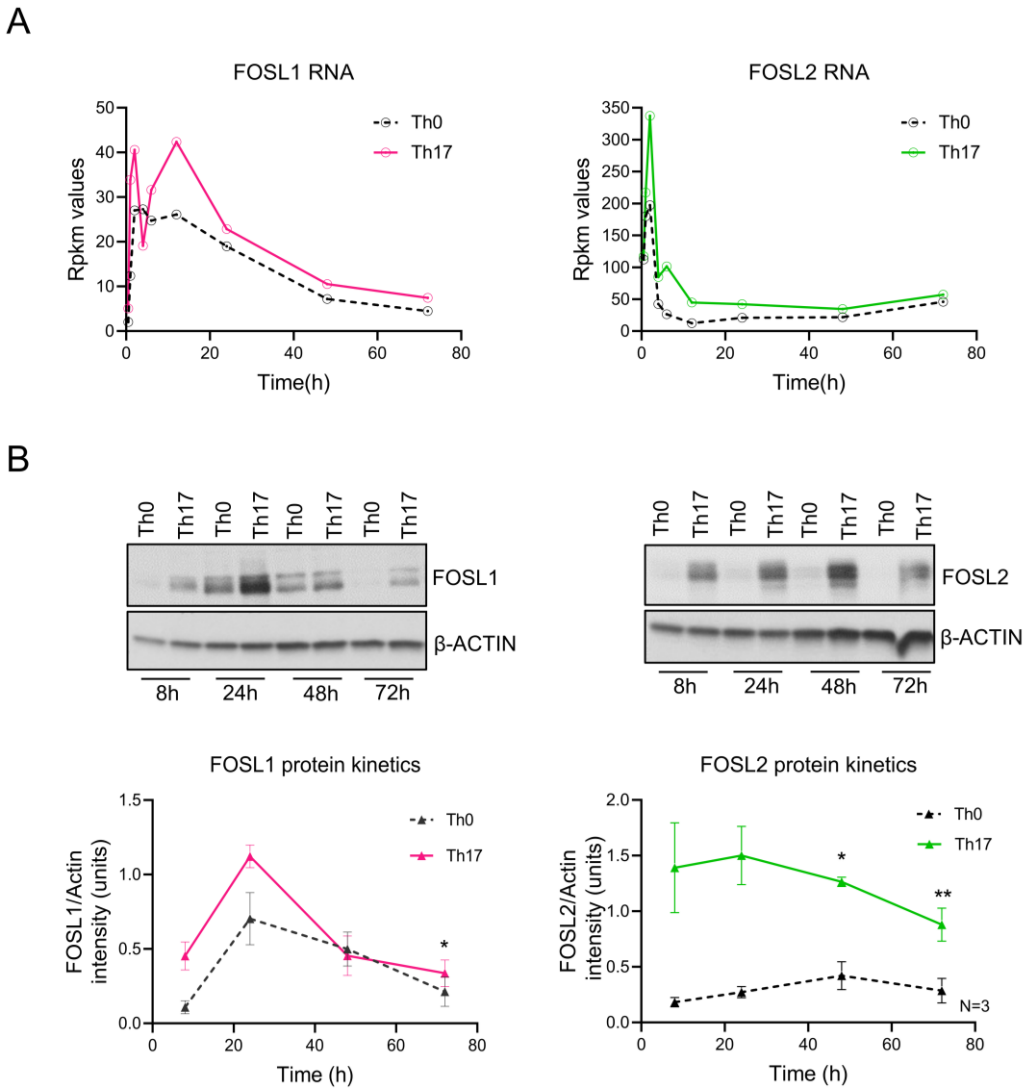


Figure 4.3.1. Expression profile of FOS-like proteins in human Th17 cells. A. Transcriptome analysis for kinetics of FOSL1 (left) and FOSL2 (right) expression under activation (Th0, dotted line) or Th17-polarizing (coloured) conditions using human cord blood derived naïve CD4 cells. Line graph represents Rpkm values from RNA-seq data of Tuomela et al. 2016 [94] **B.** Immunoblot images showing FOSL1 (bottom left) and FOSL2 (bottom right) protein levels at different time points of activation (Th0, dotted line) or Th17-polarization. Actin has been used as loading control. Blots were quantitated in ImageJ and corresponding intensity values (normalized to actin) are plotted in the adjoining graphs (above). Data shows mean ± standard error of the mean (SEM) for three biological replicates. Statistical significance is calculated using two-tailed Student's t test (*p < 0.05; **p < 0.01, **p < 0.001).

4.3.2 IL6/STAT3 signaling drives expression of FOS-like proteins

TCR activation is already known to upregulate AP-1 activity (reviewed in [95, 96]). We next focused on dissecting the Th17-polarizing cytokines that contribute to further stimulating their activation-induced levels. In order to achieve this, we performed a cytokine-induction assay and analysed for levels of FOS-like proteins using flow

cytometry (Fig 4.3.2A). Of these, IL-1β and IL-6 were seen to significantly enhance expression of both proteins (relative to Th0), with IL-6 showing the more striking trend. Interestingly, TGF-β depicted a contrasting-induction profile by suppressing FOSL1 and upregulating FOSL2 levels. A similar observation has been previously reported. [97, 98].

IL-6 cytokine has been well-documented in inducing STAT3 activation [99, 100]. Moreover, the IL-6/STAT3 signaling axis has previously been shown to drive expression of FOS-like proteins in T-cells and cancers [60, 101-104]. Considering the key role of STAT3 is establishing Th17 fate [101, 105], we wished to determine if the polarization-driven changes in FOSL expression required STAT3 function. We used immunoblotting to analyse protein levels of FOSL1 and FOSL2 in STAT3-depleted Th17-polarized cells (Fig 4.3.2B). We noticed that the loss of STAT3 significantly reduced levels of both the proteins. However, its effect on FOSL2 expression was much stronger. ChIP-seq data from a previous human Th17 study (from Lahesmaa lab) has shown STAT3 occupancy on promoter regions of *FOSL2* but not *FOSL1*, which might explain the differential influence [101].

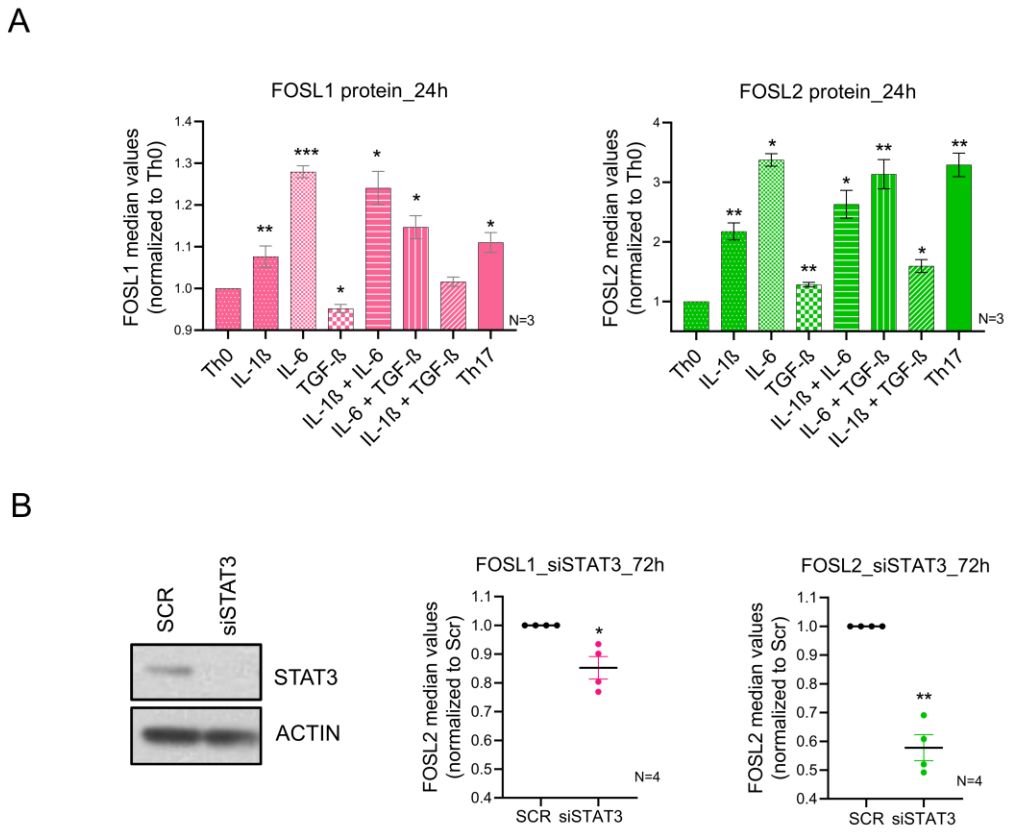


Figure 4.3.2 Induction of FOSL1 and FOSL2 in human Th17 cells. A. FOSL1 and FOSL2 protein levels were determined for cells cultured under conditions of activation (Th0), differentiation (Th17) or activation combined with each of the Th17-polarizing cytokines (either alone or in combination with each other) at 24h, using flow cytometry. Estimated median values for protein expression were normalized to Th0 and plotted for three biological replicates. Significance was calculated by comparing each condition to Th0 **B.** Immunoblot shows levels of STAT3 in naïve CD4 cells treated with STAT3-targeting siRNA, further cultured under Th17-polarizing conditions for 48h. Adjoining graphs indicate flow-cytometry analysis which was used to analyse FOSL1 (left) and FOSL2 (right) protein expression in STAT3-silenced 72h Th17 cells. Non-targeting siRNA (SCR) was used as control. Median values for protein expression were normalized to SCR and plotted for 4 biological replicates. Data shows mean ± standard error of the mean (SEM) for three biological replicates in figure A and four biological replicates in figure B. Statistical significance is calculated using two-tailed Student's t test (*p < 0.05; **p < 0.01, **p < 0.001).

4.3.3 Loss of FOSL1 or FOSL2 enhances IL-17 secretion in human Th17 cells

The early induction and sustained elevated levels shown by FOS-like proteins is indicative of a potential involvement in governing human Th17 lineage. Reports using gene-knockout mice models have previously indicated opposing functions for FOSL1 and FOSL2 in regulating murine Th17 fate. While FOSL2 inhibits, FOSL1 has been

shown to support the differentiation process in mouse [58, 60]. In order to evaluate their role in steering human Th17 polarization, we used RNAi to silence each of these proteins and examined its effect on IL-17 secretion. Two different siRNAs were used to individually target FOSL1 and FOSL2 each, in order to ensure reproducibility and rule out off-target effects. Cells were nucleofected and cultured according to the workflow described in Fig 4.3.3A. We confirmed the siRNA-efficacy based on a significant reduction seen in corresponding protein levels using immunoblot analysis (Fig 4.3.3B). We also noticed that silencing FOSL1 did not evidently alter expression of FOSL2 and vice-versa, which is in agreement with other findings in the field (Fig 4.3.4A) [106, 107]. Interestingly, FOSL1 or FOSL2 depletion, both were seen to significantly enhance IL-17 secretion at 72h of polarization. This demonstrates a negative role for these paralog proteins in regulating human Th17 effector responses. It further highlights the fact that although FOSL2 function shows agreement between human and mouse, the role of FOSL1 seems to be conflicting in the two species. Thus, our findings emphasize on the importance of validating murine gene-function studies using cells of human origin.

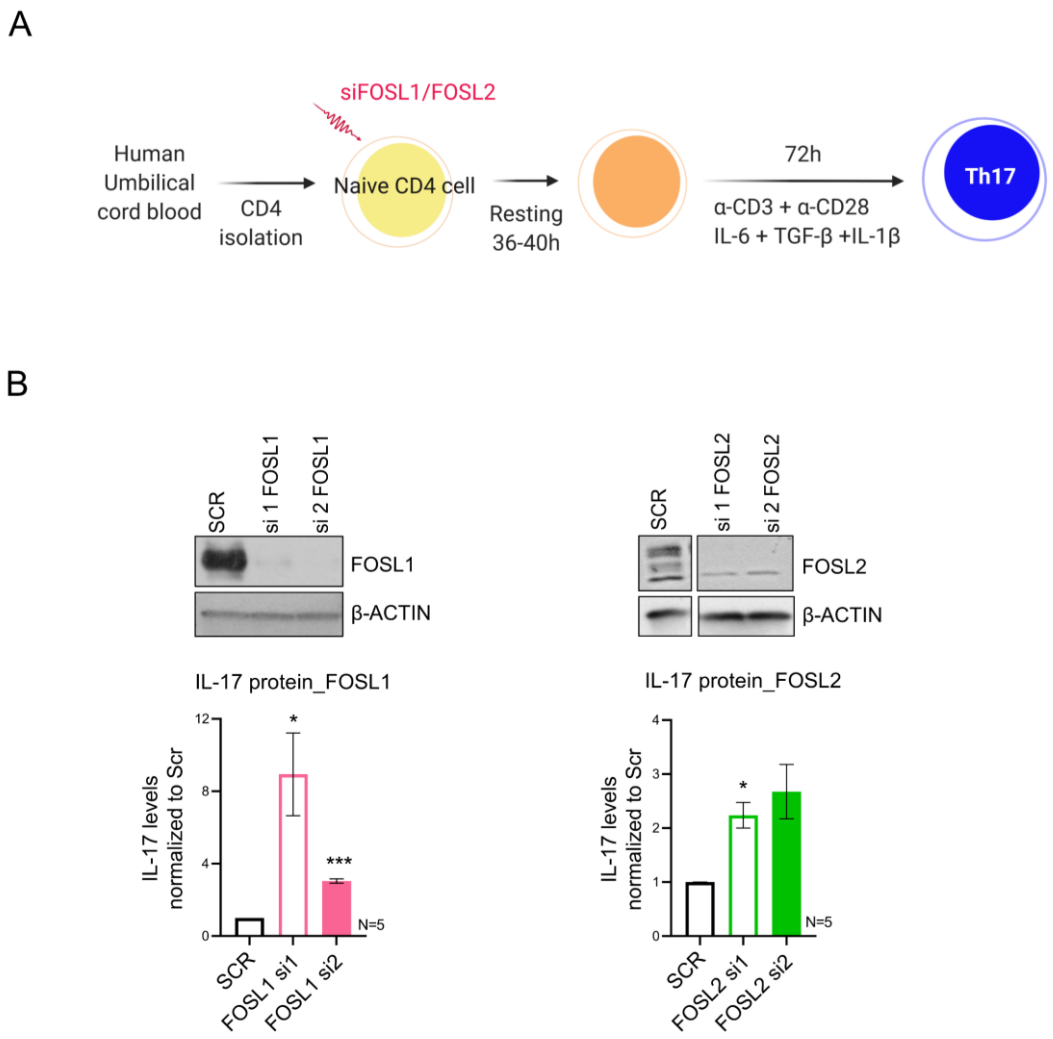


Figure 4.3.3. Effect of FOSL1 and FOSL2 silencing on human IL-17 cytokine secretion. A. Nucleofection workflow. Naïve CD4 cells are treated with indicated FOSL1 or FOSL2 targeting siRNA, rested for 36-40h and further cultured under Th0 or Th17 polarizing condition (IL-6, IL-1β and TGF-β) along with neutralizing antibodies for 72h **B.** Two different siRNAs each, were used to silence FOSL1 or FOSL2 in naïve CD4 cells and the treated cells were further differentiated to Th17 phenotype. Immunoblot analysis shows FOSL1 (left) and FOSL2 (right) protein levels in siRNA-silenced cells, cultured for 24h under Th17 polarizing conditions. Non-targeting siRNA (SCR) was used as nucleofection control. Actin is used as loading control. Blots represent three biological replicates. ELISA was used to analyse IL-17A levels in supernatants of naïve CD4 cells treated with two different FOSL1 or FOSL2 targeting siRNAs (left and right panel respectively), further cultured under Th17 polarizing conditions for 72h. Values were initially normalized to live cell count, followed by normalization with Scr. Data shows mean ± standard error of the mean (SEM) for five biological replicates. Statistical significance is calculated using two-tailed Student's t test (*p < 0.05; **p < 0.01, **p < 0.001).

4.3.4 IL-17 expression is synergistically repressed by FOSL1 and FOSL2

Functional coordination among FOS and JUN proteins during transcriptional regulation has been well studied over the years [108]. Whether such synergy or cooperativity exists between FOSL1 and FOSL2, is however yet to be explored. Since both these proteins were found to regulate IL-17 secretion in a similar fashion, we wanted to determine if their simultaneous perturbation resulted in enhanced changes. In order to achieve this, we used both RNAi-mediated silencing and RNA-based over-expression strategies. For simultaneous silencing, naïve CD4 cells were co-nucleofected with a combination of FOSL1 and FOSL2-targeting siRNAs and immunoblotting was performed to confirm the corresponding reduction in their protein levels (Fig 4.3.4A). We parallelly maintained single KD controls (Cells individually silenced for FOSL1/FOSL2) in order to enable comparison. To determine its effect on Th17 differentiation, we analysed IL-17 expression at transcript and protein level using qPCR and ELISA respectively (Fig 4.3.4C&D). We observed that co-depletion of FOSL1 and FOSL2 (Double KD) resulted in an evidently enhanced upregulation of IL-17 RNA and protein (as compared to the single KD controls).

Next, to validate the above results, we approached an alternate strategy where we simultaneously over-expressed these proteins, using in-vitro transcribed (IVT) RNA. Naïve CD4 cells were nucleofected with a combination of FOSL1 and FOSL2 IVT RNAs (Double OE) and flow cytometry analysis was performed to confirm their elevated levels (Fig 4.3.4B). Corresponding Single OE controls were also maintained. At 72h of differentiation, we observed that inducing a parallel increase in FOSL1 and FOSL2 levels results in a much-pronounced inhibition of IL-17 expression at both RNA and protein level (relative to Single OE) (Fig 4.3.4E&F). These results strengthened our knockdown-based findings and confirmed that FOS-like proteins indeed cooperatively suppress human IL-17 expression, in early-differentiating cells.

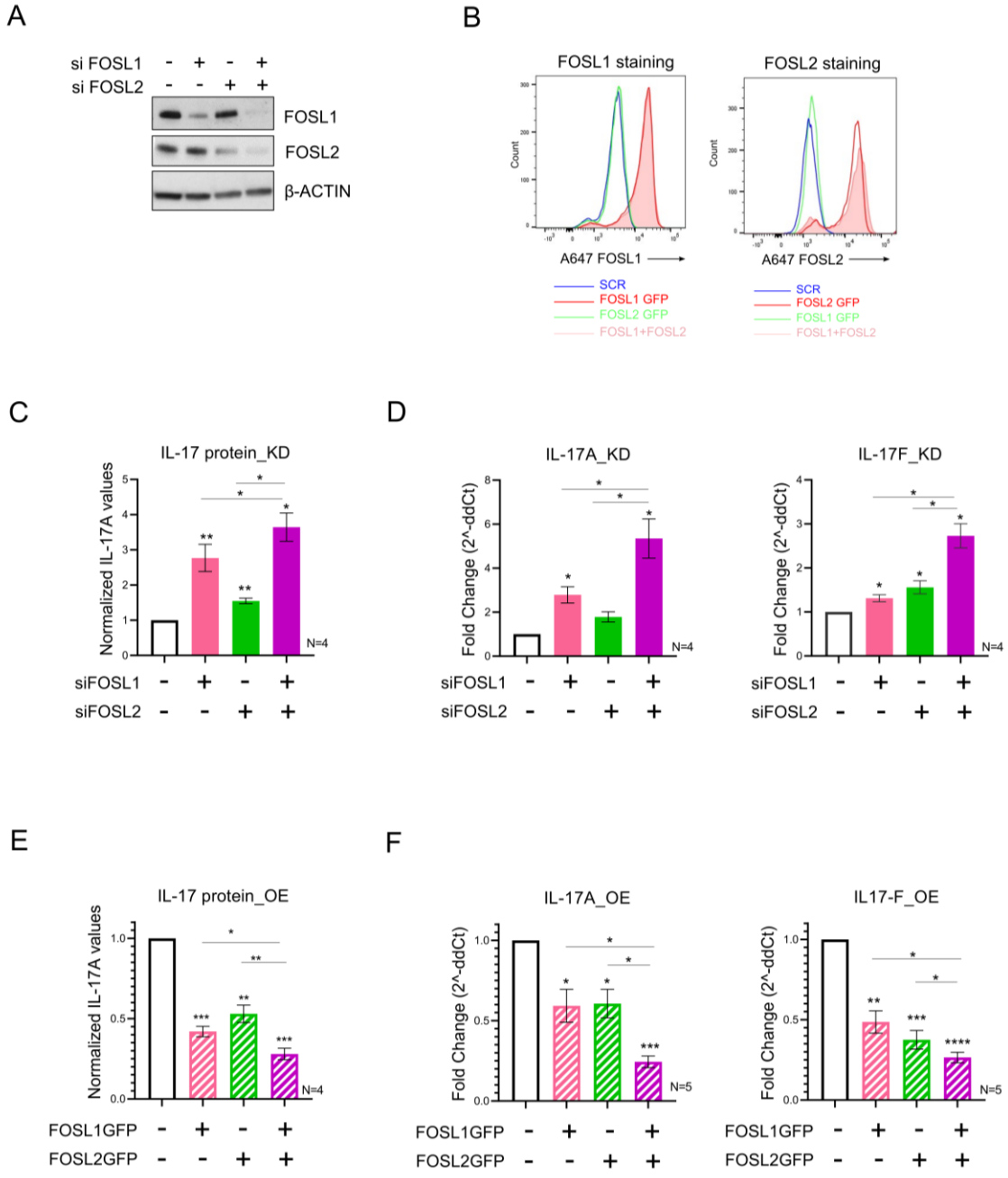


Figure 4.3.4. Cooperative-effect of FOSL1 and FOSL2 on IL-17 expression. A. Immunoblot analysis of FOSL1 and FOSL2 protein expression in naïve CD4 cells treated with either FOSL1, FOSL2 or FOSL1+FOSL2 targeting siRNA, further cultured under Th17 polarizing conditions for 24h. Non-targeting siRNA (SCR) was used as RNAi control and actin serves as loading control. Blot shown is a representative image of 3 biological replicates **B.** Overlay histograms show flow-cytometry based analysis of Alexa 647 stained total FOSL1 (left) or FOSL2 (right) protein in naïve CD4 cells treated with either GFP-FOSL1, GFP-FOSL2 or both over-expression RNAs, further cultured under Th17 polarizing conditions for 24h. Non-targeting GFP RNA is used as control. Data shown is a representative of 3 biological replicates. Legend describes color-coding for the different conditions **C&E.** ELISA was used to measure IL-17A levels in supernatants of naïve CD4 cells treated with indicated conditions of FOSL-knockdown or -over-expression and further differentiated to Th17 phenotype for 72h. Values were first normalized for live cell count at 72h and then for respective SCR or GFP IL-17 values. Data shows mean ± standard error of the mean for four biological replicates **D.** qPCR analysis was used to estimate fold changes for IL-17A (left) and IL-17F (right) RNA in naïve CD4 cells treated

with indicated conditions of FOSL knockdown, further cultured under Th17 polarizing conditions for 72h. The first bar column indicates values for non-targeting siRNA (SCR) which was used as control. Fold change normalized to SCR is plotted for four biological replicates **F.** Bar plot shows qRT-PCR analysis-based fold changes for IL-17A (left) and IL-17F (right) RNA in naïve CD4 cells treated with indicated conditions of FOSL over-expression, further cultured under Th17 polarizing conditions for 72h. All over-expression RNA was GFP-tagged and empty GFP (first bar column) was used as a control. Fold change normalized to GFP control is plotted for five biological replicates. Data in panels D&F show mean ± standard error of the mean (SEM). Statistical significance is calculated using two-tailed Student's t test (*p < 0.05; **p < 0.01, **p < 0.001).

4.3.5 Transcriptome analysis indicates that synergism between FOSL1 and FOSL2 significantly alters the Th17 lineage program

In order to globally unravel the individual and synergistic transcriptional targets of FOS-like proteins, we performed RNA-seq and differential expression (DE) analysis for Single and Double KD Th17 cells (Non-targeting/SCR siRNA was used as control). For FOSL1 KD, FOSL2 KD and Double KD conditions respectively, we detected 466, 1,529 and 1,998 DE genes at 24h and 315, 139 and 1,499 DE genes at 72h of polarization (false discovery rate; FDR < 0.1). We performed a similar analysis for Single and Double OE 72h Th17 cells (using GFP RNA as control), where we identified 31, 352 and 522 DE transcripts for FOSL1 OE, FOSL2 OE and Double OE conditions respectively (false discovery rate; FDR < 0.1). Overall, the collective number of DE targets were found to be significantly higher when both proteins were simultaneously altered, as against when only one of them was perturbed.

To meticulously dissect the targets that are cooperatively-regulated by these proteins, we compared the Logarithmic Fold changes between Single KD/OE and Double KD/OE conditions. We observed that a significant number of genes showed enhanced expression changes upon co-depletion or dual over-expression, as compared to corresponding single-factor perturbations (Heatmaps in Fig 4.3.5(I)A&B). Among these, we detected the key cytokine gene *IL17A,* which portrayed a pronounced upregulation in Double KD and an increased downregulation in Double OE Th17 cells, thereby validating our findings from Fig 4.3.4. Other prime markers of Th17 differentiation including *IL17F, IL23R and CCR6* were also seen to be cooperatively regulated in a negative fashion. Additionally, many other genes with previously-studied roles in Th17 or inflammatory responses (such as *FASLG, IL7R, NT5E, BCL2A1, STAT4, CD70, PRDM1, FGF2, DUSP2 and FUT7*) appeared to be synergistically-regulated. Apart from these observations, our analysis also underscores some

lineage-associated candidates (including *IL-21, USP18, GZMB, IFI44, IL3 and OASL*), which were either non-synergistically or oppositely regulated by FOSL1 and FOSL2. This highlights the fact that although there is an evident coordination between regulatory functions of these proteins, they are also capable of independently influencing the Th17 lineage.

Since the RNA-seq analysis for the simultaneously perturbed Th17 cells exhibited a much higher number of DE genes and also portrayed more striking fold changes for important Th17-associated molecules, we hereon focused on exploring only the Double KD and Double OE targets. We noticed that the overlap between 24h and 72h Double KD DE genes was very poor (6 genes including *TNFRSF18, ISG15, NME3, LOC606724, RN7SL2, RPL13AP3*; FDR≤0.1 and |fold change|≥1.8), suggesting a time-dependent regulatory profile for FOSL1 and FOSL2. Further, Ingenuity pathway analysis (IPA) discovered that co-depletion or dual over-expression significantly altered expression of genes involved in Mitochondrial Dysfunction, Sirtuin Signaling, T-helper cell differentiation, IL-23 signaling and Th1/Th2/Th17 activation (Fig 4.3.5(I)C&D). Moreover, autoimmune-related pathways such as 'Altered T-cell and B-cell Signaling in Rheumatoid arthritis' and 'Systemic Lupus Erythematosus in B cell signaling' were among the top-enriched processes.

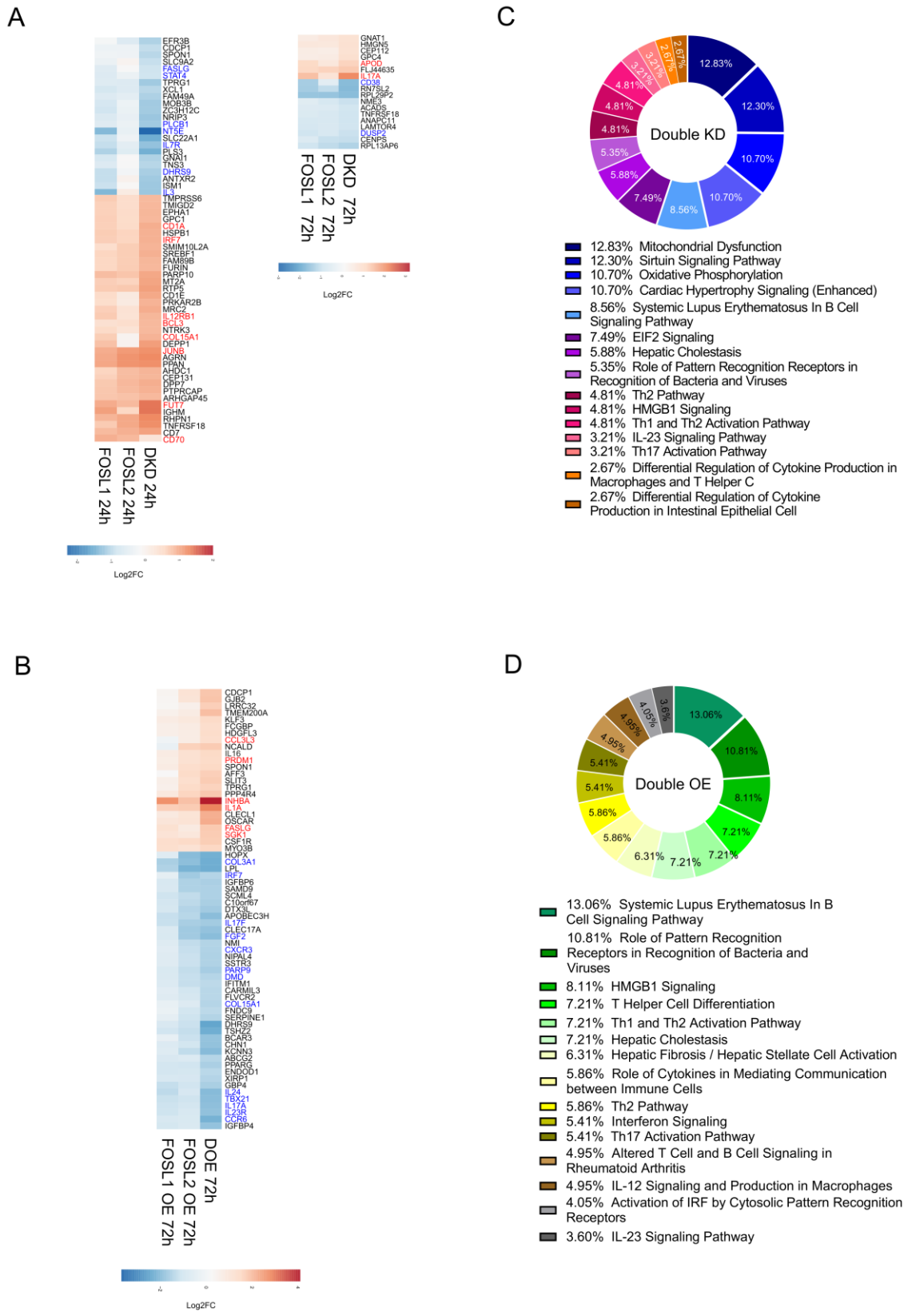


Figure 4.3.5(I). Comparison of expression changes in Th17 cells upon individual versus simultaneous perturbation of FOS-like proteins A. Functional cooperativity between FOSL1 and FOSL2. Heatmaps show significant number of targets with enhanced Log_2Fold change values in FOSL Double KD as compared to their corresponding single KD controls at 24h (upper

panel) and 72h (lower panel) of Th17 polarization. Genes with Th17-relevance are highlighted; upregulated in red and downregulated in blue. **B.** Heatmap shows significant number of targets with enhanced Log_2Fold change values for FOSL Double over-expression (OE) Th17 cells as compared to their corresponding single over-expression (OE) controls, at 72h of Th17 differentiation. Genes with Th17-relevance are highlighted; upregulated in red and downregulated in blue. For A&B, Log_2Fold change was calculated relative to respective SCR or GFP controls **C&D.** Ingenuity pathway analysis was used to identify signaling pathways that are altered upon simultaneous silencing (Fig. C) or simultaneous over-expression (Fig. D) of FOS-like proteins.

Volcano plots in Fig 4.3.5(II)A&C highlight some of the top differentially expressed transcripts in Double KD and Double OE Th17 cells. Collectively analysing information from expression changes seen in both these datasets, FOS-like proteins were seen to negatively regulate multiple (Th17) lineage-driving genes, including *CCR6 [109, 110], IL23R [111], IL21 [112], JUNB [113, 114], CD70 [115], IL12RB1 [116], CD52 [117] and CXCR3 [118]* (Heatmaps in Fig 4.3.5(II)B&D). Concomitantly, known inhibitors of inflammation or Th17 differentiation such as *PRDM1 [119], DUSP2 [120] and NT5E [121-123]*, were seen to be positively regulated. We also identified multiple known targets of human STAT3 (*CXCR5, HOPX, IL24, FNDC9, NR4A2 and GZMB*), to be oppositely influenced by FOS-like proteins [101].

It was intriguing to note that although our findings holistically illustrate FOSL proteins to restrict human Th17 fate, we also found them to negatively regulate some known-repressors of the lineage (including *TBX21 [124], IRF7 [125] and IL24 [127]*). Nonetheless, since functional studies for most of these candidates are based on murine models, it is imperative to first determine their human-specific role, before commenting on their involvement. Further, in order to determine the strongest FOSL-regulatory targets, we compared the Double KD and Double OE datasets and highlighted the candidates which were common but antagonistically regulated (Fig 4.3.5(II)E). We found 37 such statistically-significant targets, which included important Th17-relevant genes (*IL17F, IL17A, CCR6, DMD, FASLG, BCL2A1, TIGIT, NT5E, CD70, COL15A1 and IRF7*) (FDR≤0.1 and |fold change|≥1.8).

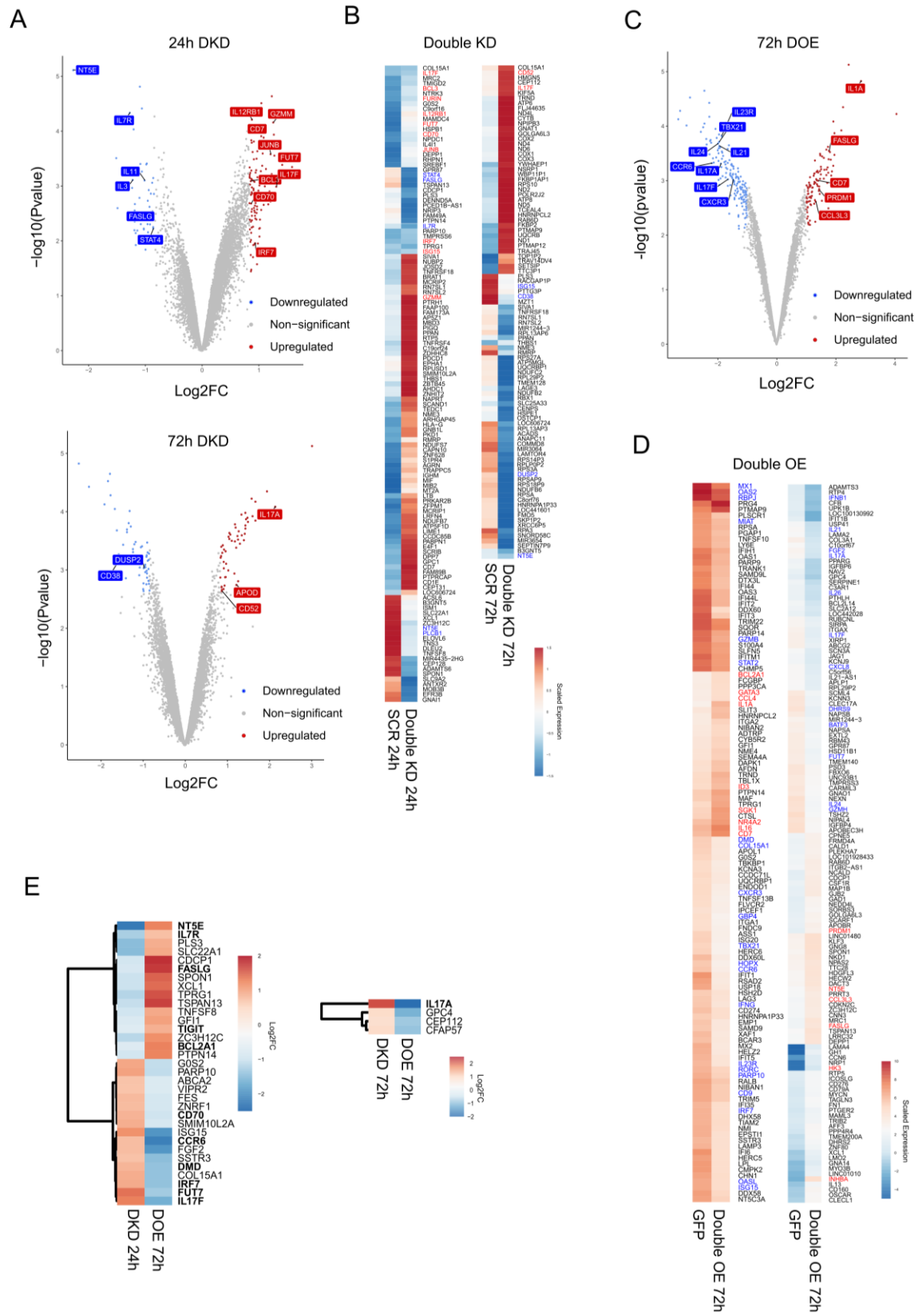


Figure 4.3.5(II) Genes altered upon co-depletion and dual over-expression of FOSL1 and FOSL2 A. Genome-wide expression analysis of human Th17 cells simultaneously silenced for FOSL1 and FOSL2. Volcano plot of Log2 fold-change (x-axis) versus Log10 p-value (y-axis) highlights Th17-relevant transcripts that are differentially expressed upon Double KD of FOSL1 and FOSL2 at 24h (above figure) and 72h (below figure) of differentiation. Red dots indicate up-

regulated genes and blue dots indicate downregulated genes with FDR≤0.1 and |fold change|≥1.8. **B.** Heatmap shows top DE gene targets (FDR≤0.1, |fold change|≥1.8) upon simultaneous silencing of FOSL1 and FOSL2 at 24h (left panel) and 72h (right panel) of Th17 polarization. Scaled expression values are plotted and genes with Th17-relevance are highlighted (Upregulated in red and downregulated in blue). **C.** Volcano plot highlighting Th17-relevant, differentially-expressed (DE) genes at 72h of differentiation upon double over-expression of FOSL1 and FOSL2. Red dots indicate up-regulated and blue dots indicate down-regulated genes with an FDR≤0.1, |fold change|≥1.8. **D.** Heatmap showing scaled expression values for top DE genes (FDR≤0.1, |fold change|≥1.8) in FOSL Double OE Th17 cells at 72h. Targets with Th17-relevance are highlighted in red (up-regulated) and blue (down-regulated). **E.** Comparative analysis of oppositely-regulated DE transcripts in FOSL double knockdown (DKD) and FOSL double over-expression (DOE) Th17 cells. Heatmap depicts DE genes with antagonistic expression profiles in 72h FOSL over-expressed versus 24h/72h FOSL-silenced Th17-polarized cells. Genes with Th17-relevance are highlighted; upregulated in red and downregulated in blue.

4.3.6 Experimental validation of FOSL1 and FOSL2 transcriptional targets in human Th17 cells

Among the genes that were oppositely regulated in Double KD and Double OE Th17 cells, we successfully validated the expression changes for CCR6 at the protein level, using flow-cytometry analysis (Fig 4.3.6A). We additionally confirmed the synergistic changes for NT5E, STAT4, CD70, APOD and JUNB at the protein level, using Single and Double KD Th17 cells (Fig 4.3.6B&C). NT5E/CD73 is an ectonucleotidase that catalyses the breakdown of AMP into free Adenosine, which is known to have anti-inflammatory effects [128]. Importance of NT5E in resolution of inflammation has been well-demonstrated in both human and mouse [121-123, 129]. Immunoblotting (at 72h) and flow cytometry analysis (at 48h) showed that co-depletion of FOSL1 and FOSL2 significantly reduces NT5E expression in Th17 cells (Fig 4.3.6B&C). This indicates that FOSL function may keep inflammatory responses in check.

We then analysed levels of JUNB and STAT4 at 72h of polarization using immunoblot analysis (Fig 4.3.6C). STAT4 levels were seen to be significantly downregulated in Double KD Th17 cells, indicating a positive correlation. Since STAT4 is a known master-regulator of human Th1 responses, this might indicate a potential mechanism for FOS-like proteins to resist Th17 induction by promoting alternate lineage-development. JUNB is known to be required for murine Th17 differentiation but its human-specific role is yet to be investigated [113, 114]. Loss of FOSL1 and FOSL2 showed upregulated JUNB levels, which could imply an already well-studied functional compensation among these AP-1 members (reviewed in [10, 15]). In a similar fashion, Double KD conditions also showed a significant rise in CD70 and APOD protein levels, both of which are known to positively correlate with Th17 development or associated inflammatory phenotypes [115, 130, 131] [104] (Fig 4.3.6B&C). Importantly, all of the above validated targets showed relatively enhanced changes upon simultaneous silencing (compared to single KD).

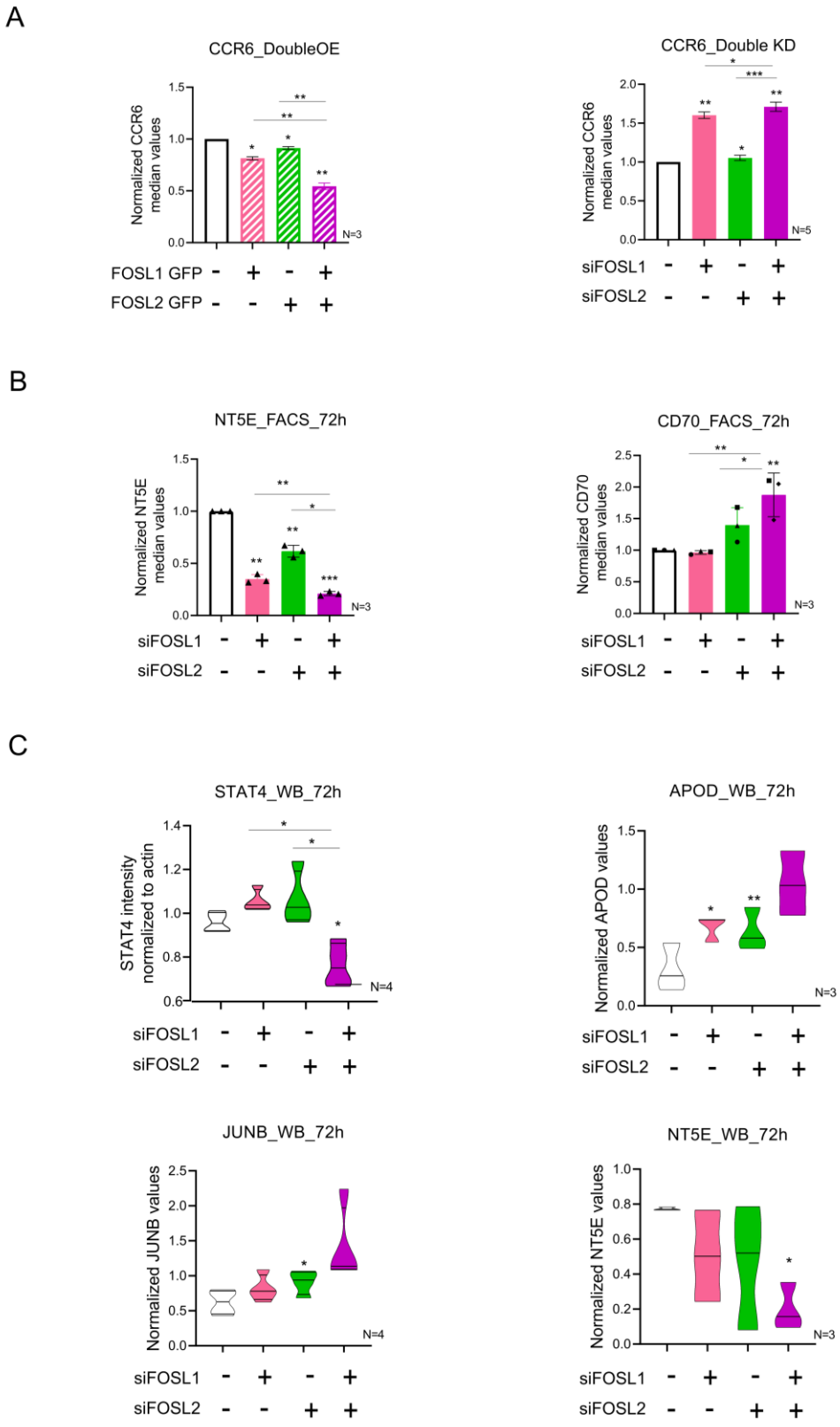


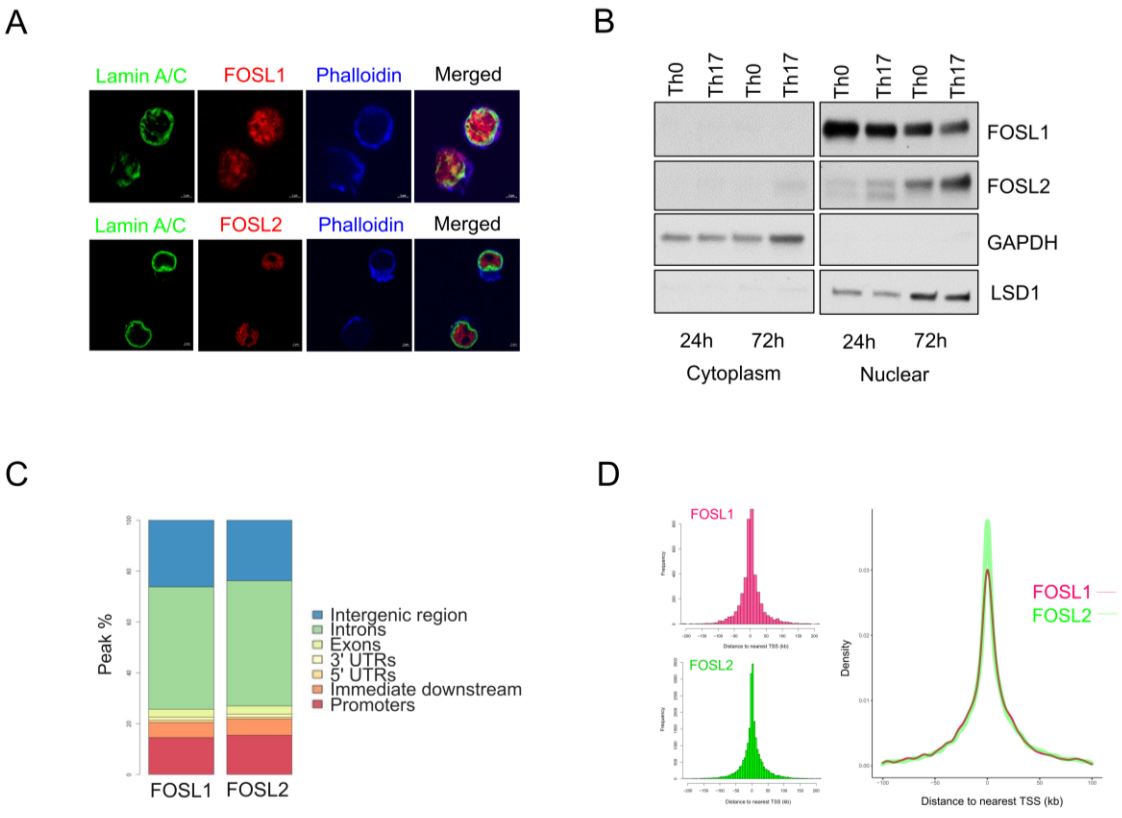
Figure 4.3.6 Validation of RNA-seq targets using immunoblot analysis. A. CCR6 expression was analyzed by flow-cytometry in FOSL single/Double KD cells (right panel) OR FOSL single/Double OE cells (left panel) at 72h of Th17 polarization. Median values for protein expression were normalized to respective control samples (SCR or Empty GFP) and plotted for five biological replicates of KD data and three biological replicates of over-expression data **B.** Flow-cytometry based analysis of NT5E (left) and CD70 (right) protein expression in FOSL-single/double KD cells at 48h and

72h of Th17 differentiation respectively. Median values for protein expression are normalized to SCR and plotted. Data is representative of three biological replicates. **C.** Protein expression for STAT4, NT5E, APOD and JUNB was analysed by immunoblotting. Blots were quantitated using ImageJ and intensity values normalized to actin are plotted in the bar graph. Data is representative of three biological replicates for NT5E & APOD and four biological replicates for JUNB and STAT4. Bar graphs in panel A show mean ± standard error of the mean (SEM) for the respective experiments. Statistical significance has been calculated using two-tailed Student's t test (*p < 0.05; **p < 0.01, **p < 0.001).

4.3.7. Nuclear proteins FOSL1 and FOSL2 show an overlapping profile of genomic occupancy

AP-1 are well-known transcriptional proteins which execute their gene-regulatory roles by occupying associated DNA targets. With a view to elucidate the global occupancy profile of FOSL1 and FOSL2, we performed ChIP-sequencing analysis using 72h Th17 cells. Since these proteins are known to portray cell type-specific cellular localization [132, 133], we initially confirmed their predominant nuclear profile using immunofluorescence and subcellular fractionation methods (Fig 4.3.7A&B). Our ChIP-seq analysis identified 22,127 peaks for FOSL2 and 4,088 peaks for FOSL1 with an IDR significance of <0.01. We found about ~75% of them to occupy intergenic/intronic regions and only about ~15% of them to lie within putative gene promoters (Fig 4.3.7C). We discovered this to be in agreement with previous findings [134-136]. Fig 4B depicts the individual distribution of FOSL1 and FOSL2 binding sites relative to the position of the closest TSS (Fig 4.3.7D, left). Interestingly, an overlay between peak distribution profiles of the two proteins depicted a highly similar trend for their occupancy (Fig 4.3.7D, right). Next, we performed a de-novo motif enrichment analysis to identify the top consensus sequences for their respective binding sites (Fig 4.3.7E). Strikingly, FOSL1 and FOSL2 were enriched as the top two known-motifs for both the ChIP-seq datasets, which highlights their tendency to bind nearly-identical sequences (Fig 4.3.7E).

Earlier studies have demonstrated AP-1/ATF proteins to co-occupy genomic sites in multiple cell-types [22, 137-139]. We also identified an analogous trend in our motif analysis, where JUNB, FOS, ATF3 and BATF were seen to portray a similar DNA-binding profile as FOS-like proteins (Fig 4.3.7E).



E

De-novo enriched motif for FOSL1

Known motifs enriched for FOSL1

FACTOR	MOTIF	P-value
FRA-1		1e-2964
FRA-2		1e-2963
FOS		1e-2960
JUNB		1e-2890
BATF		1e-2795
ATF3		1e-2776

De-novo enriched motif for FOSL2

Known motifs enriched for FOSL2

FACTOR	MOTIF	P-value
FRA-2		1e-11615
FRA-1		1e-11539
FOS		1e-11652
ATF3		1e-11365
JUNB		1e-11350
BATF		1e-11231

Figure 4.3.7. Localization and DNA-binding profile of FOSL1 and FOSL2. A. Immunofluorescence images showing localization of FOSL1 (red, above panel) and FOSL2 (red, below panel) in 72h Th17-polarized cells. Lamin A/C (in green) is used to mark the nuclear periphery whereas Phalloidin (in blue) stains the cytoplasmic actin **B.** Cellular lysates of 24h and 72h cells under Th0 and Th17 culture conditions were fractionated, followed by immunoblotting for FOSL1 and FOSL2. GAPDH and LSD1 are used as markers for cytoplasmic and nuclear fractions respectively. Data for three biological replicates are shown. **C.** ChIP-seq analysis of FOSL1 and FOSL2 using 72h cultured Th17 cells. Bar plot depicts stacked peak-annotation results for binding sites of FOSL1 and FOSL2 **D.** Figures on the left show distribution of FOSL1 and FOSL2 binding sites relative to the position of the closest transcription start site (TSS). TSS is defined to be at position zero. The adjoining figure on the right is an overlay plot comparing their individual profiles. **E.** Peaks bound by FOSL1 (left) and FOSL2 (right) were analyzed for enrichment of known motifs. Top six motifs identified using 'Homer known motif enrichment analysis' have been shown. Peaks with IDR p<0.01 were used for motif discovery.

4.3.8 FOSL1 and FOSL2 share occupancy on many of their cooperatively regulated targets that have relevance to Th17-function

Studies by Tolza et al. have previously shown FOS-like proteins to co-bind some of their target genes in human breast cancer cell-lines [106]. We wished to investigate this paradigm in human Th17 cells, particularly in the synergistic context. We began with determining the intersection between their individual ChIP peaks and discovered 3,711 binding sites to be common between the two proteins (with an overlap of 200 bases or more) (Fig 4.3.8A). We then gene-annotated the common sites (to the nearest TSS) and evaluated how many of them were synergistically regulated in our transcriptional analysis. Interestingly, 148 and 153 of these shared genomic sites were also found to be significantly altered in Double KD (24h or 72h) and Double OE Th17 cells. These were identified as the directly-bound synergistic targets of FOSL1 and FOSL2. Amongst them were positively-regulated genes such as *IL7R, JAK2, BCL2A1, FASLG, PRDM1, PLCB1* and *DPP4* as well as negatively-regulated candidates such as *FURIN, CXCR3, MIAT, IL24, ETV6, IL17F, FOSB* and *ROR1. SGK1* and *RBPJ*, which have previously been established as drivers of murine Th17 pathogenicity [140, 141], were also seen to be occupied and cooperatively-regulated by FOSL1 and FOSL2. This implies a possible role of these TFs in influencing human Th17 pathogenicity, which is still a poorly characterized area.

Notably, among the DE targets in Double KD and Double OE, we respectively detected 50 and 42 such genes, which showed shared occupancy of the two proteins over putative-promoter regions (which was defined as a 5 kb window around TSS). Volcano plots in Fig 4.3.8A, underline some of these targets (in yellow). However, we noticed that most of the synergistically-regulated genes were commonly-bound over intergenic or intronic regions (distal to promoters). Such pronounced co-occupancy of AP-1 family members on non-promoter sites has previously been shown to characterize enhancer activity that drives lineage-specification [135, 139]. IGV snapshots in Fig 4.3.8B depict a significant occupancy of both these proteins on some of their cooperatively-regulated targets that have Th17-relevance (*JAK2, IL7R, MIAT, SGK1, IL17A, IL17F, ZAP70*).

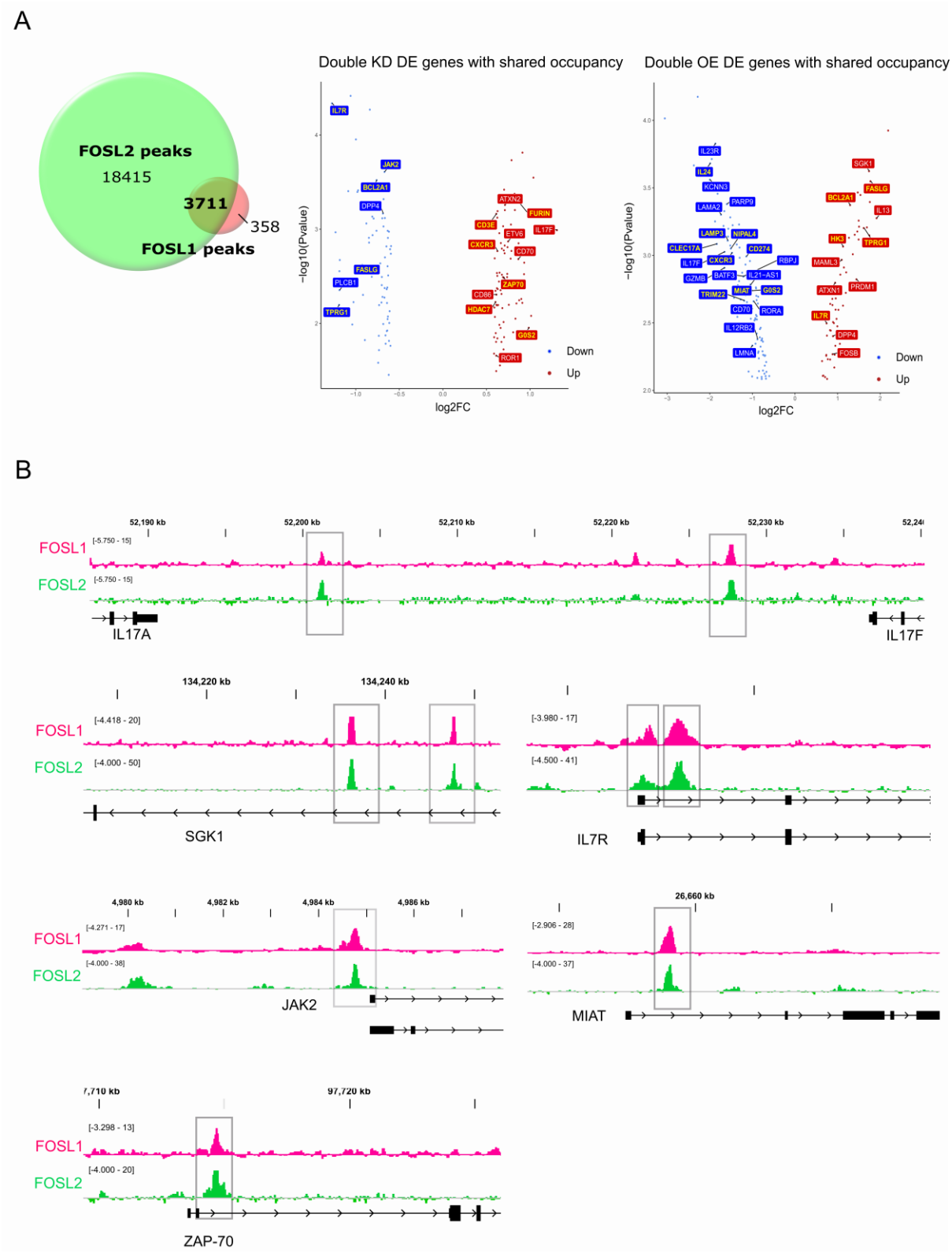


Figure 4.3.8. Directly-bound synergistic targets of FOSL1 and FOSL2. A. ChIP-peak Anno was used to determine the overlap between genome binding sites of FOSL1 and FOSL2. An overlap window of 200bp or more was used to define the shared gene targets (Peaks were annotated to the nearest gene TSS). Venn diagram shows intersection between FOSL1 and FOSL2 bound-genes. In the neighboring volcano plots, the shared genome-bound targets showing altered expression in RNA-seq under conditions of FOSL DKD (left) or FOSL DOE (right) have been shown. These were considered as shared direct targets (DE genes with FDR≤0.1, |fold change|≥1.5). Blue represents the

downregulated and red represents the upregulated genes. Further, direct targets showing shared genomic occupancy of FOSL1 and FOSL2 within 5kb around gene TSS have been highlighted in yellow **B.** Integrative Genomics Viewer (IGV) snapshots showing FOSL1 and FOSL2 occupancy over some of their shared direct targets that have known-relevance to Th17 function.

4.3.9 Interacting partners of FOSL1 and FOSL2 indicate shared and exclusive complexes for transcriptional regulation

AP-1/ATF proteins, regardless of their propensity to bind similar DNA sequences, are known to perform non-redundant roles [22, 137]. This functional diversity has been largely attributed to their dynamic interactome, which appears to be context-specific. It is widely established that FOSL1 and FOSL2, due to lack of a transactivation domain, need to heterodimerize with JUN proteins in order to influence expression of their gene-targets. Since these proteins occupy DNA as a dimer, their individual regulatory abilities are largely influenced by the interacting partner [138]. Interestingly, transcriptional synergy has also been known to be mediated through cooperative interactions between proteins within the same regulatory complex [142]. Although functions of FOS-like proteins have been extensively studied, their binding partners in the context of T helper cell differentiation have not been explored. In order to have a comprehensive understanding of their synergistic influence on Th17 responses, it is instrumental to identify their interactome.

To address this, we immunoprecipitated FOS-like proteins from 72h Th17 cells and analysed their binding partners using tools of Mass spectrometry (MS). Our MS analysis revealed a total of 173 and 77 interactors for FOSL1 and FOSL2 respectively. Heatmaps in Figures 4.3.9B&C show significance scores for enrichment of these associating proteins. We created Cytoscape networks for the interactomes and mapped them against the STRING database in order to visualise the functional relatedness between the detected candidates (Fig 4.3.9A). Interestingly, our analysis showed no interaction between FOSL1 and FOSL2. Though their physical association has been reported in lower organisms like yeast [133], we failed to find any immunological studies claiming the same, thereby supporting our results.

Strikingly, our MS profiling identified a total of 36 proteins (Fig 4.3.9D) to commonly bind to FOSL1 and FOSL2 and this included Th17-linked molecules such as JUNB [60, 113, 114], SIRT-1 [143], RUNX1[144], IFI16 [145] and EIF4E [146]. (The other

common-identifications have been listed in Figure 4.3.10A). On the other hand, even among the exclusive interactors, we found many candidates with known-implications in Th17 development and inflammatory phenotypes. These included COL1A1 [147], SMARCE1 [148], TRIM21 [149] and HDAC2 [150] for FOSL1 and MYO1D, CD48 [151], JUND, and JUN for FOSL2.

FOS-JUN interactions are one of the most widely occurring protein-protein associations which have been identified across cell types. Amid members of the JUN family, JUNB was discovered among the most significant binding partners for both FOSL1 and FOSL2. Importance of JUNB in driving murine Th17 differentiation and controlling alternative T-helper lineages has already been established [113, 114]. Further included in the shared list were RUNX1 and SIRT-1, both of which are known to execute Th17-associated functions. Interestingly, role of RUNX1 in modulating the lineage has already been attributed to its differential interactome. While its association with RORγT supports IL-17 transcription, its binding to FOXP3 is known to have a negative influence on the cytokine [144]. A similar interaction-dependent mechanism has been reported for SIRT-1, whereby its physical association with RORγT is known to deacetylate the master regulator and enhance its ability to induce Th17 responses [143]. These findings thus indicate a possible mechanism for FOS-like proteins to restrain Th17 differentiation by sequestering or altering the activity of binding partners, that are known to support the lineage.

It appears that the concordant and discordant functions of FOS and its interacting proteins, are dictated by their ability to associate with different TFs. This is in agreement with previous observations in the field.

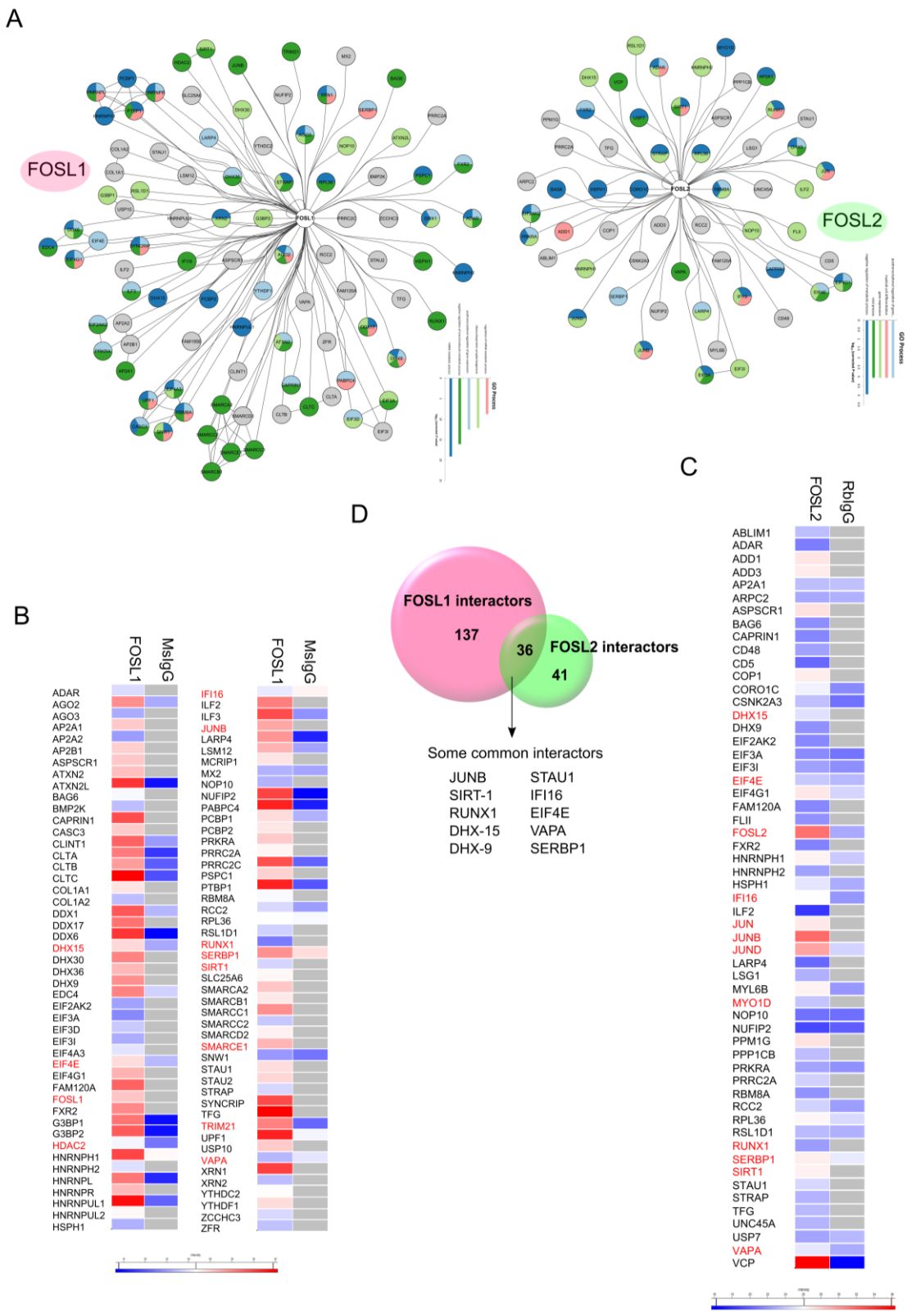


Figure 4.3.9. MS-based identification of FOSL1 and FOSL2 interactors in human Th17 cells. A. Immunoprecipitation followed by tandem mass spectrometry was used to characterize protein complexes of FOSL1 and FOSL2 in 72h Th17 cells. Cytoscape network of FOSL1 and FOSL2 interactome was created and mapped against the STRING database. The nodular colour represents the enrichment of GO process (FDR <0.05). **B&C.** Interacting partners for FOSL1 or FOSL2 are represented in the heatmap. Z-scores for interactors are plotted **D.** Venn diagram depicts the common

and exclusive interactors of FOSL1 and FOSL2. Some key Th17-relevant and other proteins which constitute a part of the shared interactome have been highlighted.

4.3.10 Validation of FOSL1 and FOSL2 binding partners in human Th17 cells

We experimentally validated the shared interactions of FOSL with JUNB, RUNX1, JUN and SIRT-1, using immunoprecipitation followed by immunoblotting (Fig 4.3.10B&C). Though JUN was detected only for FOSL2 in our MS analysis, our western blot results indicated it as a common partner. Targeted mass-spectrometry has been extensively utilized for confirming MS-based identifications. We employed a similar technique called Parallel Reaction Monitoring (PRM) (Fig 4.3.10D), to authenticate the other binding partners which were either shared (VAPA, EIF4E), or exclusive to FOSL1 (COL1A1, COL1A2, SMARCE1) and FOSL2 (SERBP1, DHX15, MYO1D).

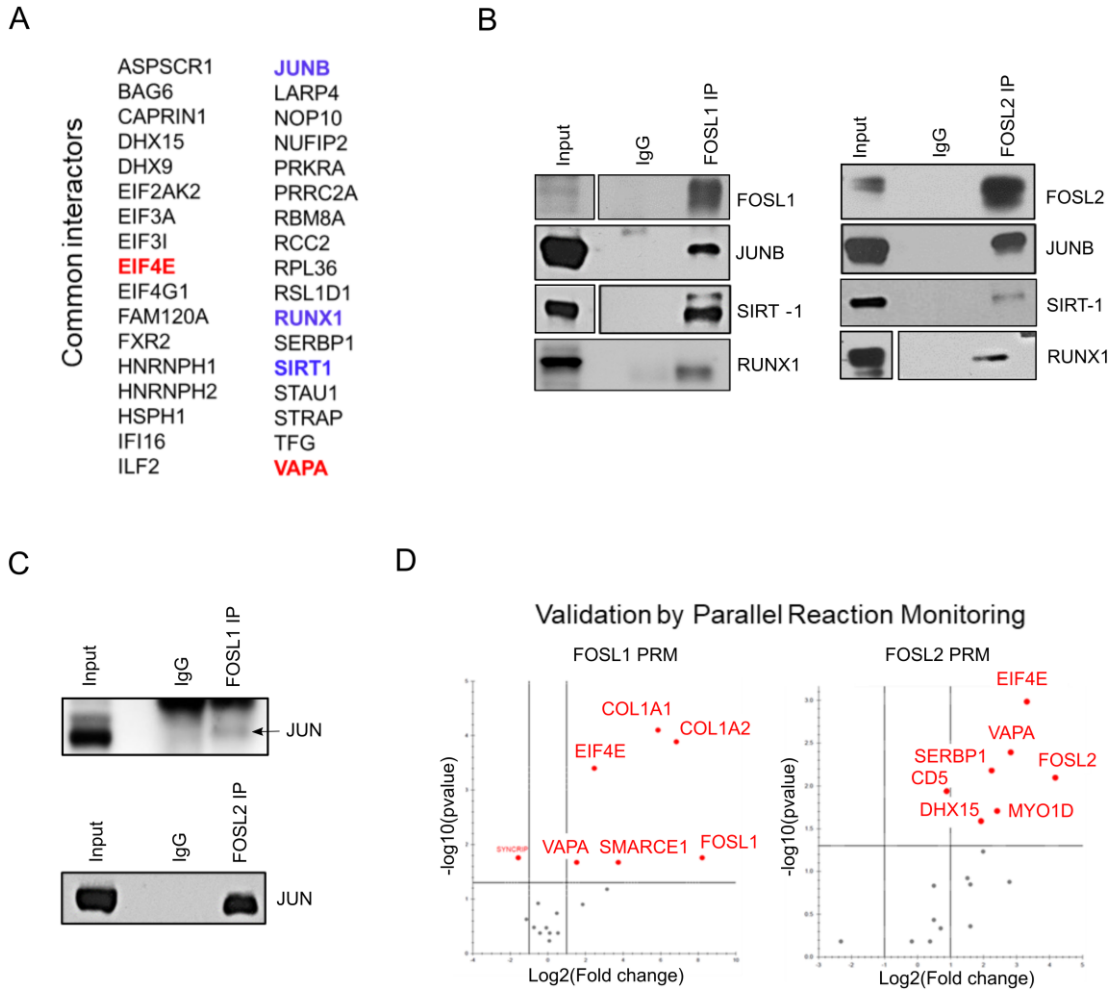


Figure 4.3.10. Validation of binding partners for FOS-like proteins. A. Complete list of common interactors for FOSL1 and FOSL2. The validated targets have been highlighted in blue **B&C.** Immunoprecipitation followed by western blotting was used to experimentally validate JUNB, RUNX1, JUN and SIRT-1 as common interactors of FOSL1 (left panel) and FOSL2 (right panel). Immunoblot analysis to confirm pull-down of FOSL1 and FOSL2 is also shown. Data is representative of three biological replicates. **D.** Parallel Reaction monitoring (PRM) analysis for additional validation of the FOSL interactome. Volcano plots depict the proteins interacting with FOSL1 or FOSL2 with significance.

4.4. Discussion

FOS-like proteins have been extensively studied as regulators of cancer signaling but their involvement in T-helper cell differentiation has been addressed only lately. Murine reports by Ciofani *et al*. and Moon *et al*. have shown that FOSL1 and FOSL2 oppositely regulate Th17-fate in mouse [58, 60]. However, based on our findings, we discover that the human counterpart reveals a different picture. While our FOSL2 findings are in agreement with those in mouse, our results for FOSL1 indicate a species-specific role. Such discrepancies in gene-function between human and mouse Th17 cells have

previously been published for molecules like SATB1 [104], Aryl Hydrocarbon Receptor (AHR) [152, 175] and PRDM1 [119, 153]. By utilizing a dual-strategy of siRNA-based silencing and RNA-based over-expression, our study conclusively proves that both FOSL1 and FOSL2 negatively influence early stages of human Th17 differentiation.

Mechanistic interplay among different members of the AP-1/ATF superfamily has been shown to exist in multiple cell types [10]. Our study reveals that changes in individual levels of FOSL1 or FOSL2, quite limitedly alters the early differentiation events in focus, potentially owing to a known-functional compensation (reviewed in [10, 24]). Hence, we invested in examining whether a perturbation of these proteins in parallel, could more strikingly influence lineage establishment. Previous studies on cancer cells have tried to explore the effects of simultaneously knocking down FOSL1 and FOSL2 on gene-expression [106]. However, a functional synergy between them has not yet been reported. Our study is the first one to employ co-depletion and dual over-expression strategies to combinatorially demonstrate an evident cooperativity between transcriptional abilities of FOSL1 and FOSL2 in human Th17 cells. Apart from our initial results on IL-17 expression, our RNA-seq analysis highlighted a distinct synergism between FOS-like proteins in regulating key Th17 marker genes (*IL17A, IL17F, CCR6 and IL23R*). We also identified a cooperative control of these TFs over many other candidates that have known functions in human Th17 responses (*IL12RB1 [116], PRDM1 [119], CD70 [115, 130], NT5E [122, 123]*). This established the fact that FOSL1 and FOSL2 jointly instruct the transcriptional initiation program for human Th17 cells.

While focusing on the transcriptional targets identified for dual-perturbation conditions, we observed important changes in multiple genes (*FUT7, DUSP2, IL7R, FURIN, ID3, FGF2, and BCL3*), whose role in human Th17 differentiation has not been determined. Nevertheless, we noticed that their functions in the murine counterpart have been studied and these appeared to be in support of our hypothesis. Among these, we found DUSP2, which is a known STAT3-phosphatase and murine Th17-inhibitor, to be positively regulated by FOS-like proteins [120]. A similar profile was identified for ID3, another known repressor of Th17 responses in mouse [154]. We also discovered a positive influence on TIGIT, whose expression in Treg cells has been shown to inhibit pro-inflammatory reactions [155]. On a parallel note, BCL3, which is reported to promote inflammation by restricting Th1 conversion to protective Th17, was observed

to be negatively influenced by FOSL1 and FOSL2 [156]. Likewise, FGF2, which synergistically acts with IL-17A to promote EAE responses, was also seen to be cooperatively inhibited [157]. In fact, we noticed multiple molecules associated with murine Th17 pathogenicity (*CCL3, GZMB, IL7R, IL23R, SGK1, RBPJ, IFN-γ, NR4A2, TBX21, IFNB1, IL24 and STAT4*) to be significantly altered in our study [158]. Concurrently, we observed a suppressive effect on genes such as *OAS2, MX1 and ISG15*, which have been associated with development of Rheumatoid arthritis in humans [147]. *ISG15*, in fact showed opposing expression changes at 24 and 72h, thereby highlighting a temporal effect. We also witnessed FOSL-mediated repression of the *IL-26* cytokine which is a marker of highly differentiated human Th17 cells [159, 160]. These findings thus implicate a potential role of FOSL proteins in tweaking the inflammatory and homeostatic balance, for early-differentiating Th17 populations.

Many receptors and ligands involved in cellular migration *(CCL3L3, CCL4, CXCL8, FUT7, CXCR3, CCR6*) were seen to be altered for their expression in our transcriptome analysis. We particularly observed a negative influence on *FUT7*, a selectin-ligand known to suppress skin-inflammation by promoting Treg recruitment [161]. However, further investigation would be required to determine whether FOS-like proteins truly influence migration of regulatory T-cells during inflammatory conditions Another chemokine ligand CCL4, which has previously been implicated in development of MS disease, was also found to be upregulated upon dual-overexpression of FOSL1 and FOSL2 [162]. Addedly, we identified interesting FOSL-dependent changes in transcription of multiple non-coding RNAs (ncRNAs) (MIR4435-2HG, MIR3654, MIR3064, MIR1244-3, LINC01010, LINC01480, MIR1244-3, MIAT). Since AP-1 mediated regulation of ncRNAs is relatively unexplored, it would be worth deciphering whether they collectively influence the Th17-initiation circuit. Apart from these targets, our RNA-seq analysis importantly discovered 37 genes which showed opposing changes between FOSL loss-of-function and gain-of-function conditions. Since multiple of them were directly associated with Th17 responses (*IL17A, IL17F, NT5E, CD70, CCR6, IL7R, IRF7*), it strengthens the idea of FOS-like proteins significantly orchestrating the early transcriptional program for Th17 fate.

Though previous studies have holistically proposed near-identical DNA-binding sequences for AP-1 family members, none of them have compared the individual occupancy of FOSL1 and FOSL2, particularly in the context of T-cell differentiation.

Our ChIP-seq analysis showed an extensive overlap in the binding sites of these two TFs, which could possibly suggest a mechanistic basis for their functional coordination. However, the co-occupancy of FOSL1 and FOSL2 at these regions needs to be confirmed by performing chromatin immunoprecipitation in sorted or uniformly-polarized Th17 cells. This would rule out the probability of an overlap arising due to occupancy of these proteins on common sites, but within different sub-populations. It is crucial to acknowledge this, knowing the widely established asynchrony that exists for in-vitro differentiated cultures.

Integrating our ChIP-seq and RNA-seq analysis, we observed a significant fraction of the Double KD or Double OE targets (~150 genes each) to be commonly-bound by FOSL1 and FOSL2. This evidently portrays a direct and well-coordinated control over their transcriptional targets. Consistent with earlier findings in the field, less than one third of the shared-direct targets were occupied around TSS regions and most others were predominantly bound within intronic/intergenic sites [106, 139]. These included well-studied Th17 genes such as *IL17F, PRDM1, RORA, SGK1, IL23R, RBPJ, BATF3* and others. Importantly, the intergenic region between *IL17A*/*IL17F locus* showed multiple sites which were commonly-bound by FOSL1 and FOSL2. This appears to be a conserved regulatory paradigm, since genomic elements amid the murine *Il17a/Il17f* locus also demonstrate overlapping AP-1 occupancy (*Junb, Jund, Batf, Fosl2 and Irf4*) [113].

Further, PRDM1, which is a known-inhibitor of human Th17 responses, was seen to be directly-bound and positively regulated by FOSL1 and FOSL2 [119]. This is consistent with previous findings in other cell-types [107, 163]. In fact, in human lung cancer cells, c-JUN has been found to cooperatively regulate *PRDM1* levels along with FOSL1 and FOSL2. Such coordination might be executed via the two conventional AP-1 binding sites that have been identified in the vicinity of the *BLIMP1/PRDM1* promoter, potentially facilitating co-binding [164]. Further experiments are required to determine if other FOS/JUN/ATF TFs are involved in the synergistic control of PRDM1 in human Th17 cells.

Among the other shared-direct targets, we discovered *FASLG, BCL2A1, NIPAL4, CLEC17A, IL7R, JAK2, DPP4, CXCR3, MIAT, ZAP-70, HK3* and others. Analysing many of these for their previously-established roles, delivered useful inferences in

favour of our hypothesis. For instance, BCL2A1, a known repressor of murine Th17 autoimmunity was found to be positively regulated by FOS-like proteins. On a parallel note, we found FOSL mediated-suppression of the *Zap-70* gene, which is a key player of T-cell activation/apoptosis and is known to drive arthritis development in mouse [165]. However, expression changes for a few of these targets assigned a more convoluted angle to FOSL function. For e.g. JAK2, which has been shown to be required for development of Th17-mediated autoimmunity, appeared to be directly induced by FOSL proteins [166].

A tendency to predominantly bind intergenic/intronic sites has previously indicated enhancer-based regulation for multiple members of the AP-1/ATF superfamily [135]. This includes a recent study that shows how AP-1 proteins imprint the enhancer landscape for cellular senescence [139]. It would be interesting to investigate whether a similar scenario exists for early-differentiating human Th17 cells. In light of this, it would be crucial to address the regulatory nature of the binding regions common to FOSL1 and FOSL2. Analysing the presence of enhancer-associated histone modifications (H3K27ac and H3K4me1), in combination with information on the chromatin-accessibility of the underlying region, would be an important study to follow-up. Our motif analysis for FOSL1 and FOSL2 bound-sites revealed enrichment of other AP-1/ATF factors (JUNB, FOS, BATF and ATF3). Of these, BATF and FOSL2 have already been shown to compete for occupancy on Th17-relevant targets and antagonistically regulate their expression in murine Th17 cells. However, the exact role of BATF in human Th17 differentiation has not been reported. It would be worthwhile to determine whether such functional competition also exists in the human counterpart of the lineage.

JUNB has been shown to positively regulate murine Th17 differentiation [114], however, its involvement in human-specific responses has not been studied. Noteworthily, JUNB appeared to functionally associate with FOSL1 and FOSL2 in multiple parts of our study. Firstly, loss of FOSL proteins upregulated JUNB levels. They were also seen to potentially occupy similar genomic sequences. This could either suggest an antagonistic equation or a well-coordinated compensatory mechanism between them. Additionally, our MS-analysis revealed JUNB among the top interacting partners for both FOSL1 and FOSL2, consistent with previous reports in the field (reviewed in [10, 15]). These findings clearly suggest a distinct molecular

interplay between the mentioned FOS and JUN family members, during early stages of human Th17 differentiation. However, a thorough investigation is needed for a better understanding on their interrelated nature.

Besides JUNB, FOSL interactomes showed many other common binding partners with Th17-relevance (JUNB, SIRT-1, RUNX-1, EIF4E). However, since FOSL1 and FOSL2 were not seen to interact with each other, this could indicate them to be a part of different regulatory modules with overlapping members. Hence, further experiments are needed to determine exactly how these shared binding partners could contribute to their transcriptional synergy. Another interesting observation from our MS profiling was the reproducible association of FOS-like proteins with multiple collagen-synthesis genes, some of which were also seen as targets in our RNA-seq data (COL15A1, COL1A1, COL1A2, COL3A1). This is intuitive owing to the well-established role of these factors in osteoclast development, which requires a significant involvement of bone matrix components like collagen [167-169]. More importantly, changes in collagen protein levels are known to have direct implications in rheumatoid arthritis and osteoarthritis [170, 171]. This might insinuate a role of FOSL proteins in dictating autoimmune-associated inflammatory phenotypes in humans. We propose that our MS-based analysis could add an important resource to the field since it is the first one to comprehensively compare interactions of FOSL1 and FOSL2 in human T cells.

Apart from binding partners, we also assessed for post translational modifications of FOS-like proteins, using tools of LC/MS-MS. This seemed important because multiple oncogenic studies have already reported a strong influence of PTMs like phosphorylation on FOSL transcriptional activity [29, 32]. For FOSL2 in particular, we discovered about 13 sites where serine/threonine residues were phosphorylated. Since PTM profiling of these proteins and their influence on T cell differentiation has not been addressed so far, investigating more on the functional relevance of the identified modifications could open a whole new area of research for human Th17 signaling.

Role of FOSL2 as a regulator of murine T-helper cell plasticity has been previously reported [58]. On similar lines, perturbing FOSL1 and FOSL2 in our human Th17 study significantly altered genes important for Th1, Th2 and Treg lineages (including *TBX21, STAT4, GATA3, IFNG, BATF3, GZMB and IL-13*). We particularly identified a FOSL-

dependent increase in levels of the Th2-promoting factors, GATA3 and IL3 [174], which indicates that these AP-1 proteins could potentially drive Th2 responses. In support of this, we also witnessed FOSL-mediated inhibition of FURIN levels, a proprotein convertase that has been shown to constrain Th2 differentiation [173]. Nonetheless, it is crucial to further dissect these findings in order to elucidate if FOS-like proteins truly restrict Th17 fate by contextually promoting alternate ones. Strikingly, *IL3* was one of the very few candidates that was seen to be oppositely regulated by FOSL1 and FOSL2, as individual factors. This potentially indicates differential functions of these AP-1 members as single and jointly co-ordinating proteins.

Elevated levels of FOSL1 have been detected in multiple human autoimmune conditions [38, 50, 51] [172] [49]. Although some of these reports suggest a positive correlation, we parallelly found studies indicating its negative influence on inflammatory phenotypes, which supports our hypothesis [48] [50]. Towards that end, we found findings which demonstrate that over-expression of FOSL1 in epithelial cells upregulates SOCS3, which is a known-inhibitor of STAT3 [49]. Similar conflicts with STAT3 function were also witnessed in our RNA-seq analysis, where FOSL1 and FOSL2 appeared to cooperatively-inhibit known STAT3-induced genes (*CXCR5, HOPX, IL23R, IL24, CCR6, FNDC9, GZMB*). This underlines their participation in restricting STAT3-mediated Th17 signaling.

Despite being a repressor of murine Th17 function, FOSL2 has been shown to positively regulate expression of molecules required for survival and maintenance of the lineage. This implies functional complexity [58]. A similar situation might be true for the human counterpart where FOSL levels are seen to be increased upon induction of human Th17 differentiation, in a STAT3-dependent manner, regardless of their inhibitory effect on the corresponding effector responses [101]. This provides evidence for a more tangled interplay between the ‘supporters’ and the ‘suppressors’ of the lineage. Furthermore, we witnessed that FOS-like proteins synergistically inhibited *IL24,* a known repressor of Th17, used for autoimmune therapy [127]. This conflicts their negative influence on Th17-transcriptional circuits and may suggest a more contextual involvement in the process. Addressing this front would be crucial for thoroughly assessing their immunotherapeutic potential.

We acknowledge that our findings contradict the results by Moon *et al*., where FOSL1 was seen to have a positive effect on human IL-17 expression [60]. The authors of this study have used human peripheral blood (PB) as a source of naïve CD4 cells, as against umbilical cord blood (UCB) that was used in our experiments. We propose that this conflict could potentially evolve from important differences that are known to exist for UCB versus PB immune cells (reviewed in [66, 68]). Our major reason for resorting to the use of UCB, was the significantly higher fraction of CD45RA+ T cells obtained, which also exhibit better 'naivety' and lesser cytotoxicity than the ones from PB, which is crucial for studying in vitro differentiation responses [176]. Further, the published study utilized viral vector-based gene perturbation strategies, which are known to cause undesirable effects on T-cell phenotypes (reviewed in [62, 63]). As against this, our study employs a non-viral approach and provides a more confirmative verdict by using both siRNA-based depletion and IVT RNA based over-expression of FOSL1 and FOSL2. Our RNA-seq and ChIP-seq datasets further highlight a significant number of targets that evidently validate our hypothesis, as against the published report, which merely quotes effects on cytokine expression.

Th17 plasticity in humans has not been very well understood. Though a number of murine studies have tried to examine the molecular network that supports the transition from homeostatic-to-pathogenic Th17 phenotype, this switch is still inadequately studied for cells of human origin. Our Ingenuity pathway analysis discovered that many of the FOSL-affected genes are involved in autoimmunity-causing Th17 signaling. A comprehensive study is required to further ascertain whether FOS proteins along with JUN and other AP-1/ATF members dictate the phenotypic switch for Th17-mediated inflammation in human Th17 cells. This could provide critical insights into the immunotherapeutic potential of AP-1 proteins.

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Chapter 5

Human BATF and FOS-like proteins contrastingly regulate factors defining the Th17 lineage

5.1 Introduction

FOS, JUN and ATF/CREB families together represent the AP-1/ATF superfamily of BZIP proteins. Members of this superfamily are characterized by highly basic, leucine zipper domains which mediate dimerization and DNA-binding function. AP-1 and ATF-like proteins have also been shown to heterodimerize with each other and regulate gene-expression in a promoter-specific and tissue-specific manner [1]. An important member of the AP-1/ATF superfamily is - BATF (Basic leucine zipper, ATF like), which is known to be a crucial regulator of lymphocyte differentiation [2].

Human BATF is a nuclear protein consisting of 125 amino acids with a serine-rich N-terminal region and a BZIP domain [3]. Lack of a transactivation domain makes it obligatory for BATF to dimerize with other proteins, in order to mediate its role as a transcriptional regulator [3]. BATF molecules are incapable of homo-dimerization and majorly heterodimerize with JUN proteins to regulate consensus AP-1 targets. Previously, BATF was known to act as a repressor of AP-1 transcription [4-11]. However recent studies on T cells contradict these findings, thereby indicating a contextual involvement of this protein in AP-1 regulation [12, 13]. Interestingly, BATF appears to be differentially expressed in different cell types, with the most abundant levels detected in lymphoid populations, particularly in B cell lymphomas. Further, role of BATF as a transcriptional regulator has been well-established in biological processes like self-renewal of HSCs [14], B-cell IgG class-switching [6], $CD8^+$ effector responses [15] and T-helper cell activation and differentiation [2, 7, 16]. Amongst these, the most profound influence of BATF function has been detected in development and cytokine secretion of T helper cell subsets.

CD4 T lymphocytes subjected to conditions of TCR activation along with specific polarizing-cytokines, undergo distinguished transcriptional programs and develop into

effector T cell subsets such as Th1, Th2, Th17, Th9, Tfh and Treg. Cytokines profiles for each of these subsets are distinct and are known to perform specialized immune functions. Role of BATF in regulation of T-helper cell responses is largely governed by the type of differentiation subset. Transcriptional profiling of murine Th0, Th1 and Th2 cells, has revealed a significant upregulation of BATF expression in these subsets. However, its role in Th1 and Th2 effector responses has been found to be dispensable [7]. Further, BATF has been shown to positively regulate development of Tfh and Th9 cells by regulating genes like Bcl-6, c-Maf and IL-9 [2, 6, 17]. Interestingly, elevated levels of BATF mRNA have also been detected during early stages of mouse and human Th17 differentiation [7, 18]. Studies using mouse models have helped in efficient characterization of BATF function in Th17 lineage-establishment.

5.1.2 Role of BATF in regulation of Th17 differentiation

Protective immune responses at mucosal barriers as well development of autoimmune diseases fundamentally require the function of Th17 cells. Pro-inflammatory cytokines like IL-17A, IL-17F, IL-21, IL-22 and GM-CSF dictate immunoprotective and immunopathogenic features of these cells. STAT3 acts as a master regulator of Th17 differentiation and has been shown to occupy *BATF* promoters in both human and mouse [19]. However, STAT3-mediated transcription of the gene has been demonstrated only in human Th17 cells [20]. In 2009, an important study by Schraml *et al*. used gene-deficient mice models to demonstrate a positive and non-redundant role of BATF in Th17 lineage specification. Although development of immune cells was found to be unaltered, *Batf* deficiency seriously impaired T-cell specific IL-17 expression in mice. This was further supported by *in vitro* findings using *Batf*$^{-/-}$ Th17 cultures which showed significantly reduced expression of IL-17 and IL-21 cytokines. Further, loss of BATF caused EAE resistance in mice in a Th17-dependent manner, thus emphasizing its requirement in development of autoimmunity [7]. Interestingly, BATF neither had an effect on proximal signaling of Th17-inducing cytokines (IL-6 and TGF-β), nor did it influence STAT3 expression/activation. This assigns it a relatively downstream role in Th17 lineage-development. Apart from Th17 cells, BATF is also known to promote IL-17 secretion in other cells like iNKT cells, thereby suggesting a global role in induction of the cytokine [21]. Nevertheless, under conditions of hepatitis

B infection in mice, BATF has been witnessed to act as a suppressor of Th17 cytokine-secretion, thus depicting exceptions to its conventional roles in gene transcription [22].

BATF is also involved in dampening FOXP3 expression and Treg features in Th17-primed cells, further promoting Th17 lineage development [7]. However, its absolute role in murine Tregs is quite the opposite, where it positively regulates Treg responses in association with JUNB and IRF4 [23]. Thus, T-helper lineage genes appear to be contextually regulated by AP-1/ATF family members and multiple supporting reports indicate that this is quite intrinsic to their function (review, [24]). Recently, multiple high-throughput methods have enabled the mechanistic dissection of BATF function in pro-inflammatory signaling responses [16]. The next section elaborates on the dynamic regulatory landscape of Th17 cells in regard to BATF proteins.

5.1.3 Th17 regulatory networks involving BATF and other AP-1/ATF family members

Key Th17 regulators such as RORγT and IRF4 have been shown to functionally synergize with BATF for induction of Th17 responses [12]. In fact, BATF:IRF4 complexes are known to act as 'pioneer factors' that bind to Th17-specific loci and provide chromatin accessibility to lineage-specifying transcriptional proteins like STAT3 and RORγT. However, this activity of regulatory initiation requires ETS1 and CTCF proteins and also occurs in Th0 cells in absence of polarizing cytokines [25]. Thus, it appears to be a general mechanism used by T-helper cells to nucleate lineage-specific regulatory complexes around corresponding effector gene loci, under differential cytokine signaling conditions [12] (review, [26]). However, assigning functional solidarity to these regulatory proteins is not an efficient way of characterizing their roles and a contextual perspective needs to be adopted while determining their involvement in T-helper cell responses. Interestingly, previous reports have confirmed interaction of BATF with multiple AP-1 proteins including c-JUN, JUNB and JUND (review, [4]). BATF/JUN transcriptional activity has previously been shown to be crucial for T-helper responses and development of autoimmune conditions like osteoarthritis [12, 17, 27, 28]. Particularly JUNB has been shown to associate with BATF/IRF4 complexes and positively regulate expression of Th17 cytokine genes, while inhibiting Th1/Treg lineage factors [7, 29]. These targets show a direct

occupancy of both JUNB and BATF on their corresponding promoters or intergenic regions.

Comprehensive analysis of DNA-binding targets for important regulatory proteins in murine Th17 cells, has revealed a significant overlap in genomic occupancy of JUNB, JUND, BATF, IRF4 and FOSL2, especially on Th17-associated gene loci [12]. While JUNB, IRF4 and BATF promote expression of Th17 lineage-genes, FOSL2 and JUND act as their repressors [12, 16, 28]. It is indeed fascinating that members of the same family with such resemblance in their genome binding profiles, regulate Th17 responses differently. For a few members like JUNB and JUND, functional antagonism has been shown to be mediated via competition for genome binding sites. Loss of JUNB enhances JUND occupancy on its previously bound targets and oppositely regulates their expression [12]. Surprisingly, JUNB also serves as a heterodimerization partner for FOSL2 in murine Th17 cells [30]. While both of them commonly suppress alternate lineage cytokines, they exert opposing effects on transcription of murine Th17 effector molecules [16, 28]. Thus, BATF and FOSL2 might be required to differentially associate with other regulatory proteins for mediating their concordant and discordant functions. Based on the above findings, the interplay between AP-1/ATF family proteins in Th17 transcriptional programming seems to be quite complex and needs to be addressed thoroughly. More of the key regulatory proteins that share occupancy on Th17-associated loci need to be characterized for mechanisms that integrate their function in Th17 signaling. Interestingly, a couple of independent studies have found such an overlap for FOSL2 and BATF genomic targets as well [12, 16]. The following section illustrates the activity and binding profile of these AP-1/ATF members in murine Th17 cells.

5.1.4 Instrumentation of murine Th17 responses by FOSL2 and BATF

In 2012, Ciofani *et al*. were the first ones to holistically analyse the function and genome occupancy profile of key murine Th17 regulators [16, 20, 31]. One of the prime findings of the study was the discovery of FOSL2 as a modulator of T-helper cell plasticity. Intriguingly, FOSL2 was shown to negatively regulate Th17 cytokine genes, while promoting factors required for its survival and maintenance. Hence, it appeared to operate antagonistically to BATF, in regard to transcription of Th17-effector

functions [7]. Further, genome-wide occupancy analysis performed in this study, demonstrated a significant overlap in DNA-binding targets of these functionally conflicting proteins. Many of the shared targets appeared to be genes associated with Th1, Th17 and Treg lineages. The authors stated this genomic overlap as co-occupancy of these factors and proposed a binding competition between them for gene targets that they could oppositely regulate. However, it is important to closely evaluate this hypothesis. The ChIP-seq analysis performed in this study utilized non-sorted, *in vitro* differentiated Th17 cells, in case of which, all the cells within the culture could never be homogeneously polarized. This would result in a mixture of differentiated, partially-differentiated and undifferentiated clones. Hence, an overlap between genome-binding sites could also be indicative of these TFs occupying the same targets, but within different subpopulations. Thus, follow-up studies are required to determine whether these AP-1 proteins actually co-occupy Th17-relevant gene targets.

5.1.5 BATF and FOS-like proteins in human Th17 cells

Functional profiling of Th17 regulators has largely been limited to mouse models and thus information pertaining to the regulatory landscape of human cells has been quite limited in the field. During early stages of human Th17 differentiation, both BATF and FOS-like proteins have been found to be significantly upregulated ([18], Figure 4.3.1). Though murine BATF has been widely characterized for its Th17-specific role, none of the studies so far have touched upon its involvement in the human counterpart. Role of human FOSL2 however has been confirmed in Section 4.3, where it appears to act as a negative regulator of Th17-associated genes. Interestingly, it functionally synergizes with its paralog protein FOSL1, in order to orchestrate lineage-specific responses. By performing RNA-seq analysis and protein-level validations for their transcriptional targets, we have further underlined their Th17-repressing abilities.

FOSL1 and BATF have been previously shown to be induced by STAT3 and also share JUNB as a common heterodimerizing partner [20, 28, 32]. However, a direct regulatory link between them has not been established yet. Determining transcriptional targets of human BATF could thus enable a proficient comparison between functional profiles of these proteins. Our genome wide-occupancy analysis for human FOSL1

and FOSL2 shows a high overlap between their binding sites, especially over Th17-effector genes (See Figure 4.3.8). Moreover, our motif analysis for their ChIP peaks identified BATF as one of the top-known motifs, indicating that they might bind to similar DNA sequences (See figure 4.3.7E). Nevertheless, the actual profile for genomic occupancy of human BATF and its correlation to binding sites of FOS-like proteins is yet to be investigated. Against the background of the potential competition proposed between these factors in the murine system [16], it is crucial to investigate whether such a conflicting interplay exists in human Th17 cells as well. Since only a handful of the Th17 regulators inducing the lineage have been characterized in human, a comparative study between these AP-1/ATF proteins could provide critical insights on molecular networks of species-specific Th17 signaling.

5.2 Materials and Methods

5.2.1 Primary Human CD4⁺ T-cell isolation and Th17 culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from the umbilical cord blood of healthy neonates (Turku University Central Hospital, Turku, Finland) by the Ficoll- Paque density gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare). Naïve CD4⁺ T-cells were further purified using CD4⁺ Dynal positive selection beads (Dynal CD4 Positive Isolation Kit; Invitrogen). CD4⁺ T-cells were stimulated with plate-bound α-CD3 (3.75 µg/ml; Immunotech) and soluble α-CD28 (1 µg/mL; Immunotech) in X-vivo 20 serum-free medium (Lonza). X-vivo 20 medium was supplemented with L-glutamine (2 mM, Sigma-Aldrich), and antibiotics (50 U/mL penicillin and 50 µg/mL streptomycin; Sigma-Aldrich). Th17 cell polarization was induced using a cytokine cocktail of IL-6 (20 ng/mL; Roche), IL-1β (10 ng/mL) and TGF-β (10 ng/mL) in the presence of neutralizing anti-IFN-γ (1 µg/mL) and anti-IL-4 (1 µg/mL) antibodies to block Th1 and Th2 differentiation, respectively. For the control cells (Th0), CD4⁺ T-cells were TCR stimulated with α-CD3 and α-CD28 in the presence of neutralizing antibodies without differentiating cytokines and cultured in parallel. All cytokines and neutralizing antibodies used in the study were purchased from R&D Systems unless otherwise stated. All cultures were maintained at 37°C in a humidified atmosphere of 5% (v/v) CO2/air.

5.2.2 siRNA mediated silencing of BATF

$CD4^+$ T-cells from umbilical cord blood were suspended in Opti-mem I (Invitrogen) and transfected with BATF targeting siRNA or non-targeting control siRNA (Sigma) using the nucleofection technique by Lonza. Four million cells were transfected with 6µg of siRNA after which the cells were rested at 37º C for 40-42h in RPMI 1640 medium (Sigma-Aldrich) supplemented with pen/strep, L-glutamine (2 mM) and 10% FCS, and subsequently activated and cultured under Th17 conditions as described above.

For identification of BATF target genes, cells were harvested at 24 and 72h post induction of polarization. Three biological replicates were prepared, each time including BATF targeting siRNA and non-targeting control siRNA. Total RNA was isolated and samples were prepared as described in 5.2.6. siRNA sequences have been provided in Appendix II. A pool of two siRNA's was used for silencing BATF.

5.2.3 Immunoprecipitation

Immunoprecipitation for BATF was performed using Pierce MS-Compatible Magnetic IP Kit (Thermo Fischer, Cat no.90409). 72h Th17 cell culture pellets were lysed in appropriate volumes of kit provided Cell-lysis buffer. All antibodies used were pre-incubated with Protein A/G beads for 4-5h to form bead-Ab complexes. Lysates were first pre-cleared with control IgG-bead complexes for 3h. Pre-cleared lysates were then incubated overnight with BATF (Cell Signaling Tech., Cat no. 8638) Ab-bead complexes. Species-specific control IgG antibodies were used as negative-IP control. Immunoprecipitated protein complexes were washed (following manufacturer's protocol) and further eluted with appropriate volume of elution buffer. Eluted protein was then analysed by immunoblotting using antibodies described in 3.2.9.

5.2.4 Quantitative Real-time PCR

Total RNA was isolated using RNeasy kit (Qiagen Cat No. 74104) which included on-column DNAse treatment. Removal of genomic DNA was further ensured using an additional treatment with Invitrogen DNAseI. cDNA was synthesized with Reverse Transcription kit (Applied Biosystems) using oligo dT primers as per manufacturer's instructions. TaqMan primers and probes were designed with Universal Probe Library

Assay Design Centre (Roche). All Taqman reactions were performed using Absolute QPCR Mix, ROX (Thermo scientific, Cat no. AB1139A). EF1α was used as endogenous control. The qPCR runs were analysed using the 7900HT Fast Real-Time PCR System (Applied Biosystems). Taqman primers and probes are listed in Appendix I.

5.2.5 Western Blotting

Cell culture pellets were lysed using RIPA buffer (Pierce, Cat no. 89901), supplemented with protease and phosphatase inhibitors (Roche) and sonicated using Bioruptor UCD-200 (Diagenode). Sonicated lysates were centrifuged at 14,000 rpm for 20 min at 4°C and supernatants were collected. Samples were estimated for protein concentration (DC Protein Assay; Bio-Rad) and boiled with 6x Laemmli buffer (330 mM Tris-HCl, pH 6.8; 330 mM SDS; 6% β-ME; 170 µM bromophenol blue; 30% glycerol). Samples were loaded on gradient PROTEAN TGX Precast Protein Gels (BioRad, Helsinki, Finland) and transferred to PVDF membranes (Trans-Blot Turbo Transfer Packs, BioRad).

The following antibodies were used for immunoblotting experiments of the study – anti-BATF (Cell Signaling Tech., Cat no. 8638), anti-STAT4 (Cell Signaling, 2653); anti-RORC (Abnova, Cat no. H00006097-D01P) and anti-β-actin (SIGMA, Cat no. A5441). HRP conjugated anti-mouse IgG (SantaCruz, Cat no. sc-2005) and anti-rabbit IgG (BD Pharmingen, Cat no. 554021) were used as secondary antibodies.

List of antibodies used for IP-immunoblotting are as follows. anti-RUNX1 A-2 (Santa Cruz, Cat no. sc-365644); JUNB C-11 (Santa Cruz, Cat no.sc-8051); anti- BATF (CST, Cat no. 8638); anti-SIRT1 (Cell signaling, Cat no.2496); anti-JUN (BD Biosciences, Cat no.610326). Conformation specific Rabbit HRP (Cell Signaling, Cat no.5127) and Mouse HRP (Cell Signaling, Cat no. 58802) were used as secondary antibodies.

5.2.6 Flow Cytometry

CCR6 surface staining was performed 72h after initiation of Th17 culture, for which cells were washed twice with FACS buffer (0.5% FBS/0.1% Na-azide/PBS) and incubated with PE anti-CCR6 antibody (BD Cat no. 559562) for 20 min at 4ºC. Suitable isotype control was maintained. Samples were acquired on LSRII (BD Biosciences,

Franklin Lakes, NJ); live cells were gated based on forward and side scattering. Acquired data was analysed with FlowJo (FLOWJO, LLC).

5.2.7 RNA-seq analysis

I. RNA Isolation and RNA-Seq Sample Preparation

RNA was isolated (RNeasy Mini Kit; QIAGEN, Hilden, Germany) and given on-column DNase treatment (RNase-Free DNase Set; QIAGEN) for 15 min. The removal of genomic DNA was ascertained by an additional treatment of the samples with Invitrogen DNase I. After RNA quantification (using Nanodrop 2000) and quality control (using BioRad Experion or Agilent Bioanalyzer), libraries for RNA-Seq were prepared. The high quality of the libraries was confirmed with Advanced Analytical Fragment Analyzer (Advanced Analytical Technologies, Heidelberg, Germany) or with Agilent Bioanalyzer, and the concentrations of the libraries were quantified with Qubit® Fluorometric Quantitation (Life Technologies, ThermoFisher). Sequencing was performed at the Finnish Functional Genomics Centre (FFGC) using HiSeq3000 Next-Generation Sequencing platform (Illumina).

II. Alignment and Differential Expression Analysis

Obtained sequencing reads were checked for quality using FastQC (v.0.11.14) [33] and MultiQC (v.1.5)[34]. High quality reads were aligned to the human reference genome (hg38) using R (v.3.6.1) [35]/ Bioconductor(v.3.9) [36] package-Rsubread (v.1.34.6)/method-align [37]. Gene-wise read counts were obtained using parameters 'strand specificity-reversely stranded, paired end reads-NO'. Statistical testing and differential expression analysis was performed using Bioconductor package ROTS (v.1.12.0) [38]. Note that for each comparison, the expressed genes (CPM expression value >1) in at least 50% of the replicates in one of the compared sample groups were included in the statistical testing. Further DE genes were detected with cut offs FDR<0.1, Fold change<1.8 (unless otherwise specified).

III. Data representation

Heatmaps showing z scores or Log2FC values for the differentially expressed genes were generated using gplots R package.

5.2.8 ChIP-seq analysis

I. Sample preparation

$CD4^+$ T-cells were cultured under Th17 cell polarizing conditions for 72 hrs. Chromatin was prepared using Diagenode Chromatin shearing optimization kit (Cat no. C01010055) and further subjected to sonication using Bioruptor sonicator (Diagenode) to obtain chromatin fragments of 100–500 bps. Fragmented chromatin was incubated with 10-12 µg of BATF (Cell signaling Tech., Cat no. 8638) antibody and incubated with magnetic beads for crosslinking (Cat no. 112.04 Dynal Biotech, Invitrogen). The crosslinks were further reversed (65°C for 12–16 h, mixer conditions), treated with Proteinase K and RNase A and then purified using QIAquick PCR purification kit, QIAGEN. DNA libraries were prepared (Fasteris Life Sciences) and sequenced using Illumina HiSeq 4000.

II. Analysis

Raw read quality control was performed with FastQC (v. 0.11.4) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The adapter sequences present in the raw reads were trimmed using TrimGalore (v. 0.4.5) (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and the trimmed reads were mapped to the hg38 reference genome using Bowtie2 [39] (v. 2.3.3.1). Duplicate reads were marked with Picard tools' (v. 2.20.2) MarkDuplicates function and reads with mapping quality < 30 were filtered out using samtools (v. 1.9). Sample quality was controlled by calculating cross-correlation scores and the non-redundant fraction with phantompeakqualtools (v. 1.2) and preseq (v. 2.0), respectively. Peaks were called using MACS2 (v. 2.1.0) and reproducible peaks were identified using IDR with a FDR cut-off of 0.01. [40-45]

5.2.9 ChIPpeakAnno

R package ChIPpeakAnno was used to annotate the peaks and identify regions common to the two transcription factors with a minimum overlap of 200 bases [46].

5.2.10 Motif analysis

Enriched transcription factor binding site motifs within the peaks were identified by Homer (v. 4.11) using both de novo and known motifs. A 200 bp window was used for motif finding.

5.2.11 Ingenuity Pathway Analysis

Pathway analysis was performed using Ingenuity Pathway Analysis (IPA, www.qiagen.com/ingenuity; Qiagen; March 2019) tool. IPA pathways with p-value <0.01 were considered as significantly enriched.

5.2.12 Volcano plots for BATF KD DE genes

List of DE targets was acquired from RNA-seq analysis of BATF-silenced (24 and 72h) Th17 cells. Volcano plots were generated using the 'Volcano Plot' function of Galaxy Europe under 'Graph/Display Data' [47]. Targets with FDR<0.1 and fold change>1.8 were highlighted using respective colors. Chosen Th17-relevant genes were represented with labelled boxes.

5.2.13 Graphical representation and Venn diagrams

All graphs were plotted using GraphPad Prism software (V8.3.0). Two-tailed students T-test was used to calculate statistical significance, unless otherwise mentioned. All Venn diagrams were generated using Biovenn [48] or Venny [49].

5.2.14 Clustered heatmap

[50] FOSL1 IDR filtered peak file was used to create matrix using 'Compute Matrix' from deepTools. Obtained matrix was used to plot heatmap with k-means clustering using function 'plotHeatmap' from deepTools (10). Genes were annotated using 'ChIPseeker' function from deepTools [51].

5.2.15 Heatmap for shared direct targets of FOSL1, FOSL2 and BATF

Common sites for FOSL1, FOSL2 and BATF obtained from ChIP-peak Anno analysis, were annotated to the nearest TSS using Homer. Of these, the genes differentially regulated in Double KD or Double OE (FDR<0.1 and fold change>1.5) were considered. Their corresponding RNA-seq expression changes were acquired using 'Joint two files' operation under 'Text manipulation' on Galaxy Europe. Subsequent heatmaps were plotted using 'plotHeatmap' function from deepTools [47].

5.2.16 Re-alignment of publicly available H3K27Ac dataset

Publicly available H3K27Ac ChIP-seq (Aschenbrenner et al. 2018) [52] data for FACS-sorted Th17 cells derived from human peripheral blood and further activated for 5

days, was acquired from GEO (GSE101389). Since the original alignment was to hg19, raw reads were obtained and re-aligned to hg38 with BWA. Bigwig files were generated using bam coverage, normalized to Rpkm. Input subtracted files were generated using Compare Utility from deepTools.

5.2.17 STRING interactome for BATF

Predictive interactome network for BATF was acquired using the database (Only 'Databases' and 'Experiments' were considered as the information source for the predicted partners). The minimum required interaction score was set to 0.7 (high confidence). The maximum number of interactors to be displayed in first shell were restricted to 10.

5.3 Results

5.3.1 Human BATF acts as a positive regulator and antagonizes FOSL function during early Th17 differentiation

Results from Section 4.3, comprehensively describe the synergistic action of FOSL1 and FOSL2 in inhibiting human Th17 responses. We wished to further study their functional correspondence to BATF, while regulating the lineage. This prompted us to first elucidate the influence of BATF on human Th17 differentiation. We addressed the same, using a RNAi-based approach. Naïve CD4 cells from human umbilical cord blood were treated with non-targeting and BATF-targeting siRNA and further cultured under Th17-polarizing conditions. Loss of BATF showed a significant reduction in expression of key Th17 markers such as CCR6 and IL-17 cytokine, at both RNA and protein level (Fig 5.3.1(I) A-D).

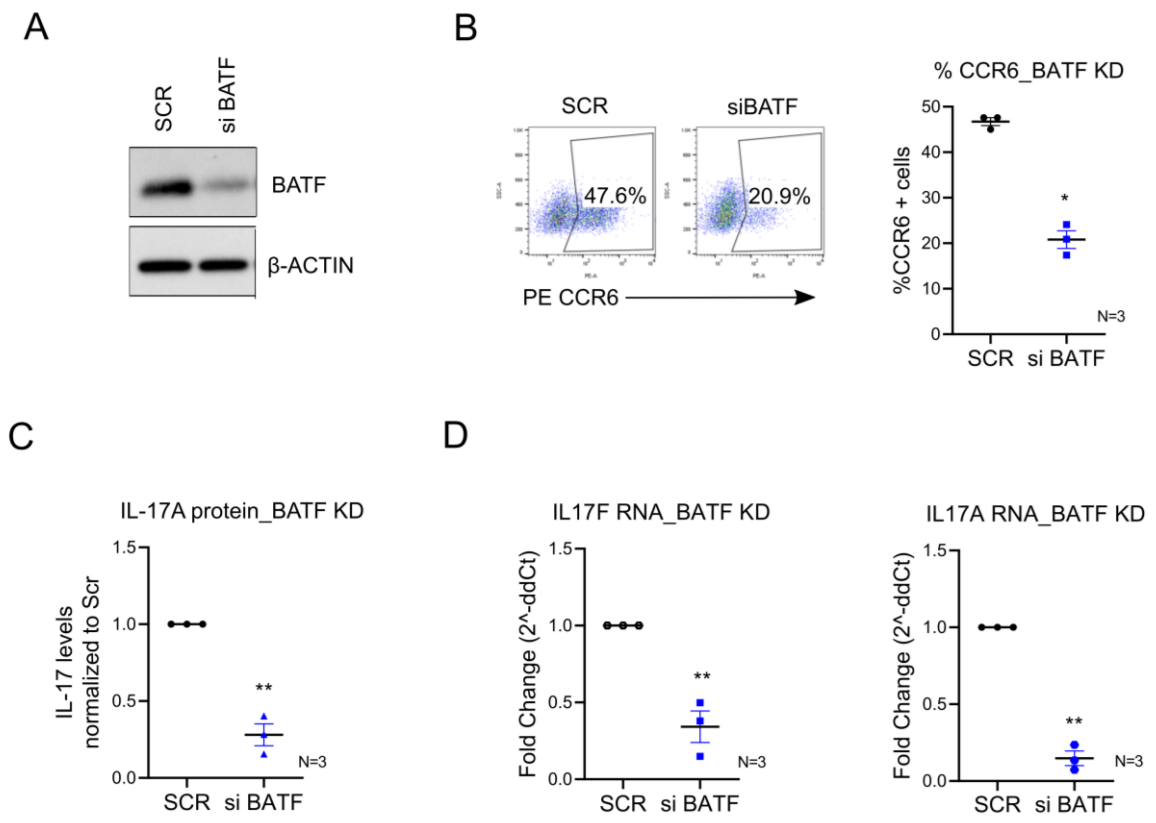

Figure 5.3.1(I). Effect of BATF-silencing on markers of human Th17 differentiation. **A.** Immunoblot showing BATF protein levels in SCR versus BATF-silenced Th17 cells. Data represents three biological replicates. **B.** FACS plot depicts percentage of CCR6 positive cells in SCR and BATF-silenced Th17 populations. Adjoining bar plots show quantitated CCR6 median values in the respective conditions, normalized to non-targeting control (SCR) **C.** ELISA was used to measure IL-17A levels in supernatants of cells treated with SCR or BATF siRNA, polarized under Th17 conditions for 72h. All ELISA readings were first normalized to live cell count, followed by normalization with values for SCR. **D.** qPCR analysis shows fold changes of IL-17F (above) and IL-17A (below) transcript levels in BATF-silenced Th17 cells at 72h of polarization. Graphs in panels B, C & D show mean ± standard error of the mean (SEM) for three biological replicates. Statistical significance has been calculated using two-tailed Student's t test (*p < 0.05; **p < 0.01, **p < 0.001)

Further bolstering these results, RNA-seq analysis of BATF-depleted Th17 cells showed a significant downregulation in expression of multiple lineage-supporting genes (IL17A, IL17F, IL23R, CCR6 and IL21) (Fig 5.3.1(II)A&B). These findings establish the importance of BATF in induction of human Th17-effector responses. Some of the most important transcriptional targets of BATF at 24 and 72h of polarization have been highlighted in the volcano plots/heatmaps shown in Fig 5.3.1(II)A&B. Further, Ingenuity pathway analysis demonstrated that the genes altered upon loss of BATF are involved in pathways such as IL23 signaling, 'Systemic Lupus Erythematosus in B cell Signaling', 'T-helper cell differentiation', 'Th1/Th2 Activation

pathway and Th17 activation', 'Role of IL17A in Arthritis' and 'Altered T cell and B cell signaling in RA' (Fig 5.3.1(II)C).

Based on these results, it is evident that the transcriptional potential of BATF functionally antagonizes that of FOS-like proteins, during early stages of human Th17 differentiation. This is consistent with earlier findings in field, which portrayed BATF as a repressor of AP-1 activity, specially FOS proteins [11]. In order to further dissect this molecular antagonism at the level of gene targets, we focused on the candidates that were oppositely regulated in FOSL Double KD and BATF KD cells (Fig 5.3.1(II)D, heatmap on the left). Likewise, we were interested in targets which were similarly altered in FOSL Double OE and BATF KD cells (Fig 5.3.1(II)D, heatmap on the right). Consequently, we found a significant number of factors known to characterize Th17 function or inflammatory responses (FUT7, IL21, RORA, IL23R and HOPX) to be positively regulated by BATF and negatively influenced by FOS-like proteins. Similarly, known inhibitors of the lineage including PRDM1 and ID3, appeared to be negatively controlled by BATF while being positively regulated by FOSL. Thus, the initiation circuit for Th17 signaling in human involves a distinct functional antagonism between the mentioned members of the AP-1/ATF superfamily.

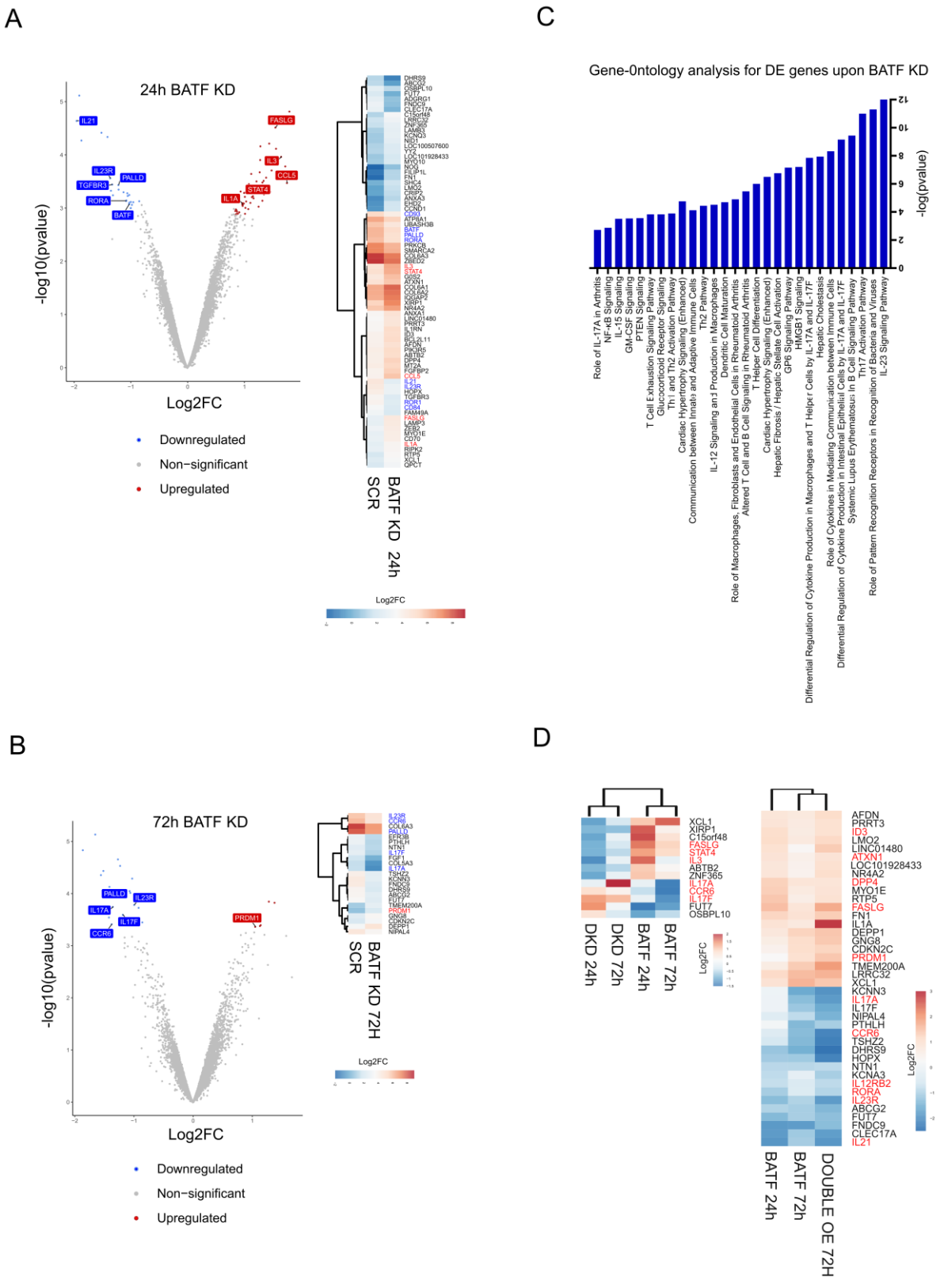

Figure 5.3.1(II) Comparing RNA-seq targets of FOS-like proteins and BATF. A&B. Heatmaps depicting top DE genes (FDR≤0.1, |fold change|≥1.8) in BATF-silenced Th17 cells at 24 and 72h of polarization. Scaled expression values are plotted and differential expression for important genes are highlighted (upregulated genes in red, downregulated genes in blue). Adjoining volcano plots highlight the significantly upregulated (red) and downregulated (blue) genes detected in 24h and 72h

BATF-silenced cells (FDR≤0.1, |fold change|≥1.8). DE genes with known relevance to Th17 function have been shown in labelled boxes **C.** IPA (Ingenuity pathway analysis) was used to identify biological pathways altered upon silencing of BATF in Th17 polarized cells at 24h and 72h **D.** Common but oppositely regulated transcriptional targets of FOS-like proteins and BATF. Heatmap on the left shows antagonistically regulated DE genes in FOSL double knock-down and BATF-silenced cells, under Th17-polarizing conditions at the specified time points. Heatmap on the right depicts DE genes that are similarly regulated in FOSL double over-expression and BATF-silenced Th17 cells, at the mentioned time points.

5.3.2 FOS-like proteins and BATF significantly share their DNA-binding sites and contrastingly regulate expression of key Th17 genes

To further investigate which of the antagonistically regulated targets are directly bound, we performed ChIP-seq analysis for BATF and then compared its occupancy to FOSL1 and FOSL2 (Fig 5.3.2). Our BATF results identified a total of 16,479 binding sites with an IDR significance <0.01. Among these, 5,492 peaks were seen to occupy putative-promoter regions of genes, of which 35 appeared to be transcriptionally altered by BATF. These included characteristic lineage-defining molecules (IL17A, CCR6, IL21 and BATF), thereby underscoring its direct role in programming the Th17-transcriptional network.

We then plotted the distribution of BATF-binding sites relative to the position of the closest TSS and compared it with the trend seen for FOS-like proteins. Interestingly, the resulting overlay graph (Fig 5.3.2A, left) clearly indicated a significant concordance in genomic occupancy of these proteins. Likewise, their stacked peak-annotation profile also appeared to be highly similar, showing predominant binding of these factors over promoter-distal regions (Fig 5.3.2A, right).

Motif analysis for BATF-bound regions discovered BATF as the strongest known-motif and identified the sequence in Fig 5.3.2B as the topmost consensus one. More importantly, FOSL1 and FOSL2 were enriched among the top-five known-motifs for BATF-occupied sites, thereby denoting similar binding sequences (Fig 5.3.2 B). This also complemented our previous motif-findings for FOS-like proteins in Chapter 4 (Fig 4.3.7E). In order to holistically view the congruence in their global-occupancy, we plotted signal intensities 2 kb upstream and downstream of the center of the peaks for FOSL1, FOSL2 and BATF (Fig 5.3.2C). The individual binding sites were further clustered within different groups. We observed that the overall appearance of the plot

indicated a high unanimity between these factors. Notably, clusters 2-4 and 7 showed higher enrichment of BATF whereas 5, 8, 9 & 10 portrayed higher signal densities for FOSL1 and FOSL2, thereby highlighting some differences that could hold regulatory importance.

We further determined the exact overlap in binding sites of these TFs using ChIPpeakAnno, and discovered a total of 2,624 regions that were shared between them (Fig 5.3.2 D). We annotated these commonly occupied sites to the nearest gene TSS and examined which of them are antagonistically regulated by FOSL and BATF. Candidates under this group were defined as their 'shared direct targets' and expression changes for some of them have been depicted in Fig 5.3.2 D. Importantly, this list included multiple genes controlling Th17-lineage establishment (RORA, IL17F, ROR1, IL23R) or its associated responses (PRDM1, FASLG, DPP4, IL12RB2). These candidates also appeared to fall within different binding-groups in the clustered heatmap (Fig 5.3.2C), suggesting minor discrepancies in the way they are bound by BATF and FOS-like proteins. Our analysis further revealed that apart from FASLG, NIPAL4 and CLEC17A, all the other shared direct targets were occupied within their intronic/intergenic regions. This consents with previous studies in the field which have reported a predominant and overlapping binding of AP-1 and its associated members over non-promoter regions of their gene-targets [53, 54].

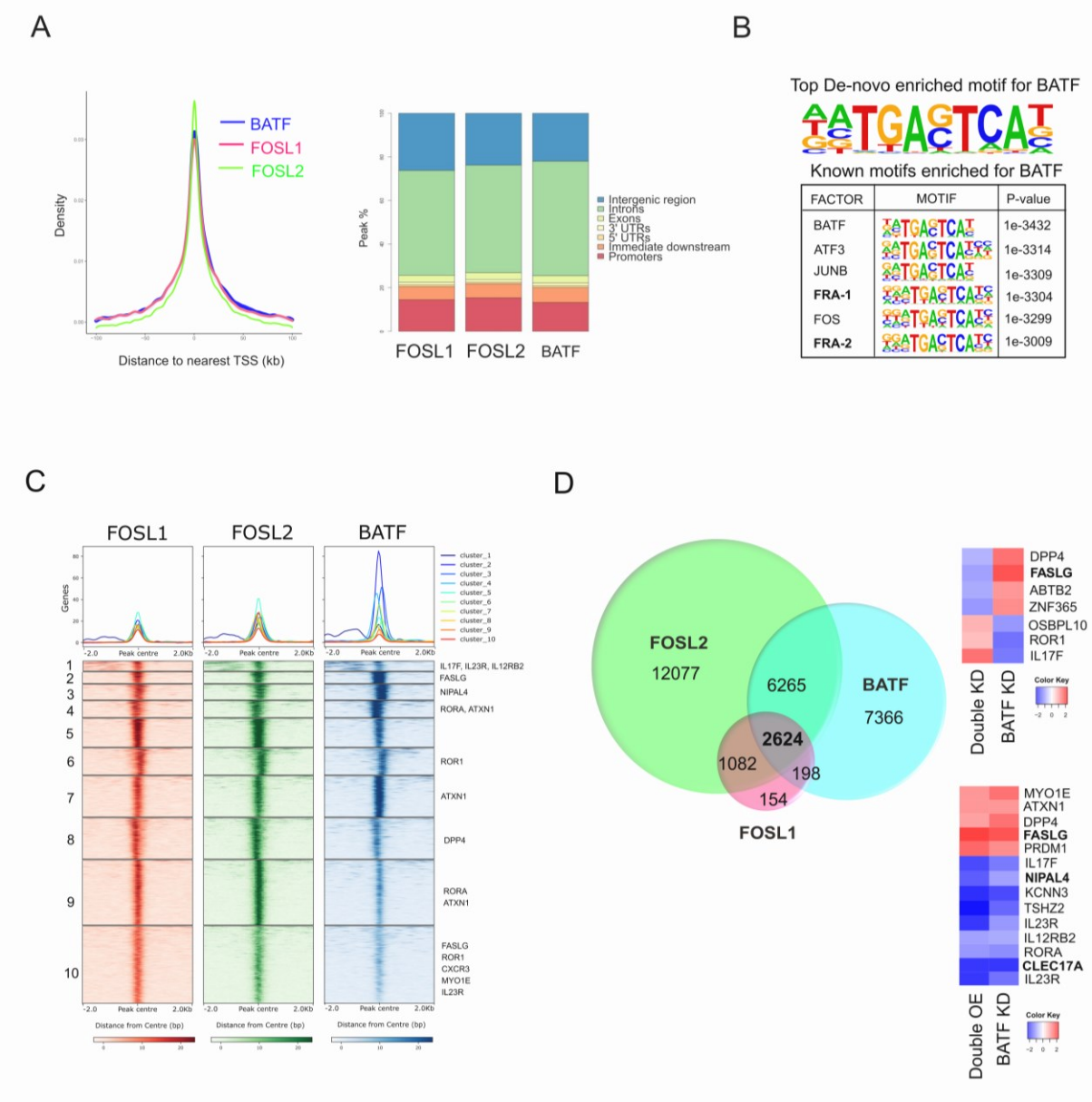

Figure 5.3.2 Correspondence between genome-binding sites of FOSL1, FOSL2 and BATF. A. ChIP-seq analysis of FOSL1, FOSL2 and BATF using 72h cultured Th17 cells. Graph shows overlay between peak distribution profiles of FOSL1, FOSL2 and BATF. TSS is defined to be at position zero. Bar plot (on the right) depicts peak-annotation results for binding sites of FOSL1, FOSL2 and BATF in 72h Th17-polarized cells. **B.** Peaks bound by BATF were analyzed for enrichment of known motifs. Top six motifs identified using 'Homer known motif enrichment analysis' have been shown. Peaks with IDR p<0.01 were used for motif discovery **C.** Heatmap with k-means clustering showing the enrichment of ChIP-seq signals at a 2Kb window upstream and downstream of the peak centre for FOSL1, FOSL2 and BATF-bound regions in the genome **D.** Venn diagram showing overlap between genomic targets of FOSL1, FOSL2 and BATF (Peaks sharing 200 bp or more across the ChIP-seq datasets were considered to be true overlaps). Adjoining heatmap depicts fold changes for gene targets which are commonly bound but oppositely regulated by FOS-like proteins and BATF. Genes showing shared occupancy of the three TFs, within a 5Kb window around their TSS have been highlighted in bold.

5.3.3 Shared genomic sites appear to be flanked by H3K27ac marks

More recently, AP-1 TFs have been shown to co-occupy DNA regions which are flanked by enhancer marks (H3K4me1/H3K27ac) and such regulatory modules appear to drive lineage-specification in other cell types [54-55]. To scrutinize our study on this front, we examined the binding sites from our ChIP-seq data for the presence of H3K27Ac, using a published human Th17 dataset ([52]; GSE101389). Interestingly, we discovered that many of the shared direct targets of FOSL1, FOSL2 and BATF portrayed a similar trend as the previous findings. Genome-viewer snapshot images in Figure 5.3.3A illustrate how H3K27ac flanks shared binding sites of these AP-1/ATF proteins over intronic/intergenic regions of key Th17 genes (IL17F, IL23R, MYO1E and PRDM1).

A similar trend for H3K27ac was identified upstream of the human *STAT4* locus (*IGV image*, Fig 5.3.3B). STAT4 was included among the synergistic targets of FOSL1 and FOSL2 and was also discovered to be antagonistically influenced by BATF in our transcriptome analysis. Studies using human CD4 cells have highlighted the importance of STAT4 in driving Th1 polarization and Th1/Th17-mediated autoimmunity [57]. Nevertheless, its exact involvement in non-pathogenic human Th17 responses remains unexplored. We used immunoblotting to validate our RNA-seq findings on STAT4 where loss of BATF was seen to upregulate its protein levels whereas co-depletion of FOSL1 and FOSL2 was seen to reduce it (Fig 5.3.3B). Such control over STAT4 expression might imply BATF/FOSL-dependent orchestration of the Th17 lineage by controlling diversification to Th1-fate.

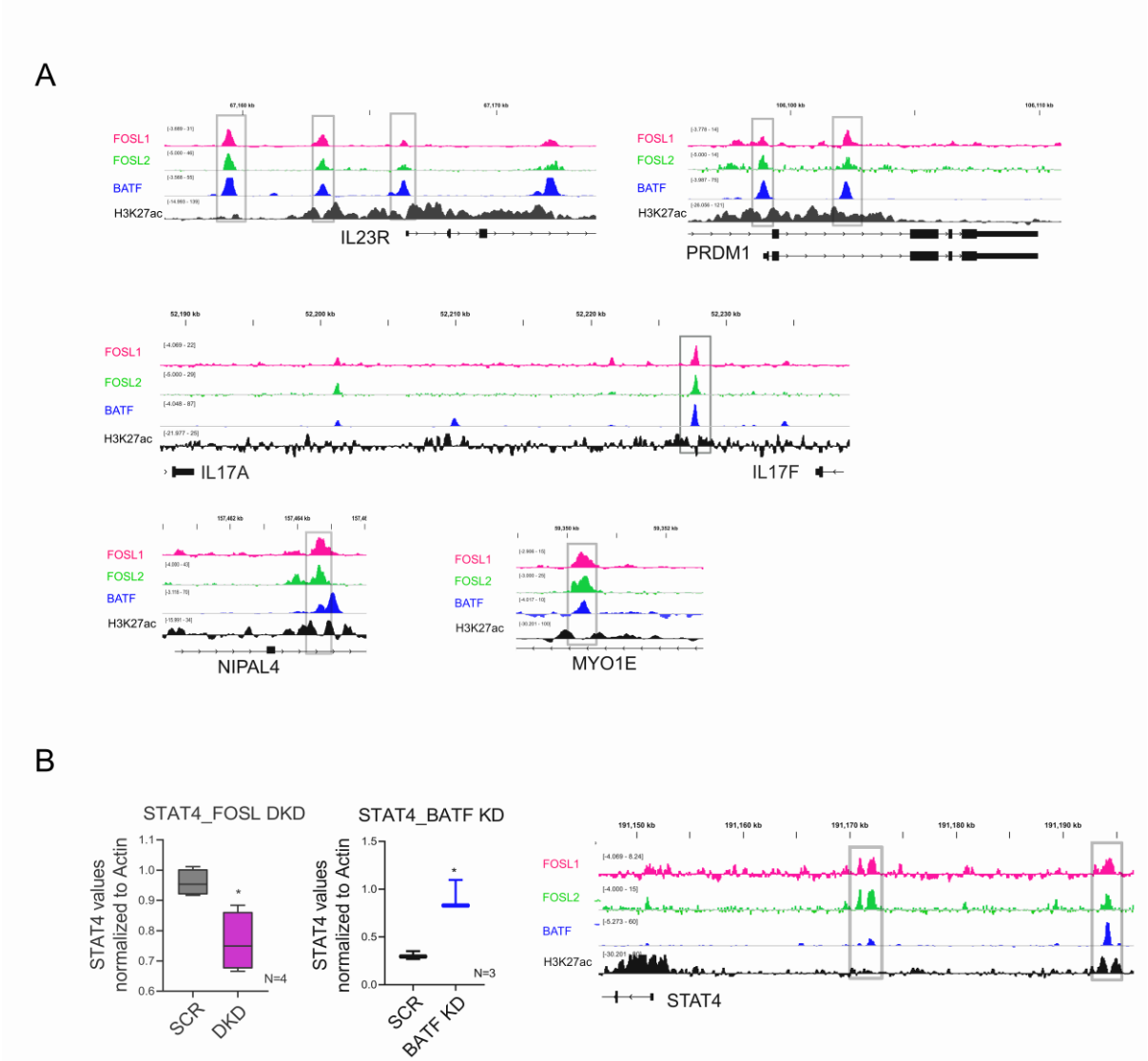

Figure 5.3.3 IGV illustrations showing overlapping occupancy of FOSL1, FOSL2 and BATF along with H3K27ac marks on their target genes. A. IGV track images display peak sharing of the FOSL1, FOSL2 and BATF on/near Th17-associated gene loci. Profile of H3K27ac marks around the commonly-bound genes has also been shown. **B.** Bar plot depicts immunoblot-based expression analysis of STAT4 in FOSL DKD (left fig.) versus BATF-silenced (right fig.) cells, cultured under Th17-polarizing conditions for 72h. Data shows mean ± standard error of the mean (SEM) for BATF KD (N=3) and FOSL DKD (N=4) experiments. Statistical significance is using two-tailed Student's t test (*p < 0.05; **p < 0.01, **p < 0.001). Adjoining IGV track shows shared occupancy of FOSL1, FOSL2 and BATF along with flanking H3K27ac marks near the STAT4 locus.

5.3.4 BATF and FOS-like proteins might compete for interaction with common binding partners

Co-expressing AP-1/ATF factors, irrespective of their widely-acknowledged similarity in genomic occupancy, are known to exhibit non-overlapping functions. Such versatility is known to arise from the dynamic protein-interaction networks of these

proteins [11, 58, 59] [60]. Context-specific and cell-type specific interactions have been a characteristic feature of members belonging to the AP-1/ATF superfamily. More importantly, competition for common binding partners has been shown to mediate functional antagonism between these proteins. For instance, BATF is known to compete with FOS for partnering with JUN proteins, thereby negatively influencing its transcriptional activity [11]. In order to address if a similar mechanism facilitates the functional conflict for human BATF and FOSL in our study, we focused on investigating their common interacting proteins in human Th17 cells.

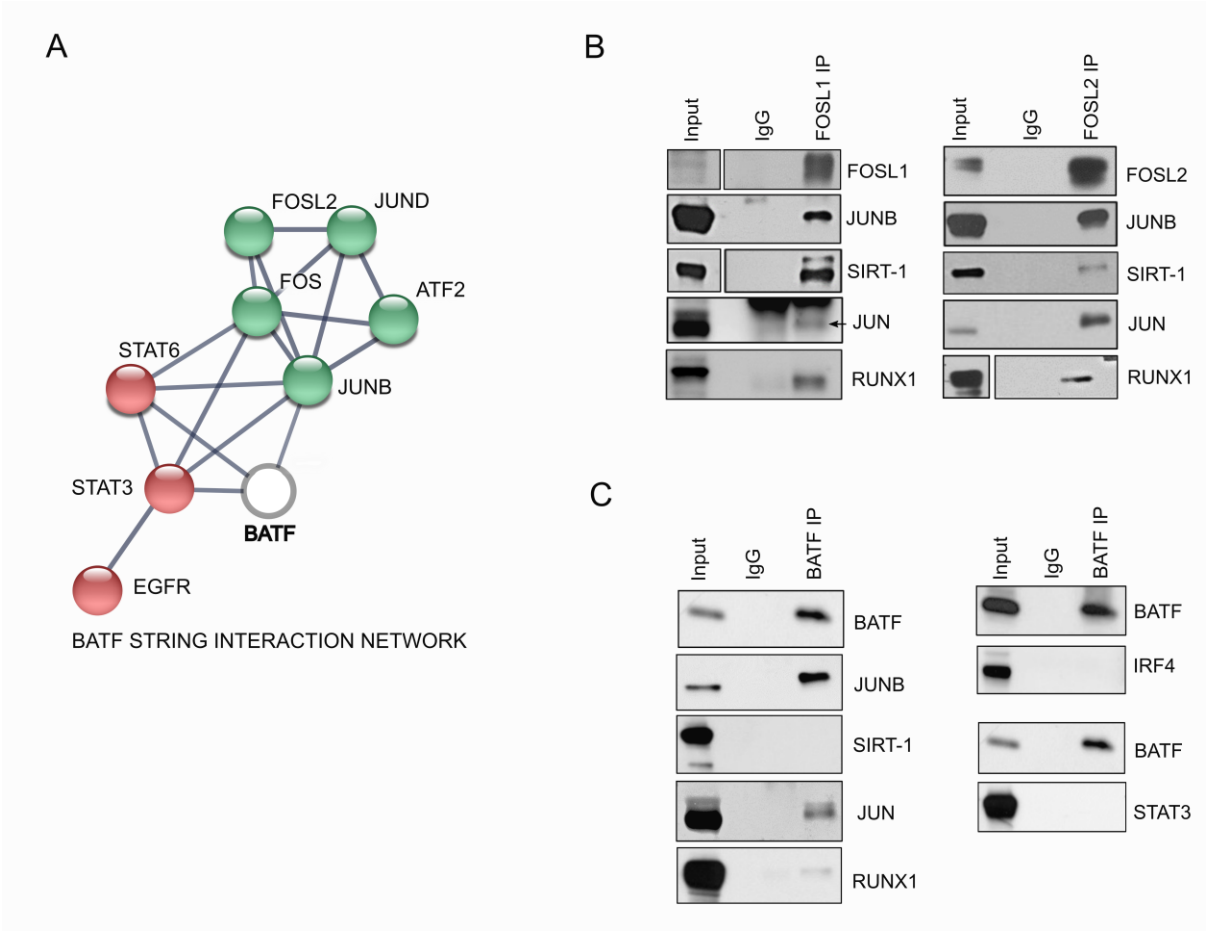

Figure 5.3.4 Common binding partners for BATF and FOS-like proteins. A. STRING network for human BATF. Width of lines between the nodes indicate confidence values for each association. Only interactions with a minimum score of 0.7 are shown (high confidence). K means clustering-3 was used to visualize the network. **B.** Immunoprecipitation followed by western blotting showing interaction of JUNB, SIRT-1, JUN and RUNX1 with FOSL1 (left panel) and FOSL2 (right panel). Immunoblot analysis to confirm pull-down of FOSL1 and FOSL2 is also shown. Data is representative of three biological replicates. **C.** Immunoprecipitated BATF was analysed for its interaction with some chosen binding partners of FOS-like proteins (JUNB, SIRT-1, JUN and RUNX1), using western blotting. Additionally, binding of BATF to STAT3 and IRF4 [16] was analysed to validate their previously-known association in mouse. Data is representative of three biological replicates.

Our MS findings from Figure 4.3.9, showed JUNB, JUN, RUNX-1 and SIRT-1 to interact with both FOSL1 and FOSL2 (Represented in Fig 5.3.4B). We wished to further determine their association with BATF. Predictive STRING network analysis for human BATF showed a predominant association with members of the JUN family (Fig 5.3.4A). This complies with other studies in the field which have portrayed heterodimerization of BATF with JUNB and c-JUN [3]. In order to investigate this in human Th17 cells, we immunoprecipitated BATF and determined its association with the mentioned factors, using immunoblotting. We confirmatively discovered a reproducible interaction of BATF with JUN and JUNB (Fig 5.3.4C). Likewise, BATF and FOSL were also found to share RUNX1 as a binding partner (Fig 5.3.4C). These findings thus propose that the functional conflict between these AP-1 proteins may be governed by their contest for overlapping interactors.

Interestingly, another identified FOSL-partner, SIRT-1, did not appear to bind to BATF, thereby parallelly highlighting an exclusive network for these TFs (Fig 5.3.4C). A thought-provoking observation was the fact that STAT3 and IRF4, which are predicted to associate with BATF (Fig 5.3.4 A) and are known to form pioneering complexes with it during lineage-induction in mouse [16, 61], did not show a positive interaction in human Th17 cells (Fig 5.3.4C). This raises challenging questions pertaining to regulatory signaling events during early stages of human Th17 differentiation and their concordance with murine findings.

5.4 Discussion

Early research in the field had introduced BATF as an inhibitor of AP-1 function [11]. However, recent advances have attributed a more context-specific perspective to this idea [17]. BATF function, in regard to Th17 differentiation, has previously been studied using murine models only [7]. In agreement with the findings in mouse, our study foremostly demonstrates BATF as a key TF required for generating human Th17 responses. Further highlighting its role in early stages of polarization, our RNA-seq analysis for BATF-silenced cells underscores its positive effect on genes driving the lineage and its negative influence on molecules inhibiting Th17 fate.

In 2012, Ciofani *et al*. reported an interesting finding in the AP-1 field, where BATF and FOSL2 were found to contrastingly regulate genes involved in murine Th17 differentiation [16]. Our study is the first one to dissect this kind of antagonism in human Th17 cells, by performing a comparative analysis for transcriptional targets of BATF and FOSL proteins. Based on this, we identified a significant fraction of genes, including the key Th17 markers (IL17A, IL17F, IL23R, CCR6, IL21), to be oppositely regulated by these AP-1 factors. We also found an evident contrast in regulation of other candidates such as IL3, FASLG, STAT4, PRDM1, IL12RB2, HOPX and DPP4, all of which have been previously studied for their involvement in Th17 function in either human, mouse or both.

Amongst these, IL3 cytokine, which is known to enhance Th2 responses, was observed to be negatively influenced by BATF and positively regulated by FOS-like proteins [62]. IL-3 expression has also been found to mark murine encephalitogenic T-cells, but with a dispensable role in EAE development [63]. Its contribution to pro-inflammatory reactions, however, needs to be validated in human Th17 cells to decipher its involvement in AP-1 mediated control of the lineage. On similar lines, we identified another candidate HOPX, with known implications in driving Th1 (and not Th17) immunopathology, to be positively regulated by BATF and negatively regulated by FOS-like proteins [64]. Based on the above-mentioned findings, it may be suggestive that these AP-1/ATF factors have the potential of orchestrating regulators of alternate T cell lineages, in early-differentiating populations.

A parallel highlight of the study by Ciofani *et al*. was the proposed genomic co-occupancy of FOSL2 and BATF, which is suggestive of a potential competition between them for binding DNA regions. It is to be importantly considered that the authors make this claim, purely on the basis of overlapping binding sites identified for the two TFs. Consistent with these findings, our analysis on human Th17 cells also demonstrated a large intersection between genomic occupancy of BATF, FOSL1 and FOSL2. However, owing to valid concerns, we refrain from assigning an angle of co-occupancy or competition, for the time being. Since the ChIP experiments performed in our study used *in vitro* differentiated Th17 clones which are known to be asynchronous, the shared peaks could easily indicate these proteins binding to a common target, but within different sub-populations. Hence, in order to claim co-binding of these factors, the analysis needs to be conducted on sorted homogenous

Th17 cultures. Furthermore, the idea of competitive-binding could only be confirmed by using gene knockout or knockdown strategies where the occupancy of one of these factors would appear elevated in absence of the other. Such findings have previously been reported for JUNB and JUND in murine Th17 cells, which are also shown to functionally oppose to each other [12]. Besides, in case these antagonistic regulators truly co-occupy DNA regions, the exact mechanism that enables either of them to dominate and dictate the expression status of the underlying gene, needs to be thoroughly investigated. One possibility could be that BATF and FOSL form a part of different regulatory modules on their common sites, which could be contextually inactivated under varied signaling conditions.

Based on our study, a total of 2624 genomic regions were found to be commonly-bound by these three TFs. Amongst these were included 18 of their antagonistically regulated targets. This signifies a direct and conflicting transcriptional control of these AP-1 factors over their shared genes, many of which were found to be associated with Th17-function (IL-17F, IL23R, FASLG, PRDM1, DPP4, RORA, IL12RB2, RORA). Of these, a key candidate was FASLG, which was found to be directly bound and positively regulated by FOS-like proteins, while being negatively regulated by BATF. Ligation of FAS/FASLG in targets cells, is known to initiate the caspase cascade for apoptosis [65]. Our findings are in concert with multiple reports in the field that have shown AP1-dependent regulation of FAS-mediated signaling and cell survival (reviewed in [66]). Though the exact role of FASLG is poorly characterized in human Th17 cells, it is known to modulate inflammatory conditions in a context-specific manner. In fact, the inherent susceptibility of inflammatory and anti-inflammatory cells to FAS signaling determines the outcome of multiple autoimmune conditions. Previous reports highlight that human patients with Acute Coronary Syndrome have enhanced FAS/FASLG expression. However, Tregs within these patients have higher susceptibility to FAS-mediated apoptotic signaling (as against Th17 cells), thereby resulting in chronic inflammation [67]. On similar lines, human patients suffering from multiple sclerosis show defects in the FAS/FASL pathway which enhances survival of pathogenic T-cells and promotes inflammatory conditions [68-70]. Having discussed these findings, it is evident that FOSL/BATF-mediated influence on expression of FASLG could have implications in immunopathology.

During murine Th17 differentiation, BATF has been shown to occupy gene promoters and regulate expression of multiple effector molecules [7]. We similarly detected many Th17-relevant candidates in our human study to be bound by BATF around their TSS sites, and be transcriptionally influenced by it (*IL21, IL17A, PRDM1, BATF, CCR6*). However, the BATF-occupied sites which overlapped with FOSL1 and FOSL2, in the vicinity of their antagonistically regulated targets, were mostly seen to occur within intronic and intergenic regions. Previously, Schraml et al. and Carr et al. have collectively shown that murine BATF and FOSL2 bind to multiple intergenic sites around *Il17a/Il17f* locus [7, 12]. These sites were found to resemble canonical AP-1 binding sequences. Our study found this scenario to be conserved in human Th17 cells where FOSL1, FOSL2 and BATF similarly intersected around the *IL17A/IL17F loci*.

Specific genomic regions portraying an overlapping occupancy of AP-1 proteins have previously been shown to define enhancer landscapes [71]. Underlining this idea, we found a flanking profile of H3K27ac marks around the FOSL/BATF peaks in our study. This suggests that a regulatory interplay at enhancer elements could be typical of members belonging to this superfamily. Further, our motif analysis for all the three TFs reproducibly suggested that proteins like ATF3, FOS and JUNB may have DNA binding motifs similar to theirs. ATF3 in particular, is differentially upregulated in human Th17 cells [73], is known to drive Th1 differentiation [74] and has recently been found to be protective against murine colitis [75,76]. Given its unexplored involvement in Th17-specification, elucidating the same could augment our understanding on interconnected AP-1 circuits governing the lineage. Addedly, early-expressing proteins like BATF have been shown to exhibit pioneering functions by occupying closed-chromatin regions where they induce nucleosomal clearance for lineage-defining factors [25]. Regardless of the well-established genomic overlap between AP-1/ATF factors, other members of the family like FOS/JUN are yet to be investigated for their ability to moderate chromatin accessibility. Addressing this could deliver novel insights into regulation of early events of transcription in T-helper differentiation.

AP-1/ATF family members execute their transcriptional activity in the form of dimers. Apart from JUN proteins which are known to homodimerize, most other members of this family heterodimerize with different partners in order to dictate gene-regulation. It

is well established that the nature of DNA-binding or gene regulation is determined by the dimer pair as a whole. On these lines, it is evident that an overarching study for functions of these TFs requires elucidation of their binding partners. Antagonism between AP-1 members is known to be mediated via modulation of their protein-protein interactions [60]. Some of the early reports have also demonstrated BATF as a dominant negative of FOS proteins. This is achieved by BATF forming transcriptionally inert or self-favouring dimers with JUN, which is a shared interactor with FOS [11]. Our Mass-spec and immunoprecipitation analyses using human Th17 cells identified JUN and JUNB as commonly associating proteins for BATF, FOSL1 and FOSL2. Though this is in agreement with other reports which suggest interaction-dependent mechanisms for conflicting AP-1 proteins, simply assuming molecular competition between them would be difficult. Additional experiments are required to address this aspect and also to further determine if contextual signaling favours either BATF or FOSL to heterodimerize with JUN proteins. Also, studies by Bitton-Worms et al. have shown interaction-based inhibition of JUN transcriptional activity by JDP2 [72]. It would be equally important to determine if the functional antagonism between BATF and FOSL involves selective inhibition of JUN activity. Furthermore, investigating the individual role of JUN proteins in human Th17 responses would be crucial for advancing our understanding on this paradigm.

Among the non-AP1 proteins, we observed RUNX1 to physically interact with all the three TFs. Intriguingly, RUNX1 has been shown to antagonistically regulate IL-17 transcription by differentially interacting with RORγT in Th17 cells and FOXP3/Tbet in Treg/Th1 cells. This is dictated by the polarizing cytokine environment and is known to control CD4 plasticity along the Treg-Th17-Th1 axis (reviewed in [26]). Based on such existing information, it is possible to consider that the BATF-RUNX1 and FOSL-RUNX1 dimers discovered in our study could be binding to common targets but oppositely regulating them in a context-specific manner. Since RUNX1, BATF and FOSL are co-expressed in human Th17 cells, addressing the exact mechanism through which these interactions come into play would be fundamental.

Functional associations between TFs, and their effect on gene-regulatory circuits have been well-studied. Research on similar lines has identified the AP-1/ATF family as one of the most dynamic TF groups, majorly owing to a highly contextual molecular

interplay between its members, that comprises of both harmonious and conflicting equations (reviewed in (Hess et al., 2004)). Our study, for the first time, comprehensively assigns such angles of functional cooperativity or antagonism between these proteins, in the context of human Th17 effector responses (reviewed in [66]). We also underline some crucial differences in this interplay for the human and mouse counterparts, thereby emphasizing on species-specific regulation.

Owing to a well-known dimer-based action of AP-1 proteins, assigning individual functions to the monomers has been a real challenge in the field. Thus, employing a holistic approach and studying AP-1 activity as a complete complex could prove to be a more beneficial strategy. Bearing this in view, we focused more on exploring the inter-relatedness between FOSL1, FOSL2 and BATF, than their individual functions. Our results thus provide useful insights into the early transcriptional networks for human Th17 fate. Nevertheless, whether these factors actually orchestrate the balance for homeostatic and pathogenic human Th17 responses still remains to be elucidated. Further, it would be equally important to determine if any of the known autoimmune-related SNPs have the potential to disrupt binding of these proteins over their shared genomic regions. This could help is opening new avenues on medicinal research and immunotherapy of autoimmune disorders.

5.5 References

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Appendix I - Taqman RT-PCR Primers

Oligonucleotide name	5' - Nucleotide sequence -3'	Roche universal library probe number
FOSL1 Primer 1	CCACTGGTACTGCCTGTGTC	12
FOSL1 Primer 2	CTAGAGCTGGTGCTGGAAGC	
FOSL2 Primer 1	ACGCCGAGTCCTACTCCA	70
FOSL2 Primer 2	TGAGCCAGGCATATCTACCC	
BATF Primer 1	ACAGAGAAGGCCGAC	85
BATF Primer 2	CTTGATCTCCTTGCG	
IL17A Primer 1	TGGGAAGACCTCATTGGTGT	8
IL17A Primer 2	GGATTTCGTGGGATTGTGAT	
IL17F Primer 1	GGCATCATCAATGAAAACCA	10
IL17F Primer 2	TGGGGTCCCAAGTGACAG	
RORC Primer 1	AGACTCATCGCCAAAGCATC	87
RORC Primer 2	TCCACATGCTGGCTACACA	
STAT3 Primer 1	ACCTAGGGCGAGGGTTCA	50
STAT3 Primer 2	CCTAAGGCCATGAACTTGA	
SATB1 Primer 1	GTACGCGATGAACTGAAACG	14
SATB1 Primer 2	TTAAAAGCCACACGTGCAAA	

Appendix II - siRNA sequences

	Target gene	siRNA sequence	Source
1	FOSL1 si 1 (Single/Double KD)	CACCAUGAGUGGCAGUCAG[dT][dT]	Sigma
2	FOSL1 si 2 (Single KD)	GGACACAGGCAGUACCAGU[dT][dT]	Sigma
3	FOSL2 si 1 (Single KD)	CUGGGUGGUCUGAAUAUUAAA[dT][dT]	Sigma
4	FOSL2 si 2 (Single KD)	GGCCCAGUGUGCAAGAUUA[dT][dT]	Sigma
5	STAT3	GGAGAAGCAUCGUGAGUGA	Sigma
6	BATF si 1	GAAACAGAACGCGGCUCUA	Sigma
7	BATF si 2	GAACGCGGCUCUACGCAAG	Sigma
8	FOSL2 Thermo siRNA (Double KD)	Sequence not provided by company	Thermo Scientific- Cat no. 115633
9	SATB1 siRNA	GCAUUAUACCUUCUGUGAUUA	Sigma

Publications and Manuscripts

1. Tripathi SK[+], Välikangas T[+], **Shetty A**[+], Khan MM, Moulder R, Bhosale SD, Komsi E, Salo V, De Albuquerque RS, Rasool O, Galande S, Elo L, Lahesmaa R. Quantitative proteomics reveals the dynamic protein landscape during initiation of human Th17 cell polarization. iScience. 2019 Jan 25;11:334-55. ([+]Equal contribution)

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3. **Shetty A**[+], Tripathi SK[+], Junttila S[+], Buchacher T[+], Bhosale S, Biradar R, Envall T, Laiho A, Moulder R, Rasool O, Galande S, Elo L and Lahesmaa R. FOSL1 and FOSL2 cooperatively suppress human Th17 responses. ([+]Equal contribution)
(Manuscript ready to be submitted)