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Snai2 and *Snai3* transcriptionally regulate cellular fitness and functionality of T cell lineages through distinct gene programs

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ABSTRACT

T lymphocytes are essential contributors to the adaptive immune system and consist of multiple lineages that serve various effector and regulatory roles. As such, precise control of gene expression is essential to the proper development and function of these cells. Previously, we identified *Snai2* and *Snai3* as being essential regulators of immune tolerance partly due to the impaired function of $CD4^+$ regulatory T cells in *Snai2/3* conditional double knockout mice. Here we extend those previous findings using a bone marrow transplantation model to provide an environmentally unbiased view of the molecular changes imparted onto various T lymphocyte populations once *Snai2* and *Snai3* are deleted. The data presented here demonstrate that *Snai2* and *Snai3* transcriptionally regulate the cellular fitness and functionality of not only $CD4^+$ regulatory T cells but effector $CD8^{\alpha+}$ and $CD4^+$ conventional T cells as well. This is achieved through the modulation of gene sets unique to each cell type and includes transcriptional targets relevant to the survival and function of each T cell lineage. As such, *Snai2* and *Snai3* are essential regulators of T cell immunobiology.

1. Introduction

Cellular immunity is regulated through the interactions of multiple cell types that constitute the innate and adaptive arms of the immune system. Within the adaptive immune system, T lymphocytes play a key role not only as immune effectors but also as immune regulators. To this end, the T lymphocyte "family" consists of multiple lineages. Such examples include $CD8^{\alpha+}$, $CD4^+$ $CD25^-$ conventional (T_{Conv}) and $CD4^+$ $CD25^+$ regulatory (T_{Reg}) cells. Within the thymus, various transcriptional regulators precisely control the decisions regarding lineage fate. Following the $CD4^+$ $CD8^{\alpha+}$ double positive T cell stage, Runx3 and ThPOK drive $CD8^{\alpha+}$ and $CD4^+$ T cell differentiation, respectively, while also antagonizing the alternative differentiation program (He et al., 2010; Woolf et al., 2003). Once the $CD4^+$ fate choice has been made, selective expression of Foxp3 "turns on" the gene program required for T_{Reg} specification (Gavin et al., 2007). While these transcriptional regulators are key to the development and function of the above mentioned cell types, the totality of transcriptional regulatory networks involved is much more complicated. This is perhaps best illustrated in the T_{Reg} lineage which modifies nuanced regulatory responses through the use of an evolving list of transcriptional regulatory mechanisms (Campbell and Koch, 2011). Not surprisingly, multiple layers of redundancy have developed to ensure the proper expression of these gene programs(Miyazaki et al., 2014; Ouyang et al., 2010). One such example includes the Id family of transcriptional regulators. These proteins negatively regulate the function of E-box DNA-binding factors such E2A and Snai1 (Chang et al., 2013; Rivera and Murre, 2001). Upon deletion of *Id2* or *Id3*, the other family member is able to functionally compensate and ensure the ability of T_{Regs} to suppress T helper (T_H2)-mediated inflammation. This specific T_{Reg} function is subsequently lost upon the deletion of both *Id2* and *Id3* (Miyazaki et al., 2014).

The Snail family consists of 3 evolutionarily conserved transcriptional regulators: *Snai1* (*Snail*), *Snai2* (*Slug*) and *Snai3* (*Smuc*) (Manzanares et al., 2001). These proteins share the same basic structure inclusive of an N-terminal SNAG (Snail/Gfi-1) domain and multiple C_2H_2-type zinc finger DNA-binding domains (DBDs) within the C-terminus (Kataoka et al., 2000). Using their DBDs, Snail factors recognize consensus E-box DNA elements (CANNTG) with a preference for G/C-rich central dinucleotides (Soleimani et al., 2012). Once bound to target genes, Snail family members augment

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transcription through the recruitment of various chromatin modifiers via the SNAG domain (Ferrari-Amorotti et al., 2013; Peinado et al., 2004). Although classically known as transcriptional repressors, a growing body of data supports the ability of Snail proteins to positively regulate their targets upon interaction with other transcription factors (Gingold et al., 2014; Hu et al., 2010).

The founding member, *Snai1*, was discovered in *Drosophila melanogaster* where it was first characterized as a key factor involved in embryonic patterning (Alberga et al., 1991; Boulay et al., 1987). Since then, Snail family members have been most well characterized in the areas of embryonic and developmental biology (Lomeli et al., 2009; Murray and Gridley, 2006; Murray et al., 2007). However, this family also plays a number of roles in the development and function of the immune system (summarized in Pioli and Weis, 2014). Recently, we demonstrated a previously unappreciated functional redundancy for *Snai2* and *Snai3* in lymphoid development (Pioli et al., 2013). Furthermore, the conditional deletion of *Snai2* and *Snai3* (cDKO) resulted in fatal autoimmunity that could be corrected by the transplantation of wildtype (WT) T_{Regs} (Pioli et al., 2015). While high levels of autoantibodies characterized this disease, these animals lacked the T cell proliferation commonly associated with many autoimmune diseases (Brunkow et al., 2001; Oliveira, 2013). This led us to hypothesize that the deletion of *Snai2* and *Snai3* not only affected T_{Regs} but also diminished the fitness of $CD8^{\alpha+}$ and T_{Conv} cells as well. Using competitive reconstitution, we demonstrated that cDKO $CD8^{\alpha+}$, T_{Conv} and T_{Reg} cells were compromised in their ability to compete with their WT counterparts. Additionally, a reduced amount of cDKO T cells was able to enter the activated, effector/memory-like pool. RNA sequencing (RNA-seq) analysis showed that Snai2 and Snai3 regulated genes essential for the cellular fitness and function of all 3 lineages. Importantly, Snai2 and Snai3 accomplished this via modulation of transcriptional targets almost completely exclusive to each individual cell type. Thus, *Snai2* and *Snai3* are key transcriptional regulators of T cell biology.

2. Materials and methods

2.1. Animal strains and care

Animals were housed in the Animal Resource Center (University of Utah Health Science Center, Salt Lake City, UT) according to the guidelines of the National Institute of Health for the care and use of laboratory animals. All animal protocols were reviewed and approved by the University of Utah Institutional Animal Use and Care Committee. *Vav-Cre* (Stock #: 008610), *Rag2*$^{-/-}$ (Stock #: 008449) and *UBC-GFP* (Stock #: 004353) mice were purchased from The Jackson Laboratory and bred in house. *Snai2/Snai3* conditional double knockout (cDKO) mice were derived from *Snai2*$^{+/-}$ *Snai3*$^{Fl/Fl}$ *Vav-Cre*$^{+/-}$ breeding pairs. *Snai3*$^{Fl/Fl}$ have been made available from the Jackson Laboratory (Stock #: 027276). Animal numbers used per experiment are noted in the figure legends.

2.2. DNA isolation and genomic DNA PCR

Approximately 5 mm portions of tail were boiled in 50 mM NaOH until fully dissolved. 1 M Tris was added to neutralize the NaOH. Following centrifugation to remove insoluble material, DNA was precipitated from supernatants following standard ethanol precipitation guidelines. *Snai2*, *Snai3* and *Vav-Cre* genotyping was performed with Thermo Scientific *Taq* DNA Polymerase (Cat. #: FEREP0402) using 2 μL of DNA per reaction. Products were electrophoresed in 2% agarose gels. Cycling parameters are available upon request. Primer sequences are provided in Supplementary Table 1.

2.3. RNA isolation and RNA sequencing (RNA-seq)

Total RNA was isolated from cells using the Qiagen miRNeasy Micro Kit (Cat. #: 217084) according to the manufacturer's instructions. Isolated RNA was utilized for RNA-seq library preparation using the Illumina TruSeq Stranded Total RNA Sample Preparation Kit with Ribo-Zero Gold treatment to eliminate ribosomal RNAs. Libraries were subjected to HiSeq2000 50 Cycle Single Read Sequencing. Greater than 2.5×10^7 reads per sample (quality score, $Q \geq 20$) were obtained and aligned to the mm10 (Ensembl build 75) transcriptome index using Novoalign. Aligned reads were further processed for splicing and expression variance using the Useq 8.7.4 software package. The data has been submitted to the NCBI GEO database (GSE74467). 4 replicates were performed for wildtype (WT) and cDKO $CD8^{\alpha+}$ and $CD4^+$ $CD25^-$ conventional (T_{Conv}) T cells. For $CD4^+$ $CD25^+$ regulatory (T_{Reg}) T cells, 4 and 3 replicates were performed for WT and cDKO genotypes, respectively. For mathematical purposes, a value of 0.0001 was added to all gene fragments per kilobase per million mapped reads (FPKM) values as to avoid "zero" values. Mean fold changes for each gene were calculated by dividing mean WT by mean cDKO FPKM values for a given T cell lineage. Significantly altered genes for $CD8^{\alpha+}$, T_{Conv} and T_{Reg} cells are listed in Supplementary Tables 3–5. Analysis for all detectable $CD8^{\alpha+}$, T_{Conv} and T_{Reg} genes can be found in Supplementary Tables 6–8. Data tracks were visualized with the University of California–Santa Cruz (UCSC) genome browser. Venn diagrams for gene expression analysis were created using the online program Venny 2.0 (http://bioinfogp.cnb.csic.es/tools/venny/index.html) (Eden et al., 2007; Eden et al., 2009). Gene ontology analysis was performed using Gorilla online software. The Benjamini and Hochberg method was used to correct *p*-values for multiple testing (FDR *q*-value). Gene Set Enrichment Analysis (GSEA) was performed using software made publicly available by the Broad Institute (http://software.broadinstitute.org/gsea/index.jsp).

2.4. Fluorescence-activated cell sorting (FACS) analysis and isolation of T cell populations

Upon dissection, the plunger of a 5 mL syringe was used to dissociate thymus, spleen and mesenteric lymph node (mLN) tissues. Cells were strained through a 100 μm filter and collected in 10 mL of FACS buffer (1x PBS + 0.1% BSA). Peripheral blood was isolated from the retro-orbital (r.o.) sinus via heparin-lined capillary tubes and complete blood counts were obtained using a Hemavet 950 FS (Drew Scientific). Remaining contents were then collected in 5 mL of FACS buffer. After centrifugation, erythrocytes were lysed on ice for 10 min using ammonium-chloride-potassium (ACK) buffer. Following lysis, cells were respun, resuspended in FACS buffer and counted using a Hemocytometer. Cells were stained on ice for 30 min using the appropriate antibody cocktail. Samples were washed with FACS buffer, centrifuged and resuspended in FACS buffer. To discriminate between live and dead cells, 4′6-diamidino-2-phenylindole (DAPI) was added at a final concentration of 3 μM. The antibodies utilized with their indicated dilutions are available in Supplementary Table 2. Population analysis was performed on the FACS Canto II (BD Biosciences) and results for a given cell type are graphically represented as mean values ± standard error measurement (SEM). Cell sorting of select populations was performed on the FacsAria Cell Sorter (BD Biosciences) at the University of Utah Flow Cytometry Core.

2.5. Bone marrow transplantation

One day prior to transplantation, 8–12 weeks old *UBC-GFP* recipient mice were lethally irradiated with 2 doses of 500 cGy split