Università degli Studi di Milano

Department of Medical Biotechnology and Translational Medicine

The long non-coding FOXP3 regulates cell identity and function of T regulatory lymphocytes stabilizing the key transcription factor Foxp3

PhD program in
Experimental Medicine and Medical Biotechnologies
XXXI cycle

Curriculum
Immunology and Hemato-Oncology

PhD Thesis:
Alessia Dardanelli

Tutor: Massimiliano Pagani
Coordinator: Massimo Locati

Accademic Year: 2017/2018

Index

Abstract

The human immune system is a suitable context to study plasticity in response to environmental stimuli. Differentiation of Naïve cells into specialized subsets guarantees the proper immune system function. These cellular subsets were once considered as terminally differentiated, but recent findings, showed that they display a high degree of plasticity, whose underlying molecular mechanisms are still poorly understood. In this context, $CD4^+$ T regulatory lymphocytes (Treg) are the principal actors in the regulation of immune responses and maintenance of immunological self-tolerance thanks to their peculiar suppressive function.

Treg cells dysfunction is associated to autoimmune pathologies, inflammatory diseases and cancer. Their inherent plasticity could be exploited as a promising therapeutic opportunity to modulate their differentiation and function in the context of several immune-mediated diseases.
A better characterization of the molecular mechanism underlying plasticity is thus compelling. Long non-coding RNAs (lncRNAs) has been identified as novel players in the modulation of cell plasticity and in the maintenance of cell identity. These features, along with their cellular specificity, brought lncRNAs to the fore as novel and promising therapeutic targets.
In this study, we demonstrate that the expression of lncFOXP3, a $CD4^+$ T regulatory specific lncRNAs located upstream of *FOXP3* gene, is crucial for the maintenance of Treg phenotype and proper function.

Expression of lncFOXP3 correlates with FoxP3, the master transcription factor of Treg cells, but the two transcripts are not co-regulated. Functional experiments revealed that lncFOXP3 down-regulation reduces FoxP3 protein levels and, moreover, impacts on Treg suppressive activity.

Finally, the analysis of lncFOXP3 protein interactors highlighted the association with Ubiquitin-specific-processing protease 7 (USP7), indicating its putative role in the maintenance of FoxP3 protein stability. Our results suggest a direct involvement of lncRNAs in the maintenance of Treg cell proper function. Therefore, modulation of lncRNAs could potentially be exploited to either enhance or quench Treg cell suppressive function in the treatment of autoimmune diseases, cancer or immunodeficiencies.

Introduction

1. Long non-coding RNAs: the power of heterogeneity

With the advent of new high-throughput technologies, it was discovered that the fraction of transcribed genome is higher than expected, with the majority of transcripts not encoding for proteins. In particular, against a total of 62.1% of the human genome covered by processed transcript (74.7% by primary transcripts), exons of protein-coding genes cover only the 2.94% of the genome (Derrien et al., 2012). These findings are explained by a change of paradigm in the way we think of gene expression control that includes the expansion of non-coding regulatory RNAs (ncRNAs) (Taft, Pheasant, & Mattick, 2007). Therefore, we should reassess the centrality of protein-coding RNAs in favor of non-coding ones. Non-coding RNAs are mainly classified based on their size into "small" ncRNAs, being less than 200 nucleotides in length, and "long" or "large" ncRNAs (lncRNAs), ranging from more than 200 to tens of thousands nucleotides (Table 1). Some of these RNAs have general housekeeping functions, such as ribosome-associated RNA (rRNA), transfer RNA (tRNA) and small nuclear/nucleolar RNA (sn/snoRNAs). Some classes of short RNAs like miRNAs, siRNAs and piRNAs have regulatory functions in several cellular processes. The class of long non coding RNAs (lncRNAs) includes different functional transcripts with no potential to encode for functional proteins longer than 30 aminoacids (Mercer, Dinger, & Mattick, 2009).

ncRNA*	No. of known transcripts[†]	Transcript lengths (nucleotides; nt)[‡]	Functions
Precursors to short RNAs			
miRNA	1,756	>1,000	Precursors to short (21–23 nt) regulatory RNAs
snoRNA	1,521	>100	Precursors to short (60–300 nt) RNAs that help to chemically modify other RNAs
snRNA	1,944	1,000	Precursors to short (150 nt) RNAs that assist in RNA splicing
piRNA	89	Unknown	Precursors to short (25–33 nt) RNAs that repress retrotransposition of repeat elements
tRNA	497	>100	Precursors to short (73–93 nt) transfer RNAs
Long ncRNAs			
Antisense ncRNA	5,446	100–>1,000	Mostly unknown, but some are involved in gene regulation through RNA interference
Enhancer ncRNA (eRNA)[§]	>2,000	>1,000	Unknown
Enhancer ncRNA (meRNA)[‖]	Not fully documented	As variable as the length of mRNAs	Unknown, but they resemble alternative gene transcripts
Intergenic ncRNA	6,742	10^2–10^5	Mostly unknown, but some are involved in gene regulation
Pseudogene ncRNA	680	10^2–10^4	Mostly unknown, but some are involved in regulation of miRNA
3′ UTR ncRNA	12	>100	Unknown

*miRNA, microRNA; snoRNA, small nucleolar RNA; snRNA, small nuclear RNA; piRNA, piwi-interacting RNA; tRNA, transfer RNA; antisense ncRNA, transcripts mapping and overlapping coding and non-coding RNAs; enhancer ncRNA (eRNAs and meRNAs), transcripts that initiate within regions that regulate specific genes; intergenic ncRNA, transcripts that map to genome regions between annotated genes; pseudogene ncRNA, transcripts that come from processed or unprocessed pseudogenes; 3′ UTR ncRNA, 3′-untranslated regions of ncRNA.

Table 1- Main classes and functions of mammalian ncRNAs. Different type of non-coding RNAs classified based on their length. In table the # of known transcripts, their length and relative functions are reported (Kowalczyk & Higgs, 2012) .

Besides the size-based classification, non-coding RNAs can be grouped according to their position relative to known sequences of the genome. In particular, lncRNAs are usually classified relative to neighbouring protein-coding genes (Figure 1) as:

- *Sense* lncRNAs if they originate from the same strand of protein-coding genes and partially overlap with them
- *Antisense* lncRNAs, if they are transcribed in the opposite direction and overlap, at least, one coding exon
- *Intronic* lncRNAs, originating from a protein-coding gene intron, in either direction and terminate without overlapping exons
- *Intergenic* lncRNAs, if they lie within a sequence between two protein-coding genes
- *Enhancer* lncRNAs, arising from the enhancer region of a protein-coding gene
- *Circular* lncRNAs which usually arise from un-canonical splicing events of protein-coding genes

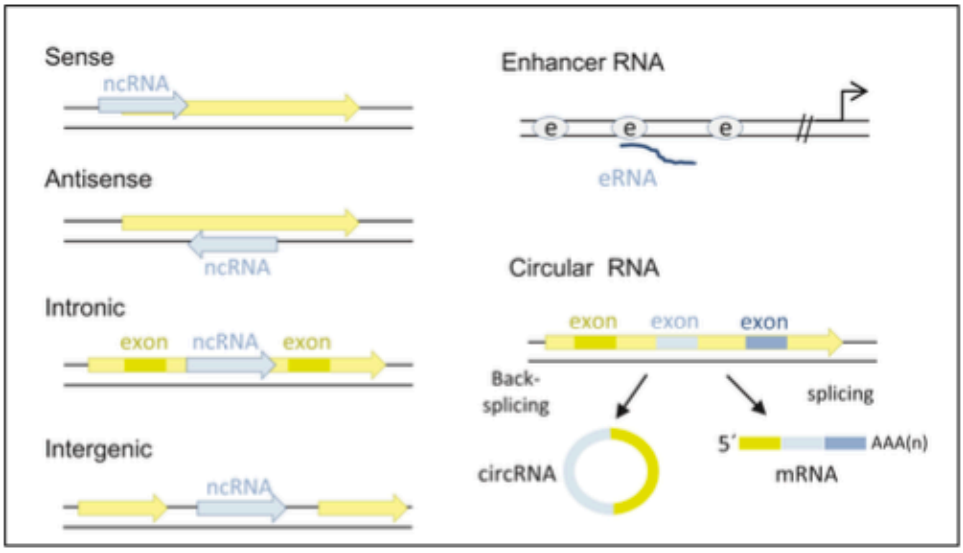


Figure 1- Classification of long non-coding RNAs (lncRNAs). They can be classified relative to neighbouring protein-coding genes as: sense, antisense, intronic, intergenic enhancerRNA and circular RNA (Uchida & Dimmeler, 2015).

The majority of lncRNAs are generated by the same transcriptional machinery of mRNAs, as demonstrated by the presence of RNA polymerase II and histone modifications, such as H3K4me3 at promoters and H3K36me3 within gene bodies, histone marks associated with transcription initiation and elongation, respectively (Guttman et al., 2009).They are capped by methylguanosine at their 5' end, often spliced, 3' polyadenylated and are devoid of evident open reading frames

(ORF)(Kapranov et al., 2007). Nevertheless, there are some characteristics that can be used to discriminate lncRNAs from mRNAs: they are shorter than mRNAs (average length of 1kb compared with 2.9 kb), they have fewer exons (an average of 2.9 compared with 10.7), show poorer primary sequence conservation and are expressed at tenfold lower level (Cabili et al, 2011).

The low expression of lncRNAs in whole organ tissues may be partially explained by their cell-type-specific expression, at least in complex tissues, consistent with lncRNAs having tissue-defining roles (Melé et al., 2017). Intriguingly, it has been shown that many lncRNA TSSs overlap with repetitive elements or RNA-derived transposable elements (TEs), suggesting that these components could be important drivers for lncRNA evolution (Kapusta et al., 2013; Kelley & Rinn, 2012).

In the last years, there was intense debate about the functions of these molecules. Due to their relatively low expression level, unknown functions and low sequence conservation, they were first considered as the sub-product of a transcriptional noise resulting from low RNA Polymerase fidelity and incidental transcription at enhancer regions (de Santa et al., 2010; Struhl, 2007).

LncRNAs, though, have a precise patterns of expression during differentiation and development, display distinct transcription factors on their promoters, peculiar signatures at the chromatin level, as well as specific histone modifications like H3K9Ac, H3K4me3, and H3K36me3, as mentioned before (Guttman et al., 2009).The interest toward lncRNAs has rapidly grown and their expressions have been quantified in many different tissues and cell types by high-throughput RNA sequencing (RNA-seq); notably, lncRNA number is still growing, in contrast to the number of protein-coding genes that is remarkably stable over years.

Thanks to these studies, it has been also found that lncRNA expression is highly tissue specific, displaying more cell-specificity than protein coding genes, and they have been related to several pathological stages (Shi, Sun, Liu, Yao, & Song, 2013; Tang et al., 2013)

Notably, at various differentiation stages, they are more dynamically expressed than protein coding genes, suggesting they can contribute to regulation of gene expression. These unique properties hint to lncRNA involvement in pluripotency (Lin et al., 2015), commitment and differentiation (Klattenhoff et al., 2014), maintenance of cell identity and cell fate determination (X. Lu et al., 2014). They are also involved in apoptosis (Rossi & Antonangeli, 2014), imprinting (Autuoro, Pirnie, & Carmichael, 2014), cell growth (Yin et al., 2014), and dosage compensation (Gendrel & Heard, 2014) (Figure 2).

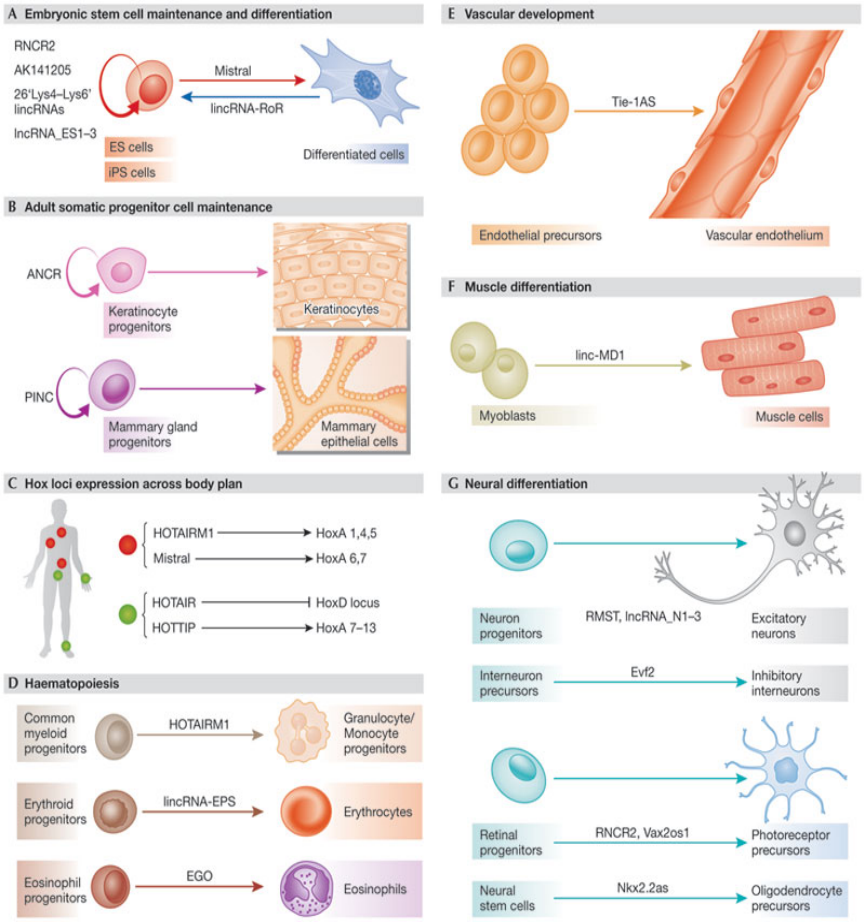


Figure 2- Regulation of mammalian cell differentiation by lncRNAs. a) Some lncRNAs are involved in the maintenance of the pluripotent state of ES cells and differentiation; **b)** lncRNAs involved in the maintenance of adult epidermal lineage progenitor cells; **c)** Many lncRNAs regulate the transcription of Hox gene; **d)** Some of them are involved in haematopoiesis; **e)** Others are involved in vascular developmet; **f)** Some lncRNAs control muscle differentiation; **g)** examples of lncRNAs playing a role in neural tissues, during development and disease (Hu, Alvarez-Dominguez, & Lodish, 2012).

Despite their biological functions have begun to be understood only in the last few years, many lncRNAs have been identified as crucial players in almost every biological process.

Recent works proposed a wide variety of mode of actions for lncRNAs, and further mechanisms still to be discovered cannot be excluded. Frequently, lncRNAs act by interacting with chromatin or DNA modifiers and transcription factors to modulate gene expression at the transcriptional level; competing with microRNAs, acting as sponges to regulate gene expression at the post-transcriptional level or modulating sub-cellular trafficking, translation, splicing, and post-transcriptional modifications (Figure 3, Tables 2-3).

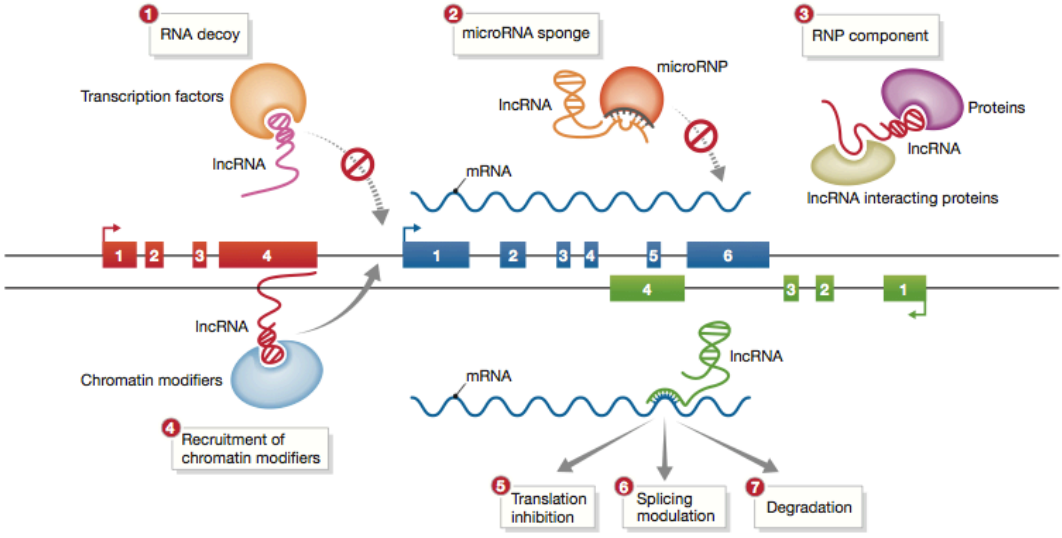


Figure 3 - Mechanisms of lncRNA function. Different mechanisms have described by which lncRNAs can regulate their targets. They can act at transcriptional and post-transcriptional level. They can bind to regulatory proteins; they are able to recruit chromatin-modifying complexes or their DNA targets *in cis*. Some of them seem are also involved in translation, splicing and degradation (Hu et al., 2012).

lncRNA	Function	Mechanism
Regulation of mRNA transcription		
XIST	X inactivation	Chromatin-mediated repression
HOTA1R	Repression at the *HOXD* locus	Chromatin-mediated repression
HOTT1P	Activation at the *HOXA* locus	Chromatin-mediated activation
KCNQIOT1	Imprinting at the *KCNQ1* cluster	Chromatin-mediated repression
ANR1L	Repression at the *lNK4b ARF-INK4a* locus	Chromatin-mediated repression
AIRN	Imprinting at the *IGF2R* cluster	Chromatin-mediated repression, transcription interference
ME4 antisense	Repression of *ME4* mRNA	Transcription interference
IRT1	Repression of *IME1* mRNA	Chromatin-mediated repression
GAL10 lncRNA	Repression of *GAL1* and CAL10 mRNAs	Chromatin-mediated repression
PH084 antisense	Repression of *PH084* mRNA	Chromatin-mediated repression
ICR1	Repression of *FLO11* mRNA	Modulation of transcription factor recruitment
PWR1	Activation of *FLO11* mRNA	Modulation of transcription factor recruitment
SRG1	Repression of *SER3* mRNA	Nucleosome remodelling
fbp1 ncRNA	Activation of *fbp1*	Chromatin remodelling
UNOCR	Activation of lysozyme mRNA	Nucleosome remodelling
Alu repeat-containing RNA	Transcriptional repression during heat shock	Inhibition of Pol II
HSR1	Activation of the HSF1 transcription factor	Allosteric activation together with eEFIA
Non-coding *DHFR*	Transcriptional repression of DHFR	Inhibition of pre-initiation complex formation
CAS5	Repression of glucocorticoid receptor-mediated transcription	DNA mimicry
EVF2	Transcriptional activation of DLX2 targets, transcriptional repression of MeCP2 targets	Recruitment of DLX2 or MeCP2
CCND1 promoter RNA	Repression of *CCND1* transcription	Allosteric activation of TLS
NRON	Repression of NFAT-mediated transcription	Inhibition of transcription factor nucleocytoplasmic shuttling

Table 2 - LncRNA-mediated regulation of proteins (Geisler & Coller, 2013).

Regulation of mRNA processing		
Neuroblastoma *MYC* (NAT)	Inhibition of neuroblastoma *MYC* intron 1 splicing	Unknown mechanism involving the inhibition of splicing via RNA-RNA duplex formation
*Rev-ErbA*alpha	Inhibition of the *c-ErbAalpha 2* splice isoform	Unknown mechanism involving the inhibition of splicing via RNA-RNA duplex formation
ZEB2 (NAT)	Activation of *ZEB2* translation	Unknown mechanism involving regulated splicing of an IRES-containing intron
MALAT1	Ser/Arg splicing factor regulation	Scaffolding of subnuclear domains
Sas10 mRNA 3′UTR	Repression of *Rnp4F* mRNA	Unknown mechanism involving RNA editing

Modulation of mRNA post-transcriptional regulatory pathways		
Antisense *UCHL1*	Upregulation of UCHL1 protein production	SINE2B element-mediated translational upregulation
KCS1 antisense	Production of truncated KCS1 protein	Unknown mechanism involving base pairing
1/2-sbsRNA1	Down-regulation of *SERPINE1* and *FLJ21870* mRNAs	Staufen-mediated decaythrough Alu element base pairing
BACE1AS	Up-regulation of *BACE1*	Stabilization of BACE1 mRNA by blocking miRNA-induced repression
UNCMD1	Control of muscle differentiation through upregulation of MAML1 and MEF2C transcript ion factors	Sequestration of miRNAs
HULC	Downregulation of miRNA-mediated repression	Sequestration of miRNAs
PTENP1 pseudogene	Upregulation of PTEN	Sequestration of miRNAs
IPS1	Downregulation of miRNA-mediated repression	Sequestration of miRNAs
CDR1as	Downregulation of miRNA-mediated repression	Sequestration of miRNAs

Table 3 - LncRNA-mediated regulation of gene expression (Geisler & Coller, 2013).

LncRNAs can exert their function through their intrinsic property to fold into thermodynamically stable secondary structures. Each module can also fold independently from another, forming bonds at the level of Watson-Crick, Hoogsteen and ribose face (Cruz & Westhof, 2009; Lescoute & Westhof, 2006). These RNAs can allow allosteric transitions that can act as switches in response to environmental stimuli. They are processed faster than mRNA, given that they must not be translated, allowing a rapid response to signals. LncRNAs can be regulated via a hundred nucleotide modifications that can modulate their function and structure, as occurs for tRNAs, rRNAs and snoRNAs.

LncRNAs are plastic and show a versatile structure that can contribute to lncRNA functions by binding to proteins, other RNAs, and also DNA (Engreitz et al., 2014).

1.1 LncRNA subcellular localization

In the last years, the development of many innovative high-sensitivity techniques allowed the establishment of functional classes of lncRNA depending on their subcellular localization. Indeed, recent studies revealed that lncRNAs have different subcellular localization patterns, allowing them to carry their different and specific functions (Figure 4).

Cytoplasmic lncRNAs

Cytoplasmic lncRNAs can stabilize or de-stabilize mRNA partners by interacting with RNA binding proteins. As examples, lncRNA *BACE1-AS* (BACE1 antisense) enhances the stability of BACE1 mRNA through a region of partial complementarity, following exposure to various cell stressor (Faghihi et al., 2010), while the lncRNA *TINCR*, by associating directly with STAU1 (Staufen1) forms a ribonucleoprotein (RNP) complex that stabilizes a subset of mRNAs necessary for epidermal differentiation (Kretz et al., 2013). On the contrary, lncRNA *1/2 sbsRNAs* recruits STAU1 resulting in the degradation of target mRNAs via a process called STAU1-mediated mRNA decay, thus promoting myogenesis and adypogenesis (Gong & Maquat, 2011). Another well characterized mechanism of action is the regulation of mRNA translation by complementary base pairing; examples include the regulation mediated by *Uchl1-AS1*(Carrieri et al., 2012) and *p21* lncRNA that, respectively, exert a positive or negative control on their targets. Yoon et al., showed that lncRNA-*p21* binds the RBP HuR (human antigen–R) and leads to the recruitment of microRNA let-7 along with RNA-induced silensing complex (RISC) onto lncRNA-*p21*, triggering its degradation (Yoon et al., 2012). Conversely, lncRNA *Uchl1-AS1* under stress conditions binds the 5'UTR of Uchl1 mRNA triggering the translation of UCHL1 protein (Carrieri et al., 2012). Recently, an additional mechanism of action for the cytoplasmic *lnc-31* has been reported: *lnc-31*, required for myoblast proliferation, promotes ROCK1 protein synthesis, by stabilizing its translational activator, YB-1 (Dimartino et al., 2018). Some of the cytoplasmic lncRNAs can act as competing endogenous RNAs, the so-called "ceRNAs". CeRNAs display miRNA responsive elements (MREs) along their sequence, therefore, they are able to sequester miRNAs and to act as miRNA "sponges", thus protecting miRNA targets from repression (Salmena

et al 2011). An example of a lncRNA acting as a sponge is *linc-MD1*, that contributes to muscle differentiation by sponging miR-133 and miR-135, unleashing MAML-1 and MEF2C expression (Cesana et al., 2011) (Figure4).

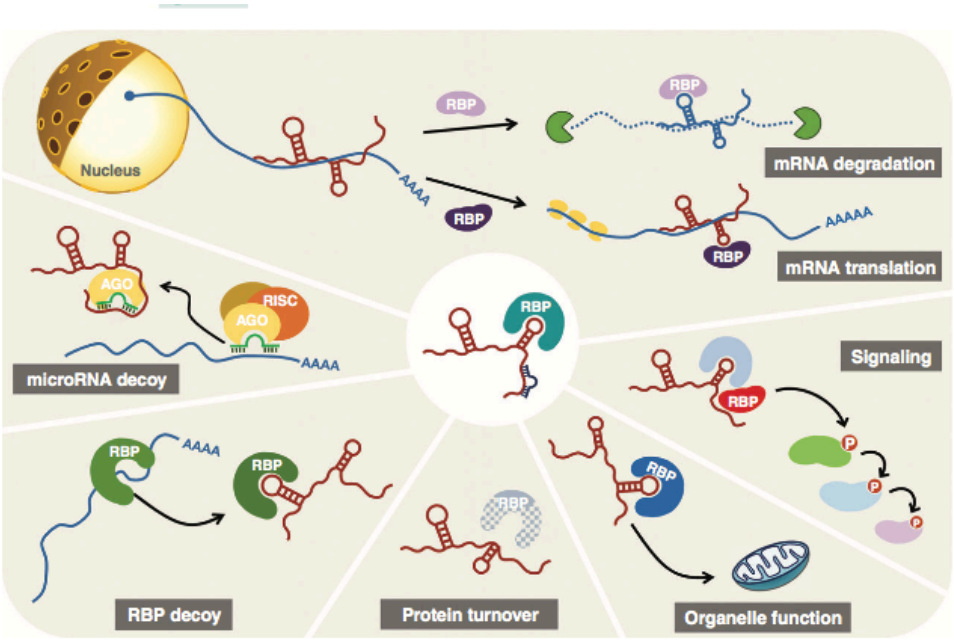


Figure 4- Mechanisms of action of cytoplasmic lncRNAs. Associated with RNA-binding proteins (RBPs) or with partially complementary mRNAs lncRNAs can regulate the stability and translation of specific mRNAs. They can also be involved in activation of signalling molecules or serves as platforms facilitating protein degradation. Finally they can reduce the obtainability of RBP or microRNAS to mRNAs. (Noh, Kim, McClusky, Abdelmohsen, & Gorospe, 2018).

Nuclear lncRNAs

LncRNAs are preferentially enriched in the nucleus relative to the cytoplasm, and in particular they seize the chromatin fraction. Indeed, many lncRNAs are engaged in epigenetic and transcriptional regulation of gene expression (Fatica & Bozzoni, 2014). Nuclear lncRNAs can work *in cis* if they act nearby their transcriptional locus, or *in trans* if they activate or repress the expression of genes located in independent genomic loci (Chu, Zhong, Artandi, & Chang, 2012). Nuclear lncRNAs do not have standard mechanisms of action because they are versatile molecules, but different hypotheses have been proposed (Figure 5):

- *Scaffolds*: when they are able to form complex simultaneously with several molecular components resulting in activation or repression of gene expression (Y. Zhang et al., 2008). An example of lncRNA acting as a scaffold is NEAT1, an highly abundant lncRNA that is crucial for the sequestration of proteins involved in the formation of paraspeckles within nuclei (Clemson et al., 2010; Imamura et al., 2014). Another example of nuclear lncRNA that acts as scaffold is HOTAIR, that is able to interact with PRC2 silencing complex at its 5'- end and with the activating epigenetic complex LSD1/CoREST/REST at its 3'-end. (Raj, van den Bogaard, Rifkin, van Oudenaarden, & Tyagi, 2008).

- *Guides*: if they recruit functional protein complexes and tether them to specific target genes (Tarailo-Graovac & Chen, 2009). This can happen in the case of recombination events that mediate genetic diversity in developing lymphocytes as class switch (CSR) and V (D)J recombination, that seems to be mediated by sense and antisense transcripts dictating the locations of combinatorial events (Abarrategui & Krangel, 2007; Bolland et al., 2004; Verma-Gaur et al., 2012).

- *Enhancer*: if they cooperate for the binding between enhancer region and the promoter of genes (Das et al., 2016).

- *Decoy*: when they sequestrate protein factors in specific nuclear compartment and compete for their binding to other DNA or RNA targets (Tarailo-Graovac & Chen, 2009)

- *Chromosomal architect*: the peculiar function of some lncRNA to contribute to the dynamic structure of the nucleus by the establishment and the maintenance of chromosomal domains responsible for the spatial coordination of gene expression (Harrow et al., 2012) .

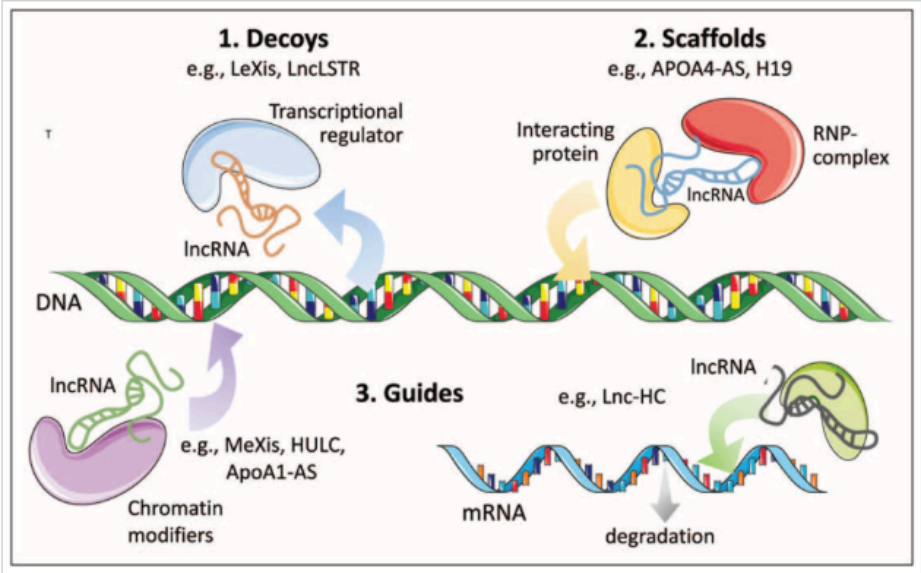


Figure 5- Schematic representation of nuclear lncRNA mechanisms of action. 1) lncRNAs can act as decoys to move proteins away from a specific DNA location; 2) they can serve as molecular scaffolds to bring proteins into stable complexes modulating gene expression;3) lncRNAs can also guide proteins to mRNAs influencing the stability of these transcripts (Van Solingen et al., 2018).

2. Long non-coding RNAs in the immune system

With the advent of RNA-seq technologies and their application in the study of the "non-coding world", the interest toward lncRNAs has been growing as well as the understanding of their multiple cellular functions and possible involvement in different pathologies. In the last years, many studies focused their attention on lncRNAs quantification in different tissues and cell types. Many lncRNA catalogs were generated and all these studies revealed that the expression of lncRNAs is highly cell-type specific (Panzeri, Rossetti, Abrignani, & Pagani, 2015), and this specificity is conserved across evolution. One of the best context to study lncRNAs is the immune system, and in particular in $CD4^+$ T cell differentiation where lncRNAs have been shown to play a role for this process (Ranzani et al., 2015). Upon antigen recognition, $CD4^+$ Naive T cells differentiate into distinct T helper subsets characterized by the expression of specific master transcription factors and by the release of different cytokines. Recently, this simple scenario has been subjected to debate and the idea of distinct T helper cell subset as terminally differentiated lineages has been revisited. Increasing evidences demonstrate that $CD4^+$ T cells exhibit an high degree of plasticity, producing different patterns of cytokines and transcription factors in response

to environmental stimuli (Shea & Paul, 2010). Moreover, in some cases these cells can concomitantly express a subset of different cytokines and transcription factors, together with their prototypical set. For example, IFN-γ, the main Th1 cytokine, is frequently released by Th17 cells simultaneously with IL-17 (Wilson et al., 2007), or IL-10, specifically expressed by Th2 subsets, can be also produced by Th1, Treg and Th17 cells (Hedrich & Bream, 2010). Similar to cytokines, transcription factors can be flexibly expressed in $CD4^+$ T cells. For example, Treg cells express their master regulator Foxp3, but can also express retinoic acid receptor-related orphan receptors γt (RORγt), the principal transcription factor of Th17 and Runx3 (F. Zhang, Meng, & Strober, 2008); on the other hand, TFh cells can differentiate from Foxp3 positive cells also expressing Bcl6, the TFh specific transcription factor (Chung et al., 2011). In this context, lncRNAs play a key role in controlling plasticity and maintaining cell identity, as well as transcription factors and other ncRNAs. In particular, ncRNAs seems to act as fine-tuners of fate choices: they are involved in changes of extrinsic signals causing the alteration of phenotype (Turner, Galloway, & Vigorito, 2014). A lot of single-case or genome wide studies on lncRNAs in murine immune system are now available in literature, but there are only few studies in human context (Table 4) (Heward & Lindsay, 2014). The first functional study on Th1 and Th2 lymphocytes, engage lncRNA *Tmevpg1*, that is specifically expressed in both human and mouse Th1 cells. It is involved in the induction of IFN-γ expression in response to Th1 differentiation program only in this cellular context, underlying the specificity of action of lncRNAs (Collier et al., 2012). Another paper found lncRNA *GATA3-AS1* is selectively expressed in primary Th2 cells and is involved in a co-regulation with GATA3 (H. Zhang et al., 2013). This few examples are just clues of the importance of lncRNAs in human immune system and further analyses are necessary for an in-depth characterization of lncRNA function in the immune system.

LncRNA	Model system	Observation
Innate immune response		
Multiple	Coronavirus infection in mouse lung	RNA-seq demonstrated widespread differential expression of lncRNAs following lung infection with severe acute respiratory syndrome coronavirus in four mouse strains (129/S1, CAST, PWK, and WSB)
Multiple	LPS-stimulated mouse macrophages	Identification of multiple lincRNAs and eRNAs using pol II and H3K36me3 epigenetic marks. Eight of 11 lincRNAs were validated by qRT-PCR
LincRNA-Cox2	LPS-stimulated mouse bone marrow-derived dendritic cells	Identification of 20 lincRNAs including *lincRNA-Cox2* using deposition of epigenetic marks of active transcription (H3K4me3 at their promoters and H3K36me3 within the transcribed region)
LincRNA-Cox2	$Pam3CSK_4$-stimulated mouse bone marrow-derived macrophages	Revealed that *lincRNA-Cox2* repressed the expression of 787 genes in non-stimulated cells and the increased expression of 713 genes following exposure to $Pam3CSK_4$. The actions of *lincRNA-Cox2* were mediated through interaction with hnRNP-A/B and hnRNP-A2/B1
THRIL	$Pam3CSK_4$-stimulated human monocytic THP-1 cells	Microarray analysis identified 159 differentially expressed lincRNAs including down-regulation of antisense lncRNA *THRIL* (TNFα and hnRNPL related immunoregulatory lincRNA). *THRIL* was shown to regulate both basal and $Pam3CSK_4$-stimulated gene expression through an interaction with hnRNPL
Lethe	TNFα-stimulated mouse embryonic fibroblasts	RNA-seq identified 112 lncRNAs and 54 transcribed pseudogenes that were differentially expressed including *Rps15a-ps4* (renamed *Lethe*). *Lethe* was induced in response to IL1β and dexamethasone and shown to interact and block the binding of the RelA (p65) subunit of NF-κB
NEAT1	Poly(IC)- or influenza-stimulated HeLa and human epithelial A549 cells	Increased *NEAT1* expression induced the formation of paraspeckle formation. Redistribution of SFPQ from the *CXCL8* promoter to the paraspeckles following *NEAT1* binding leads to increased *CXCL8* expression
Ptprj-as1	LPS-stimulated mouse bone marrow-derived macrophages	Induced in response to LPS
IL1β-RBT46 and *IL1β-eRNA*	LPS-stimulated human monocytes and monocytic THP-1 cells	RNA-seq identified 76 eRNAs, 40 lincRNAs, 65 antisense RNAs, and 35 regions of bidirectional
Unnamed	LPS-stimulated K562 leukemias cells	Multiple lncRNAs were located upstream of *TNF* and shown to negatively regulate *TNF* expression, possibly through binding to the transcriptional repressor, LRRFIP1 [leucine rich repeat (in FLII) interacting protein 1]
Lnc-IL7R	LPS-stimulated monocytic THP1 cells	*Lnc-IL7R* is transcribed from the 3′-UTR of *IL7R* in the sense orientation. Induced following LPS stimulation and negatively regulates IL7R, IL8, IL-6, VCAM-1, and E-selectin expression, a process associated with diminished H3K27me3 levels
PACER	PMA- and LPS-stimulated human U937 monocytic cell line	*PACER* (p50-associated COX-2 extragenic RNA) is expressed upstream of the *Cox2* promoter and positively regulates COX2 production. *PACER* binds to, and drives the release of, the repressive p50 dimer of NF-κB from the *Cox2* promoter
Lnc-DC	Differentiation of human and mouse dendritic cells	*Lnc-DC* (LOC645638) is required for monocyte differentiation into dendritic cells (DC). *Lnc-DC* promotes phosphorylation and activation of STAT3, a transcription involved in DC differentiation, by blocking its dephosphorylation by SHP1
Adaptive immune response		
Multiple	Human $CD8^+$ T cells	Microarray studies identified 100s of lymphoid-specific lncRNAs and showed differential expression during $CD8^+$ T cell activation and following differentiation into $CD8^+$ memory and effector T cells

LncRNA	Model system	Observation
NTT	Human T cell lines	*NTT* (noncoding transcript in $CD4^+$ T cells) was identified in activated T cells
Gas5	Human primary T cells and T cell lines (CEM-C7 and Jurkat)	*Gas5* (growth arrest specific transcript-5) levels increase upon growth arrest and inhibit cell-cycle progression and promote apoptosis
Gas5	Human primary T cells	Inhibition of T cell proliferation through the mTOR antagonist rapamycin is mediated by upregulation of *Gas5*
NRON	Human Jurkat T cell	*NRON* (noncoding repressor of NFAT) blocked the nucleocytoplasmic transport and therefore the transcriptional activity of NFAT through interaction with multiple proteins including members of the importin-β superfamily
NRON	Human Jurkat T cells and mouse T cells	*NRON* shown to attenuate NFAT dephosphorylation and thereby block NFAT nuclear translocation, activation, and induction of IL-2
NeST	Transgenic mouse infected with *Salmonella* and Theiler's virus	Overexpression of *NeST* (Nettoie Salmonella pas Theilers's) was shown to increase clearance of bacterial *Salmonella* infection but reduce resistance to the mouse Theiler's picornavirus. *NeST* induced the expression of IFN-γ through an interaction with WD repeat domain 5 (WDR5), a core subunit of the MLL histone H3 lysine 4 (H3K4) methyltransferase complex
LincR-Ccr2-5′AS	Mouse $CD4^+$ T_H2 cells	RNA-seq studies identified 1524 lincRNAs in 42 mouse T cell subsets. *LincR-Ccr2-5′AS* was located at the 5′-end of *Ccr2* in $CD4^+$ T_H2 cells and was shown to regulate both the induction and suppression of gene expression during T_H2 differentiation. *LincR-Ccr2-5′AS* is also implicated in chemokine-mediated signalling including cell migration
Multiple	Mouse T and B cells	LncRNAs shown to regulate chromatin remodelling associated with variable, diversity, and joining (V(D)J) recombination required to produce antigen receptors (Ig or TCR)
Multiple	Mouse B cells	Transcription of antisense and sense lncRNAs is associated with looping of V_H regions into close proximity with the DJ_H region during recombination in pro-B cells, a process that occurs within transcription factories
Pathogen-associated		
PAN	KSHV-infected B cell lines	PAN (polyadenylated nuclear) RNA expression from KSHV was shown to modulate host cell response including downregulation of IFNγ, IL18, and α-interferon 16
PAN	KSHV-infected B- and T cell lines	PAN RNA-mediated suppression of host genes is mediated through polycomb repression complex 2 (PRC2)-mediated histone methylation

Table 4 - LncRNAs and immune response (Heward & Lindsay, 2014) .

2.1 Novel lncRNA signature in human primary lymphocytes

In our lab we published a broad analysis of lncRNA transcriptome of human lymphocytes, providing the first comprehensive catalogue of lncRNAs expressed in 13 distinct human lymphocyte subsets (Ranzani et al., 2015). This analysis highlighted that lncRNAs display high cell specificity. More in detail, this study defined the profile of lncRNAs by RNA-seq from $CD4^+$ Naive, Th1, Th2, Th17, T regulatory (Tregs), Central memory (CM), effector memory (EM), $CD8^+$ Naive, CM, EM, B Naive, B memory, and B $CD25^+$ lymphocyte subsets purified by FACS from PBMC of healthy donors (Table 5). In this work, an RNA-seq analysis pipeline for the identification of both novel and already annotated long non-coding RNAs expressed in these

subsets was exploited, resulting in a collection of almost 5000 lncRNAs genes. To identify new lncRNAs expressed in human primary lymphocytes, three *de novo* transcriptome reconstruction strategies were used, combining two different sequence mappers (TopHat and Star) with two different tools for *de novo* transcripts assembly (Cufflinks and Trinity). Through this kind of analysis, 563 novel lncRNA genes were identified, increasing by 11.8% the total number of lncRNAs known to be expressed in human lymphocytes (Figure 6).

Subset	Purity (%)	Sorting phenotype	Genes
$CD4^+$ naïve	99,8 ± 0,1	$CD4^+$ $CCR7^+$ $CD45RA^+$ $CD45RO^-$	20061
$CD4^+$ T_H1	99,9 ± 0,05	$CD4^+$ $CXCR3^+$	20855
$CD4^+$ T_H2	99,7 ± 0,3	$CD4^+$ $CRTH2^+$ $CXCR3^-$	19623
$CD4^+$ T_H17	99,1 ± 1	$CD4^+$ $CCR6^+$ $CD161^+$ $CXCR3^-$	20959
$CD4^+$ T_{reg}	99,0 ± 0,8	$CD4^+$ $CD127^-$ $CD25^+$	21435
$CD4^+$ T_{CM}	98,4 ± 2,8	$CD4^+$ $CCR7^+$ $CD45RA^-$ $CD45RO^+$	20600
$CD4^+$ T_{EM}	95,4 ± 5,5	$CD4^+$ $CCR7^-$ $CD45RA^-$ $CD45RO^+$	19800
$CD8^+$ T_{CM}	98,3 ± 0,8	$CD8^+$ $CCR7^+$ $CD45RA^-$ $CD45RO^+$	20901
$CD8^+$ T_{EM}	96,8 ± 0,9	$CD8^+$ $CCR7^-$ $CD45RA^-$ $CD45RO^+$	21813
$CD8^+$ naïve	99,3 ± 0,2	$CD8^+$ $CCR7^+$ $CD45RA^+$ $CD45RO^-$	20611
B naïve	99,9 ± 0,1	$CD19^+$ $CD5^-$ $CD27^-$	21692
B memory	99,1 ± 0,8	$CD19^+$ $CD5^-$ $CD27^+$	21239
B $CD5^+$	99,1 ± 0,8	$CD19^+$ $CD5^+$	22499

Table 5. Purification and RNA-sequencing of human primary lymphocyte subsets: purity obtained by sorting 13 human lymphocyte subsets using different combination of surface marker (sorting phenotype) and number of expressed genes (FPKM >0.21).

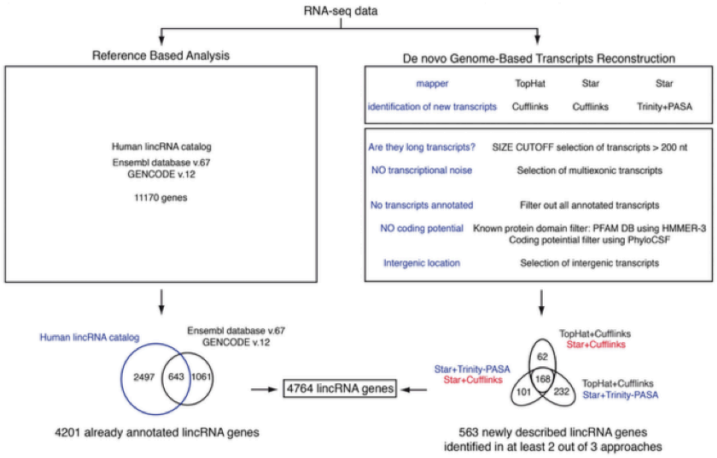


Figure 6. Identification of lncRNAs expressed in human lymphocyte subsets. RNA-seq data were processed according to two different strategies: quantification of lncRNAs already annotated and *de novo* Genome Based Transcripts Reconstruction for the quantification of newly identified lncRNAs expressed in

human lymphocytes. The number of lncRNA genes and transcripts identified in lymphocytes subsets is indicated (Ranzani et al., 2015).

As already found in other cellular systems, these lncRNAs showed a much higher cell specificity (73%) than their coding counterpart (31%) and notably this feature is maintained even when lncRNAs were compared to genes encoding for membrane receptor (40%), that are generally considered the main marker of different lymphocyte subsets (Figure 7). These findings suggest that lncRNAs might contribute to the definition of lymphocytes identity and to the modulation of their functional plasticity. Starting from these datasets, we extracted signatures for lncRNAs specifically expressed in the different lymphocyte subsets.

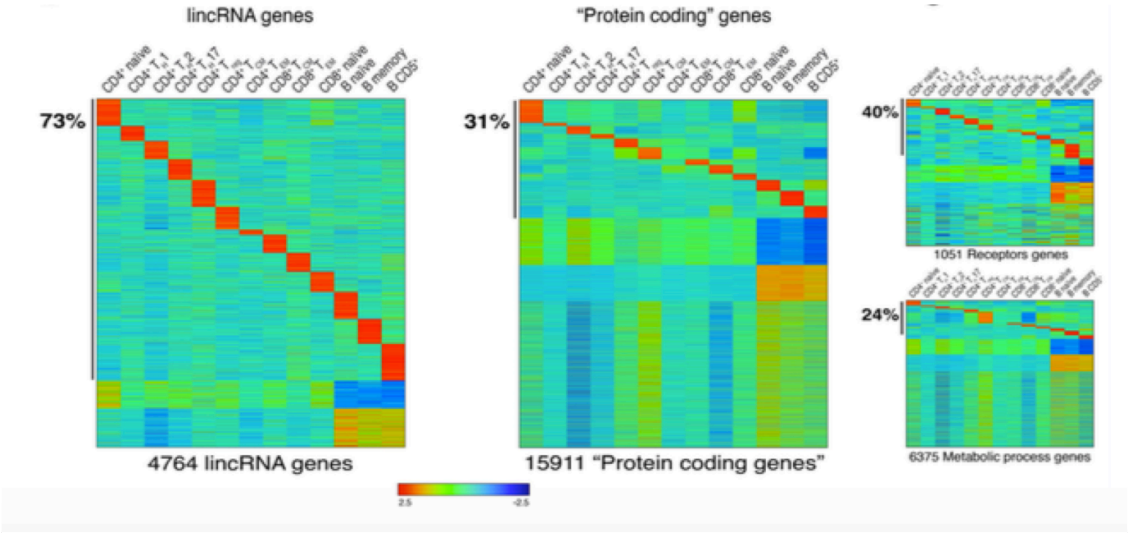


Figure 7. Definition of transcript clusters in human lymphocytes. K-means clustering and cell specificity of lncRNAs, coding and receptors genes across 13 human lymphocyte subsets. Color intensity represents the raw log-normalized FPKM counts estimated by Cufflinks (Ranzani et al., 2015).

By analysing lncRNA signatures in sixteen different human tissues, it was demonstrated that lncRNAs belonging to lymphocytes signatures are very poorly expressed in non–lymphoid tissues and, interestingly, they are not detectable when the whole lymphoid tissue is analysed (Figure 8). All these findings revealed the importance to assess the expression of lncRNAs in purified primary cells rather than in total tissues, where one cell-specific transcript can be diluted by the transcripts of all the other cell types of the tissues.

It is important to note that these newly identified lncRNAs are more expressed and more cell-specific than the already annotated lncRNAs defined as signatures.

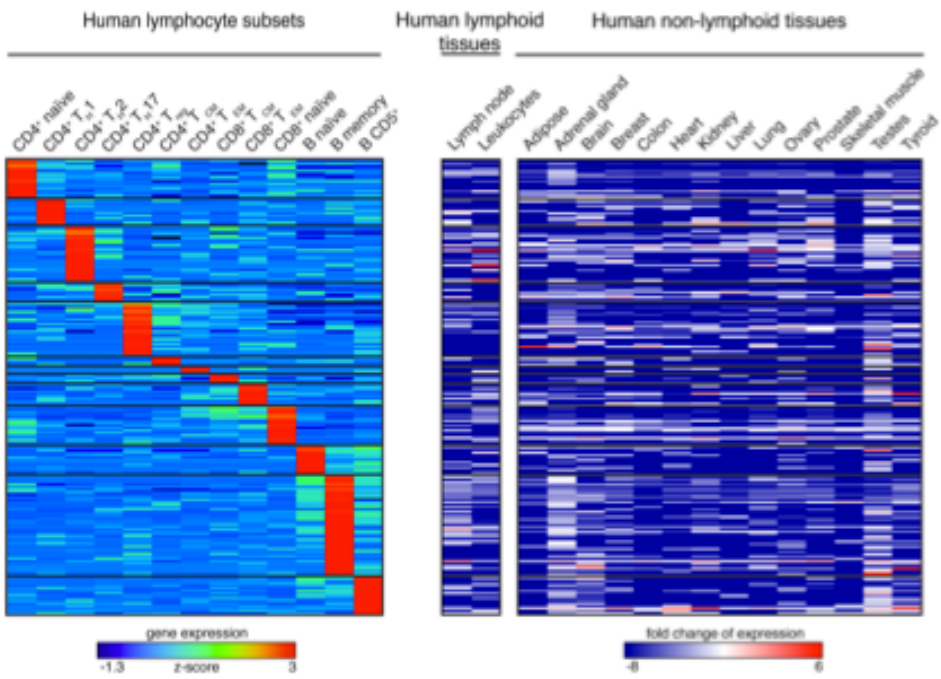
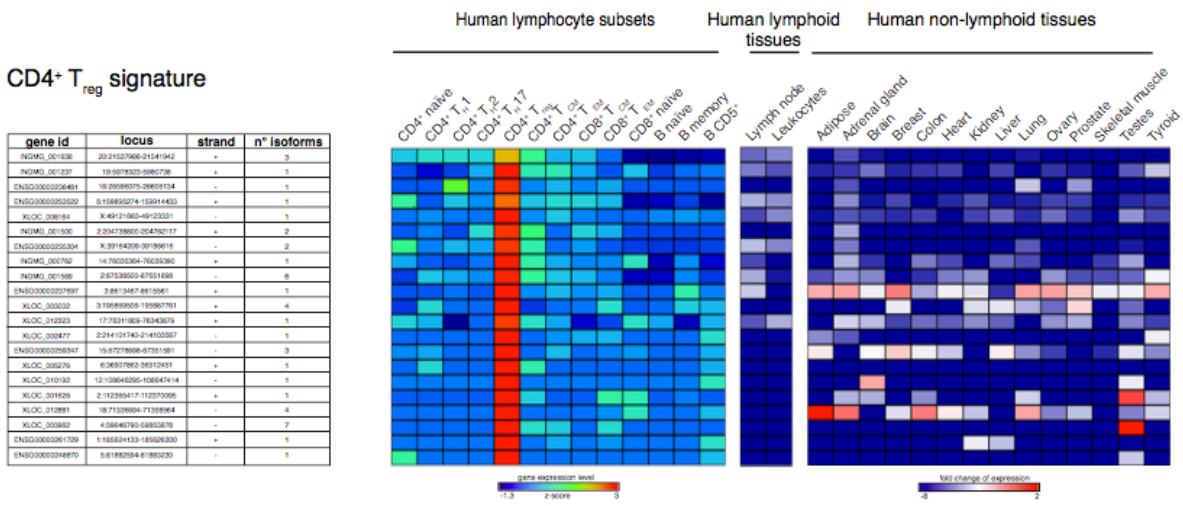


Figure 8. LncRNA signature of human lymphocyte subsets. Heatmap of normalized expression values of lymphocyte lncRNA signature (fold change > 2-5 respect to all the other subsets). Signature lncRNAs relative expression values calculated as $\log_2$ ratios between lymphocyte substes and a panel of human lymphoid and non-lymphoid tissues (Ranzani et al., 2015).

Starting from the list of lncRNAs, Ranzani et al. also provided evidences of the functional role of a chromatin associated $CD4^+$ Th1 specific lincRNA, named linc-MAF-4 due to its proximity to MAF gene, that inversely correlates with the transcription factor MAF and whose down-regulation skews $CD4^+$ T cell differentiation toward Th2 phenotype.

Besides the functional characterization of linc-MAF4 function in Th1 cells, a great number of newly identified, $CD4^+$ subset-specific lncRNAs still needs to be investigated. In particular, a list of 21 specific $CD4^+$ Treg cell signature lncRNAs have been defined, whose functional relevance is currently unknown (Figure 9).

One of these uncharacterized $CD4^+$ Treg specific lncRNA, named lncFOXP3, appeared to be more relevant than the other, due to its close proximity with *FOXP3* gene. Foxp3 is the key transcription factor, located on the X chromosome, stably expressed in Treg cells and fundamental for their development and proper function. More accurate analysis of this Treg specific lncRNA could be fundamental to assess if this lncRNA can contribute to Treg cell identity and functions.



gene id	locus	strand	n° isoforms
NGMG_001838	20:21537966-21541942	+	3
NGMG_001237	19:5078323-5980736	+	1
ENSG00000236491	16:29596075-29605134	-	1
ENSG00000252622	5:158695274-159914433	+	1
XLOC_008184	X:49121663-49123331	-	1
NGMG_001500	2:204739605-204762117	+	2
ENSG00000235304	X:39164208-39186616	-	2
NGMG_000762	14:76035364-76039360	+	1
NGMG_001588	2:87539503-87551898	-	6
ENSG00000237897	3:8613467-8615561	+	1
XLOC_000002	3:195689505-195887761	+	4
XLOC_012323	17:7831169-76343679	+	1
XLOC_000477	2:214101743-214103557	-	1
ENSG00000258347	15:67278698-67351561	-	3
XLOC_000276	6:36937662-36912451	+	1
XLOC_010192	12:108646295-108647414	-	1
XLOC_001526	2:112389417-112573095	+	1
XLOC_012891	18:71336694-71368964	-	4
XLOC_000952	4:58646793-58653878	-	7
ENSG00000261729	1:185624133-185626300	+	1
ENSG00000248870	5:81882584-81883230	-	1

Figure 9. **LncRNA signature in CD4$^+$ Treg subset** Heatmaps of signature lncRNAs expression for CD4+Treg cells subsets. For each lncRNA gene id, locus, strand prediction and number of isoforms are also reported (Ranzani et al., 2015).

3. CD4$^+$ T regulatory cells in immune system

Since early 1970s, it has been known that the T lymphocyte compartment contains some cells indispensable for the maintaining of immune homeostasis and tolerance (Sakaguchi, Wing, & Miyara, 2007). This T-cell population was intensively studied over the following years in various fields of immunology but was finally abandoned much due to the lack of good phenotypic markers (Sakaguchi et al., 2007). T cell-mediated suppression was slowly resurrected in the 1980s and early 1990s, as demonstrated by the publication in 1995 by Shimon Sakaguchi and colleagues, describing the regulatory properties of a sub-population of CD4$^+$ T lymphocytes in mice that constitutively expressed high amounts of the interleukin-2 receptor α–chain (IL-2Rα, CD25). They showed that the passive transfer of T cell suspensions depleted of CD4$^+$ CD25$^+$ T cells into athymic nude mice resulted in the spontaneous development of various T-cell mediated autoimmune diseases, whereas co-transfer of a small number of CD25$^+$ CD4$^+$ T cells clearly inhibits the development of autoimmunity. Six years later, probably due to the gloomy memory of the suppressor cell debacle, equivalent cells were described also in humans. To get off to a good start, they were renamed regulatory T cells (Treg) (Sakaguchi, Yamaguchi, Nomura, & Ono, 2008)) (Figure 10).

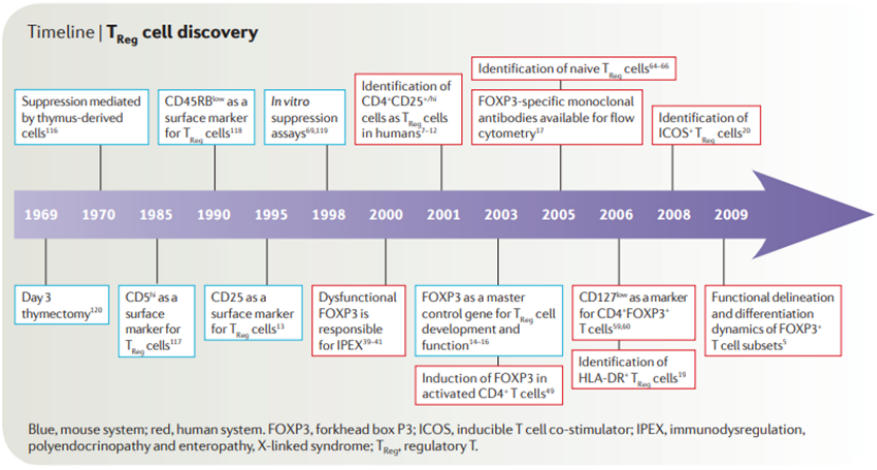


Figure 10. Treg cell discovery over the years (Sakaguchi, Miyara, Costantino, & Hafler, 2010)

These cells play an important role in controlling on-going immune responses and silencing self-reactive T cells. Indeed, Treg cells act as "policemen" of the immune system by actively controlling the proliferation and activation of cells of both the adaptive and innate immune systems (Gondek, Lu, Quezada, Sakaguchi, & Noelle, 2005). In addition Treg cells are important to maintaining tolerance to self-tissues, in sustaining fetal and transplanted tissues and in promoting tissue repair. Furthermore, these cells promote tolerance to components of the 'extended self', such as nutrients and other environmental exposures. As a result, to the great scientific interest in the Treg field over the past decades, the knowledge has vastly expanded together with the number of described Treg subtypes. There are two different type of $CD4^+$ Treg cells: 'natural' Treg (nTreg) and 'induced' Treg (iTreg), defined by where they develop, which have complementary and overlapping functions in the controlling of immune responses. NTreg cells develop in the thymus during the course of positive and negative selection, while iTreg cells develop in the periphery from conventional $CD4^+$ T cells following antigenic stimulation (Workman et al., 2009) (Figure 11).

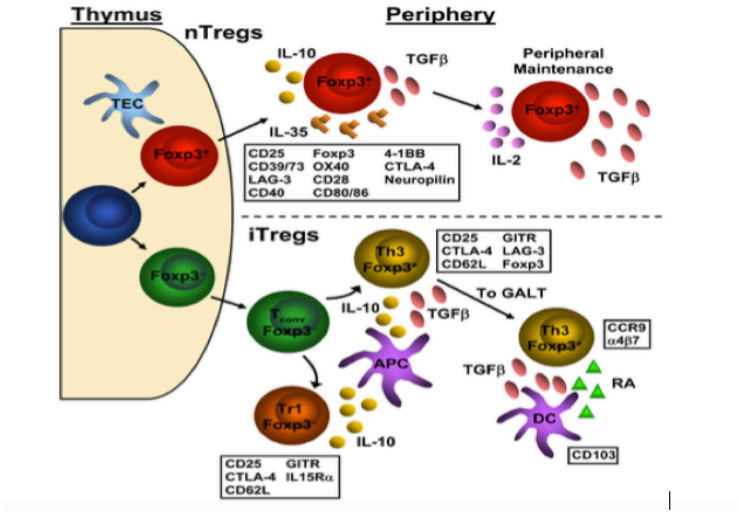


Figure 11. Development of nTreg and iTreg cells. nTreg cells differentiate from Naïve conventional T cells to Foxp3$^+$ Tregs in the thymus. In the periphery, nTreg start to expess a number of cell surface markers. On the other side iTreg can be generated from conventional T cell precursors. Once in the periphery, Naïve conventional T cells can be induced to become Foxp3$^-$ Tr1 cells or Foxp3$^+$ Th3 cells (Workman et al., 2009)**.**

3.1 Natural CD4$^+$ CD25$^+$ Treg cells

Natural CD4$^+$ CD25$^+$ T regulatory, as all the other T cells, arise from progenitor cells in the bone marrow and undergo their lineage commitment and maturation in the thymus. They represent a small population of peripheral CD4$^+$ T cells, but their presence is crucial (Sakaguchi, 2004). Once generated, the thymic Treg cells are exported to peripheral tissues, where they function normally to prevent the activation of other, self-reactive T cells that have the potential to develop into effector cells. As previously mentioned, Treg population was first defined as a subpopulation of CD4$^+$ T lymphocytes constitutively expressing high levels of the IL-2 receptor α chain (CD25), differently from conventional T cells, which express CD25 transiently after activation (Sakaguchi et al., 1995). Together with β chain (CD122) and the common cytokine receptor γ-chain (CD132), CD25 forms the high- affinity IL-2R. Interestingly, IL-2R signalling has been shown to be important for the development and maintenance of Treg cells (Shevach et al., 2006). Mice lacking both CD25 (and CD122) and IL-2 are defective in Treg cells, suggesting a functional role for CD25 and the requirement for IL-2 by Treg. Intriguingly, Treg cells have no capacity to produce IL-2 and they rely on IL-2-secreting activated T effector cells (Teffs).

Thus, by expressing high levels of CD25, Tregs can deprive Teffs of IL-2 and impair their proliferation and survival. In addition, it has been shown that in human only the CD25high population correlates with suppressive capacity, whereas the CD25low/intermediate cells did not suppress T lymphocyte proliferation (Baecher-Allan, Brown, Freeman, & Hafler, 2001). However, the CD25 molecule cannot be used as a differentiation marker for Treg cells due to its expression by other T cells when activated by T cell receptor (TCR) ligation. The comparison between genes expressed by $CD25^+$ Treg cells versus genes expressed by $CD4^+$ T cells allowed the identification of the X chromosome-encoded forkhead transcription factor Foxp3 as a key controller of the development and function of nTregs. It was shown that Foxp3 is a specific lineage marker fundamental for Treg cells suppressive activity (Fontenot, Gavin, & Rudensky, 2003). Despite its specificity and pivotal role for Treg cells, because of its intracellular localization its impossible to use it as potential marker for the isolation and purification of viable Treg cells (Workman et al., 2009). Treg phenotype has been also linked to the expression of other surface markers, like CTLA-4 and glucocorticoid-induced tumor necrosis receptor (GITR) (Ronchetti et al., 2015). CTLA-4 is implied to play a crucial role in Treg-mediated suppression *in vivo* and *in vitro.* CTLA-4 binds CD80 and CD86 on antigen presenting cells (APCs) inducing an indirect suppressive effect through blockade of CD28 co-stimulation. Although the majority of human Treg cells constitutively express high levels of intracellular CTLA-4, the expression of surface CTLA-4 is induced on all $CD4^+$ T cells upon activation. For this reason, CTLA-4 expression cannot distinguish Treg cells from activated conventional T cells (Tconv) during immune activation, moreover as occur for CD25, is not possible to use it for the identification of live Treg cells. The same problem occurs with GITR, a member of tumor-necrosis-factor receptor (TNFR) superfamily that is expressed on the surface of $CD4^+$ $CD25^+$ Treg cells. Depletion of GITR-expressing T cells can cause organic-specific autoimmune diseases in normal mice. However, like CTLA-4, GITR is also upregulated upon $CD4^+$ T cell activation, and thus, these molecules have limited utility as marker for isolation of Treg cells. In 2006 Liu et al., discovered the surface molecule CD127, interleukin 7 receptor- α (IL7R-α) chain, that became an important sign for human Treg cells identification. They showed that the expression of CD127 is down-modulated on Treg cell and it inversely correlates with Foxp3 expression and suppressive activity of Tregs (Liu et al., 2006). $CD4^+$ $CD25^{hi}$ CD127- isolated Treg cells showed the best purity, function, stability and *in vitro* expansion capacity and promising isolation of pure Treg cells with high suppressive activity. Later on, Yu et al., found that $CD4^+$ $CD25^+$ $CD127^{low/-}$ T cells expressed the highest level of Foxp3 and had the strongest correlation with $CD4^+$ $CD25^+$ $Foxp3^+$ T cells, the accepted identifying characteristics for "real" natural Treg population (Yu et al., 2012). Moreover, was showed that $CD4^+$ $CD25^+$ $CD127^{low/-}$ T cells could suppress the proliferation of $CD4^+$ CD25- T cells, suggesting that these cells perfectly fit the definition of naturally regulatory T cells in human

peripheral blood. Therefore, CD127 expression alone cannot accurately discriminate Treg cells from activated T cell *ex vivo*. CD62L (L-selectin) expression can also be used to differentiate between Treg cells, which are $CD25^{hi}CD127^{low}CD62L^{+}$, and recently activated conventional $CD4^{+}$ T cells, which are $CD62L^{low}$ (Schmetterer, Neunkirchner, & Pickl, 2012). Moreover, in healthy individuals, $CD4^{+}$ $CD25^{+}$ Treg cells have also an activated/memory phenotype, predominantly expressing $CD45RO^{+}$, indicating that these cells have previously encountered antigen *in vivo*. The $CD45RO^{+}$ $CD25^{hi}$ $FOXP3^{hi}$ cells are activated and functionally differentiated subset of Treg cells. They are highly proliferative *in vitro* and *in vivo* and have more potent suppressive activity. On the other hand, "Naive" Treg cells are characterized by the surface expression of CD45RA and low levels of Foxp3. Expression of CD45RA without concomitant expression of CD45RO is a phenotypic marker for Naïve T cells that have not experienced TCR stimulation-mediated maturation. Contrary to $CD45RO^{+}$ Treg cells, most of $CD45RA^{+}$ $FOXP3^{low}$ Naïve Treg cells also express CD31, a cell surface marker specific for recent thymic emigrants. Therefore, it can be concluded that the majority of thymus-derived Treg cells found in the periphery are $CD45RA^{+}$ $FOXP3^{low}$ Naïve Treg cells. The absence of Ki67, a nuclear proliferation marker, indicates that these Naïve Treg are quiescent, the reason why are also defined as 'resting' Treg cells. Naïve Treg cells proliferate after in vitro TCR stimulation and are highly resistant to apoptosis, which is in contrast to $CD45RO^{+}$ $CD25^{hi}$ $FOXP3^{+}$ Treg cells, which tend to be hyper-responsive and apoptotic after activation *in vitro* (Figure 12).

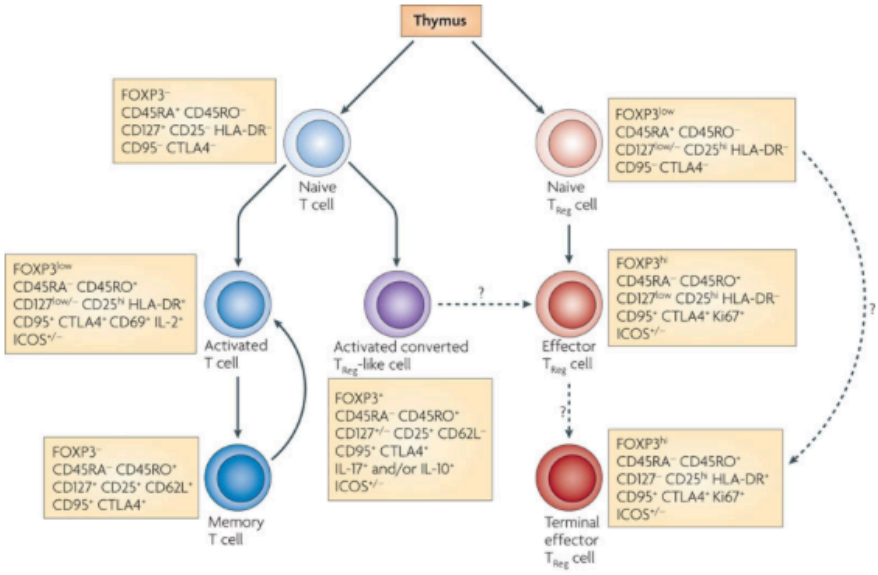


Figure 12. Treg cell differentiation and specific markers for each population. Different phenotypic markers can be expressed during $CD4^+$ T cell differentiation into conventional T cell and regulatory T (Treg) cell lineage (Sakaguchi et al., 2010).

3.2 FoxP3 is the master regulator of Treg cell phenotype and function

The master regulator for the development and function of Treg cells is the transcriptional factor FoxP3 (Forkhead box P3), a member of the forkhead/winged-helix family of transcription factors that controls a gene regulatory network essential for Treg suppressive functions. The *FOXP3* gene was identified as the defective gene in the mouse strain Scurfy, an X-linked recessive mutant exhibiting hyper-activation of $CD4^+$ T cells and overproduction of pro-inflammatory cytokines (Brunkow et al., 2001). Mutations of the human *FOXP3* gene causes the genetic disease IPEX (immune dysregulation, polyendocrinopathy, enterophaty, X-LINKED syndrome), (Sakaguchi et al., 2008). *FOXP3* gene, consists of 11 exons, located in the p-arm of the X chromosome (L. Lu et al., 2018) and a high degree of conservation is shared between human and mouse genes. *FOXP3* gene contains three conserved functional domains: a carboxy-terminal forkhead domain (FKH) which mediates DNA binding and nuclear localization, a leucine-zipper domain necessary for homo-and-hetero dimerization and finally a zinc-finger domain that seems to be dispensable for dimerization and repressive function (Vent-Schmidt, Han, Macdonald, & Levings, 2014). Contrary to the other FOXP family members, *FOXP3* encodes for a proline-rich N-terminal region and does not contain a C-terminal binding protein 1 transcriptional repressor domain. This N-terminal region is sufficient to promote the suppressive actions of *FOXP3* and the minimal repressor domain has been mapped to the N-terminal 67–132 amino acids (Lopes et al., 2006).

Interestingly, whereas mouse T cells have a single isoform of *FOXP3*, human T cells express different splicing isoforms of this protein. The best-characterized are known as FOXP3b and FOXP3a expressed at the same levels (Allan et al., 2005). Even if ectopic expression of the FOXP3b isoform is sufficient to convert T conventional cells into suppressive Treg cells, it is not able to interact with the retinoic-acid related orphan receptor alpha (ROR-α) since this association occurs via the LxxLL motif in exon 2 (Du, Huang, Zhou, & Ziegler, 2008). FOXP3a can be present in the cytoplasm or nucleus whereas FOXP3b is localized only in the nucleus, since it lacks the nuclear-export signal encoded in a lysine-rich region on exon 2 (Figure 13).

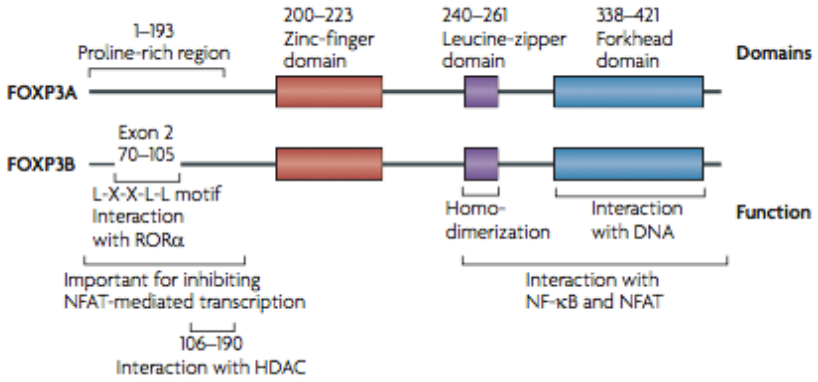


Figure 13. Schematic representation of Foxp3 protein. Isoform A (FOXP3a) and isoform B (FOXP3b) protein structure, with all domains and relative putative functions (Sakaguchi et al., 2010)

Three highly conserved non-coding regions in *FOXP3* locus are involved in the regulation of its transcription. The first one (CNS1) is the promoter region located upstream of the first exon of Foxp3 that is activated in response to TCR signalling through binding of transcription factors like nuclear factor of activated T cells (NFAT) and AP-1 (Mantel et al., 2006). The second highly conserved non coding region (CNS2) has been identified as TGFβ-sensitive element and contains binding sites for NFAT and SMADs (Tone et al., 2008). The third conserved region (CNS3) in *FOXP3* locus has been identified in CpG-rich enhancer and/ or stabilizer region that is fully demethylated in Treg cells and methylated in conventional T cells and normally referred as the Treg-cell-specific-demethylated region (TSDR) (Baron et al., 2007) (Figure 14).

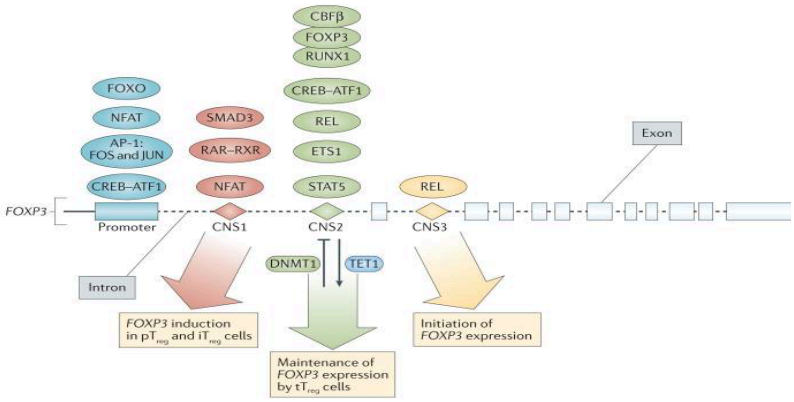


Figure 14: The control of *FOXP3* by transcription factors and regulatory elements. Transcription factors involved in the activation and maintenance of *FOXP3,* binding its promoter or the three conserved non-coding sequence (CNS1, CNS2 and CNS3) (L. Lu et al., 2018).

Foxp3 was found to interact with multiple transcription factors involved in activation, differentiation and response of $CD4^+$ T cell to TCR stimulation, such as NFAT (Wu et al., 2006), nuclear factor kappa-B (NF-kB) (Bettelli, Dastrange, & Oukka, 2005), runt-related transcription factor 1 (RUNX1)(Ono et al., 2007), RORs (Zhou et al., 2008), IFN regulatory factor 4 (IRF4) (Zheng et al., 2009), signal transducer activator of transcription 3 (STAT3) (Chaudhry et al., 2009) and Jun (Lee, Gao, & Fang, 2008). Genome wide analysis has shown that Foxp3 binds the promoter region of many genes associated with TCR signalling. A large number of Foxp3-boud genes were up-regulated or down-regulated in $Foxp3^+$ T cells, indicating that this protein can acts as both a transcriptional activator and repressor, and is thought to lock the regulatory program in place by amplifying and stabilizing transcriptional profile of Treg precursor cells (Marson et al., 2010). Many transcriptional targets of human and murine FoxP3 protein were revealed, including genes whose expression is up-regulated like CD25, CTLA4 and GITR, or repressed as IL-2 and PTPN22 (Zheng et al., 2007). Constitutive expression of FoxP3 is fundamental for the maintenance of Treg cells suppressor function. Indeed, the constitutive expression of FoxP3 in mature Treg cells was demonstrated to be indispensable for the maintenance of tolerance mediated by these cells (Williams & Rudensky, 2007)(Zheng et al., 2009).

The precise molecular mechanisms regulating Foxp3 expression are still not completely understood, but it has been reported that TGF-β, IL-2, or TCR stimulation of T cells can result in increased Foxp3 expression (Kim & Leonard, 2007; Yao et al., 2013). In Treg cells, the expression of Foxp3 is not unique, given that *in vitro* TCR stimulation of $CD4^+$ $CD25^-$ T cells results in the transient expression of Foxp3 mRNA and protein. However, the vast majority of cells do not exhibit a

suppressive phenotype, and it is possible that Foxp3 acts here to prevent T cell hyper-activation (J. Wang, Ioan-Facsinay, van der Voort, Huizinga, & Toes, 2007). In contrast, a small population of these TCR-stimulated $CD4^+$ $CD25^-$ cells express both high and stable FoxP3 protein, thus acquiring suppressive capacity (Allan et al., 2005). All these studies have shown that the persistent expression of FoxP3 is essential for the maintenance of suppressor function of Treg cells.

3.3 Post-translational modification of FoxP3

The post-translational modification (PTM), like acetylation, phosphorylation and ubiquitylation, play an important role on FoxP3 protein regulation and consequently in the control of Treg cell function (Figure 15)

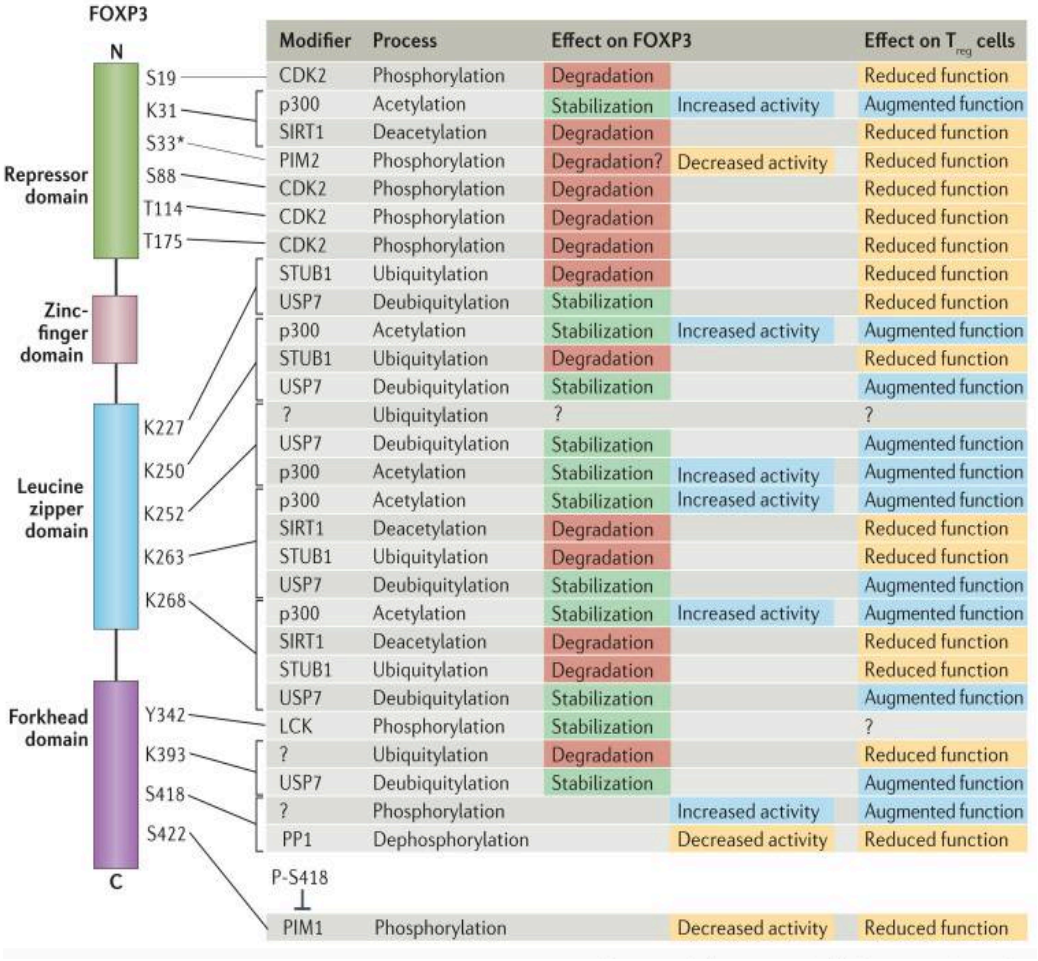
Acetylation is mediated by enzymes called lysine acetyltransferases (KATs). This kind of post-translational modification occurs at specific lysine residues and neutralizes the positive charges required for histones to compact chromatin structure. Acetylated histones are generally associated with decondensation of DNA and activation of gene transcription. Acetylation makes FoxP3 more stable, improves its ability to bind chromatin and to carry out its functions as a transcriptional regulator (Van Loosdregt et al., 2010). In particular TIP60 and p300 are the two principal KATs responsible for the acetylation of K63, K263 and K268 of FoxP3, favouring its association with the promoters of its target genes (Van Loosdregt et al., 2010). Inhibition or deletion of p300 reduces levels of acetylated and total Foxp3 in Treg cells, negatively affecting the viability and function of these cells (Xiao et al., 2014). The deletion of TIP60 also decreases FoxP3 expression levels, leading to autoimmune disease (L. Wang et al., 2016). Conversely, Lysine deacetylases (KDACs) and HDACs, such as the well known SIRT1, remove acetyl groups from FoxP3, negatively affecting its protein levels and Treg cells function (Beier et al., 2011).

FoxP3 is also subjected to phosphorylation. The effect of this PTM depends on the protein domain that is affected; in particular the C terminus of FoxP3 can be modified by an unknown kinase at S418; this phosphorylation increases FoxP3 ability to bind to DNA. Conversely, the phosphorylation of FoxP3 at other sites can limit its ability to promote Treg cell activity.

Cyclin-dependent kinase 2 (CDK2) is another protein able to phosphorylate four CDK motifs within the N-terminal domain of FoxP3, negatively affecting Foxp3 levels and function (Morawski et al,. 2013). It was shown that CDK2-deficient Treg cells are more suppressive than wild type control (Chunder, Wang, Chen, Hancock, & Wells, 2012). The kinase PIM1, which is highly expressed by human Treg cells, can phosphorylate the S422 at C-terminal domain of FoxP3

interfering with FoxP3 activity and limiting the expression of CD25, CTLA4 and GITR (Z. Li et al., 2014).

Several studies have shown that ubiquitylation is another PTM occurring on FOXP3 protein: when FoxP3 is ubiquitylated, it is prone to undergo proteosomal degradation, thus modulating Treg cell functions (Ben-Neriah, 2002). During $CD4^+$ T cells differentiation, Hypoxia-inducible factor 1(HIF1) interacts with FoxP3 and triggers K48 polyubiquitulation, leading to the proteosomal degradation of FoxP3 (Dang et al., 2012). Another specific interactor of FoxP3 is the chaperone molecule heat shock 70 kDa protein (HSP70), which recruits the stress-activated U-box domain type E3 ubiquitin ligase STUB1 that mediates the proteosomal degradation of some proteins including FoxP3, reducing its levels and consequently leading to Treg inactivation (Chen et al., 2014). As polyubiquitylation negatively affects the stability of FoxP3, deubiquitinases (DUBs) maintain the levels of FoxP3 protein, stabilizing Treg cells phenotype. Indeed, DUBs catalysing the removal of ubiquitin from specific protein substrates can prevent protein degradation (Nijman et al., 2005). The DUB ubiquitin-specific peptidase 7 (USP7) is expressed in different type of T cells, including Treg cells. In particular, it has been shown that USP7 is upregualted and active in Treg cells and it catalyse the deubiquitylation of FoxP3 (Loosdregt et al., 2014). Ectopic expression of USP7 specifically decreased FoxP3 polyubiquitination, resulting in increased FoxP3 protein expression. Conversely, knock-down of USP7 reduces FOXP3 levels in Treg cells and inhibits their suppressive function (Loosdregt et al., 2014). A recent study demonstrated that the deletion of USP7 in mouse Treg cells cause a deregulation of immune system, leading to a lethal auto immunity within a month after birth (L. Wang et al., 2016). This phenotype is associated with unstable FoxP3 protein levels, hyper-proliferation, alteration of Treg cell gene expression patterns, and inhibition of their suppressive activity. Surprisingly, the authors of this study found that *in vivo* administration of a specific USP7 antagonist inhibited FoxP3 expression and the suppressive activity of Treg cells in tumor-bearing mice. Also, they showed that the antagonist suppressed the growth of tumors by augmenting antitumor immunity (L. Wang et al., 2016). This suggest that targeting USP7 could have therapeutic potential in cancer, due to its control on FoxP3 protein levels. The regulation of FoxP3 by PTMs represent a new layer in the study of immune regulation.



Modifier	Process	Effect on FOXP3		Effect on T_{reg} cells
CDK2	Phosphorylation	Degradation		Reduced function
p300	Acetylation	Stabilization	Increased activity	Augmented function
SIRT1	Deacetylation	Degradation		Reduced function
PIM2	Phosphorylation	Degradation?	Decreased activity	Reduced function
CDK2	Phosphorylation	Degradation		Reduced function
CDK2	Phosphorylation	Degradation		Reduced function
CDK2	Phosphorylation	Degradation		Reduced function
STUB1	Ubiquitylation	Degradation		Reduced function
USP7	Deubiquitylation	Stabilization		Reduced function
p300	Acetylation	Stabilization	Increased activity	Augmented function
STUB1	Ubiquitylation	Degradation		Reduced function
USP7	Deubiquitylation	Stabilization		Augmented function
?	Ubiquitylation	?		?
USP7	Deubiquitylation	Stabilization		Augmented function
p300	Acetylation	Stabilization	Increased activity	Augmented function
p300	Acetylation	Stabilization	Increased activity	Augmented function
SIRT1	Deacetylation	Degradation		Reduced function
STUB1	Ubiquitylation	Degradation		Reduced function
USP7	Deubiquitylation	Stabilization		Augmented function
p300	Acetylation	Stabilization	Increased activity	Augmented function
SIRT1	Deacetylation	Degradation		Reduced function
STUB1	Ubiquitylation	Degradation		Reduced function
USP7	Deubiquitylation	Stabilization		Augmented function
LCK	Phosphorylation	Stabilization		?
?	Ubiquitylation	Degradation		Reduced function
USP7	Deubiquitylation	Stabilization		Augmented function
?	Phosphorylation		Increased activity	Augmented function
PP1	Dephosphorylation		Decreased activity	Reduced function
P-S418 ⊥ PIM1	Phosphorylation		Decreased activity	Reduced function

Figure 15. List of post-translational modifications of FoxP3. Schematic representation of FOXP3 structure with its functional domains and post-translational modification sites. On the right of the table are listed different type of modifications, their effects on FoxP3 protein stability and function and consequently, the impact on suppressive function of Treg cells (L. Lu et al., 2018)**.**

3.4 Treg cell suppressive function

Upon antigen exposure in the regional lymph nodes, natural Foxp3$^+$ Treg cells become activated and exert their suppressive function. Different mechanisms have been proposed for how Tregs can exert their suppression on other immune cells, mostly on the basis of *in vitro* suppression assays (Figure 15).

Treg cells can exert their suppressive function secreting soluble inhibitory cytokines such as TGF-β, IL-10 and IL-35. The cytokine IL-10 exerts immunosuppressive effects on different cell types. It is able to inhibit the production of specific pro-inflammatory cytokines like IL-12 and after the binding to its receptor IL-10R, it blocks the proliferation of effector T and DC cells. Similar to IL-10, the multifunctional cytokine TGF-β is able to inhibit the production of IL-12, resulting in the suppression of effector T cells differentiation and proliferation.

Finally, IL-35 is an immune-modulatory cytokine predominantly expressed by Treg cells. It is ables to suppress the proliferation of helper T cells and to promote the conversion of naive T cells into Treg suppressive cells (Arce-Sillas et al., 2016; Taylor, Verhagen, Blaser, Akdis, & Akdis, 2006).

Another suppressive mechanism relies on the production of cytotoxic molecules. Indeed, similar to NK and CD8$^+$ lymphocytes, also Treg cells are able to produce perforin and granzyme A or B and it has been demonstrated that Treg cells exert a perforin-dependent cytotoxicity against target cells (Gondek et al., 2005; Grossman et al., 2004). A third suppressive mechanism of Treg cells include the surface expression of inhibitory molecules such as CTLA4 and LAG-3. Regarding CTLA4, it can compete with the co-stimulatory molecule CD28 for the binding to CD80 and CD86, inducing cell cycle arrest, preventing IL-2 secretion and limiting T cell contact with APCs (Schneider et al., 2006). Moreover, CTLA4 interactions with APCs has been demonstrated to induce secretion of indoleamine 2,3-dioxygenase (IDO), that catalyzes degradation of tryptophan, resulting in metabolic disruption and starvation of effector T lymphocytes (Munn et al., 1999). Furthermore, the adhesion molecule LAG-3 (or CD-223) is expressed on Treg cells surface and can inhibit dendritic cells (DCs) maturation and activation upon interaction with MHC-II molecules on these cells (Liang et al., 2008). Finally, Treg cells can exert suppressive functions by the elevation of intracellular cAMP levels in responder cells. Indeed, Treg can generate a local anti-inflammatory environment through the activation of adonosine signalling system that has several implications in immune system. Local adenosine production limits T lymphocyte immune responses that leads to increased cAMP levels in the target cells (Deaglio et al., 2007) (Figure 16).

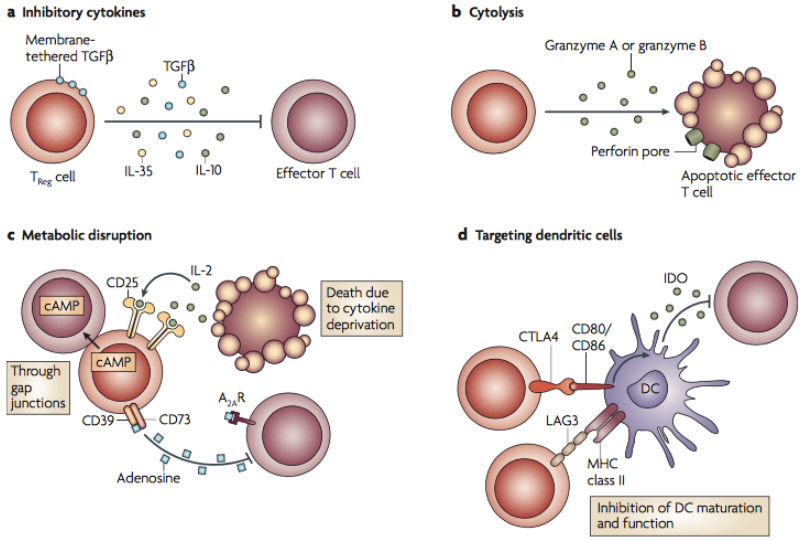


Figure 16. Mechanisms of Treg cells suppressive function (Vignali, Collison, & Workman, 2009). Schematic representation of four different mechanisms of Treg suppressive function: a) Inhibitory cytokine production such as TGF- β; b) Cytolysis; c) Metabolic disruption such as deprivation of IL-2 and release of inhibitory molecule like adenosine; d) Inhibition of DC maturation and function through IDO and LAG3 release.

4. CD4$^+$ T regulatory cells as new therapeutic target

Every year an increasing number of people are affected by autoimmune diseases and cancer. The onset of these pathologies is tightly related with immune system failure to regulate auto-reactive responses and/or to control tumor cell growth. In this context, CD4$^+$ Treg cells have attracted considerable attention because they play a crucial role in the regulation of immune responses and in the maintenance of immunological self-tolerance. Indeed, Treg cells deficiency or dysfunction have been associated to several autoimmune and inflammatory diseases (Oda, Hirata, Guembarovski, & Watanabe, 2013). In contrast, increased Treg cells numbers and/or function is not always beneficial. Indeed, in tumour immunology, increased Treg cell activity contribute to the establishment of conditions that facilitate immune evasion and tumor progression through the inhibition of antitumor effector T cell activity (Steer, Lake, Nowak, & Robinson, 2010). Therefore, Treg cells functional modulation might provide novel therapeutic opportunities to treat several immune mediated diseases. In the case of autoimmune diseases, different approaches have been tried over the years to manipulate activation, expansion or suppressive functions of these cells and several compounds are approved for clinical use (Chang, 2014).

Nonetheless, pharmaceutical companies are still investing money to identify novel approaches for Treg cells functional modulation. As an example, a humanized CD4-specific monoclonal antibody, called Tregalizumab, has been developed and tested in clinical trials. It acts by activating Treg cells and showed promising effects from Phase II trials in psoriasis and rheumatoid arthritis (König, Rharbaoui, Aigner, Dälken, & Schüttrumpf, 2016). The idea that modulation of Treg cells within the tumor environment can lead to improved tumor therapy has been a matter of debate until recently, but, in the last years, evidence is being accumulated to support this hypothesis. Compelling evidence came from a study by Simpson et al., where the mode of action of a monoclonal antibody specific for cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) has been clarified (Simpson et al., 2013). Anti-CTLA-4 treatment, that demonstrated significant antitumor activity in clinical trials for metastatic melanoma (Hodi et al., 2010), has been shown to enhance intratumoral effector T cells activity by selective depletion of Treg cells in the tumor microenvironment. Despite the encouraging results, many current therapies based in modulation of immune responses still use broad-spectrum drugs with serious side-effects. Therefore, the quest for highly specific and less toxic therapies is still an open challenge and a relevant medical need aimed at improving therapies efficacy and reducing unwanted adverse drug events that are detrimental to the patients.

Aim of the project

The human immune system is a suitable context for the study of cell plasticity in response to environmental stimuli. The differentiation of Naïve cells into highly specialized subsets guarantees the proper immune system function. While these subsets were once considered as terminally differentiated cells, they recently demonstrated a high degree of plasticity, whose underlying molecular mechanisms are still poorly understood.

$CD4^+$ T regulatory cells (Treg) thanks to their peculiar suppressive function play a crucial role in the regulation of immune responses and maintenance of immunological self-tolerance. Indeed, Treg cells dysfunction is associated to autoimmune pathologies, inflammatory diseases and cancer. Their inherent plasticity could be exploited as a valuable and promising therapeutic opportunity to modulate their differentiation and function in the context of several autoimmune mediated diseases.

Different compounds are already approved for clinical use to manipulate activation, expansion or suppressive functions of Treg cells. Despite the encouraging results, many current therapies based on modulation of immune responses still use drugs that act systemically with serious side-effects.

We contributed to highlight that long non-coding RNAs (lncRNAs) are key in the modulation of cell plasticity within the human immune system. These molecules proved to be highly specific and fundamental for the maintenance of cell-identity and are reported to be aberrantly expressed in a plethora of human diseases. These features brought lncRNAs to the fore as novel and promising therapeutic targets. On these premises, we are now collecting evidences regarding lncRNAs specifically expressed in human Treg lymphocytes.

In particular, we investigated the role and function of lncFOXP3, a specific Treg long non-coding RNA transcribed upstream *FOXP3* gene, the master transcription factor of Treg cells and fundamental for their proper functions. Due to the close proximity of lncFOXP3 to *FOXP3* gene and the preliminary evidence we collected on its specific Treg expression, here we aim at investigating whether lncFOXP3 plays a role in the maintenance of human Treg cell identity and function, making this lncRNA a promising therapeutic target for Treg cell modulation in immune mediated diseases.

Material and Methods

Purification of human lymphocyte subsets

Buffy coats were obtained from healthy donors at Istituto di Ricovero e Cura a Carattere Scientifico Policlinico Cà Granda, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque centrifugation. $CD4^+$ T cells were purified using Naive $CD4^+$ T cell isolation kit by Miltenyi (130-104-453) and then Naïve and naïve Treg cell population were isolated by sorting on a FACS Aria flow cytometer (BD) by different combinations of surface markers (Table 1).
The ethical committee of Istituto di Ricovero e Cura a Carattere Scientifico Policlinico Cà Granda approved the use of mononuclear cells for research purposes, and informed consent was obtained from all subjects. Sorted human $CD4^+$ Naïve and Treg cells were then expanded *in vitro* by stimulation with IL-2 (20U/ml), anti-human CD3 (OKT3) (30ng/ml), T irradiated PBMC and Rosi-EBV B cells for 3 days and then cultured in complete RPMI 1640 (Euroclone) + IL-2 (20U/ml).

RNA isolation and Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using TRIzol Reagent (Invitrogen) according to manufacturer's instructions and treated with DNase (DNA-free Kit Invitrogen). For RNA pull-down analysis: equal volumes of each RNA sample was reverse transcribed using SuperScript III First-strand Synthesis Super-Mix (Invitrogen) according to the standard protocol. RT products were diluted in PCR reaction mix and amplified using TaqMan Gene expression assay (Applied Biosystems) on a Quantstudio realtime PCR. Enrichemnt was evaluated as quantity relative to the INPUT sample.

For gene expression analyses: 500ng of total RNA was reverse transcribed using SuperScript III First-strand Synthesis Super-Mix according to the standard protocol. RT products were diluted in PCR reaction mix and amplified using TaqMan Gene expression assay on a Quantstudio realtime PCR. The $2^{-\Delta Ct}$ method was used to calculate the expression for each target gene relative to an endogenous control. qRT-PCR program used was the following: single denaturation step of 95°C for 10 min ; 40 cycles of amplification at 95°C for 15 sec and 60°C for 1 min. Each qRT-PCR was performed in triplicate. TaqMan probes used for this study are: LncFOXP3 (AJD1TH7), FOXP3 (Hs01085834_m1), Malat1 (Hs01910177_s1) GAPDH (Hs02758991_g1).

3’ RACE PCR

We employed the technique switching mechanism at the 3’ end of the RNA transcript-rapid amplification of cDNA ends (SMARTer RACE) amplification kit (Clontech, Mountain View, CA), following the manufacturer's instruction. Sequence of 3’ RACE-PCR primers used are listed in Table 2.

Chip

At 14th day of Rapid Expansion Protocol, 10^7 *in vitro* expanded Treg cells from healthy donors were crosslinked in their medium with 1/10 of fresh formaldehyde solution (50 mM Hepes- KOH pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 11%formaldehyde) for 12 minutes. Then they were treated with 1/10 of 1.25 M glycine for 5 minutes and centrifuged at 1350 g for 5 minutes at 4°C. Cell membranes were lysated in LB1 (50 mM Hepes-KOH pH7.5, 10 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40 and 0.25% Triton X-100 supplemented with Protease Inhibitor Cocktail Tablets cOmplete, EDTA-free (Roche) and Phenylmethanesulfonyl fluoride (Sigma) at 4°C.
Nuclei were pelletted at 1350 g for 5 minutes at 4°C and washed in LB2 (10 mM Tris-HCl pH 8.0, 200 mM NaCl, 1mM EDTA, 0.5 mM EGTA) supplemented with protease inhibitors.
Nuclei were again pelleted at 1350 g for 5 minutes at 4°C and resuspended with a syringe in 200ul LB3 (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate, 0.5% Nlauroylscarcosine) supplemented with protease inhibitors.
Chromatin was fragmented by ultrasound and cell debris were pelleted at 20000 g for 10 minutes at 4°C. Chromatin Immunoprecipitation was set up in LB3 supplemented with 1% Triton X-100, protease inhibitors and antibodies against H3K3Me1 (Abcam), H3K4me3 (Abcam), H3K27me3 (Millipore), H3K36me3 or no antibody (as negative control) o/n at 4°C. The day after Dynabeads® Protein G (Novex®) were added at left at 4°C rocking for 2 hours. Then the beads were washed twice with Low salt wash buffer (0.1% SDS, 2 mM EDTA, 1% Triton X-100, 20 mM Tris-HCl pH 8.0, 150 mM NaCl) and with High salt wash buffer (0.1% SDS, 2 mM EDTA, 1% Triton X-100, 20 mM Tris-HCl pH 8.0, 500 mM NaCl). Histones IPs were also washed with a LiCl solution (250 mM LiCl, 1% NP-40, 1 mM EDTA, 10 mM Tris-HCl pH 8.0). All samples were finally washed with 50 mM NaCl in 1X TE. Elution was performed o/n at 65°C in 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS. Samples were treated with 0.02 ug/ul RNase A (Sigma) for 2 hours at 37 °C and with 0.04 ug/ul proteinase K (Sigma) for 2 hours at 55°C. DNA was purified with phenol/chloroform extraction and quantified with QuantiFluor dsDNA System (Promega). qPCR

was performed on diluted cDNA with PowerSyberGreen (LifeTechnologies) and specificity of the amplified products was monitored by performing melting curves at the end of each amplification reaction.

Northern blot analysis

For lncFOXP3 and Foxp3 Northern blot, 20 ug of total RNA from Naive and Treg cells was analysed. In parallel, 20ug of total RNA from HEK cells over-expressing lncFOXP3 was used as a positive control. RNA was mixed with 3 volumes of RNA loading dye (Sigma R1386) containing 10ug/ml ethidium bromide and incubated for 10 minutes at 75°C to denature. RNA was loaded onto a 1% denaturing agarose gel containing formaldehyde and run in MOPS 1X. Capillary transfer of RNA to a positively charged nylon membrane (Amersham Hybond-N+, GE Healthcare RPN1520B) was performed overnight. After UV-crosslinking, membrane was pre-hybridized for 2 hours at 42°C in ULTRAhyb™ Ultrasensitive Hybridization Buffer (Invitrogen AM8670) and incubated overnight at 42°C with radioactively labelled probes (see paragraph "Radioactive probe production" for details). Membrane was washed twice in 2X SSC, 0.1% SDS for 15mins and exposed overnight. RNA signal was detected using a FLA-9000 Starion (Fujifilm).

Radioactive probe production

Radioactively labelled probes specific for lncFOXP3, *Foxp3* mRNA and *Gapdh* mRNA were produced by random priming (Random Primed DNA Labeling kit – Merck 11004760001). Specifically, 25ng of PCR products were incubated with 20uCi of alpha-^{32}P- Deoxyadenosine (PerkinElmer NEG512H250UC) according to manufacturer instructions. Unincorporated alpha-^{32}P-Deoxyadenosine was removed using Microspin™ G-50 Columns (GE27-5330-01 Sigma).Oligonucleotides used for PCR amplifications are reported in Table 3

Naïve T cell Activation using anti-CD3/CD28 beads

Naïve CD4$^+$ T cells were *in vitro* activated in 6 well plates at density of 5*10^6 cells per well in RPMI 10% FBS medium (P/S, Sodium pyruvate, non-essential aa, glutamine) in presence of Dynabeads human T activator CD3/CD28 at bead-to-cell ration 1:1 (Thermo Fischer Scientific) and cytokine IL-2 (20U/ml). After 5-6 days, beads were removed and resulting activated CD4$^+$ Naïve T cell were maintained in colture and expanded for the following experiment in presence of IL-2 (20U/ml).

Cell Fractionation

Cell fractionation was performed as described in Gagnon et al.,(Gagnon, Li, Janowski, & Corey, 2014). Treg cells expanded *in vitro* were centrifugated at 500 *g* at 4° C for 5 minutes and then washed in ice-cold 1X PBS. To obtain cytoplasmatic extract, cellular pellet was resuspended in ice-cold HLB Buffer (10 mM Tris pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.3% NP-40 and 10% glycerol) for hypotonic lysis and incubated on ice for 10 minutes. After 8 minutes centrifugation at 4°C, the supernatant (cytoplasmatic fraction) was collected, supplemented with 140 mM NaCl and left on ice until the end of the procedure. The resulting nuclei pellet was washed 4 times by adding HLB Buffer, by pypetting and centrifuging at 200 *g* at 4°C for 2 minutes. For protein analysis, after the last wash the nuclear fraction was resuspended in RIPA Buffer supplemented with 30U /ml of DNAse and incubated on ice for 30 minutes. The cytoplasmatic and nuclear fractions were then centrifugated at 18.000*g* for 15 minutes at 4°C and finally the supernatants were collected and analyzed by western blot. To check the quality of the fractionation Lamin A/C antibody (Sc-376248) was used as a nuclear marker and Tubulin (T8328) as a cytoplasmatic marker. For RNA analysis, expanded *in vitro* Treg were centrifugated at 500 *g* at 4° C for 5 minutes, resuspended in ice-cold HLB supplemented with 100 U of SUPERase-In and leaved on ice for 10 minutes. After 1000 *g* centrifugation at 4°C for 3 minutes the resultig supernatant (cytoplasmatic fraction) was added with 1 ml of RNA precipitation solution (RPS)(3M sodium acetate pH 5.5, 100% ethanol) and stored at -20°C at least for 1 h. The pellet (semipure nuclei) was washed 3 times with ice-cold HLB by pipetting and centrifuging at 200 g at 4°C for 2 minutes. The pellet was then further fractionate into nucleoplasmic and chromatin-associated RNA fractions by adding MWS buffer (10mM TrisHCl pH 7, 4 mM EDTA, 0.3 M NaCl, 1 M urea, 1% NP-40) supplemented with 100 units of SUPERase-in and leaved on ice for 5 minutes. The nuclei were then vortexed for 30 sec and incubated on ice for additional 10 minutes. After 1000 g centrifugation at 4°C for 3 minutes, the resulting supernatants rapresent nucleoplasmic fraction that was immediatly resuspended in 1 ml of RNA precipitation solution (RPS) and stored at -20°C for at least 1h. The resulting pellet, rapresenting the chromatin-bound RNA fraction, was washed 3 times in ice cold MWS, centrifuged at 500 g at 4 °C for 2 minutes and finally resuspended in 1 ml of TRIzol. Nucleoplasmic and cytoplasmic fractions that have been incubated in RPS at -20°C for 1h were then centrifuged at 18.000 g at 4°C for 15 minutes. The pellet were washed in ice cold 70% ethanol, centrifuged at 18.000 at 4°C for 5 minutes and finally resuspended in 1 ml of TRIzol.

Western blot

Total proteins were heated at 95°C for 5 minutes in 4X Laemmly sample buffer and then separated onto 4-12% Bis-Tris protein gels (1% MOPS running buffer) and transferred to PVDF membranes (Thermo Scientific) at 4°C overnight (30Volt constant). Membranes were then blocked using 5% non-fat milk for 2h and incubated with primary antibody at 4°C overnight. Anti-USP7 (D17C6) was used as primary antibody at 1:1000 dilution. After wash, membranes were incubated with secondary antibody Alexa Fluor 680 goat anti-Rabbit IgG 1:10.000 (LOT 1655809) for 1h at room temperature. The protein expression was normalized to β-Tubulin levels. Detection was perfomed using iBright imaging system.

RNA FISH: RNAscope technology

RNA FISH experiments were performed using the RNAscope technology (Wang et al., 2012). A series of target probes were custom-designed to hybridize specific RNA target regions (*Foxp3*, lncFOXP3 and Malat1 sequences). Each probe contains an 18 to 25-base region complementary to the target RNA, a spacer sequence, and a 14-base tail sequence (Z sequence). For fluorescent detection, label probes were conjugated to Alexa Fluor 488 or 647 to detect *Foxp3* and LncFOXP3, respectively. RNA FISH was performed on *in vitro* expanded Naive and Treg cells. For each condition, 200 x 10^3 cells were immobilized on glass slide using cytospin 4 cytocentrifuge (ThermoFisher) at 800 *rpm* for 6 minutes. Cells were fixed in 4% of PFA 1h at 4°C and then washed 3 times with PBS 1X supplemented with 0.01 U/ul of SUPERase-In for 5 minutes. Cells were washed with a specific scale of ethanol (50%-70%-100%-100%-70%-50%) followed by protease digestion (Protease #3) 10 minutes at RT. After that, slides were incubated at 40°C with the following solution: target probes previously diluted in Hybridization buffer A for 3 h; preamplifier in Hybridization Buffer B for 30 minutes; amplifier 1 in Hybridization Buffer B for 15 minutes; amplifier 2 in Hybridization Buffer B for other 30 minutes; and label probe in Hybridization buffer C for 15 minutes. After each hybridization step, slides were washed with wash buffer 3 times at room temperature for 2 minutes. For multiplex detection, equimolar amounts of target probes, preamplifier, amplifier, and label probe of each amplification system were used. Samples were analysed using a Confocal Leica SP5 microscope using MarkAndFind automated acquisition mode.

Objective 63x plus Zoom 3.5x
Sequential-multiparametric laser scanning as following:

Scan1: 405 Laser Diode at 15% Gain 800V Offset -2%

Scan2: 514 laser at 15% Gain 800V offset -2%

Scan3: 488 laser and 633 laser at 20% Gain 800V Offset: -2%

Image format 1024x1024 acquired at laser speed with 400Hrz frequency

82.2 micron pinhole used for all detections

shRNA oligonucleotides designing and cloning

Specific shRNAs targeting different sites of lncFOXP3, *Foxp3* and *Usp7* transcripts were designed by using the GPP Web Portal (https://portals.broadinstitute.org/gpp/public/seq87search), an algorithm that ranks potential 21mer targets within each human and mouse Refseq transcript. The "candidates" targeting transcripts at different position with the higher intrinsic score (a number from 0 to 15 predicting the knockdown successfulness) were selected. ShRNA as well as shRNA-non-targeting control were cloned into pLV [shRNA]-EGFP/Neo-U6 and pLV [shRNA]-Mcherry/Neo-U6 lentivector. Oligos containing a sense and an antisense sequence targeting mRNA, were annealed in presence of NEB Buffer2 (50mM NaCl, 10mM Tris-HCl, 10mM $MgCl_2$, 1mM DTT,pH 7.9@25°C). Once annealed, the dsDNA molecule obtained had at the 5' a sticky end compatible with an AgeI digested site, while at the 3' the end was suitable for ligation with an EcoRI digested site. The sense and the antisense sequences are connected by a spacer capable to forming a loop. The digested fragments were then purified and ligated into pLV cloning vector using T4 DNA ligase (Promega) and then transformed into STBL3 bacteria, following the manufacturer's protocol. Plasmids were the selected by PCR and validated by sequencing. Sequences of shRNAs used in this study are reported in Table 4.

Lentivirus production

Lentiviral particles were produced according to a standard protocol (System Biosciences User Manual). HEK293T cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 unit/ml penicillin, 100 ug/ml streptomycin in 37°C incubator with 5% CO_2. Cells were plated at 30-40% confluence 24h before transfection (70-80% confluence at time of transfection). 25 ug of lentiviral vector with the appropriate insert, 16 ug of psPAX2, 8.4 ug of pMD2.G were co-transfected into HEK293T cells using the calcium phospate precipitation method. The day after transfection, the medium was replaced with fresh medium. Supernatants containing lentiviral particles were collected 42h after transfection.

The supernatant was passed through a 0.45 um SFCA syringe filter and ultracentrifuged through a polyallomer tube at 20000 rpm at 4°C for 2h with SW28 rotor (Beckman Coulter). The concentrated virus was stored at -80° C until use. To determine the vector titer, serial diluted lentiviral particles were transduced to HEK293T cells in 12 well plate. Two days after transduction, cells were trypsinized and the percentage of GFP- or mCherry- positive cells was determined by FACS Canto II. FlowJo887 software was used for data acquisition and analysis.

Treg and HEK293T cellular transduction

$300*10^3$ Treg cells and HEK293T were transduced with either negative control lentiviral vector (LVV MOCK) and lentiviral vector expressing specific shRNA at multiplicity of infection of $1x10^8$ transducing units per ml. in the presence of IL-2 20 U/ml (Miltenyi). The percentage of GFP or Mcherry positive cells was assessed by flow cytometric analysis at FACS CantoII and the resulting positive cells were sorted 5-6 days after transduction using FACS Aria (BD). Total RNA was then isolated with mirVana miRNA Isolation Kit (Ambion) and the knock down was assessed by TaqMan Gene Expression Assay (Applied Biosystems) as well as part of the Treg cell was used for suppression assay.

Molecular Biology

Backbone plasmid for lentiviral vectors comes from System Biosciences (SBI): pCDH vector contains a CMV promoter, a multiple cloning site and a EF1α promoter controlling copGFP expression (SBI, #CD511B). This plasmid was used for the overexpression of FOXP3 in HEK293T cells. Furthermore, to overexpress lncFOXP3 in HEK293T cells this vector was engineered by the substitution of the copGFP portion with the non-signaling version of the human nerve growth factor receptor gene (ΔNGFR).

Establishment of HEK293T cell line stably express lncFOXP3 and *FOXP3*

$300*10^3$ HEK293T cells were transfected using calcium phosphate co-precipitation method as explained before and then, the viruses were purified and concentrated by centrifugation. HEK293T cells were transduced with LVV MOCK and LVV expressing lncFOXP3 and FOXP3 at MOI

$1x10^8$, cultured for 10 days and then single-cell sorted as GFP^{high} and $\Delta NGFR^{high}$ cells, using FACSAria flow cytometer (BD).

Suppression Assay

(CFSE)-labeled responders $CD4^+$ $Naive^+$ T cells (effector) from healthy donors were activated in vitro with Dynabeads αCD3/αCD28 (Thermo Fisher Scientific) and co-cultured with unlabeled $CD4^+$ Treg downregulated (for lncFOXP3 and FOXP3) cells sorted using FACS Aria II (BD Biosciences) at different ratio (1:1,1:05,1:025) (# of $CD4^+$ $Naive^+$ T cells remain constant). CFSE dye will be diluted with each cell division and therefore fluorescence intensity in highly proliferating cells will be lower. Consequently, the suppressive effects of Treg cells correlates with a decrease in the proliferation rate of naive T cells. Proliferation of CFSE-labeled cells was assessed after 3-5 days by FACS Canto.

Biotin RNA endogenous pull-down assay

LncFOXP3 endogenous pull-down assay was performed using a specific pool of biotinylated (3'-SS-biotin) probes to capture the endogenous lncFOXP3. This assay was performed on total extract obtained from $100*10^6$ nTreg cells expandend *in vitro* for 14 days. nTreg cells were washed once with PBS and then UV crosslinked twice on ice using 4000x100 $\mu J/cm^2$ at 254 nm energy in a CL-1000 crosslinker. Pellet was resuspended in Lysis Buffer (25mM TrisHCl, 150 mM Nacl, 0.5% NP-40, 1% TRITON X-100, 0.5 mM B-mercaptoethanol) supplemented with RNase and protease inhibitors and incubated on ice for 2-4h. Nuclear membrane and debris were pelleted by centrifugation at 13.000 g for 10 min at 4°C. 10% of cellular extract was saved as INPUT sample for both RNA and protein analysis. Total extract was subsequently resuspended in Hybridization Buffer (750 mM Nacl, 50mM TrisHCl, 1mM EDTA, 1% SDS, 15% Formamide, and inhibitors) and incubated slowly rotating at 37°C overnight with 100 uM of the specific pool of biotinylated probes (see Table …). The day after, 100 ul/sample of specific magnetic beads (Dynabeads MyOne Streptavidin T1 LifeTech) were pre-cleared in Lysis Buffer for 5 min at room temperature and added to cellular extract for RNA capturing 1h at 37°C in rotation. Beads were then washed with ice-cold Wash Buffer (150 mM NaCl, 100 mM Tris-HCl, 0.005% B-mercaptoethanol) for 5 times (5 minutes each). Co-precipitated proteins were isolated by resuspending 2/3 of the beads in 30µl of elution buffer (35ul of 1X RNaseH Buffer, 3.5ul of RNaseH (NEB)) and analyzed by Western Blot or Mass-spectrometry. 1/3 of beads were resuspended in 1ml of Trizol for RNA analyses. Specific

biotinilated probes are listed in Table 5.

Mass Spectrometry

Protein samples were loaded on an SDS-PAGE precast gel (NuPage Novex 4-12%, 1,5 mm, Invitrogen). Gel-separated proteins were processed for LC-MS/MS analysis as previously described (Shevchenko, Tomas, Havliš, Olsen, & Mann, 2007). Briefly, four gel bands were sliced for each sample, de-stained in 50% v/v ethanol-Ammonium Bicarbonate (AmBic) 50 mM, reduced with 10 mM DTT in 50 mM AmBic for one hour at 56°C and subsequently alkylated with 55 mM iodoacetamide in 50 mM AmBic for 45 min at RT in the dark. Subsequently, gel pieces were extensively washed with 50 mM AmBic, alternated with ethanol, and digested with 12.5 ng/mL trypsin (Promega V5113) in 50 mM AmBic overnight at 37°C. After one overnight, digested peptides were acidified with tri-fluoro acetic acid (TFA, final concentration 3%) and extracted from gel slices with two rounds of washes (in 3% TFA, 30% ACN and then in 100% ACN, respectively). Lyophilized samples were desalted and concentrated on C18-Stage Tips (Rappsilber, Mann, & Ishihama, 2007). The elution was carried out with a highly organic solvent (80% ACN) followed by lyophilisation. Prior to LC-MS/MS analysis, samples were resuspended in 1% TFA in ddH2O.

Liquid chromatography and tandem mass spectrometry (LC-MSMS)

Peptide mixtures were separated by online nano-flow liquid chromatography using an EASY-nLC™ 1000 system (Thermo Fisher Scientific, Odense, Denmark) directly connected to a QExactive instrument (Thermo Fisher Scientific) through a nanoelectrospray ion source. The nano LC system was operated in a one-column set-up with a 25 cm analytical column (75 µm inner diameter, 350 µm outer diameter) packed with C18 resin (ReproSil, Pur C18AQ 1.9 µm, Dr. Maisch, Germany).

Protein identification by MaxQuant software and data analysis

The mass spectrometric raw data were analyzed with the MaxQuant software (version 1.5.2.8) (http://www.maxquant.org/downloads.htm), using the Andromeda search engine (Cox et al., 2011; Cox & Mann, 2008). Peptides were filtered with a false discovery rate (FDR) of 1% for proteins and a minimum peptide length of 6 amino acids. The HUMAN database was used for peptide identification. Peptide and protein identifications were performed automatically with MaxQuant using default settings. Additional option for Match between runs and LFQ were selected. Proteins with p-value ≤ 0.05 were selected as statistically significant.

Table 1

Subset	Purity(%)	Sorting phenotype	Genes
CD4+ naive	99.8 ± 0.1	CD4+ CCR7+ CD45RA+ CD45RO-	20061
CD4+ Treg	99.8 ± 0.1	CD4+ CD127- CD25+	21435

Table 2

Name	Type	Sequence
GSP2_foxp3	3' RACE PCR	CTGCATCGTAGCTGCTGGCAGCCAAG
GSP2_foxy	3' RACE PCR	CAAAGCCAGGCCATCAGGCCCATTC
NGSP2_foxp3	3' NESTED RACE PCR	CCTGTTTGCTGTCCGGAGGCACCTG
NGSP2_foxy	3' NESTED RACE PCR	TGGACGACTAGAACCCTGGGCTTTGC

Table 3

Name	Type	Sequence
LFP3_F	FW	CTTTTCCAGAAGGGTCTGAAGC
LFP3_R	RW	GTGATCATGTTCAATCCTCACC
Foxp3_F	FW	TCCCACCTGGGATCAACGTGGC
Foxp3_R	RW	GTGCTGTTTCCATGGCTACCC
Gapdh_F	FW	CCCCTTCATTGACCTCAACTAC
Gapdh_R	RW	GGCCATCACGCCACAGTTTC

Table 4

shRNA name	Target gene	Sequence	Reporter
MOCK		CCTAAGGTTAAGTCGCCCTCG	GFP/Mcherry
1	lncFOXP3	CGTGAGGATGGATGCATTAAT	GFP
2	lncFOXP3	CAAGTTGCTTGACTACTT	GFP
3	lncFOXP3	GAGATGATGGCGGATATTT	GFP
shFOXP3	FOXP3	AGAGGAACTACTAATTTATTT	GFP
5	USP7	TGTATCTATTGACTGCCCTTT	MCherry

Table 5

Name	Sequence
LFP3_1	5'- ATG GCC CCA AGG TTA ATG AC-Biotin -3',
LFP3_2	5'- TCC TGG GGA GCT GAT TCT AG-Biotin -3'
LFP3_3	5'- GAG CAT GCA TGT CAG GAA GG-Biotin -3'
LFP3_4	5'- CAC CAA GGT GGG ATG AGG TC-Biotin -3'
LFP3_5	5'- AAT GGC TTG GGT GTG TTG GA-Biotin -3'
LFP3_6	5'- GGG ACA CAT CTG AGA CCC AA-Biotin -3'
LFP3_7	5'- TGG TTG AGG CTT CTG AGT TG-Biotin -3'
LFP3_8	5'- AGA CTT GAA GCT TGT GAG GC-Biotin -3'
LFP3_9	5'- TGG GAT TTG GAG AGT CCT TG-Biotin -3'
LFP3_10	5'- GAT GTG ATG GCA GGG AGA TC-Biotin -3'
LFP3_11	5'- GTT CTC AGG TTT TAA ATT CT-Biotin -3'
LFP3_12	5'- CCA AGC TTT CCT GAA CTT GA-Biotin -3'
LFP3_13	5'- TTG GTG CTG GGC TTT GAA AT-Biotin -3'
LFP3_14	5'- CAA ATA TCC GCC ATC ATC TC-Biotin -3'
LFP3_15	5'- CCA GTT TCC AAG GAT TTA GG-Biotin -3'
LFP3_16	5'- CCA GTT TCC AAG GAT TTA GG-Biotin -3'
LFP3_17	5'- ATC ATG GCC TGA TGC TTC TG-Biotin -3',
Malat_1	5'-CAAGGACTCTGGGAAACCTG-Biotin-3'
Malat_2	5'-AGGACAGCTAAGATAGCAGC-Biotin-3'
Malat_3	5'-CTAAATACCACCACCTGGAA-Biotin-3'
Malat_4	5'-ACACCCAGAAGTGTTTACAC-Biotin-3'
Malat_6	5'-CTAAGCGAATGGCTTTGTCT-Biotin-3'
Malat_7	5'-CAAGGCAAATCGCCATGGAA-Biotin-3'
Malat_8	5'-CAAGGCAAATCGCCATGGAA-Biotin-3'
Malat_9	5'-GTGATAGTTCAGGGCTTTAC-Biotin-3'
Malat_12	5'-CATCACCGGAATTCGATCAC-Biotin-3'
Malat_13	5'-GCGAGGCGTATTTTATAGACG-Biotin-3'
Malat_14	5'-CTCCCAATTAATCTTTAìCCAT-Biotin-3'
Malat_15	5'-TCTCCAAATTGTTTCATCCT-Biotin-3'
Malat_17	5'-TACTTCCGTTACGAAAGTCC-Biotin-3'
Malat_18	5'-CTGGGTCAGCTGTCAATTAA-Biotin-3'
Malat_21	5'-AGTCATTTGCCTTTAGGATT-Biotin-3'
Malat_22	5'-AACTGTAAACCTGTGGTGGT-Biotin-3'
Malat_23	5'-CCAAGGATAAAAGCAGCTCC-Biotin-3'
Malat_25	5'-ACTGCCAACTAATTGCCAAT-Biotin-3'
Malat_28	5'-CCCAATGGAGGTATGACATG-Biotin-3'
Malat_29	5'-ATCTCTCATTTATTTCGGCT-Biotin-3'
Malat_30	5'-GATACCTGTCTGAGGCAAAC-Biotin-3'
Malat_32	5'-TCTTTCCTGCCTTAAAGTTA-Biotin-3'
Malat_33	5'-TGTCAATTTATAGACCCCTG-Biotin-3'
Malat_34	5'-AAAGATTGCCTACCACTCTA-Biotin-3'
Malat_35	5'-CCTGAATGGCTTCATGAAGG-Bioton-3'
Malat_36	5'-TGCATTTACTTGCCAACAGA-Biotin-3'
Malat_37	5'-GTCGTTTCACAATGCATTCT-Biotin-3'
Malat_40	5'-CCACTGGTGAATTCAACTGG-Biotin-3'

Malat_41	5'-TTGTCCCATAACTGATCTGA-Biotin-3'
Malat_42	5'-AACACAGTTTGCTCACATGC-Biotin-3'
Malat_43	5'-TGACACTTCTCTTGACCTTA-Biotin 3'
Malat_44	5'-CACTCCAGAAAGAGGGAGTT-Biotin-3'
Malat_46	5'-CATCGTTACCTTGAAACCGA-Biotin-3'
Malat_48	5'-TTGCAGGCAAATTAATGGCC-Biotin-3'
LacZ_1	5'-AATGTGAG GAGTAACAACC-Biotin3'
Lacz_2	5'-ATT AAG TTG GGT AAC GCC AG-Biotin-3'
LacZ_3	5'-AAT AAT TCG CGT CTG GCC TT-Biotin-3'
Lacz_4	5'-AAT TCA GAC GGC AAA CGA CT-Biotin-3'
LacZ_5	5'-ATC TTC CAG ATA ACT GCC GT-Biotin-3'

Results

1. LncFOXP3 is a novel lncRNA of the CD4$^+$ T regulatory cell signature

In our laboratory we performed a broad analysis of lncRNA transcriptome of human lymphocytes, providing the first comprehensive catalogue of lncRNAs expressed in 13 human lymphocyte subsets (CD4$^+$ Naïve, Th1, Th2, Th17, Treg, CM, EM, CD8$^+$ Naïve, CM, EM, B Naïve, B memory and B CD25$^+$) purified by FACS from PBMC of healthy donors (Ranzani et al., 2015).

To increase the knowledge on the functional role of lncRNAs in human lymphocytes, we developed a RNA-seq analysis pipeline for the identification and quantification of both novel and already annotated coding and non-coding transcripts, resulting in a collection of almost 5000 lncRNA genes expressed by human lymphocyte subsets.

Interestingly, this analysis showed that lncRNAs display higher cell specificity than their coding counterpart. From these datasets we extracted signatures for lncRNAs specifically expressed in different lymphocyte subsets.

LncRNA function cannot be easily determined by looking at their primary sequence, indeed lncRNAs folding seems to be more relevant to infer their functional role. There are different approaches for the functional characterization of lncRNAs, we decided in the first place to follow a "guilt by association" approach. Since lncRNAs have been reported to influence the expression of neighbouring genes, we asked whether protein-coding genes proximal to lymphocytes signature lncRNAs were involved in key cell functions. To this purpose, specific bioinformatics functional enrichment analysis revealed that coding genes in close proximity to signature lncRNAs strongly correlated with lymphocyte T cell activation, pointing out a possible role of these novel lncRNAs in lymphocyte functions.

Among the lncRNA signature, we found 71 lncRNAs showing a correlated expression to neighbouring protein coding gene, while 46 showed an anti-correlated expression.

To obtain proof of concept of this hypothesis, we chose to characterize in depth a specific lncRNA, hereafter renamed as lncFOXP3, due to the close proximity to *FOXP3* gene, the master regulator of T regulatory cells. LncFOXP3 belongs to the list of 21 specific CD4$^+$ Treg lncRNAs whose functional relevance is currently unknown (Figure 1a).

Differential expression analysis among 13 cell subsets profiled confirmed a high specificity of lncFOXP3 within Treg cells and showed that its expression correlates with FOXP3 levels only in this lymphocyte subset (Figure 1b). Moreover, RNA-seq data were confirmed by qRT-PCR in a new set of independent samples of human primary CD4$^+$ Treg, Th1 and Th17, as well as in Naïve CD4$^+$ T cells expanded *in vitro* using Rapid expansion protocol (REP) (Figure 1c).

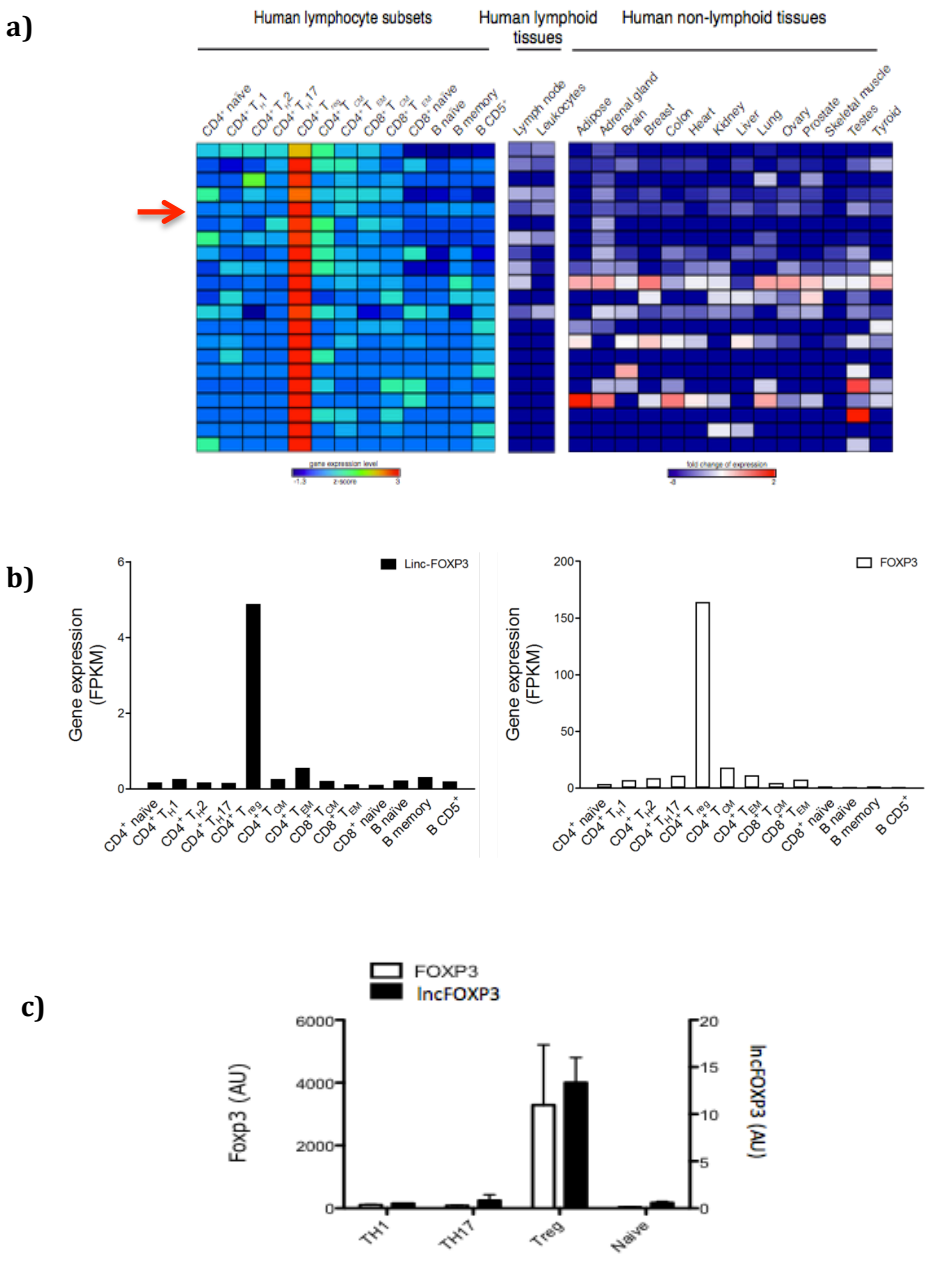


Figure 1- lncFOXP3 is specifically expressed in CD4$^+$ Treg cells. a) Heatmaps of signature lncRNA expression for CD4$^+$ Treg cell subset. Fold expression >2.5 relative to all other subsets. These lncRNAs are expressed in at least 3/5 samples (intra-population consistency). Arrow in red indicate lncFOXP3 **b)** Expression level (FPKM) of lncFOXP3 and its neighbouring protein coding gene *FOXP3* in 13 cell subsets. Differential expression analysis confirmed the high specificity of lncFOXP3 in CD4$^+$ Treg cells. **c)** Validation of RNA-seq data in Th1, Th17, Treg and Naïve cells by qRT-PCR.

2. Genomic characterization of lncFOXP-*FOXP3* locus

In the genomic region where lncFOXP3 is located a structurally similar transcript was previously annotated as TCONS_00017321 (Pj et al., 2012), with genomic coordinates spanning from 49,266,868 to 49,265,202 on ChrX strand - in 5' to 3' direction (GRCh38/hg38, released in 2013), showing a gap of 396 bp between the 3'end of the lncRNA and *FOXP3* TSS. Differently, our RNA-seq data showed a continuous track between lncFOXP3 and *Foxp3* mRNA, suggesting a partial overlap between the two transcripts (Figure 2a). To validate lncFOXP3 end, we performed a 3'RACE experiment (Figure 2b), that confirmed an overlap of 20nt between lncFOXP3 and *Foxp3* mRNA. Therefore, we were able to define novel precise genomic coordinates on ChrX strand - for lncFOXP3, that are: Exon1: 49,266,868 – 49,264,448 Exon2: 49,266,298 – 49,264,806.

This finding strongly hints to a potential implication of lncFOXP3 in the regulation of *FOXP3* expression in T regulatory cells and possibly in the maintenance of their cell identity and function. Based on these findings we decided to pursue the characterization of lncFOXP3.

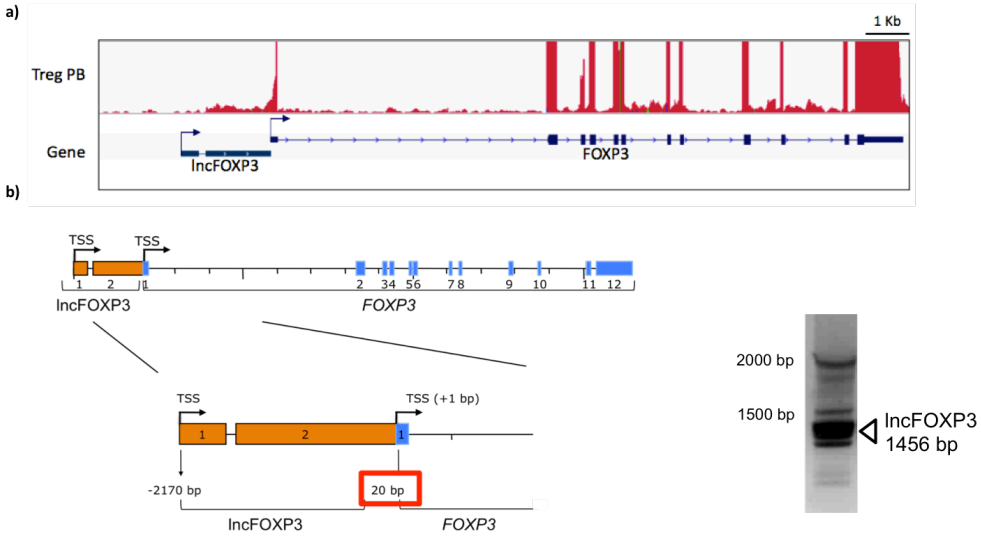


Figure 2- Structural characterization of lncFOXP3-*FOXP3* locus. a) Schematic representation of RNA-seq analysis on lnFOXP3-*FOXP3* locus in Treg cells. **b)** lncFOXP3 3' end identification by 3' RACE showed a partial overlap with *FOXP3* TSS. Agarose gel of lncFOXP3 3' end is reported on the right. In detail, the amplified product of 1456 bp revealed that lncFOXP3 3'end overlap for 20 nt with *FOXP3* TSS.

3. lncFOXP3 and *Foxp3* are two independent transcriptional units

Based on our previous findings, we asked whether lncFOXP3 and *Foxp3* are effectively two independent transcriptional units. Indeed, the partial overlap between the two transcripts in Treg cells gave rise to the possibility that lncFOXP3 is a novel *Foxp3* isoform in this specific cellular context. To solve this issue and conclusively demonstrate that lncFOXP3 is an independent transcript, we performed Northern blot analysis. LncFOXP3 probe was produced by amplifying a 500bp region spanning lncFOXP3 splice junction. The probe is specific for the mature lncRNA and it is far from its 3' end that partially overlap with *Foxp3* 5' UTR. Moreover, a *Foxp3* specific probe, able to recognize both isoforms of *Foxp3* expressed in Treg cells, was produced (Figure 3a). Northern blot analyses performed on *in vitro* expanded Treg cells and Naïve T cells, confirmed that lncFOXP3 and *Foxp3* are two independent transcriptional units. Indeed, as shown in Figure 3b, the expected band of lncFOXP3 (1915 nt) mRNA is clearly detectable and distinguishable from *Foxp3* transcript (2382 nt) in Treg sample. As a further control, we also loaded RNA extracted from HEK 293T cells ectopically expressing lncFOXP3, and do not express *Foxp3* (Figure 3c), in which we can observe the presence of lncFOXP3 expected band (Figure 3b).

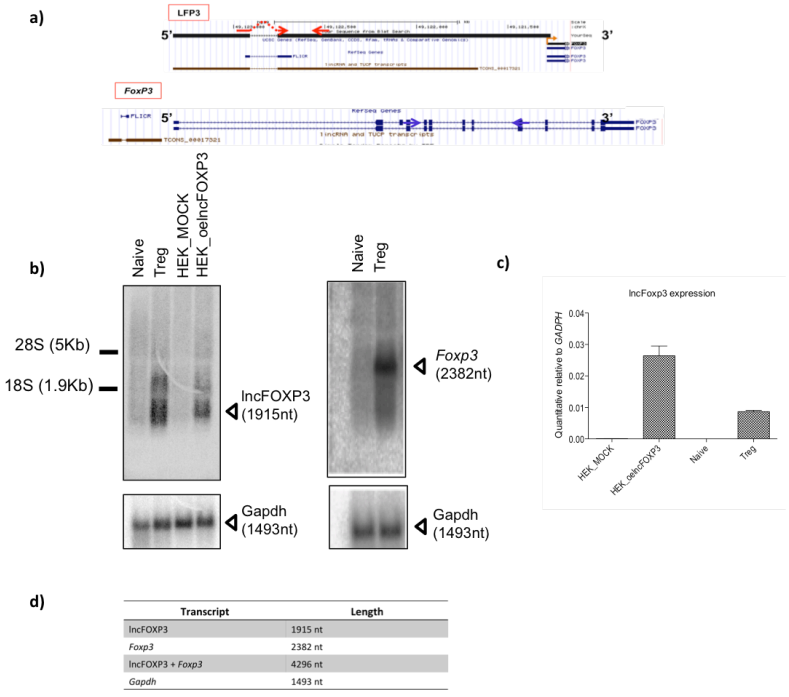


Figure 3- lncFOXP3 and *Foxp3* are two independent transcription units. a) Schematic representation of lncFOXP3 genomic locus. Arrows in red (for lncFOXP3) and in blu (for *Foxp3*) indicate primers used to produce probes used in Northern blot. **b)** Northern blot analysis performed in Naïve, Treg cells expanded *in vitro* and HEK293T cells over-expressing lncFOXP3 (qRT-PCR as a control is also reported in panel **c)** used as positive control, demonstrate that lncFOXP3 and *Foxp3* are two independent transcription units. Gapdh mRNA was used as loading control **d)** Table indicating the attended length for each transcripts.

4. lncFOXP3-*FOXP3* locus is an active chromatin region in Treg cells

LncRNAs are usually considered as key regulators of the expression of their neighbouring genes. As lncFOXP3 is located in close proximity to *FOXP3* gene, we decided to investigate this region from an epigenetic point of view performing ChIP-Seq analysis on *in vitro* expanded Treg cells.

Using ChIP-Seq we profiled genomic occupancy of H3K4me3 and H3K27Ac, associated with active promoters and transcriptionally active chromatin, H3K4me1, related to regulatory regions including enhancers, and H3K27me3 that marks repressive chromatin.

We first check histone modification enrichments on control genes: IL2RA (CD25) that should display epigenetic modifications associated with active transcription, and HOXD11, known to be switched off in Treg cells (Figure 4a). Then, we used the high-throughput ChIP-Seq approach to generate genome-wide H3K4me3, H3K27Ac and H3K4me1 maps of lncFOXP3-FOXP3 region. Treg cells displays an epigenetic configuration that favors active gene expression, while, the same locus, in Naïve T cells, displays a "paused" chromatin configuration (Figure 4b). More in detail, in Treg cells the H3K4me3 and H3K27Ac peaks are located around the TSS of *FOXP3* gene and also in lncFOXP3 locus, while repressive histone marks are absent. Overall, the epigenetic configuration indicates that *FOXP3* and lncFOXP3 are accessible loci in Treg cells. Indeed, these histone modifications can promote the binding of PolII. Moreover, the presence of H3K4me1 that spreads among lncFOXP3 locus can indicate a more active regulatory region. This highly active chromatin configuration at *FOXP3* locus promotes gene expression of this gene and of the lncFOXP3 associated.

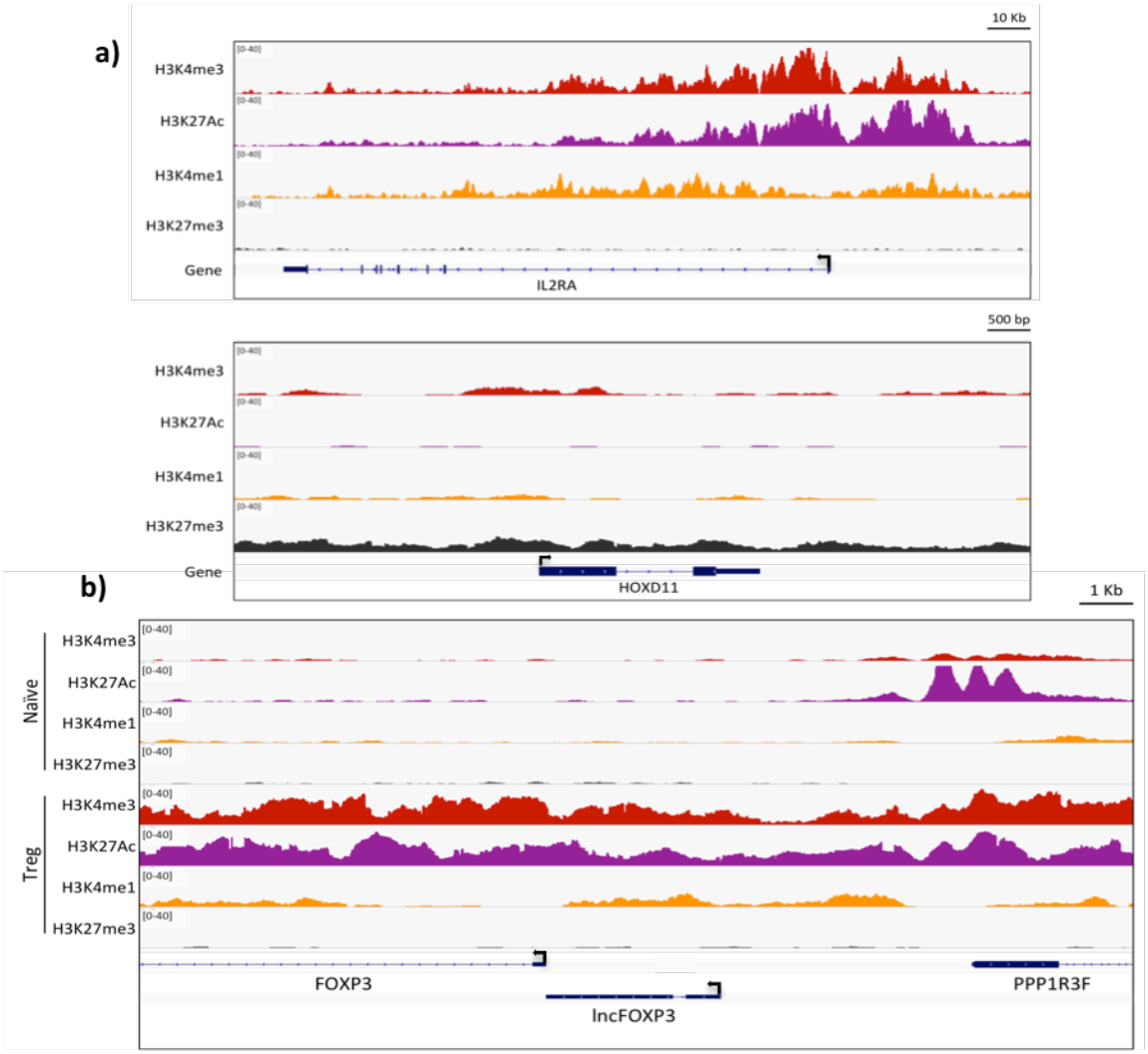


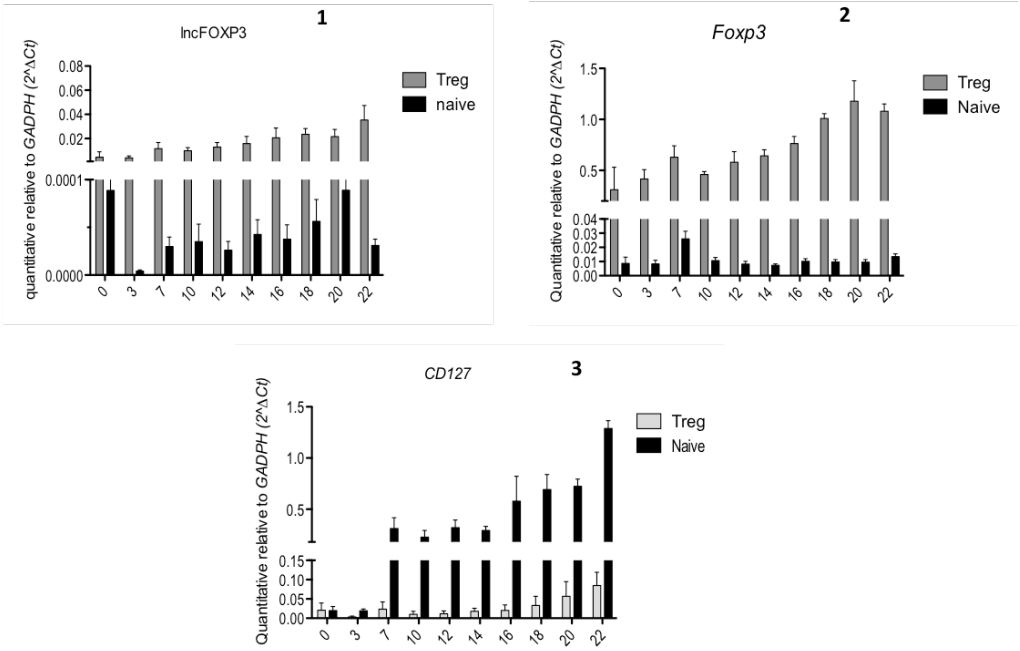
Figure 4- Chip-Seq analysis on lncFOXP3-*FOXP3* genomic locus. a) ChIP-Seq profiling of control genes IL2RA (CD25) and HOXD11. **b)** Chip-Seq profiling of specific histones modifications illustrated by UCSC genome browser graph of 1Kb region of lncFOXP3-*FOXP3* locus in Naïve and Treg cells. On the bottom of the image the genomic localization of *FOXP3* and lncFOXP3 is represented.

5. LncFOXP3 and *FOXP3* are transcriptionally uncoupled

To better understand the expression profile of lncFOXP3 within Treg cells, the expression levels of lncFOXP3 were analysed in human $CD4^+$ Treg and Naive T cells, used as negative control, at different time points after *in vitro* expansion protocol, collecting RNA from day 3 to day 25. Quantitative PCR analysis revealed that lncFOXP3 is expressed at low level in Treg cells in the first days, but its expression increases progressively up to day 20, similarly to what observed for *Foxp3* transcript. As control of canonical Treg phenotype, we checked the expression level of *CD127*.

As expected, it is expressed at low level in Treg cells and, contrary to lncFOXP3 and *Foxp3,* its expression does not increase over time, confirming that only Treg specific genes are upregulated during the *in vitro* expansion protocol (Figure 5a). Furthermore, we performed a time course analysis in human *in vitro* activated $CD4^+$ Naive T cells to assess the expression level of lncFOXP3 and *Foxp3* transcripts (Figure 5b). Human $CD4^+$ Naive T cells showed a transient expression of *Foxp3* upon activation, which has been previously reported in other studies, that is not associated with the up-regulation of lncFOXP3. This result confirmed that these genes are two independent transcriptional units that can be independently transcribed, depending on the cellular context.

a)



b)

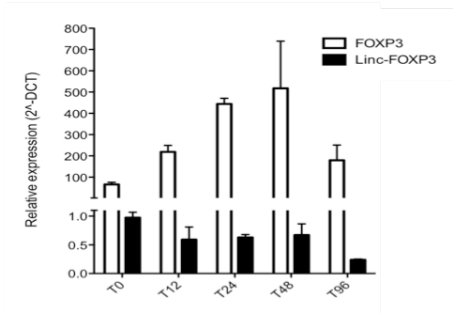


Figure 5- lncFOXP3 and FOXP3 expression profile. a) Expression profile of lncFOXP3 (panel 1a), *Foxp3* (panel 2a) and *CD127* (panel 3a) in $CD4^+$ Naïve T and Treg cells expanded *in vitro* using REP protocol. qRT-PCR shows the relative amount of the three transcripts at different time points, demonstrating that only Treg specific genes are upregulated. Histograms shows means ± sem from three independent experiments.
b) Time course analysis in human *in vitro* activated Naïve T cells demonstrate that the two transcripts are not transcriptionally co-regulated.

6. LncFOXP3 localizes both in the cytoplasm and nucleus of Treg cells

Many evidences demonstrate that lncRNA function is linked to their sub-cellular localization. Therefore, hints of lncFOXP3 putative functions in $CD4^+$ T regulatory cells can be obtained by a detailed analysis of its localization. To determine lncFOXP3 sub-cellular localization, *in vitro* expanded $CD4^+$ Treg cells were biochemically fractionated into nuclear, cytoplasmic and chromatin fractions and lncFOXP3 levels were analysed by qRT-PCR.

To evaluate the quality of the isolated sub-cellular fractions, we tested sub-cellular enrichment of three known RNAs: RNU2.1 (RNA, U2 small nuclear 1) is the RNA component of the US2 snRNP that interacts with 3' region of the intron during splicing events and localizes in the chromatin fraction; Malat1 is a long non coding RNA retained in the nucleus forming molecular scaffolds for ribonucleoprotein complexes and acting as transcriptional regulator for numerous genes; Linc00339 is a long intergenic non coding RNA mainly localized in the cytoplasm. Furthermore, we also added *Gapdh* that preferentially localized in the cytoplasmatic compartment.

Interestingly, this analysis revealed that lncFOXP3 is localized both in the cytoplasmic and in the nucleoplasmic compartments (Figure 6a). As a quality control of biochemical fractionation, we also performed western blot to analyse Lamin A/C and B-Tubulin localization, as nuclear and cytoplasmic controls, respectively (Figure 6b).

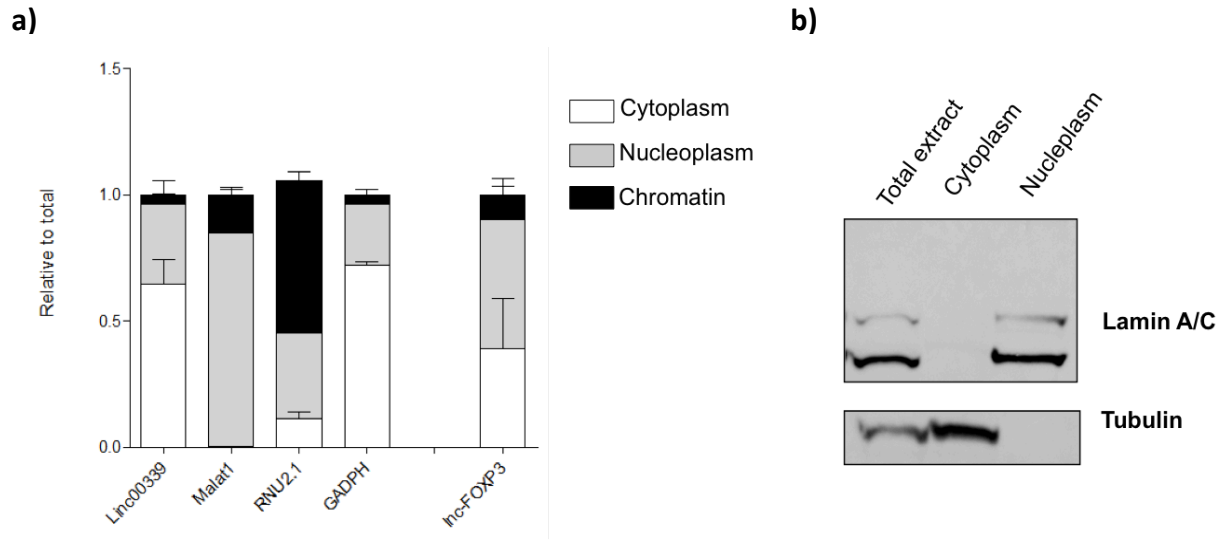


Figure 6- lncFOXP3 has nuclear and cytoplasm localization. a) 10^7 *in vitro* expandend $CD4^+$ Treg cells were biochemically fractionated. The different cellular fractions are indicated (chromatin in black bars; nucleus in grey bars and cytoplasm white bars). The histogram shows lncFOXP3 levels in the three fractions, evaluated by qRT-PCR. GAPDH and linc00339 were used as cytoplasmatic controls, Malat1 as nuclear control and RNU2.1 as chromatin control. Histograms shows mean values ± sem from three independent experiments **b)** Representative result of Immunoblotting for Lamin A/C and Tubulin used as qualitative control of biochemical fractionation.

Another method to assess lncRNA subcellular localization at single molecule resolution is based on RNA-FISH protocols. To investigate lncFOXP3 sub-cellular localization, we exploited the RNAScope technology (F. Wang et al., 2012). RNAscope is a novel RNA-FISH technology with a unique probe design strategy that allows simultaneous signal amplification and background suppression to achieve single-molecule visualization while preserving tissue morphology.

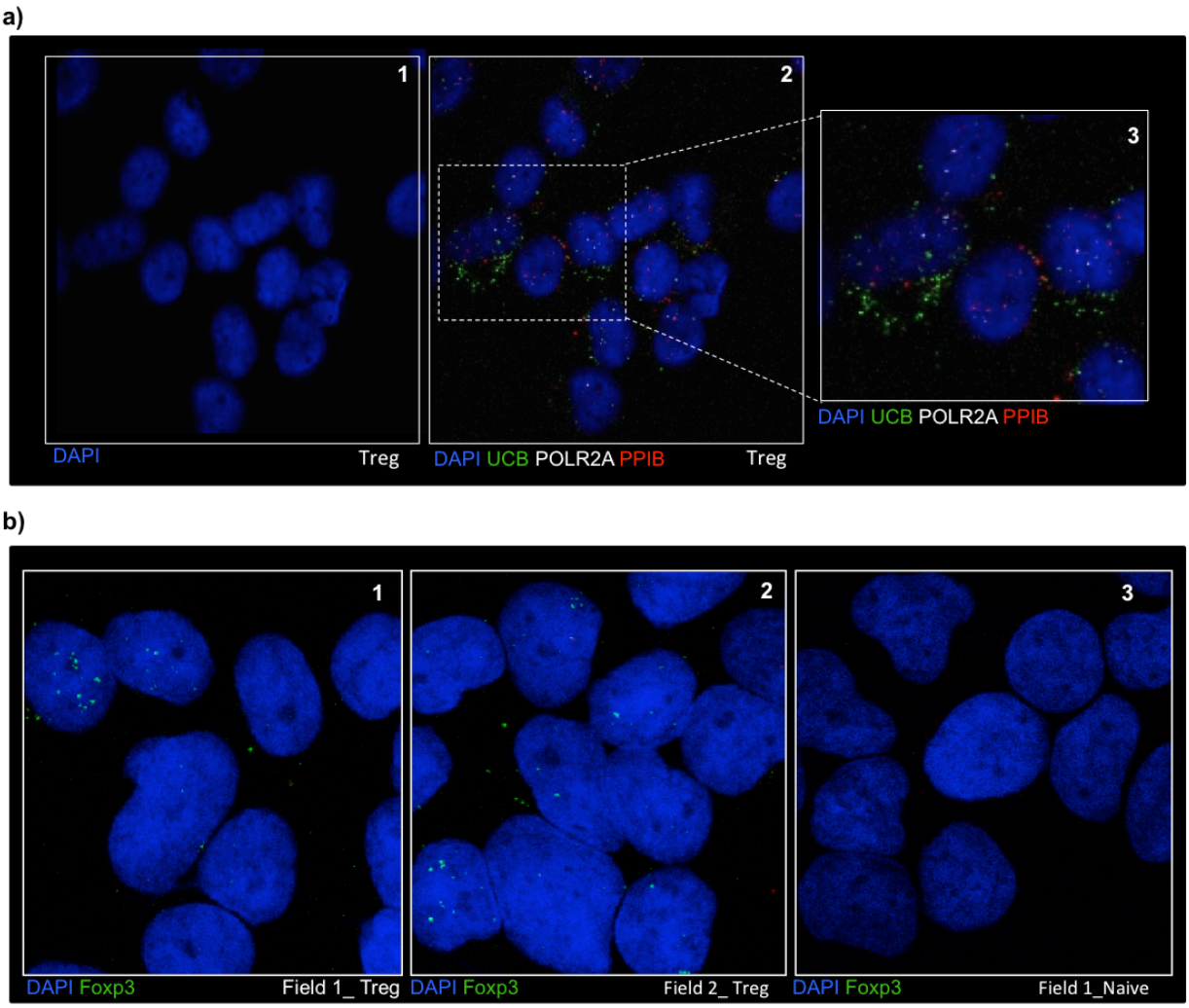
First, we set up the methodology in human primary $CD4^+$ Naive and Treg cells using positive control probes provided by RNAscope kit (Figure 7a). Once the methodology was set up, lncFOXP3, *Foxp3* and Malat1 transcripts were visualised in both $CD4^+$ Naive and Treg cells expanded *in vitro* (Figure 7b,c and d). As control, we also performed RNA-FISH on HEK293T cells stably expressing lncFOXP3 and on its relative control (HEK Mock) (Figure 7e).

In line with biochemical fractionation data, RNA-FISH analyses revealed a well-defined punctuate localization of lncFOXP3 in the nucleus and in the cytoplasm of $CD4^+$ T regulatory cells (red signal), and, as expected, the absence of any specific signals in $CD4^+$ naive T cells, that do not express lncFOXP3 (Figure 7c panel 1 and 2 versus panel 3). This technique confirms the presence of *Foxp3* transcripts only in Treg cells (Figure 7b panel 1 and 2 versus panel 3).

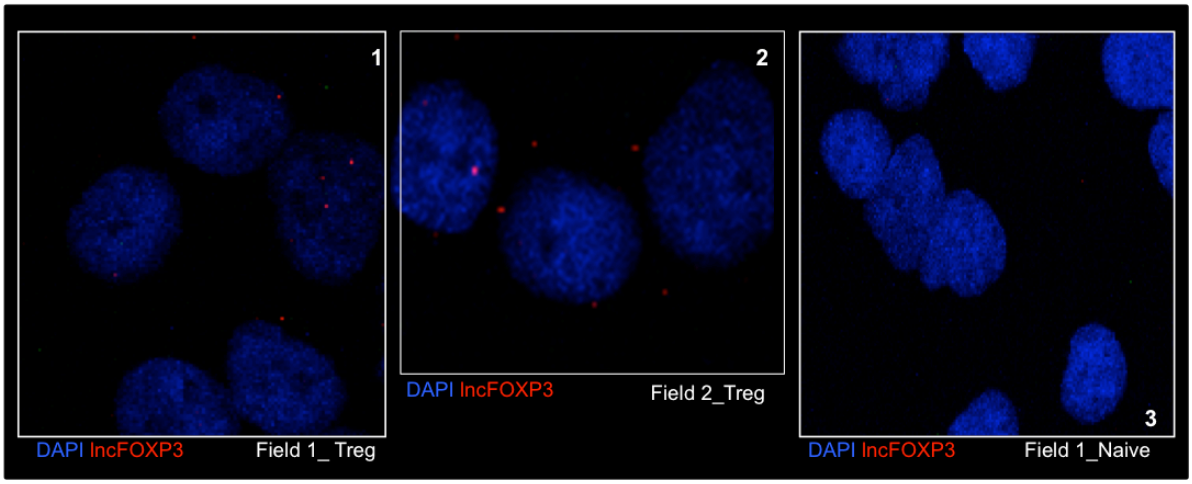
Besides the qualitative result, RNAscope technology also provides quantitative information regarding the transcripts analyzed. Indeed, a strong difference between Malat1 signals in Treg cells (Figure 7d panel 1) versus Naïve cells was detected (Figure 7d panel 2).

In order to verify if lncFOXP3 co-localizes with *Foxp3* mRNA, combined RNA-FISH analysis with specific probes against *Foxp3* (green signal) and lncFOXP3 (red signal) transcripts was performed. Data obtained from double RNA-FISH confirmed that lncFOXP3 and *Foxp3* are two independent transcripts and indicate that lncFOXP3 does not localize in close proximity to *Foxp3* mRNA. Indeed, both signals are clearly distinguished as independent spots within Treg cells (Figure 7f).

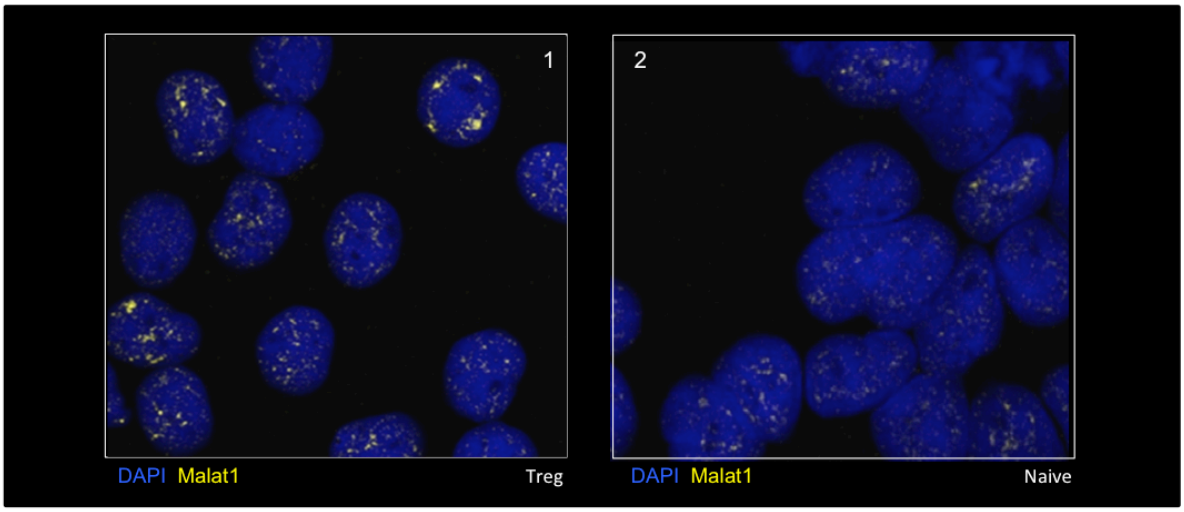
Moreover, for each experiments qRT-PCR was performed to verify the specificity and the expression levels of each transcripts in different cellular context (Treg, Naïve and HEK293T cells) (Figure 7g).



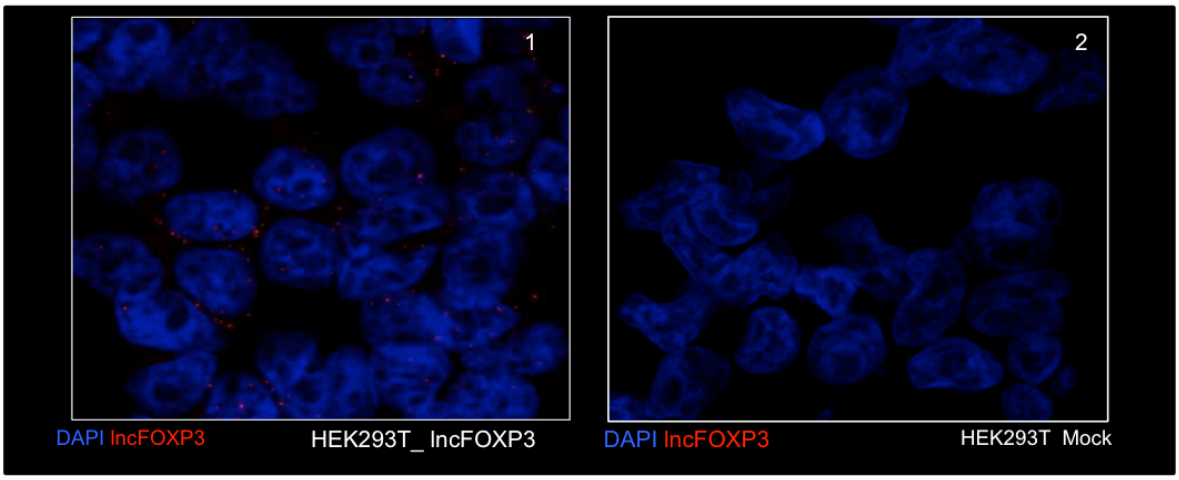
c)


1
DAPI lncFOXP3
Field 1_ Treg
2
DAPI lncFOXP3
Field 2_Treg
3
DAPI lncFOXP3
Field 1_Naive

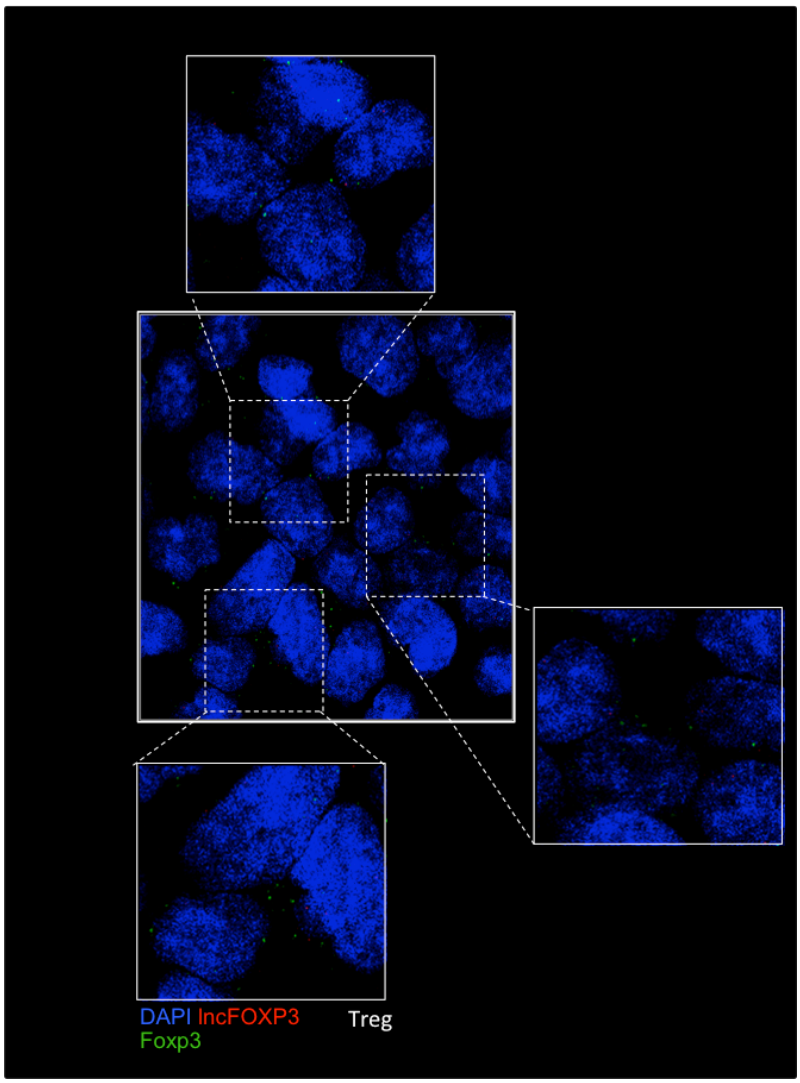
d)


1
DAPI Malat1
Treg
2
DAPI Malat1
Naive

e)


1
DAPI lncFOXP3
HEK293T_ lncFOXP3
2
DAPI lncFOXP3
HEK293T Mock

f)



g)

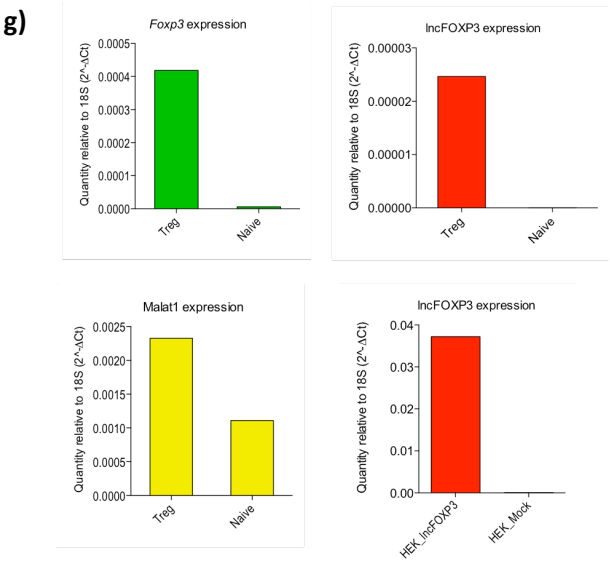


Figure 7-lncFOXP3 and *Foxp3* are two independent transcripts with a specific localization within Treg cells. a) RNAscope positive controls tested on Treg cells: Ubiquitin C (UCB) in green localized in the cytoplasm; RNA polymerase II subunit A (POLR2A) in white localized in the nucleus and Peptidylprolyl isomerase B (PPIB) in red preferentially localized near endoplasmatic reticulum. The positive controls provided by the kit were used to test the integrity of the RNA and the specificity of this technique.

The number and the intensity of the dots are directly correlated to the number of the transcripts present within analyzed cells. **b)** *Foxp3* transcript (green dots) localizes within nucleus and cytoplasm of Treg cells (panel 1b and 2b) and it is not expressed in Naïve cells (panel 3b) **c)** lncFOXP3 transcripts (red dots) localizes within nucleus and cytoplasm of Treg (panel 1c and 2c) and it is not expressed in Naïve cells (panel 3c). **d)** Malat1 transcripts (orange dots) localizes within the nucleus of Treg and Naïve cells (panel 1d and 2d).**e)** RNA-FISH performed in HEK293T cell line stably expressing lncFOXP3. Red dots indicate lncFOX3 transcript localizes in the nucleus and in the cytoplasm of HEK293T cells (panel 1e) and the absence of the specific signal in HEK293T Mock cells (panel 2e). **f)** Double RNA-FISH confirmed that lncFOXP3 (red dots) and *Foxp3* (green dots) are two independent transcripts and they do not co-localize. **g)** To assess the presence of each target transcripts (*Foxp3*, lncFOXP3 and Malat1). qRT-PCR analyses were performed for each samples. All images showed in Figure 7 were obtained using Leica SP5 confocal in fixed mode. Nuclei were counterstained using DAPI

7. Functional characterization of lncFOXP3 in Treg cells

To investigate whether lncFOXP3 plays a crucial role in the regulation of FoxP3 protein levels and consequently in Treg cell suppressive activity, we undertook systematic loss-of-function experiments in human *in vitro* expanded Treg cells. Indeed, loss-of-function models are invaluable tools to assess the physiological function of any gene product. This approach is based on lentiviral transduction of different shRNAs specifically targeting lncFOXP3 and *Foxp3* transcripts.

To this purpose, all the sequences of selected shRNAs were cloned in an optimized vector (pLKO.1 GFP shRNA)(Sancak et al., 2008), that encodes shRNA of interest under a PolIII promoter and constitutively expresses a GFP protein, used as selection marker. We first screened for the efficacy of 8 shRNAs in downregulating lncFOXP3 and *Foxp3* transcripts by qRT-PCR (data not shown); then we selected the best three shRNAs for lncFOXP3 (shRNA#1, shRNA#2, shRNA#3) and one shRNA for *Foxp3*, as functional control (shFoxp3)(Figure 8a).

As a first functional readout, we measured FoxP3 protein levels in Treg cells knocked-down for lncFOXP3 by FACS analysis. Interestingly, we found that lncFOXP3 knock-down with 3 different shRNAs is able to reduce FoxP3 protein levels in Treg cells, compared to cells transduced with shMock (Figure 8b). In particular, shRNA#2 and shRNA#3, that do not affect *Foxp3* mRNA levels (Figure 8a), are able to produce a significant reduction of FoxP3 protein levels.

Since FOXP3 is crucial for the maintenance of Treg suppression ability, we decided to test the functional consequence of lncFOXP3 down-regulation by *in vitro* suppression assay. In this assay, Treg and *in vitro* activated Naive T cells, preloaded with CFSE dye, are mixed and the suppressive effects of Treg cells correlate with decrease in the proliferation rate of Naïve T cells measured by

FACS. CFSE dye is diluted with each cell division and therefore fluorescence intensity in highly proliferating cells is lower. As shown in Figure 9, upon lncFOXP3 down-regulation obtained by using shRNA#2 (Figure 9b) and shRNA#3(Figure 9c), the suppressive ability of Treg cells is strongly reduced if compared to negative control (Mock)(Figure 9a).

These data suggest that lncFOXP3 effect impacts on Treg suppressive function possibly through modulation of FOXP3 protein.

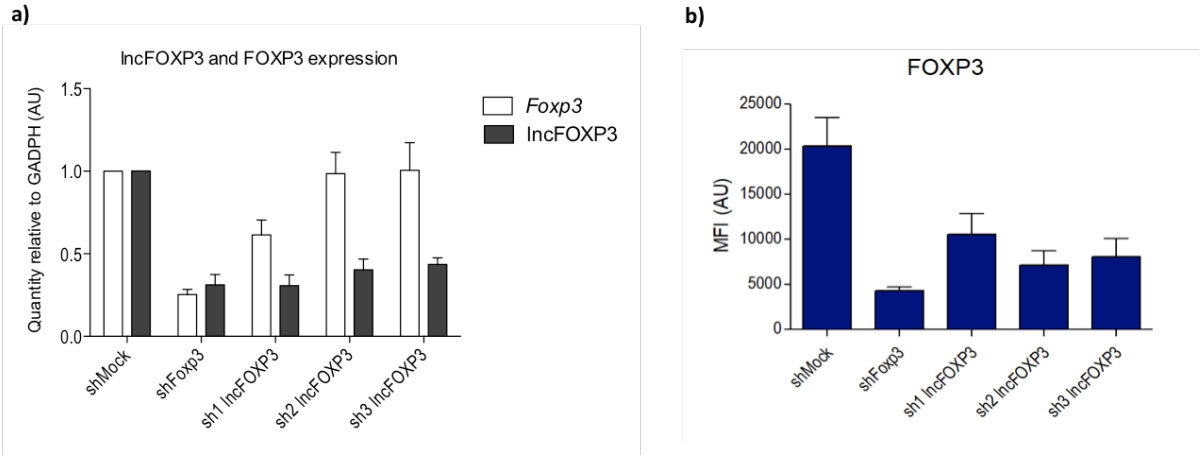


Figure 8- lncFOXP3 knock-down analysis. a) lncFOXP3 and *Foxp3* mRNA levels after down regulation of Treg cells with specific shRNAs. Results are representative of three independent experiments.**b)** FoxP3 protein levels after down-regulation of lncFOXP3 using three different shRNAs. shFoxp3 was used as positive control. Data shown here are normalized to Mock control. Results are representative of three independnet experiments.

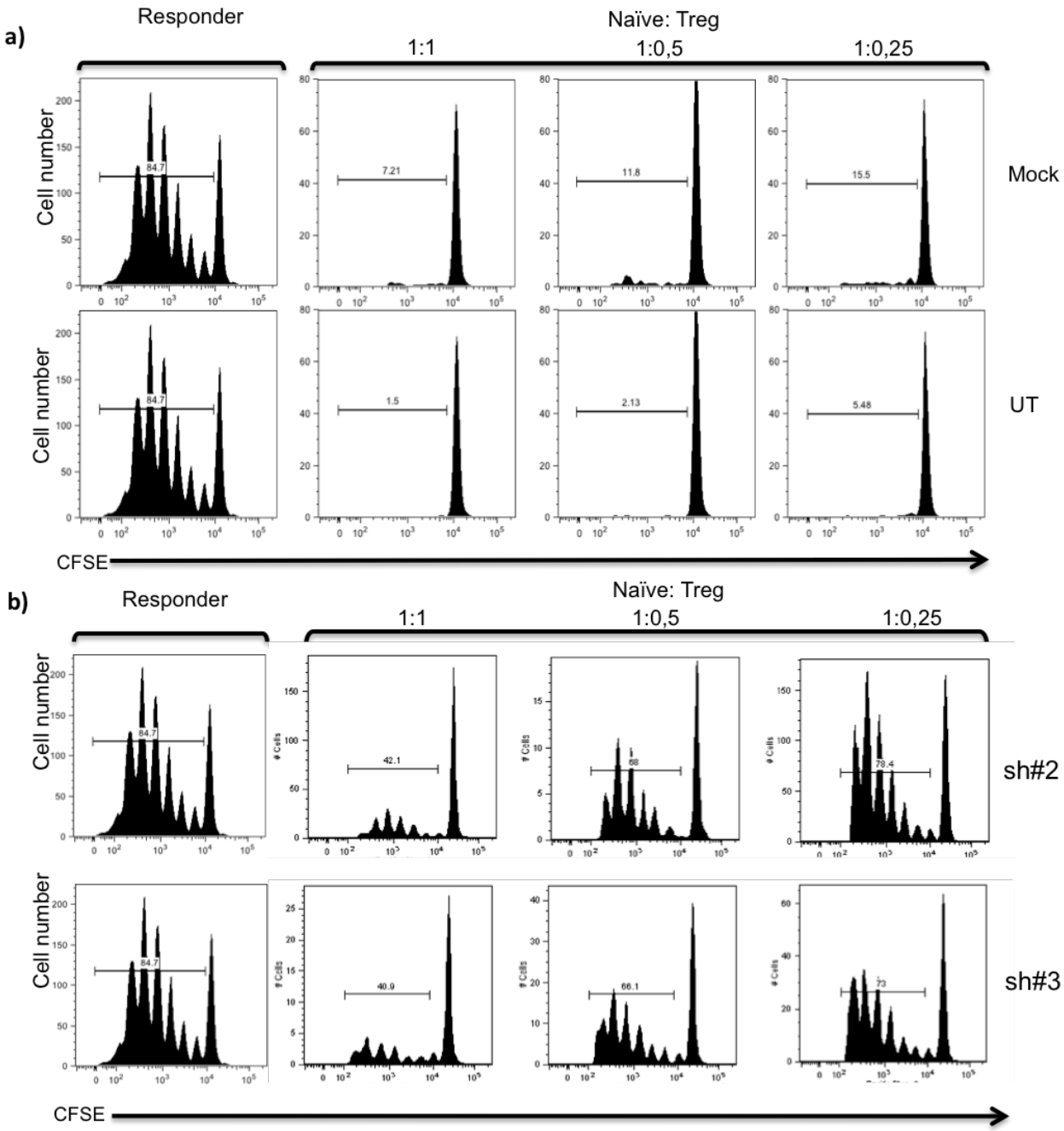


Figure 9- Analysis of Treg cell suppressive ability after lncFOXP3 knock-down. a) Histogram representing the percentage of Treg suppression activity in sh Mock-transduced cells (Mock) and in untreated cell (UT). **b)** Histogram representing the percentage of Treg suppression activity after down-regulation of lncFOX3 with shRNA#2 and shRNA#3 compared to untreated and Mock cells. Results are representative of three different experiements.

8. Identification of lncFOXP3 protein interactors

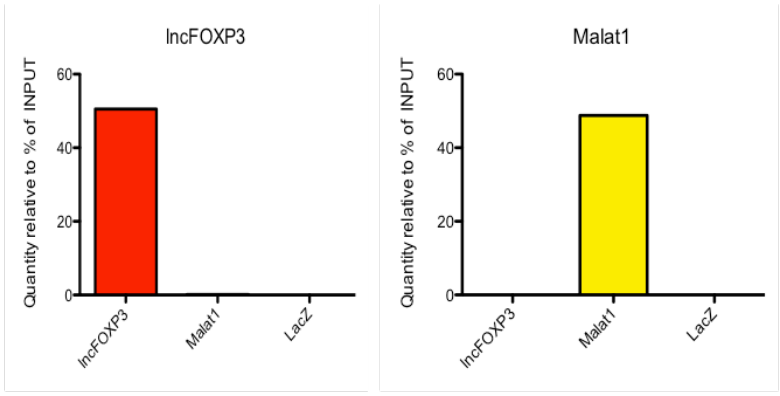
Previous studies showed that lncRNAs can regulate gene expression via interactions with chromatin-modifying complexes and potentially (L. Wang et al., 2016) modifying chromatin at gene promoters to affect their transcriptional output. Alternatively, they can also form ribonucleoprotein (RNP) complexes both in the nucleus and in the cytoplasm. Thus, the study of a lncRNA "interactome" contributes to uncover the mechanisms through which lncRNAs impact on a biological process. Based on our previous findings revealing the fundamental role of lncFOXP3 in maintaining FoxP3 protein levels and consequently, the suppressive ability of Treg cells, we decided to perform a global investigation of lncFOXP3 protein interactors to explain its possible mechanism of action. To this aim, we set up and optimized a protocol to perform endogenous lncFOXP3 pull-down on UV-crosslinked $CD4^+$ Treg cells.

Specific pool of short biotinylated DNA antisense probes for lncFOXP3 were designed and selected in order to exclude off-targets. Size-matched probes specific for the long non-coding RNA Malat1 or for LacZ gene were used as controls. qRT-PCR analysis of the RNA fraction revealed an efficient and specific enrichment of lncFOXP3 and Malat1 transcripts compared to negative control (Figure10a).

We then analyzed the protein fraction co-purified with the lncRNAs from 3 independent pull-down experiments by mass-spectrometry (MS). Among other proteins, lncFOXP3 was consistently co-precipitated with USP7, a de-ubiquitinase whose function is known to be correlated with FOXP3 stabilization in Treg cells (L. Wang et al., 2016). In addition our mass-spectrometry data revealed that USP7 can also interact with MALAT1 (Figure 10b).

The interaction between lncFOXP3-USP7 in *in vitro* expanded Treg cells was then validated in 3 independent pull-down experiments using USP7 specific antibody (Figure 10c).

a)



b)

Protein names	Gene names
SUMO-activating enzyme subunit 1	SAE1
Echinoderm microtubule-associated protein-like 3	EML3
Aspartyl aminopeptidase	DNPEP
Nuclease-sensitive element-binding protein 1;Y-box-binding protein 2;Y-box-binding protein 3	YBX1;YBX2;YBX3
Histone H1.4;Histone H1.2;Histone H1.3	HIST1H1E;HIST1H1C;HIST1H1D
cAMP-dependent protein kinase type II-alpha regulatory subunit	PRKAR2A
Proteasome subunit beta type-8	PSMB8
Transgelin-2	TAGLN2
Serine--tRNA ligase, cytoplasmic	SARS
Nck-associated protein 1-like	NCKAP1L
Serine/threonine-protein kinase 4	STK4
Protein transport protein Sec23B	SEC23B
Sister chromatid cohesion protein PDS5 homolog A	PDS5A
DNA damage-binding protein 2	DDB2
ATP-dependent RNA helicase DDX1	DDX1
Golgi apparatus protein 1	GLG1
Protein disulfide-isomerase TMX3	TMX3
FAS-associated factor 1	FAF1
Transient receptor potential cation channel subfamily V member 2	TRPV2
Ubiquitin carboxyl-terminal hydrolase 7;Ubiquitin carboxyl-terminal hydrolase	USP7

c)

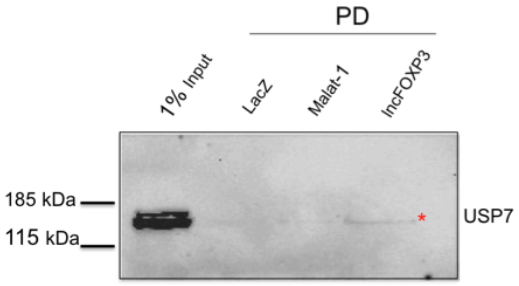


Figure 10-Identification of lncFOXP3 interactors. a) qRT-PCR quantification of lncFOXP3 and Malat1 in pull-down samples relative to INPUT. LacZ serve as negative control. **b)** Top protein candidates from mass spectrometry analysis of lncFOXP3 pull-down are listed. For each protein, the protein and gene names are reported. In red is highlighted USP7 protein, our selected candidate. **c)** Representative western blot analysis of USP7 in (1%) Input, lacZ, Malat1 and lncFOXP3 pull-down samples. Red asterisk indicates the specific signal detected in lncFOXP3 pull-down.

9. lncFOXP3-USP7 interaction stabilizes FoxP3 protein

To investigate the role of lncFOXP3-USP7 interaction we decided to produce a HEK293T cell line that stably express lncFOXP3 and *Foxp3*. To this purpose, lncFOX3 sequence was cloned in an optimized lentiviral vector (pCDH_CMV_MCS_EF1α_DNGFR), while *Foxp3* sequence was stably expressed by another optimized lentiviral vector (pCDH_CMV_MCS_EF1α-copGFP).

The resulting constructs were then transduced individually and co-transduced in HEK293T cells, giving rise HEK293T cell lines that stably express lncFOXP3 (HEK293T_lncFOXP3), *FOXP3* (HEK293T_FOXP3) and both of them (HEK293T_lncFOXP3_FOXP3).

Cells were cultured and selected for ten days. Finally, cells were single-cell sorted as GFP^{high} and $DNGFR^{high}$ to select clones expressing FoxP3 and lncFOXP3 respectively, and as double positive $GFP/DNGFR^{high}$ to identify clones expressing both lncFOXP3 and *FOXP3* genes. This experimental design should allow us to verify whether lncFOXP3 together with USP7 may play a role in promoting the stabilization of FoxP3 protein.

As shown in Figure 11a, qRT-PCR analysis revealed that lncFOXP3 and *Foxp3* transcripts show similar expression levels in both cell lines (HEK293T_FOXP3 and HEK293T_lncFOXP3_FOXP3) compared to HEK293T cells. Interestingly, densitometric analysis of Western blot (Figure 11b) revealed an increase of FoxP3 protein when lncFOXP3 and Foxp3 are co-expressed in HEK293T cells. This result suggest that in this model lncFOXP3 contributes to FoxP3 protein stabilization.

To verify if the cytoplasmic fraction of lncFOXP3 acts as stabilizer of FoxP3 protein together with USP7 we verified the effect of the downregulation of USP7 in HEK293T_FOXP3 and in HEK293T_lncFOXP3_FOXP3.

As shown in Figure 12, we observed that the downregulation of USP7 in HEK293T_FOXP3 causes a 36% decrease of FoxP3 protein level (panel a). Conversly, the same downregulation in HEK293T_lncFOXP3_FOXP3 is not sufficient to decrease FoxP3 protein (panel b), suggesting that in this context lncFOXP3 play a key role in the stabilization of Foxp3 protein.

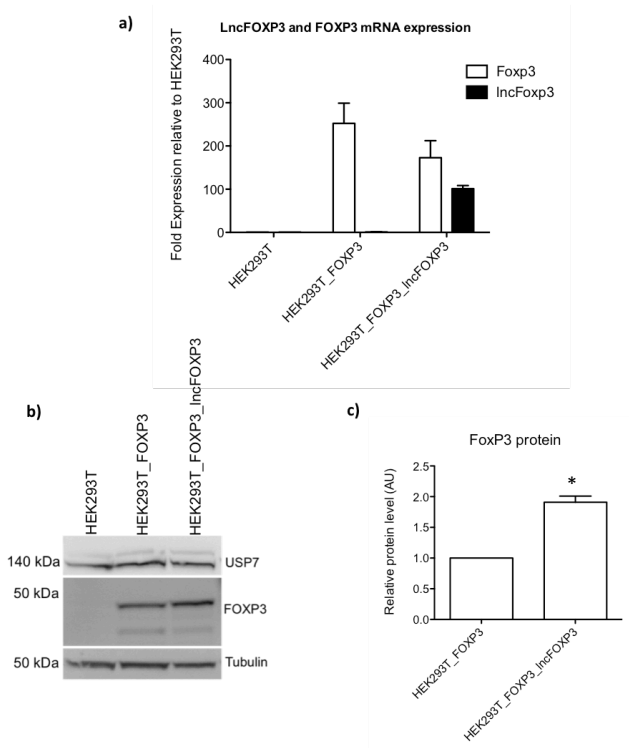


Figure 11- lncFOXP3 stabilizes FoxP3 protein a) Level expression of lncFOXP3 and FOXP3 mRNA in HEK293T overexpressing lncFOXP3 and FOXP3 evaluated by qRT-PCR**. b)** Western blot analysis shows an increase of FoxP3 protein level when lncFOXP3 is co-expressed with FOXP3 in HEK293T cells compared with HEK293T expressing only FOXP3 and negative control (HEK293T) as demonstrated also by densitometric analysis in panel **c).** Histograms show Means ± SEM of three independent experiements.
* p< 0.05.

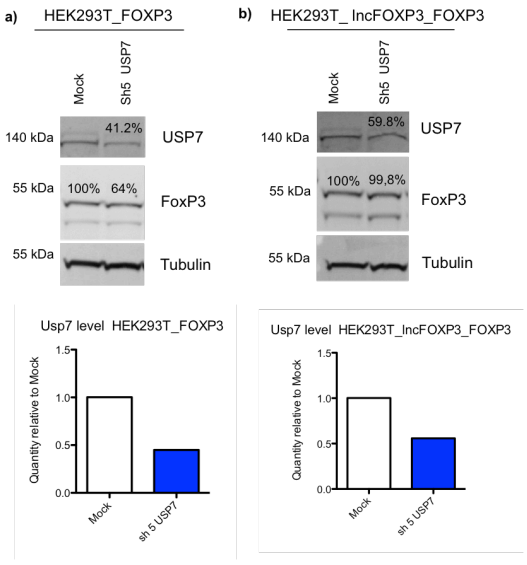


Figure 12- lncFOXP3 stabilizes FoxP3 protein in HEK293T_lncFOXP3_FOXP3 a) Western blot densitometric analysis show a 36% decreased of FoxP3 protein in HEK293T_FOXP3 after USP7 down-regulation, compared to Mock control. Data are representative of FoxP3/Tubulin protein levels relative to Mock (set as 100%). **b)** The downregulation of USP7 in HEK293T lncFOXP3_FOXP3 doesn't affect FoxP3 level. In the bottom of the figure are reported the qRT-PCR graphs showing the level of USP7 transcripts after its downregulation in HEK293T_FOXP3 and HEK293T_lncFOXP3_FOXP3 cell lines.

Discussion

High-throughout analyses of the transcriptomes have revealed that Mammalian genomes are pervasively transcribed, producing thousands of long non-coding RNAs (lncRNAs), that are now emerging as versatile regulators of gene expression involved in different biological pathways (Djebali et al., 2012; van Heesch et al., 2012). LncRNAs act as fine tuners of cellular functions throughout the human body (Shirasawa et al., 2004). Given these observations, it is not surprising that lncRNAs altered expression has been linked to many different pathologies. Indeed, it has been found that >90% of disease-associated genetic variants identified by Genome Wide Association Studies (GWAS) are located outside protein coding regions, in sequences implicated in transcription control (promoters and enhancers) or in non-coding genes (Hindorff et al., 2009). This is particularly evident in immune-mediated pathologies where at least 10% of SNPs occurs in non-coding regions. Moreover, non-coding transcript display striking expression specificity compared to coding genes. Therefore, a thorough investigation of lncRNAs involvement in immune system function is of central importance for the identification of novel and more specific therapeutic-targets for immune-related diseases.

In 2015, we published a comprehensive landscape of lncRNA expression in thirteen subsets of human primary lymphocytes (Ranzani et al., 2015). This study confirmed the high tissue specificity of lncRNAs also in the immune system and allowed the identification of lncRNAs whose expression is restricted to a given lymphocyte subset. Interestingly, it was found that lncRNAs define cellular identity better than protein coding genes that are generally considered the most precise markers of lymphocytes subsets. By exploiting three different *de novo* transcriptome reconstruction strategies 563 novel lncRNAs were identified increasing by 11.8% the number of known lncRNAs expressed in human lymphocytes. Based on these findings, signature lncRNAs might be exploited to discriminate at the molecular level those cell subsets that cannot be distinguished easily based on their cell surface markers because of their cellular heterogeneity, such as $CD4^+$ T regulatory cells (Treg). Treg cells play a crucial role in the maintenance of immunological self-tolerance thanks to their peculiar suppressive function. Recently, a high plasticity of this cell subset was demonstrated, but the molecular mechanisms underlying this plasticity are still poorly understood. Their remarkable plasticity could be exploited to modulate their differentiation and function in several immune-mediated diseases.

Among the novel and already annotated lncRNAs revealed by RNA-seq analysis in different lymphocytes subsets, in this study we decided to study one previously uncharacterized lncRNA

specifically expressed in Treg cells. This lncRNA was renamed lncFOXP3 due to the close proximity with *FOXP3* gene, the master regulator of Treg cells phenotype and function.

Differential expression analyses among thirteen cell subsets confirmed a high specificity of lncFOXP3 within Treg cells and moreover, that its expression correlates with *Foxp3* levels only in this lymphocyte subset.

Using human *in vitro* expanded Treg cells, we discovered that lncFOXP3 displays a comparable expression profile to *Foxp3* transcript. Indeed, both of them are expressed at low levels during the first days upon expansion stimulus, whereas their expression progressively increases up to day 20. We also found that on *in vitro* activated Naïve T cells, lncFOXP3 and *Foxp3* display different expression patterns: while *Foxp3* shows a rapid wave of expression, lncFOXP3 is barely detectable and is not induced upon activation. These findings suggested that lncFOXP3 and *Foxp3* are two independent transcripts and they are not co-transcriptionally regulated. Indeed, while 3' RACE experiment revealed an overlap of 20 bp between lncFOXP3 3' end and *FOXP3* 5' end, Northern Blot experiments provided conclusive evidence that lncFOXP3 is an independent transcriptional unit.

Starting from these findings we hypotized that lncFOXP3 can be involved in the regulation of *FOXP3* expression in Treg cells and consequently, can contribute to the maintenance of their identity and functions.

Biochemical subcellular fractionation allowed us to establish that lncFOXP3 is localized both in the cytoplasmatic and in the nucleoplasmic compartment of Treg cells. Moreover, as further demonstration that they are two independent transcripts, combined RNA-FISH analyses showed that lncFOXP3 does not localize in close proximity with *Foxp3* mRNA.

To assess the functional role of lncFOXP3 in Treg cells, we undertook systematic loss-of-function experiments in human *in vitro* expanded Treg cells. Using this approach, we discovered that lncFOXP3 is involved in the stabilization of FoxP3 protein since its downregulation causes a decrease of FoxP3 protein. Consequently, lncFOXP3 downregulation impairs Treg suppression ability. These results suggest that cytosolic lncFOXP3 might be functionally involved in the regulation of FoxP3 protein stability. This hypothesis is also supported by the observation that FoxP3 protein expression is transiently induced upon activation of $CD4^+$ Naive T cells, but in these cells, that do not express lncFOXP3, *Foxp3* expression is rapidly lost. LncRNAs function cannot be predicted just by the analysis of their primary sequence, thus, the study of a lncRNA "interactome" contributes to uncover the mechanisms through which lncRNAs impact on specific biological process.

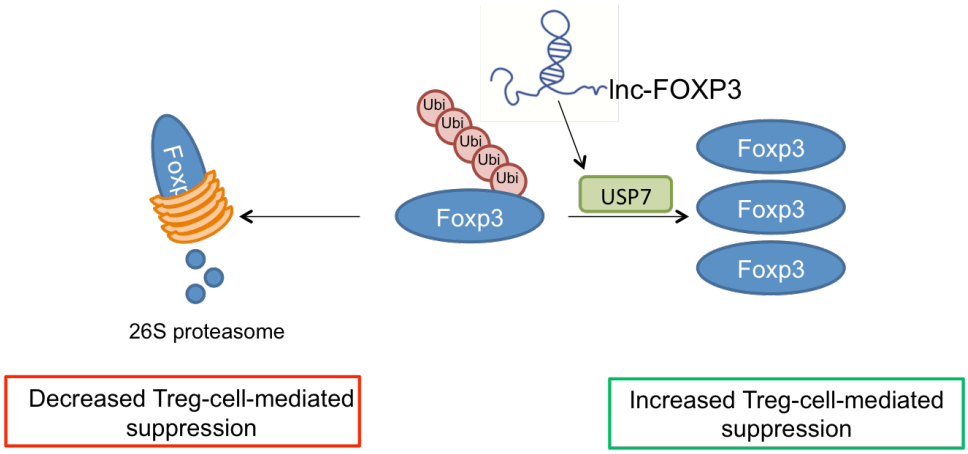
The interactome of the endogenous lncFOXP3 was established by mass-spectrometry and identified USP7 as a putative interactor of lncFOXP3. The relevance of this finding caught our attention since USP7 has been previously described as a FoxP3 protein stabilizer, through its de-ubiquitinase

activity (Wang et al., 2016). In particular, from a functional point of view, it has been shown that USP7 knockdown in Treg cells is associated to a decrease of FoxP3 protein and on an impairment of Treg cell suppressive functions, similar to what we observed when we modulated lncFOXP3. Moreover the added value of our analysis is that we performed pull-down experiments of endogenous lncFOXP3 from Treg cells, thus supporting the hypothesis that such interaction occurs *in vivo*.

By now, we have accumulated evidences indicating the key role of lncFOXP3 in the stabilization of FoxP3 protein. Indeed, we observed that lncFOXP3 ectopic expression in HEK293T is correlated with increased Foxp3 protein level. In this way, we have demonstrated that the knock-down effect of USP7, known to be the stabilizator of FoxP3, is correlated with a consistent reduction of Foxp3 protein only when lncFOXP3 is not expressed.

These findings represent still circumstantial evidence on the potential role of cytoplasmatic lncFOXP3 fraction in the stabilization of FoxP3 protein, probably mediated by the interaction with USP7. We can hypnotize that lncFOXP3 is involved in bringing USP7 in close proximity of the transcription factor *Foxp3*, consequently ensuring its stabilization.

Our working hypothesis will have to be proved through experiments that better investigate the FoxP3 protein stability following lncFOXP3 modulation. If our guess is correct, we will not only have understood the molecular mechanism that drives FoxP3 stabilization through lncFOXP3, but we will also have discovered another function of lncRNAs that will likely work also for other non-coding RNAs and their own target proteins.



Bibliography

Abarrategui, I., & Krangel, M. S. (2007). Noncoding transcription controls downstream promoters to regulate T-cell receptor α recombination. *EMBO Journal*, *26*(20), 4380–4390.

Allan, S. E., Passerini, L., Bacchetta, R., Crellin, N., Dai, M., Orban, P. C., … Levings, M. K. (2005). The role of 2 FOXP3 isoforms in the generation of human CD4+ Tregs. *Journal of Clinical Investigation*, *115*(11), 3276–3284.

Arce-Sillas, A., Alvarez-Luquin, D. D., Tamaya-Domïnguez, B., Gomez-Fuentes, S., Trejo-Garcia, A., Melo-Salas, M., Adalid-Peralta, L. (2016). Regulatory T Cells: Molecular Actions on Effector Cells in Immune Regulation. *Journal of Immunology Research*, *2016*.

Autuoro, J. M., Pirnie, S. P., & Carmichael, G. G. (2014). Long noncoding RNAs in imprinting and X chromosome inactivation. *Biomolecules*, *4*(1), 76–100.

Baecher-Allan, C., Brown, J. A., Freeman, G. J., & Hafler, D. A. (2001). CD4+CD25high Regulatory Cells in Human Peripheral Blood. *The Journal of Immunology*, *167*(3), 1245–1253.

Baron, U., Floess, S., Wieczorek, G., Baumann, K., Grützkau, A., Dong, J., … Huehn, J. (2007). DNA demethylation in the humanFOXP3 locus discriminates regulatory T cells from activated FOXP3+ conventional T cells. *European Journal of Immunology*, *37*(9), 2378–2389.

Beier, U. H., Wang, L., Bhatti, T. R., Liu, Y., Han, R., Ge, G., & Hancock, W. W. (2011). Sirtuin-1 Targeting Promotes Foxp3+ T-Regulatory Cell Function and Prolongs Allograft Survival. *Molecular and Cellular Biology*, *31*(5), 1022–1029.

Ben-Neriah, Y. (2002). Regulatory functions of ubiquitination in the immune system. *Nature Immunology*, *3*(1), 20–26.

Bettelli, E., Dastrange, M., & Oukka, M. (2005). Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(14), 5138–5143.

Bolland, D. J., Wood, A. L., Johnston, C. M., Bunting, S. F., Morgan, G., Chakalova, L., … Corcoran, A. E. (2004). Antisense intergenic transcription in V(D)J recombination. *Nature Immunology*, *5*(6), 630–637.

Brunkow, M. E., Jeffery, E. W., Hjerrild, K. A., Paeper, B., Clark, L. B., Yasayko, S. A., …

Ramsdell, F. (2001). Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nature Genetics*, *27*(1), 68–73.

Carrieri, C., Cimatti, L., Biagioli, M., Beugnet, A., Zucchelli, S., Fedele, S., … Gustincich, S. (2012). Long non-coding antisense RNA controls Uchl1 translation through an embedded SINEB2 repeat. *Nature*, *491*(7424), 454–457.

Cesana, M., Cacchiarelli, D., Legnini, I., Santini, T., Sthandier, O., Chinappi, M., … Bozzoni, I. (2011). A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. *Cell*, *147*(2), 358–369.

Chang, C. (2014). Unmet needs in the treatment of autoimmunity: From aspirin to stem cells. *Autoimmunity Reviews*, *13*(4–5), 331–346.

Chaudhry, A., Rudra, D., Treuting, P., Samstein, R., Liang, Y., Kas, A., & Rudensky, A. (2009). CD4+ Regulatory T Cells Control TH17 Responses in a Stat3-Dependent Manner. *Science (New York, NY)*, *326*(5955), 986–991.

Chen, Z., Barbi, J., Bu, S., Yang, H., Li, Z., Gao, Y., … Loser, K. (2014). cell Suppressive Activity by Promoting Degradation of the, *39*(2), 1–23.

Chu, C., Zhong, F., Artandi, S. E., & Chang, H. Y. (2012). Genomic maps of lincRNA occupancy reveal principles of RNA- chromatin interactions. *Mol Cell*, *44*(4), 667–678.

Chunder, N., Wang, L., Chen, C., Hancock, W. W., & Wells, A. D. (2012). Cyclin-dependent kinase 2 controls peripheral immune tolerance. *Journal of Immunology (Baltimore, Md. : 1950)*, *189*(12), 5659–5666.

Chung, Y., Tanaka, S., Chu, F., Nurieva, R., Martinez, G. J., Rawal, S., … Dong, C. (2011). Follicular regulatory T (Tfr) cells with dual Foxp3 and Bcl6 expression suppress germinal center reactions. *Nature Medicine*, *17*(8), 983–988.

Clemson, C. M., Hutchinson, J. N., Sara, S. A., Alexander, W., Fox, A. H., Chess, A., & Lawrence, J. B. (2010). is Essential for the Structure of Paraspeckles, *33*(6), 717–726.

Collier, S. P., Collins, P. L., Williams, C. L., Boothby, M. R., & Aune, T. M. (2012). Cutting Edge: Influence of Tmevpg1, a Long Intergenic Noncoding RNA, on the Expression of Ifng by Th1 Cells. *The Journal of Immunology*, *189*(5), 2084–2088.

Cruz, J. A., & Westhof, E. (2009). The Dynamic Landscapes of RNA Architecture. *Cell*, *136*(4),

604–609.

Dang, E. V, Barbi, J., Yang, H., Jinasena, D., Yu, H., Bordman, Z., … Pan, F. (2012). NIH Public Access, *146*(5), 772–784.

Das, R., Dimitrova, N., Xuan, Z., Rollins, R. A. R., Haghighi, F., Edwards, J. J. R., … Bird, A. A. (2016). An Improved. *Nature*, *7*(1), 705–708.

de Santa, F., Barozzi, I., Mietton, F., Ghisletti, S., Polletti, S., Tusi, B. K., … Natoli, G. (2010). A large fraction of extragenic RNA Pol II transcription sites overlap enhancers. *PLoS Biology*, *8*(5).

Deaglio, S., Dwyer, K. M., Gao, W., Friedman, D., Usheva, A., Erat, A., … Robson, S. C. (2007). Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *The Journal of Experimental Medicine*, *204*(6), 1257–1265.

Derrien, T., Johnson, R., Bussotti, G., Tanzer, A., Djebali, S., Tilgner, H., … Guigo, R. (2012). The GENCODE v7 catalog of human long noncoding RNAs : Analysis of their gene structure , evolution , and expression, 1775–1789.

Dimartino, D., Colantoni, A., Ballarino, M., Martone, J., Mariani, D., Danner, J., … Bozzoni, I. (2018). The Long Non-coding RNA lnc-31 Interacts with Rock1 mRNA and Mediates Its YB-1-Dependent Translation. *Cell Reports*, *23*(3), 733–740.

Du, J., Huang, C., Zhou, B., & Ziegler, S. F. (2008). Isoform-Specific Inhibition of ROR -Mediated Transcriptional Activation by Human FOXP3. *The Journal of Immunology*, *180*(7), 4785–4792.

Engreitz, J. M., Sirokman, K., McDonel, P., Shishkin, A. A., Surka, C., Russell, P., … Lander, E. S. (2014). RNA-RNA interactions enable specific targeting of noncoding RNAs to nascent pre-mRNAs and chromatin sites. *Cell*, *159*(1), 188–199.

Faghihi, M. A., Modarresi, F., Khalil, A. M., Wood, D. E., Sahagan, B. G., Morgan, T. E., … Paul, J. (2010). Expression, *14*(7), 723–730.

Fatica, A., & Bozzoni, I. (2014). Long non-coding RNAs: New players in cell differentiation and development. *Nature Reviews Genetics*, *15*(1), 7–21.

Fontenot, J. D., Gavin, M. A., & Rudensky, A. Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nature Immunology*, *4*(4), 330–336.

Geisler, S., & Coller, J. (2013). RNA in unexpected places: Long non-coding RNA functions in diverse cellular contexts. *Nature Reviews Molecular Cell Biology*, *14*(11), 699–712.

Gendrel, A.-V., & Heard, E. (2014). Noncoding RNAs and Epigenetic Mechanisms During X-Chromosome Inactivation. *Annual Review of Cell and Developmental Biology*, *30*(1), 561–580.

Gondek, D. C., Lu, L.-F., Quezada, S. A., Sakaguchi, S., & Noelle, R. J. (2005). Cutting Edge: Contact-Mediated Suppression by CD4+CD25+ Regulatory Cells Involves a Granzyme B-Dependent, Perforin-Independent Mechanism. *The Journal of Immunology*, *174*(4), 1783–1786.

Gong, C., & Maquat, L. E. (2011). lncRNAs transactivate Staufen1-mediated mRNA decay by duplexinwith 3ÚTRs via Alu elements. *Nature*, *470*(7333), 284–288.

Grossman, W. J., Verbsky, J. W., Barchet, W., Colonna, M., Atkinson, J. P., & Ley, T. J. (2004). Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity*, *21*(4), 589–601.

Guttman, M., Amit, I., Garber, M., French, C., Lin, M. F., Huarte, M., … Eric, S. (2009). NIH Public Access, *458*(7235), 223–227.

Harrow, J., Frankish, A., Gonzalez, J. M., Tapanari, E., Diekhans, M., & Kokocinski, F. (2012). GENCODE: The Reference Human Genome Annotation for The ENCODE Project. *Genome Research*, *22*, 1760–1774.

Hedrich, C. M., & Bream, J. H. (2010). Cell type-specific regulation of IL-10 expression in inflammation and disease. *Immunologic Research*, *47*(1–3), 185–206.

Heward, J. A., & Lindsay, M. A. (2014). Long non-coding RNAs in the regulation of the immune response. *Trends in Immunology*, *35*(9), 408–419.

Hodi, F. S., Day, S. J. O., Mcdermott, D. F., Weber, R. W., Sosman, J. A., Haanen, J. B., … Quirt, I. (2010). NIH Public Access. *N Engl J Med*, *363*(8), 711–723.

Hu, W., Alvarez-Dominguez, J. R., & Lodish, H. F. (2012). Regulation of mammalian cell differentiation by long non-coding RNAs. *EMBO Reports*, *13*(11), 971–983.

Imamura, K., Imamachi, N., Akizuki, G., Kumakura, M., Kawaguchi, A., Nagata, K., … Akimitsu, N. (2014). Long Noncoding RNA NEAT1-Dependent SFPQ Relocation from Promoter Region to Paraspeckle Mediates IL8 Expression upon Immune Stimuli. *Molecular Cell*, *53*(3), 393–406.

Kapranov, P., Cheng, J., Dike, S., Nix, D. A., Duttagupta, R., Willingham, A. T., … Gingeras, T. R. (2007). RNA maps reveal new RNA classes and a possible function for pervasive transcription. *Science*, *316*(5830), 1484–1488.

Kapusta, A., Kronenberg, Z., Lynch, V. J., Zhuo, X., Ramsay, L. A., Bourque, G., … Feschotte, C. (2013). Transposable Elements Are Major Contributors to the Origin, Diversification, and Regulation of Vertebrate Long Noncoding RNAs. *PLoS Genetics*, *9*(4).

Kelley, D. R., & Rinn, J. L. (2012). Transposable elements reveal a stem cell specific class of long noncoding RNAs. *Genome Biology*, *13*(11), R107.

Kim, H.-P., & Leonard, W. J. (2007). CREB/ATF-dependent T cell receptor–induced *FoxP3* gene expression: a role for DNA methylation. *The Journal of Experimental Medicine*, *204*(7), 1543–1551.

Klattenhoff, C., Scheuermann, J. C., Surface, L. E., Robert, K., Fields, P. A., Steinhauser, M. L., … Boyer, L. A. (2014). Lineage Commitment, *152*(3), 570–583.

König, M., Rharbaoui, F., Aigner, S., Dälken, B., & Schüttrumpf, J. (2016). Tregalizumab – A Monoclonal Antibody to Target Regulatory T Cells. *Frontiers in Immunology*, *7*(January), 1–7. Kowalczyk, M. S., & Higgs, D. R. (2012). NEWS & VIEWS FORUM Molecular biology RNA discrimination. *Nature*, 6–7.

Kretz, M., Siprashvili, Z., Chu, C., Webster, D. E., Zehnder, A., Qu, K., … Khavari, P. A. (2013). Control of somatic tissue differentiation by the long non-coding RNA TINCR. *Nature*, *493*(7431), 231–235.

Lee, S. M., Gao, B., & Fang, D. (2008). FoxP3 maintains Treg unresponsiveness by selectively inhibiting the promoter DNA-binding activity of AP-1. *Blood*, *111*(7), 3599–3606.

Lescoute, A., & Westhof, E. (2006). Topology of three-way junctions in folded RNAs. *Rna*, *12*(1), 83–93.

Li, B., Samanta, A., Song, X., Iacono, K. T., Bembas, K., Tao, R., … Greene, M. I. (2007). FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. *Proceedings of the National Academy of Sciences*, *104*(11), 4571–4576.

Li, Z., Lin, F., Zhuo, C., Deng, G., Chen, Z., Yin, S., … Li, B. (2014). PIM1 kinase phosphorylates the human transcription factor FOXP3 at serine 422 to negatively regulate its activity under inflammation. *Journal of Biological Chemistry*, *289*(39), 26872–26881.

Liang, B., Workman, C., Lee, J., Chew, C., Dale, B. M., Colonna, L., … Clynes, R. (2008). Regulatory T Cells Inhibit Dendritic Cells by Lymphocyte Activation Gene-3 Engagement of MHC Class II. *The Journal of Immunology*, *180*(9), 5916–5926.

Lin, N., Chang, K., Li, Z., Gates, K., Rana, Z. A., Zhang, D., … Rana, T. M. (2015). Controls Pluripotency and Neural Lineage Commitment, *53*(6), 1005–1019.

Liu, W., Putnam, A. L., Xu-yu, Z., Szot, G. L., Lee, M. R., Zhu, S., … Bluestone, J. A. (2006). CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4 $^{+}$ T reg cells. *The Journal of Experimental Medicine*, *203*(7), 1701–1711.

Loosdregt, J. Van, Fleskens, V., Fu, J., Brenkman, A. B., Cornelis, P. J., Pals, C. E. G. M., … Coffer, P. J. (2014). NIH Public Access, *39*(2), 259–271.

Lopes, J. E., Torgerson, T. R., Schubert, L. A., Anover, S. D., Ocheltree, E. L., Ochs, H. D., & Ziegler, S. F. (2006). Analysis of FOXP3 Reveals Multiple Domains Required for Its Function as a Transcriptional Repressor. *The Journal of Immunology*, *177*(5), 3133–3142.

Lu, L., Barbi, J., Pan, F., Hospital, F. A., Streets, C., Division, H., & Kimmel, S. (2018). The regulation of immune tolerance by FOXP3, *17*(11), 703–717.

Lu, X., Sachs, F., Ramsay, L. A., Jacques, P. É., Göke, J., Bourque, G., & Ng, H. H. (2014). The retrovirus HERVH is a long noncoding RNA required for human embryonic stem cell identity. *Nature Structural and Molecular Biology*, *21*(4), 423–425.

Mantel, P.-Y., Ouaked, N., Ruckert, B., Karagiannidis, C., Welz, R., Blaser, K., & Schmidt-Weber, C. B. (2006). Molecular Mechanisms Underlying FOXP3 Induction in Human T Cells. *The Journal of Immunology*, *176*(6), 3593–3602.

Marson, A., Kretschmer, K., Frampton, G. M., Elizabeth, S., Polansky, J. K., Macisaac, K. D., … Young, R. A. (2010). NIH Public Access, *445*(7130), 931–935.

Melé, M., Mattioli, K., Mallard, W., Shechner, D. M., Gerhardinger, C., & Rinn, J. L. (2017). Chromatin environment, transcriptional regulation, and splicing distinguish lincRNAs and mRNAs. *Genome Research*, *27*(1), 27–37.

Mercer, T. R., Dinger, M. E., & Mattick, J. S. (2009). Long non-coding RNAs: Insights into functions. *Nature Reviews Genetics*, *10*(3), 155–159.

Morawski, P. A., Mehra, P., Chen, C., Bhatti, T., & Wells, A. D. (2013). Foxp3 protein stability is regulated by cyclin-dependent kinase 2. *Journal of Biological Chemistry*, *288*(34), 24494–24502.

Munn, D. H., Shafizadeh, E., Attwood, J. T., Bondarev, I., Pashine, A., & Mellor, A. L. (1999). Inhibition of T Cell Proliferation by Macrophage Tryptophan Catabolism. *The Journal of*

Experimental Medicine, *189*(9), 1363–1372.

Nijman, S. M. B., Luna-Vargas, M. P. A., Velds, A., Brummelkamp, T. R., Dirac, A. M. G., Sixma, T. K., & Bernards, R. (2005). A genomic and functional inventory of deubiquitinating enzymes. *Cell*, *123*(5), 773–786.

Noh, J. H., Kim, K. M., McClusky, W. G., Abdelmohsen, K., & Gorospe, M. (2018). Cytoplasmic functions of long noncoding RNAs. *Wiley Interdisciplinary Reviews: RNA*, *9*(3), 1–15.

Oda, J. M. M., Hirata, B. K. B., Guembarovski, R. L., & Watanabe, M. A. E. (2013). Genetic polymorphism in FOXP3 gene: Imbalance in regulatory T-cell role and development of human diseases. *Journal of Genetics*, *92*(1), 163–171.

Ono, M., Yaguchi, H., Ohkura, N., Kitabayashi, I., Nagamura, Y., Nomura, T., … Sakaguchi, S. (2007). Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. *Nature*, *446*(7136), 685–689.

Panzeri, I., Rossetti, G., Abrignani, S., & Pagani, M. (2015). Long intergenic noncoding RNAs: Novel drivers of human lymphocyte differentiation. *Frontiers in Immunology*, *6*(MAR), 1–14.

Raj, A., van den Bogaard, P., Rifkin, S. A., van Oudenaarden, A., & Tyagi, S. (2008). Imaging individual mRNA molecules using multiple singly labeled probes. *Nature Methods*, *5*(10), 877–879.

Ranzani, V., Rossetti, G., Panzeri, I., Arrigoni, A., Jp, R., Francesco, R. De, … Abrignani, S. (2015). Europe PMC Funders Group LincRNA landscape in human lymphocytes highlights regulation of T cell differentiation by linc-MAF-4, *16*(3), 318–325.

Ronchetti, S., Ricci, E., Petrillo, M. G., Cari, L., Migliorati, G., Nocentini, G., & Riccardi, C. (2015). Glucocorticoid-induced tumour necrosis factor receptor-related protein: A key marker of functional regulatory T cells. *Journal of Immunology Research*, *2015*.

Rossi, M. N., & Antonangeli, F. (2014). LncRNAs: New players in apoptosis control. *International Journal of Cell Biology*, *2014*.

Sakaguchi, S. (2004). Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annual Review of Immunology*, *22*(1), 531–562.

Sakaguchi, S., Miyara, M., Costantino, C. M., & Hafler, D. A. (2010). FOXP3 + regulatory T cells in the human immune system. *Nature Reviews Immunology*, *10*(7), 490–500.

Sakaguchi, S., Wing, K., & Miyara, M. (2007). Regulatory T cells - A brief history and perspective. *European Journal of Immunology*, *37*(SUPPL. 1), 116–123. Sakaguchi, S., Yamaguchi, T., Nomura, T., & Ono, M. (2008). Regulatory T Cells and Immune Tolerance. *Cell*, *133*(5), 775–787.

Schmetterer, K. G., Neunkirchner, A., & Pickl, W. F. (2012). Naturally occurring regulatory T cells: markers, mechanisms, and manipulation. *The FASEB Journal*, *26*(6), 2253–2276.

Schneider, H., Downey, J., Smith, A., Zinselmeyer, B. H., Rush, C., Brewer, J. M., … Rudd, C. E. (2006). Signal by CTLA-4. *Science*, *313*(September), 1972–1975.

Shea, J. J. O., & Paul, W. E. (2010). NIH Public Access. *Science*, *327*(5969), 1098–1102.

Shevach, E. M., DiPaolo, R. a, Andersson, J., Zhao, D.-M., Stephens, G. L., & Thornton, A. M. (2006). The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells. *Immunological Reviews*, *212*, 60–73.

Shi, X., Sun, M., Liu, H., Yao, Y., & Song, Y. (2013). Long non-coding RNAs: A new frontier in the study of human diseases. *Cancer Letters*, *339*(2), 159–166.

Simpson, T. R., Li, F., Montalvo-Ortiz, W., Sepulveda, M. A., Bergerhoff, K., Arce, F., … Quezada, S. A. (2013). Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti–CTLA-4 therapy against melanoma. *The Journal of Experimental Medicine*, *210*(9), 1695–1710.

Steer, H. J., Lake, R. A., Nowak, A. K., & Robinson, B. W. S. (2010). Harnessing the immune response to treat cancer. *Oncogene*, *29*(48), 6301–6313.

Struhl, K. (2007). Transcriptional noise and the fidelity of initiation by RNA polymerase II. *Nature Structural and Molecular Biology*, *14*(2), 103–105.

Taft, R. J., Pheasant, M., & Mattick, J. S. (2007). The relationship between non-protein-coding DNA and eukaryotic complexity. *BioEssays*, *29*(3), 288–299.

Tang, J. Y., Lee, J. C., Chang, Y. T., Hou, M. F., Huang, H. W., Liaw, C. C., & Chang, H. W. (2013). Long noncoding RNAs-related diseases, cancers, and drugs. *The Scientific World Journal*, *2013*.

Tarailo-Graovac, M., & Chen, N. (2009). Using RepeatMasker to identify repetitive elements in genomic sequences. *Current Protocols in Bioinformatics*, (SUPPL. 25), 1–14.

Taylor, A., Verhagen, J., Blaser, K., Akdis, M., & Akdis, C. A. (2006). Mechanisms of immune

suppression by interleukin-10 and transforming growth factor-β: The role of T regulatory cells. *Immunology*, *117*(4), 433–442.

Tone, Y., Furuuchi, K., Kojima, Y., Tykocinski, M. L., Greene, M. I., & Tone, M. (2008). Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nature Immunology*, *9*(2), 194–202.

Turner, M., Galloway, A., & Vigorito, E. (2014). Noncoding RNA and its associated proteins as regulatory elements of the immune system. *Nature Immunology*, *15*(6), 484–491.

Uchida, S., & Dimmeler, S. (2015). Long noncoding RNAs in cardiovascular diseases. *Circulation Research*, *116*(4), 737–750.

Van Loosdregt, J., Vercoulen, Y., Guichelaar, T., Gent, Y. Y. J., Beekman, J. M., Van Beekum, O., … Coffer, P. J. (2010). Regulation of Treg functionality by acetylation-mediated Foxp3 protein stabilization. *Blood*, *115*(5), 965–974.

Vent-Schmidt, J., Han, J. M., Macdonald, K. G., & Levings, M. K. (2014). The role of FOXP3 in regulating immune responses. *International Reviews of Immunology*, *33*(2), 110–128.

Verma-Gaur, J., Torkamani, A., Schaffer, L., Head, S. R., Schork, N. J., & Feeney, A. J. (2012). Noncoding transcription within the Igh distal VH region at PAIR elements affects the 3D structure of the Igh locus in pro-B cells. *Proceedings of the National Academy of Sciences*, *109*(42), 17004–17009.

Vignali, D. a, Collison, L. W., & Workman, C. J. (2009). How regulatory T cells work. *Nature Reviews: Immunology*, *8*(7), 523–532.

Wang, J., Ioan-Facsinay, A., van der Voort, E. I. H., Huizinga, T. W. J., & Toes, R. E. M. (2007). Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *European Journal of Immunology*, *37*(1), 129–138.

Wang, L., Kumar, S., Dahiya, S., Wang, F., Wu, J., Newick, K., … Hancock, W. W. (2016). Ubiquitin-specific Protease-7 Inhibition Impairs Tip60-dependent Foxp3ï¿½+ T-regulatory Cell Function and Promotes Antitumor Immunity. *EBioMedicine*, *13*, 99–112.

Williams, L. M., & Rudensky, A. Y. (2007). Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nature Immunology*, *8*(3), 277–284.

Wilson, N. J., Boniface, K., Chan, J. R., McKenzie, B. S., Blumenschein, W. M., Mattson, J. D., … Malefyt, R. de W. (2007). Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nature Immunology*, *8*(9), 950–957.

Workman, C., Szymczak-Workman, a L., Collison, L. W., & Pillai, M. R. (2009). *The Development and Function of Regulatory T Cells*. *Cell Mol Life Sci* (Vol. 66).

Wu, Y., Borde, M., Heissmeyer, V., Feuerer, M., Lapan, A. D., Stroud, J. C., … Rao, A. (2006). FOXP3 Controls Regulatory T Cell Function through Cooperation with NFAT. *Cell*, *126*(2), 375–387.

Xiao, Y., Nagai, Y., Deng, G., Ohtani, T., Zhu, Z., Zhou, Z., … Greene, M. I. (2014). Dynamic interactions between TIP60 and p300 regulate FOXP3 function through a structural switch defined by a single lysine on TIP60. *Cell Reports*, *7*(5), 1471–1480.

Yao, Z., Kanno, Y., Kerenyi, M., Stephens, G., Durant, L., Watford, W. T., … Laurence, A. (2013). Nonredundant roles for Stat5a / b in directly regulating Foxp3 Nonredundant roles for Stat5a / b in directly regulating Foxp3. *Blood*, *109*(10), 4368–4375.

Yin, D., He, X., Zhang, E., Kong, R., De, W., & Zhang, Z. (2014). Long noncoding RNA GAS5 affects cell proliferation and predicts a poor prognosis in patients with colorectal cancer. *Medical Oncology*, *31*(11), 1–8. https://doi.org/10.1007/s12032-014-0253-8

Yoon, J. H., Abdelmohsen, K., Srikantan, S., Yang, X., Martindale, J. L., De, S., … Gorospe, M. (2012). LincRNA-p21 Suppresses Target mRNA Translation. *Molecular Cell*, *47*(4), 648–655.

Yu, N., Li, X., Song, W., Li, D., Yu, D., Zeng, X., … Li, X. (2012). CD4+CD25+CD127low/− T Cells: A More Specific Treg Population in Human Peripheral Blood. *Inflammation*, *35*(6), 1773–1780.

Zhang, F., Meng, G., & Strober, W. (2008). Interactions among the transcription factors Runx1, RORγt and Foxp3 regulate the differentiation of interleukin 17-producing T cells. *Nature Immunology*, *9*(11), 1297–1306.

Zhang, H., Nestor, C. E., Zhao, S., Lentini, A., Bohle, B., Benson, M., & Wang, H. (2013). Profiling of human CD4+T-cell subsets identifies the TH2-specific noncoding RNA GATA3-AS1. *Journal of Allergy and Clinical Immunology*, *132*(4), 1005–1008.

Zhang, Y., Liu, T., Meyer, C. A., Eeckhoute, J., Johnson, D. S., Bernstein, B. E., … Shirley, X. S. (2008). Model-based analysis of ChIP-Seq (MACS). *Genome Biology*, *9*(9).

Zheng, Y., Chaudhry, A., Kas, A., DeRoos, P., Kim, J. M., Chu, T. T., … Rudensky, A. Y. (2009). Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control TH2 responses. *Nature*, *458*(7236), 351–356.

Zheng, Y., Josefowicz, S. Z., Kas, A., Chu, T. T., Gavin, M. A., & Rudensky, A. Y. (2007). Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature*, *445*(7130), 936–940.

Zhou, L., Lopes, J. E., Chong, M. M. W., Ivanov, I. I., Min, R., Victora, G. D., … Littman, D. R. (2008). TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature*, *453*(7192), 236–240.