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Identification of proteins regulating TGF-β1 production in human regulatory T lymphocytes

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Février 2015

Thèse présentée en vue de l'obtention du grade
de docteur en Sciences Biomédicales et Pharmaceutiques
Secteur des sciences de la santé

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Je souhaite tout d'abord remercier ma promotrice pour ces 5 années passées dans son équipe. Merci Sophie d'avoir pris le temps de me transmettre tes connaissances, d'avoir eu confiance en moi et de m'avoir toujours poussée à aller plus loin! Je garderai un très bon souvenir de ma thèse, j'ai énormément appris et j'espère qu'on continuera à se voir! Merci aussi pour ton soutien dans tous les projets dans lesquels je me suis investie en dehors du labo!

Je tiens également à remercier l'ensemble de mon jury de thèse pour son soutien tout au long de mon parcours. Vous m'avez encouragé à persévérer malgré les embûches de ce projet, et s'il a abouti c'est aussi grâce à vous! J'adresse également un remerciement particulier à Pierre Coulie et à tout le corps enseignant du cours d'immunologie de m'avoir transmis le goût de l'immunologie et de la démarche scientifique.

Mes remerciements vont ensuite à l'équipe que j'ai côtoyée, chanceuse que je suis, au cours de ces années et qui sont devenus bien plus que des collègues! Julie, merci de m'avoir accueillie à bras ouverts dès mon arrivée au labo! Merci pour ta disponibilité, pour ton aide au quotidien ainsi que pour la relecture de ce manuscrit! Olivier, merci pour ta générosité et pour ton aide! Nos échanges scientifiques, idéologiques et politiques pendant que nous cultivions nos cellules vont me manquer! Stéphanie L. merci à toi d'avoir participé avec énergie au projet LAPTM4B pendant ton mémoire! Ton enthousiasme a fait énormément de bien à ce travail! Merci aussi à Stéphanie D. pour sa participation à ce projet. Ce fut un plaisir de travailler avec toi! Merci également à toi Maria pour l'aide considérable que tu apportes à notre travail au quotidien! Merci aussi pour ta bonne humeur! Julia, merci pour nos discussions scientifiques ou non autour d'un bon café! Merci à toi Emilie d'avoir contribué à ce projet et à toi Axelle de me faire rire aux éclats! Je souhaite à Stéphanie L., Olivier et Sara une excellente suite et fin de thèse! Bon courage et bonne chance!

Je souhaite également remercier les différents groupes avec lesquels nous partageons le 3è étage. D'abord, l'équipe de Pierre Coulie pour leur aide et leur amitié. Merci à Nicolas, Nathalie R., Tiphanie, Orian, Charlotte, David, Gérald et à Téofila. Un merci tout particulier à Catherine et à Suzanne pour leur gentillesse et leur aide précieuse durant ces années! J'adresse également mes remerciements au groupe d'Etienne De Plaen! Merci vous Etienne pour vos conseils pour les cultures de levures! Merci à vous Marc, Juliette, et Nathalie pour votre aide lors de mes recherches de divers produits chimiques et merci aussi pour toutes nos conversations (rarement) scientifiques! Merci aussi à Marjorie, Aurélie, Marie-Sophie, Luc, Délia, Alexandre, Nathalie V., Rui, Aline,

Florence, Joanna, Wenbin et Céline pour la bonne ambiance au travail et au traminot le vendredi soir! Merci aussi à toute l'équipe technique de l'Institut Ludwig qui nous permet de travailler dans des conditions idéales!

J'aimerais également remercier tous les membres de l'Institut de Duve et de l'Institut Ludwig. Chaque rencontre et collaboration a été bénéfique pour mon projet! Je pense particulièrement à Donatienne, Antoine, Vitalina, Anabelle, Sandrine,... Nous avons la chance de travailler dans un environnement qui favorise les échanges d'idées, de protocoles et de produits! Ce fut un honneur de travailler à l'Institut imaginé par Christian de Duve.

Ensuite je voudrais remercier mes amis qui ont été présents et m'ont soutenue tout au long de ma thèse. D'abord les SBIMs! Merci pour vos encouragements Jess, Nath (il fallait au moins te citer deux fois dans mes remerciements!), Val, Alex, Berny, Antoine, Fab, Laura, Auré et Lorraine! Ca a été un plaisir de partager les auditoires et l'expérience de la thèse avec vous! Je sais que nous continuerons à nous voir! Merci à ma nouvelle bande de copines : Franci, Violetta, Eva et Maria! Vous êtes devenues pour moi, plus que des collègues ou des partenaires de hockey, j'adore discuter avec vous et préparer notre monde de demain! Merci à toute l'équipe de dames 3 du LLNHC, anciennement les cougars maintenant devenues les zébras, vous avez été ma bouffée d'oxygène pendant ma thèse! A très vite sur le terrain ou au bar pour discuter tactique! Merci à mes amis de looongue date Perrine, Arabelle, Séverine, Dé, Jul, Cel, Anne-so, Emi, Mike, Den, Jem, Bibo...! Vous citer tous ici serait impossible mais merci à tous pour votre amitié de ces 20 dernières années, et pour vos messages d'encouragements lors de la rédaction du manuscrit et lors de ma défense! Ca y est, on peut le boire ce champagne! Merci enfin à JAVVA et à tous ses membres de m'avoir offert la possibilité de prendre du recul pendant ma thèse, de m'avoir enrichi et de contribuer à rendre le monde meilleur!

Finalement, je remercie toute ma famille d'avoir été à mes côtés pendant ces années! Mes parents qui m'ont offert la possibilité d'arriver là aujourd'hui et qui m'ont soutenue dans les moments joyeux ou difficiles toute ma vie! Merci à mes soeurs d'être des personnes humaines et joyeuses! Ne changez surtout pas! Merci aussi à toi Mamy pour ton soutien et ton enthousiasme! Merci pour tout!

Je dédie cette thèse à mon Bon-Papa.

Table of contents

Abbreviations **13**

Summary **15**

Introduction **17**

1. Regulatory T lymphocytes and cancer **18**

1.1. Cancer immunotherapy 18

1.2. Regulatory T cells 19

1.3. Tregs in cancer 22

1.4. Mechanisms of immunosuppression by murine and human Tregs 23

2. TGF-β1, an immunosuppressive cytokine **26**

2.1. TGF-β1 synthesis, processing and activation 26

2.3. TGF-β signal transduction 31

2.4. TGF-β functions in the immune system 33

2.4.1. TGF-β production by T cells 34

2.4.2. T cells as targets of TGF-β in the immune system 35

2.4.3. Other roles of TGF-β in T cell differentiation and function 36

3. GARP, a transmembrane protein expressed by activated human Tregs **39**

3.1. Structure of GARP (or LRRC32) 39

3.2. GARP expression pattern and regulation 40

3.3. Functions of GARP in Tregs 40

4. How to study protein-protein interactions? **45**

4.1 PPI detection by «in-cell» methods 46

4.1.1 Yeast Two-Hybrid systems 46

4.1.2 Mammalian Two-Hybrid systems 56

4.1.3 Proximity Ligation Assay 62

4.2 PPI detection by biochemical methods 64

4.2.1. Co-immunoprecipitation and co-Affinity Purification 64

4.2.2. Luminescence-based mammalian interactome mapping 67

4.2.3. Overlay or Far Western 68

4.2.4. Phage Display 69

4.3 The Interactome 71

Aim of the study **73**

Results **75**

1. LAPTM4B interacts with GARP and decreases TGF-β1 production in human regulatory T cells **76**

2. Identification of proteins interacting with GARP in human Tregs by immunoprecipitation followed by mass spectrometry **97**

Discussion and Perspectives **111**

1. Two distinct approaches to identify GARP partners in human Tregs **112**

2. Seven candidates identified by IP-MS **116**

3. LAPTM4B, a candidate identified by MYTH **118**

3.1. Regulation of LAPTM4B expression 118

3.2. Roles of LAPTM4B in human Tregs 119

3.3 Other described functions of LAPTM4B, and LAPTM family members 119

3.4. LAPTM4B in Treg suppressive function 121

4. TGF-β activators in Tregs **123**

References **125**

Abbreviations

5-FOA	5-Fluoroorotic Acid
$A_{2A}R$	Adenosine A2A Receptor
AD	Activating Domain
AP	Affinity Purification
BiFC	Bimolecular Fluorescence Complementation
BMP	Bone Morphogenetic Protein
BRET	Bioluminesence Resonance Energy Transfer
cAMP	cyclic Adenosine Monophosphate
CD	Cluster of Differentiation
cDNA	complementary DeoxyriboNucleic Acid
CpG	Cytosine Guanine dinucleotide
co-IP	co-ImmunoPrecipitation
co-SMAD	common SMAD
CTL	Cytotoxic T Lymphocyte
CTLA4	Cytotoxic T Lymphocyte-associated Antigen 4
Cys	Cysteine
DC	Dendritic Cell
DBD	DNA Binding Domain
dn	dominant negative
DNA	DeoxyriboNucleic Acid
ER	Endoplasmic Reticulum
FACS	Fluorescence-Activated Cell Sorting
FDA	Food and Drug Administration
FOXP3	Forkhead box P3
FRET	Fluoresence Resonance Energy Transfer
GAP	GTPase-activating protein
GARP	Glycoprotein A Repetition Predominant
GDF	Growth and Differentiation Factor
GEF	Guanine nucleotide Exchanging Factor
GFP	Green Fluorescent Protein
GST	Glutathione S-Transferase
GTP	Guanosine TriPhosphate
GVHD	Graft Versus Host Disease
IDO	Indoleamine 2,3-dioxygenase
IL	Interleukin
IPEX	Immune dysregulation Polyendocrinopathy Enteropathy X-linked syndrome
I-SMAD	inhibitory SMAD
iTreg	induced Treg
JAK	Janus Kinase
KO	Knock Out
LAP	Latency-Associated Peptide
LAPTM4B	Lysosomal-Associated Protein Transmembrane 4B
LRRC32	Leucine Rich Repeat Containing Protein 32
LTBP	Latent TGF-β Binding Protein
M2H	Mammalian two-Hybrid
mAbs	monoclonal Antibodies
MAGE	Melanoma Antigen-encoding Gene
MAPPIT	Mammalian Protein-Protein Interaction Trap
mRNA	messenger RNA
MS	Mass Spectrometry

MS-qPCR	Methylation-Specific qPCR
MYTH	Membrane Yeast Two-Hybrid
NK	Natural Killer cell
NSG	NOD/Scid/Il2rg$^{-/-}$
PAGE	PolyAcrylamide Gel Electrophoresis
PBMC	Peripheral Blood Mononuclear Cells
PCA	Protein fragment Complementation Assay
PCR	Polymerase Chain Reaction
PD1	Programmed Death 1
PD-L1	Programmed Death Ligand 1
PLA	Proximity Ligation Assay
PPI	Protein-Protein Interaction
qPCR	quantitative PCR
Rag	Recombination activating gene
RGD	Arginine-Glycine-Aspartic acid
RNA	Ribonucleic Acid
RRS	Ras Recruitment System
R-SMAD	Receptor-associated SMAD
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SCINEX-P	Screening for Interactions between Extracellular Proteins
siRNA	small interfering Ribonucleic Acid
Smurf	Smad ubiquitination regulatory factor
snRNA	small nuclear Ribonucleic Acid
SRS	SOS Recruitment System
STAT	Signal Transducer and Activator of Transcription
TAP	Tandem Affinity Purification
TCR	T Cell Receptor
TEV	Tobacco Etch Virus
TF	Transcription Factor
TGF-β	Transforming Growth Factor beta
TGFβR	Transforming Growth Factor beta Receptor
Th	T helper lymphocyte
TRAMP	Transgenic Adenocarcinoma of Mouse Prostate
Treg	regulatory T lymphocyte
TSP-1	Thrombospondin 1
tTreg	thymic Treg
UAS	Upstream Activating Sequence
UPR	Unfolded Protein Response
UPRE	Unfolded Protein Response Element
WB	Western Blot
WT	Wild-Type
xGVHD	xenogeneic Graft Versus Host Disease
Y2H	Yeast two-Hybrid
YFP	Yellow Fluorescent Protein

Summary

Regulatory T lymphocytes (Tregs) are a subset of $CD4^+$ T cells endowed with immunosuppressive activity. They inhibit immune responses through various mechanisms, which include the production of the immunosuppressive cytokine Transforming Growth Factor beta 1 (TGF-β1).

Many cell types produce TGF-β1 in a latent form, in which the mature TGF-β1 cytokine is non-covalently associated to the Latency Associated Peptide (LAP). In this latent conformation, mature TGF-β1 is prevented from binding to its receptor by LAP. To become active, mature TGF-β1 must be released from LAP, a process referred to as TGF-β1 activation.

Human Tregs, but not other types of T cells, produce active TGF-β1 upon TCR stimulation. The molecular mechanisms leading to TGF-β1 activation by Tregs are not yet fully elucidated. TCR-stimulated Tregs present latent TGF-β1 on their surface through binding to a transmembrane protein called GARP. Antibodies against GARP block active TGF-β1 production by Tregs, demonstrating that GARP is required for TGF-β1 activation by these cells. However, forced expression of GARP in non-regulatory T cells is not sufficient to induce active TGF-β1 production. We thus hypothesised that additional proteins, present in Tregs but not in other types of T cells, associate with GARP/latent TGF-β1 complex and contribute to TGF-β1 activation. The aim of our project was to identify such protein(s).

To achieve our objective, we used two different approaches. In the first, we used GARP as bait to screen a Treg cDNA library by yeast two-hybrid. This lead to the identification of LAPTM4B (Lysosomal-Associated Protein Transmembrane 4B), a multipass transmembrane protein encoded by a gene expressed at higher levels in Tregs than in other T cells. We show that LAPTM4B decreases cleavage of pro-TGF-β1, secretion of soluble latent TGF-β1 and surface presentation of GARP/TGF-β1 complexes by Tregs, but does not contribute to TGF-β1 activation. We thus concluded that LAPTM4B is a negative regulator of TGF-β1 production by human Tregs. In a second approach, we identified proteins that co-immunoprecipitate with GARP in human Tregs by Mass Spectrometry. This allowed for the identification of 7 candidates that display higher expression in Tregs compared to other T cells. To date, we excluded 4 of these candidates from further analysis, because they did not induce TGF-β1 activation in 293T cells expressing GARP and TGF-β1. The remaining 3 candidates have not been tested in this assay yet and may represent Treg proteins that cooperate with GARP to activate TGF-β1.

Altogether, we have identified LAPTM4B, a Treg protein which may play a role in regulating immune responses by decreasing TGF-β1 production by, and thus function of human Tregs. We have also identified 3 additional putative GARP-binding proteins which could also regulate Treg function. Further analyses are ongoing to determine what role, if any, these proteins play in the control of active TGF-β1 production by human Tregs.

Introduction

1. Regulatory T lymphocytes and cancer

1.1. Cancer immunotherapy

The development of immunotherapies against cancer emerged from the discovery of T lymphocytes directed against tumours in cancer patients. Despite the development of spontaneous T cell responses against their tumours, melanoma patients fail to reject their cancers. Immunotherapies aiming at increasing the frequency and/or the effector functions of tumour-specific T lymphocytes could overcome this inefficient anti-tumour response and lead to tumour rejection. They are particularly attractive since they are expected to yield less side effects than conventional anti-cancer treatments (Boon *et al.* 2006, Coulie *et al.* 2014). For this reason a huge effort has been made over the last decades to develop immunotherapeutic strategies against cancers.

The first gene encoding a human tumour-specific antigen was identified in 1991 by the group of T. Boon and called *MAGEA1* (van der Bruggen *et al.* 1991). This gene was found to be expressed by tumours but not by other healthy tissues, and was therefore an ideal target for immunotherapeutic strategies based on the use of vaccines. Since this discovery, various other genes encoding tumour specific antigens were identified. These findings paved the way to the development of therapeutic vaccines using antigens to boost anti-tumour immunity. Various vaccination modalities were tested in patients, including injections of peptides, recombinant proteins or viruses, and dendritic cells pulsed with peptides (Boon *et al.* 2006, Coulie *et al.* 2014). Unfortunately, clinical responses to the vaccines were lower than expected, and occurred in only 5 to 10% of cancer patients.

Besides vaccination approaches, other immunotherapeutic strategies to boost pre-existing T cell function were recently elaborated. These therapies, used alone or in combination with vaccinations, consist in injecting antibodies blocking T-cell receptors whose involvement with their cognate ligand induces inhibitory signals in T cells. They were termed immune checkpoints therapies. Anti-CTLA4 (Cytotoxic T lymphocyte Antigen 4) antibodies were the first «checkpoint» inhibitor tested in clinical trials for treatment of metastatic melanoma (Hodi *et al.* 2010). Given the improvement of overall survival of melanoma patients who received anti-CTLA4 treatment, this treatment received Food and Drug Administration (FDA) approval for advanced melanoma treatment (Pardoll 2012, Page *et al.* 2014). The benefits of this therapy was considerable and lead to the development of a second generation of checkpoint inhibitors, namely anti-Programmed Death 1 (PD1) and anti-

Programmed cell Death Ligand 1 (PD-L1) antibodies. These antibodies have recently shown clinical activity and safety in phase I trials (Brahmer *et al.* 2012, Topalian *et al.* 2012).

Notwithstanding successes of anti-CTLA4, anti-PD1 or anti-PD-L1 therapies in some patients, many do not respond to these immunostimulatory therapies, and this appears to result from the immunosuppressive environment that prevails inside tumours. Numerous mechanisms to inhibit anti-tumour immunity are elaborated by cancer cells. Cancer cells themselves produce soluble immunosuppressive molecules like Transforming Growth Factor beta 1 (TGF-β1), IL-10, galectins and Indoleamine 2,3-Dioxygenase (IDO) (Chen *et al.* 1994, Gorelik and Flavell 2001, Uyttenhove *et al.* 2003, Demotte *et al.* 2008, Coulie *et al.* 2014). In addition, non-cancerous cells displaying immunosuppressive functions are also recruited within the tumour (Nishikawa and Sakaguchi 2014). These include regulatory T lymphocytes, or Tregs. It is thought that blocking immunosuppression in the tumor environment could improve the efficiency of cancer vaccines or immunostimulatory antibodies.

1.2. Regulatory T cells

The existence of a T cell population displaying suppressive activity was initially proposed by Nishizuka and Sakakura in 1969 (Nishizuka and Sakakura 1969). These cells were named «Suppressor T cells», but were renamed «Regulatory T lymphocytes» or Tregs by Sakaguchi in 1995 (Sakaguchi *et al.* 1995). Tregs participate to the maintenance of tolerance to self. Tolerance of T cells against self-antigens is ensured at two levels. Negative selection of developing self-reactive T cells in the thymus is often referred to as a «central» mechanism of tolerance, whereas «peripheral» mechanisms also exist to control self-reactive T cells that escape negative selection and reach peripheral organs. Tregs play an indispensable role in the maintenance of peripheral tolerance, by keeping in check autoreactive T cells (Josefowicz *et al.* 2012). Tregs appear also to restrain immune responses against pathogens to avoid excessive immune reactions and damage to host tissues (Belkaid and Tarbell 2009).

Tregs represent a small fraction of the circulating $CD4^+$ T cell population. Several protein markers have been proposed to distinguish them from conventional, non-regulatory $CD4^+$ T cells. In order to study their function, researchers in the field put a lot of effort into the discrimination of Tregs from other conventional T cells (Figure 1). In 1995, Sakaguchi and co-workers showed that $CD4^+$

T cell constitutively expressing high levels of CD25, the α chain of the IL-2 receptor, displayed suppressive activity (Sakaguchi *et al.* 1995). Unfortunately, CD25 is not strictly specific of Tregs since all T cells acquire surface CD25 upon TCR stimulation. Other surface markers like CD127 and CTLA4 were also proposed to discriminate the Tregs from other T cells but, as for CD25, their expression is modulated upon TCR stimulation, precluding their use as strictly specific Treg markers. In the early 2000s, mutations in a gene called *Foxp3*, located on the X-chromosome, were identified as responsible for the development of a severe lymphoproliferative autoimmune disease in both mice and humans (Chatila *et al.* 2000, Bennett *et al.* 2001, Brunkow *et al.* 2001, Wildin *et al.* 2001). In humans, this disease is known as IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome). Shortly after this finding, three groups showed that gene *foxp3* codes a transcription factor that is indispensable for Treg development and function and that the phenotype of mice with spontaneous or targeted *Foxp3* mutations is due to a Treg deficiency (Fontenot *et al.* 2003, Hori *et al.* 2003, Khattri *et al.* 2003). Expression of Foxp3 is specific of the Treg lineage in mice. In humans however, FOXP3 expression is not strictly limited to Tregs as TCR stimulation induces transient FOXP3 expression in non-regulatory T cells. Therefore, FOXP3, although essential for Treg differentiation, maintenance and function, cannot serve as a reliable Treg marker in humans (Morgan *et al.* 2005, Roncador *et al.* 2005).

To date, the only molecular marker that unambiguously distinguishes human Tregs from other T cells is the demethylation of a regulatory region in intron 1 of the *FOXP3* gene. This region, termed *FOXP3i1*, *CNS2* or *TSDR*, contains CpGs that are demethylated in human and murine Tregs while they are methylated in non-regulatory T cells. Demethylation of *FOXP3i1* ensures stable FOXP3 expression. The methylation status of this region can be assessed by sequencing or by methylation-specific quantitative PCR (MS-qPCR) on sodium bisulfite-treated genomic DNA (Baron *et al.* 2007, Floess *et al.* 2007, Wieczorek *et al.* 2009, Stockis *et al.* 2009a). This molecular marker, although highly reliable to discriminate Tregs in humans, cannot be used to sort cells from tissue or blood samples, but can serve to measure the proportion of Tregs in a given sample. It can also serve to characterise clones of human $CD4^+$ T cells, to distinguish Treg from non-regulatory T helper (Th) clones (Stockis *et al.* 2009a).

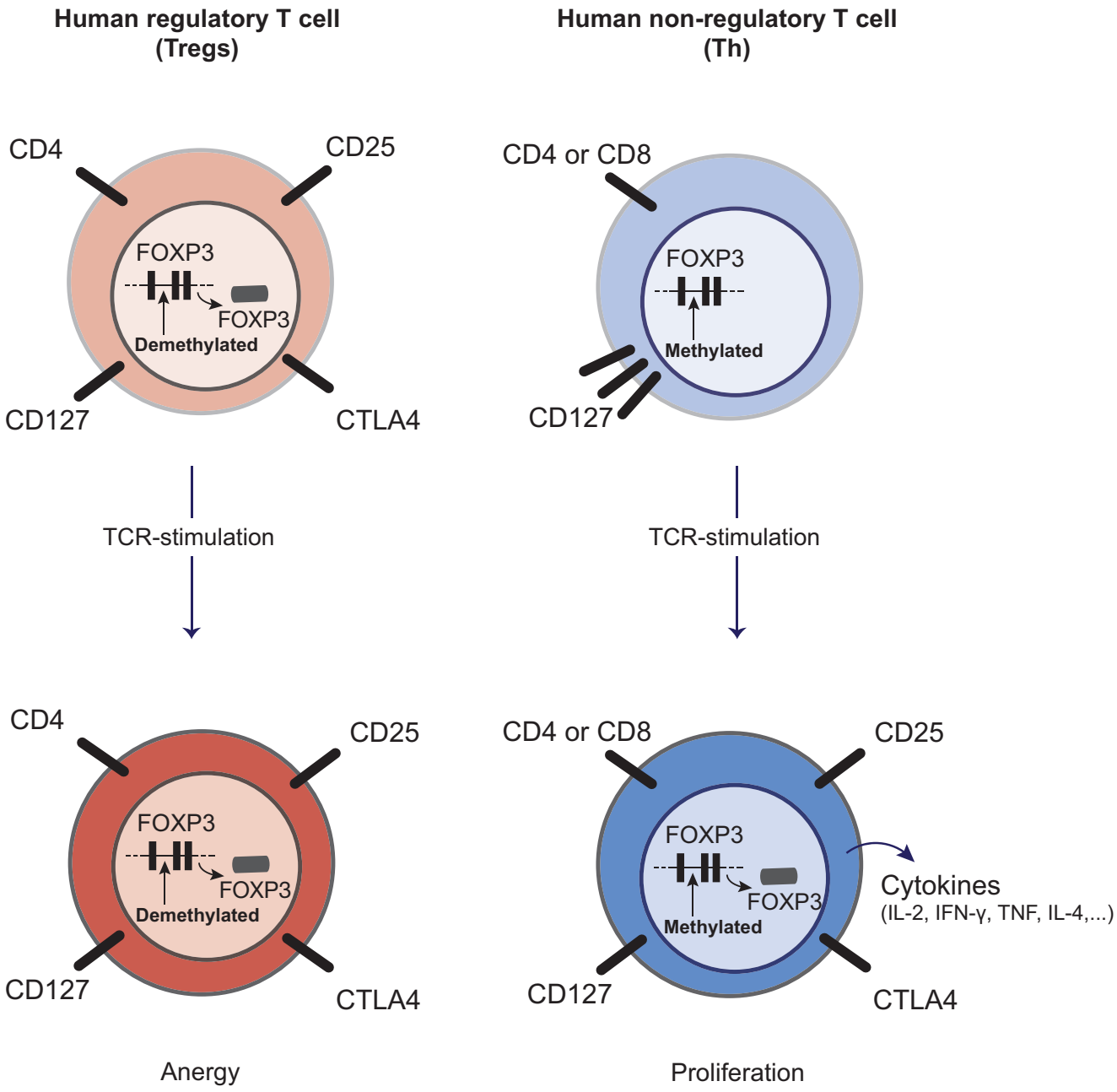

Figure 1. Schematic representation of regulatory and non-regulatory markers.
Regulatory T cells (Tregs) are characterised by their suppressive function and their anergy in the absence of exogenously added cytokine. Molecules such as CD25, CTLA-4 and CD127 are differentially expressed on Tregs by comparison to other $CD4^+$ T cells. However, their expression is also modified during non-regulatory T cell activation, rendering them inadequate to unambiguously discriminate Tregs from recently activated T cells. The most specific molecular marker of human Tregs is the stable demethylation of a region of the intron 1 of gene *FOXP3*.

1.3. Tregs in cancer

Because Tregs are a T cell subset specialised in the inhibition of immune responses, they are thought to play a deleterious role in cancer. Evidence for this was provided in multiple murine models. In 1999, Onizuka and co-workers demonstrated that, reduction of Treg numbers by administration of a depleting anti-CD25 antibody reduced the growth of transplanted tumors in six out of eight models tested (Onizuka *et al.* 1999). The same year, the group of Sakaguchi used a model in which *nude* mice (athymic mice) were co-transplanted with syngeneic leukemia and splenic cells (Shimizu *et al.* 1999). They observed that when splenocytes were depleted from $CD25^+$ T cells prior to transfer, mice succeeded in eradicating the transplanted leukemia. From these initial studies and all the evidence accumulated over the last 15 years, it is now clear that Tregs favours cancer development (Nishikawa and Sakaguchi 2014). Interestingly, recent studies in mouse melanoma models revealed that anti-CTLA4 antibody treatment thought so far to block inhibitory effect of CTLA4 on T cells, appears to act also partially by depleting tumor-infiltrating Tregs (Simpson *et al.* 2013).

In humans, the lack of a reliable protein marker of Tregs constitutes a major obstacle to the analysis of their role in cancer. Many studies report increased frequencies of $FOXP3^+$ cells in the blood of cancer patients by comparison to healthy controls, and some show correlations between high $FOXP3^+$ T cells/Th or $FOXP3^+$ T cells/CTL ratios and poor prognosis (Colombo and Piconese 2007). However, as stated above, FOXP3 is also expressed in activated T cells, rendering the interpretation of these observations difficult. Our group used methylation-specific PCR for *FOXP3i1* to evaluate frequencies of Tregs with a demethylated *FOXP3i1* in the blood of cancer patients having received immunomodulatory drugs (de Vries *et al.* 2011). These drugs were expected to induce reductions in Treg numbers hoping to favour tumour rejection. They included anti-CD25 monoclonal antibodies, low dose cyclophosphamide and IL-2/diphteria toxin fusion protein . Our results indicated that none of the Treg-depleting therapies led to a significant decrease in Treg frequencies in the blood of treated patients. Thus, it remains still difficult to evaluate the role of Tregs in human cancer patients.

1.4. Mechanisms of immunosuppression by murine and human Tregs

Many different suppression mechanisms have been described for mouse Tregs. They include expression of inhibitory surface receptors and secretion of inhibitory soluble molecules. Treg suppression can be due to a direct effect on effector T cells, or to indirect effects on antigen presenting cells, leading to decreased effector T cell activation (Vignali *et al.* 2008). Which mechanism is used by human Tregs in various physiological or pathological contexts is not known. We will summarise below the major suppressive mechanisms described for mouse Tregs.

A first mechanism that appears to play an important role in murine Treg suppression is mediated by surface CTLA4. CTLA4 is a receptor expressed at the surface of activated T cells which binds B7.1 (CD80) and B7.2 (CD86) molecules on the surface of antigen presenting cells. Engagement of CTLA4 induce inhibitory signals in T cells. The role of CTLA4 in Treg function was underlined by the *Foxp3-Cre* x *Ctla4*$^{fl/fl}$ mouse model. These mice lack CTLA4 expression on Tregs. They display signs of autoimmunity, suggesting a role for CTLA4 in Treg function. However their phenotype is milder than that of *Foxp3* knock-out mice: *Foxp3-Cre* x *Ctla4*$^{fl/fl}$ die within seven weeks of age, whereas *Foxp3*$^{-/-}$ die within 3 weeks (Wing *et al.* 2008). This indicates that CTLA4 is probably not the only molecule important for proper Treg function. In addition, how CTLA4 expression on Tregs could mediate Treg function was not clear. A molecular explanation was proposed by Qureshi and co-workers, who showed that B7 expression on dendritic cells (DCs) is reduced upon engagement of CTLA4 expressed on Tregs (Qureshi *et al.* 2011). A second explanation is that interaction between CTLA4 on Tregs and B7 on DCs induces a production of IDO by the latter providing an immunosuppressive environment (Fallarino *et al.* 2003, Shevach 2009).

A second proposed mechanism is the «IL-2 sink». Because Tregs constitutively express high levels of CD25 (the high affinity subunit of the IL-2 receptor), they may locally deprive effector T cells of IL-2. In this manner, IL-2 is prevented to stimulate proliferation of effector T cells (de la Rosa *et al.* 2004). However, this mechanism remains controversial and difficult to prove *in vivo*.

Third, Tregs were shown to mediate cytolysis of target cells through secretion of Granzyme A, Granzyme B and perforin (Grossman *et al.* 2004, Cao *et al.* 2007, Zhao *et al.* 2007, Boissonnas *et al.* 2010).

A fourth suppression mechanism described is the production of cAMP and adenosine. Both molecules have anti-proliferative effects on T cells but can also negatively regulate DCs (Josefowicz *et al.* 2012). Bopp *et al.* observed that Tregs express high levels of intracellular cAMP which can be transferred to target effector T cells by gap junctions (Bopp *et al.* 2007, Huang *et al.* 2009). cAMP then inhibits T cell proliferation by decreasing IL-2 production (Bodor *et al.* 2001). Adenosine is also produced abundantly by Tregs. It binds to adenosine $A_{2A}R$ receptors on neighbouring T cells and induces T cell growth inhibition *in vitro*. This mechanism was demonstrated to play a role in two reports. A defect in Treg suppressive function *in vivo* was observed when Tregs are unable to produce adenosine in a skin allograft rejection model (Deaglio *et al.* 2007), and T cells from $A_{2A}R^{-/-}$ mice failed to be suppressed by Tregs in a colitis model (Naganuma *et al.* 2006).

Finally, Tregs produce well-known immunosuppressive molecules that may also inhibit immune cell function. Mainly, IL-10 and TGF-β1 were shown to play crucial roles in Treg-mediated suppression (Rubtsov *et al.* 2008). A detailed chapter on TGF-β will follow.

Mechanisms of suppression by human Tregs are more difficult to study, notably because of the lack of an appropriate protein marker to identify these cells. Our group is trying to study human Treg suppression, and has chosen to use clones of human Tregs characterised by demethylated *FOXP3i1* as a source of stable, and molecularly defined Treg cells. By comparing transcriptional profiles of human Treg clones to those of clones of non-regulatory T cells (Th and CTL clone, characterised by a methylated *FOXP3i1)*, we found that Tregs produce the active form of the immunosuppressive cytokine TGF-β. Active TGF-β produced by Tregs acts in autocrine fashion in Tregs themselves, but also in paracrine fashion on Th cells co-cultured with Tregs. The immunosuppression exerted by human Treg clones *in vitro* can be prevented at least in part with neutralising anti-TGF-β antibodies (Stockis *et al.* 2009a). We thus decided to focus our analyses of human Treg suppression by studying the mechanism by which Tregs produce the active form of TGF-β.

2. TGF-β1, an immunosuppressive cytokine

2.1. TGF-β1 synthesis, processing and activation

Transforming Growth Factor-β1 (TGF-β1) belongs to the TGF-β superfamily that comprises in total 33 ligands encoded by the human genome. It includes TGF-βs, the Bone Morphogenetic Proteins (BMPs), activins, Growth and Differentiation Factors (GDFs) and Nodal (Chang *et al.* 2002). Mammals express three isoforms of TGF-β (TGF-β1, 2 and 3) encoded by three different genes. TGF-β ligands as well as their receptors are expressed by almost every cell in the organism. They have pleiotropic effects on cell proliferation, differentiation, migration and survival and affect many biological processes. TGF-β1 is the predominant isoform expressed in the immune system. T cells, B cells, NK cells, DCs and platelets produce this cytokine (Assoian *et al.* 1983, Li *et al.* 2006). TGF-β production is a tightly regulated process. Although little is known about the regulation of the transcription of the *TGFB1* gene, the steps leading to the production of bioactive TGF-β molecule have been extensively studied.

TGF-β is produced as an inactive precursor, the pre-pro-TGF-β (Figure 2). Pre-pro-TGF-β include, a signal peptide allowing targeting to the endoplasmic reticulum (ER). After cleavage of the signal peptide, the resulting pro-TGF-β dimerise and undergoes a second proteolytic cleavage by Furin (Dubois *et al.* 1995, Dubois *et al.* 2001). This leads to a large N-terminal fragment called the latency-associated peptide (LAP), and a short C-terminal fragment corresponding to the mature TGF-β cytokine (Gentry *et al.* 1988, Miyazono *et al.* 1988, Travis and Sheppard 2014). Mature TGF-β remains non covalently bound to the LAP and in this conformation, called «latent TGF-β», the mature TGF-β is prevented from binding to its receptor and exerting an activity (Travis and Sheppard 2014).

In some cell types, latent TGF-β is covalently bound to Latent TGF-β Binding Protein (LTBP). One LTBP molecule associates to a latent TGF-β dimer by forming disulphide bonds with Cysteines 33 (Cys33) of each LAP monomer. Not all cell types produce latent TGF-β in complexes with LTBP.

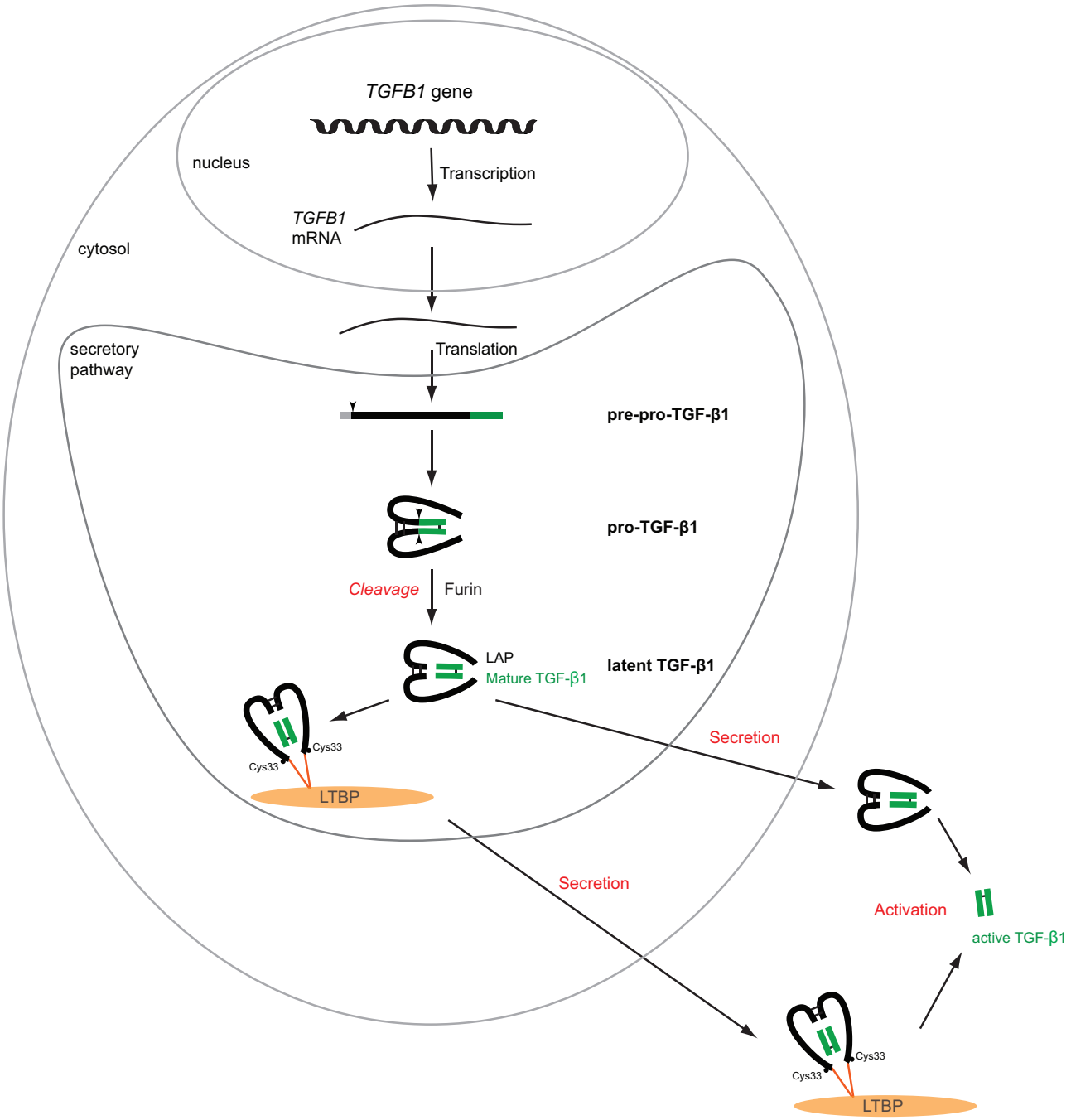

Figure 2. Schematic representation of TGF-β1 processing.
TGFB1 gene is transcribed and translated as a pre-pro-protein. Once it has reached the ER, the signal peptide is cleaved and two TGF-β1 molecules dimerise forming the pro-TGF-β1 homodimer. Pro-TGF-β1 is further cleaved by Furin into 2 parts: the N-terminal fragment or the Latency-Associated Peptide (LAP), and the C-terminal fragment or mature TGF-β1. After cleavage, LAP and mature TGF-β1 remain non-covalently associated to each other forming the latent TGF-β1. This complex is inactive and can either be secreted as such or as part of a larger complex in which latent-TGF-β1 is covalently bound to Latent TGF-β Binding Protein (LTBP). Latent TGF-β1 secreted alone or associated with LTBP is inactive and require an additional step of activation that releases the active TGF-β1 from the LAP.

The tridimensional structure of latent TGF-β1 was recently revealed by X-ray crystallography (Shi *et al.* 2011 and Figure 3). The latent TGF-β1 has a ring-like shape in which two «arms», corresponding to the two LAP monomers encircle the mature TGF-β1 dimer. The two LAP monomers overlap with one another at their extremities. On their C-terminus, they are linked to each other by two interchain disulphide bounds, forming a so-called «neck» in the structure. On their N-terminus, they overlap to form a «straitjacket-like» structure surrounding the mature TGF-β dimer. An RGD motif (arginine-glycine-aspartic acid) is present at the «shoulders» of the structure (close to the C-terminus) and the Cys33 residues, which can bind LTBP, are at the opposite side in the straitjacket, close to the N-terminus. In this rigid structure, all the contact sites of mature TGF-β with its receptor are rendered inaccessible by LAP (Shi *et al.* 2011, Travis and Sheppard 2014).

All cells secrete TGF-β in its latent inactive form. To exert its function through binding to its receptor, mature TGF-β must be released from the LAP, a process referred to as TGF-β activation. Several mechanisms of TGF-β activation, which appear to vary from one cell type to another, were described in *in vitro* and *in vivo* models (Travis and Sheppard 2014).

Activation of TGF-β *in vitro* is easily achieved by inducing protein denaturation. For example, heat, extremes of pH, ionizing radiation and chaotropic agents can all activate TGF-β (Travis and Sheppard 2014). In the blood flow, latent TGF-β bound to LTBP is produced mainly by platelets, and undergoes activation due to action of shear forces (Ahamed *et al.* 2008). Reactive oxygen species were also shown to activate TGF-β *in vitro* (Barcellos-Hoff and Dix 1996).

The mechanisms involved in TGF-β activation *in vivo* are of course more difficult to study. Several mechanism were proposed. One appears to involve Thrombospondin-1 (TSP-1), a large homotrimeric protein produced by many cell types and secreted in the extracellular matrix. A short peptide in TSP-1 (KRFK) was shown to bind to a specific sequence of LAP (LSKL), and interfere with LAP binding to mature TGF-β, hence activating the cytokine through release of mature TGF-β (Schultz-Cherry *et al.* 1995, Ribeiro *et al.* 1999). This activation mechanism was first identified *in vitro* but was later confirmed to be relevant *in vivo* by the group of N. Bouck (Crawford *et al.* 1998). *Tsp1*$^{-/-}$ mice manifest inflammation in several organs that is similar to that observed in *Tgfb1*$^{-/-}$ mice. Interestingly, treating *Tsp1*$^{-/-}$ mice with a KRFK peptide suppressed inflammation in lung and pancreas (Crawford *et al.* 1998). In the same line, wild-type mice treated with a LSKL peptide inhibiting Tsp-1 mediated activation induced inflammation in lung and pancreas. Those

data strongly suggests an involvement of Tsp-1 in TGF-β1 activation *in vivo*. However, the phenotype of the *Tsp1*$^{-/-}$ mice is not as severe as that of *Tgfb1*$^{-/-}$ mice, suggesting that other mechanisms play important roles in TGF-β1 activation *in vivo*.

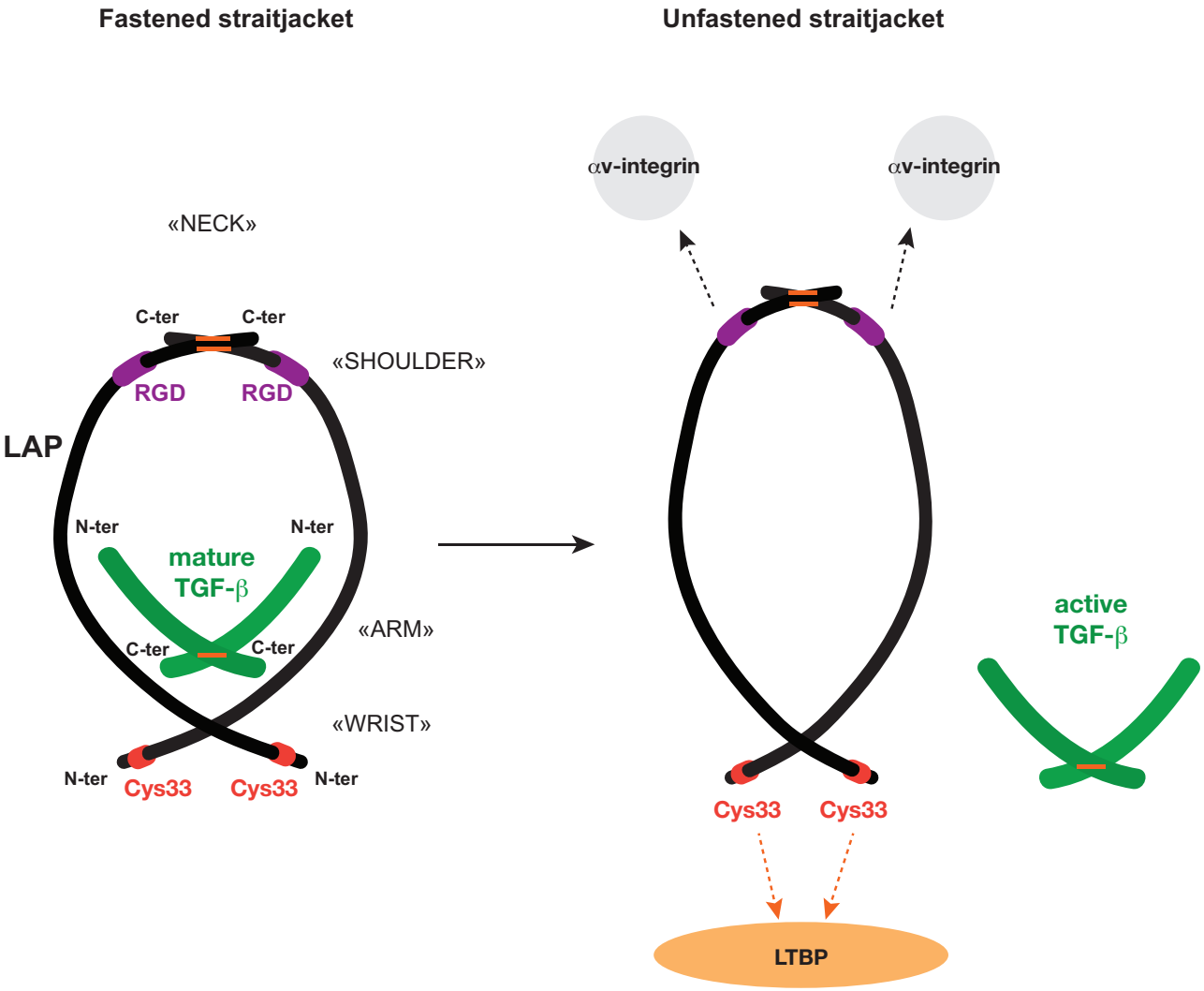

Figure 3. Schematic representation of the latent TGF-β1 complex.
Latent-TGF-β1 complex is composed of a dimer of LAP and a dimer of mature TGF-β1. As revealed in its crystal structure (Shi *et al.* 2011), latent TGF-β1 forms a ring-shaped complex, where the LAP dimer encircles the mature TGF-β1 dimer. LAP monomers bind to each other through disulfide bonds at their C-termini (C-C bonds are represented by orange lines), forming the «neck» of the complex. RGD sequences can be found at the «shoulders», while the N-terminal parts form the «arms», bearing cysteine residues (Cys33) at their «wrists». LTBP can form disulfide bounds with these two Cys33 in LAP. Release from the LAP of mature TGF-β1 can occur when tensile forces are powered on the LAP by integrins through binding to the RGD motifs on one side and by binding to LTPB proteins on the other side.

Integrins are a family of heterodimeric transmembrane receptors comprising an α and a β chain that are involved in various cellular processes including cell adhesion, proliferation and migration. A subset of integrins bind to proteins containing an RGD motif. At least eight integrins (αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, αIIbβ3, α5β1, α8β1) of the 24 mammalian integrins bind to RGD-containing proteins. The presence of an RGD motif in the LAP suggests a potential interaction between latent TGF-β and integrins. The role of integrins in TGF-β1 activation *in vivo* was studied in several mouse models. In 2007, the group of J. Munger created a mouse with a knock-in mutation in the *Tgfb1* gene changing the TGF-β1 RGD motif into an RGE non-functional motif. So-called *Tgfb1*$^{RGE/RGE}$ mutant mice display the lethal multi-organ inflammation and autoimmunity phenotype of the *Tgfb1*$^{-/-}$ mice, despite production of normal levels of latent TGF-β1 (Yang *et al.* 2007). Authors concluded that RGD-binding integrins are requisite latent TGF-β1 activators in the immune system. However which integrins are implicated is not completely understood yet. Two integrins, avβ6 and avβ8, were clearly shown to activate TGF-β in some cell types (Munger *et al.* 1999, Mu *et al.* 2002).

Expression of αvβ6 was reported to be restricted to epithelial cells. *In vitro*, cells expressing αvβ6 were shown to activate TGF-β in co-cultures with a reporter cell line expressing the luciferase gene under the control of a TGF-β responsive promoter. TGF-β signalling in this system was abolished with anti-αvβ6 antibodies. Separating reporter cells from αvβ6 expressing cells with a semi-permeable membrane also prevented TGF-β signalling in the reporter cells, suggesting a requirement for cell-cell contact to achieve paracrine action of TGF-β (Huang *et al.* 1996, Munger *et al.* 1999). *In vivo,* involvement of the β6 integrin subunit in TGF-β activation was demonstrated in *Itgb6*$^{-/-}$ mice, which display exaggerated inflammatory responses and are protected from tissue fibrosis at multiple sites, a process known to rely on TGF-β production (Munger *et al.* 1999). These phenotypes revealed an important role of the β6 subunit in TGF-β activation *in vivo*. However, the pathology developed in *Itgb6*$^{-/-}$ mice is again less severe than that of *Tgfb1*$^{-/-}$ mice, meaning that other integrins are involved in TGF-β activation *in vivo*.

Itgb8$^{-/-}$ mice show abnormal vascular development and die during embryonic development or shortly after birth, precluding analyses of the role of β8 integrins in TGF-β activation after birth. Tissue specific deletions of *Itgb8* were therefore developed (Travis *et al.* 2007). Mice lacking integrin β8 in leukocytes (*Vav1-Cre* x *Itgb8*$^{fl/fl}$) develop severe autoimmunity over time. To discriminate which immune cells were responsible for this phenotype, the authors generated

CD11c-Cre x *Itgb8*$^{fl/fl}$ to create a DC specific deletion of *Itgb8*. They also generated *CD4-Cre* x *Itgb8*$^{fl/fl}$ *mice* to create a T cell-specific deletion of integrin β8 (Travis *et al.* 2007). Whereas mice lacking *Itgb8* in DCs suffered from severe autoimmunity, identical to that of mice with an *Itgb8* deficiency in all leukocytes, mice lacking *Itgb8* on T cells were undistinguishable from WT mice for over 14 months of age. Those results strongly suggest that the autoimmune phenotype of mice lacking integrin β8 is largely due to a lack of *Itgb8* in DCs. Integrin β8-deficient DCs were also shown to fail to induce Treg and Th17 differentiation *in vivo*, two differentiation processes known to depend on TGF-β signalling (Travis *et al.* 2007, Acharya *et al.* 2010, Worthington *et al.* 2011, Kudo *et al.* 2012).

The crystal structure of latent TGF-β provided a model for a mechanical description of integrin-mediated TGF-β activation (Shi *et al.* 2011). A traction on the LAP could be exerted by integrins through their binding to the RGD motifs at the C-terminus of the LAP. Disulphide links between Cys33 at the opposite N-terminus and LTBP proteins tether LAP to the extracellular matrix, allowing the tensile force exerted by integrins to release the mature TGF-β from the LAP straitjacket (Figure 3).

Altogether, these models point to a role for Tsp-1, αvβ6 and αvβ8 integrins in latent TGF-β activation *in vivo*. But none indicate which molecule is implicated in active TGF-β production by Tregs.

2.3. TGF-β signal transduction

Upon release from the LAP, mature TGF-β binds to its receptors expressed in virtually all cell types. All three isoforms of TGF-β bind to a common receptor complex, a hetero-tetrameric structure composed of two «type I» and two «type II» TGF-β receptor chains (TGFβRI and TGFβRII, Figure 4). TGF-β first binds to a TGFβRII homodimer, which then recruits a TGFβRI homodimer. Both TGFβRI and TGFβRII are serine/threonine kinases. The TGFβRII chains are constitutively active. Upon TGF-β engagement, TGFβRI is recruited, phosphorylated and activated by TGFβRII.

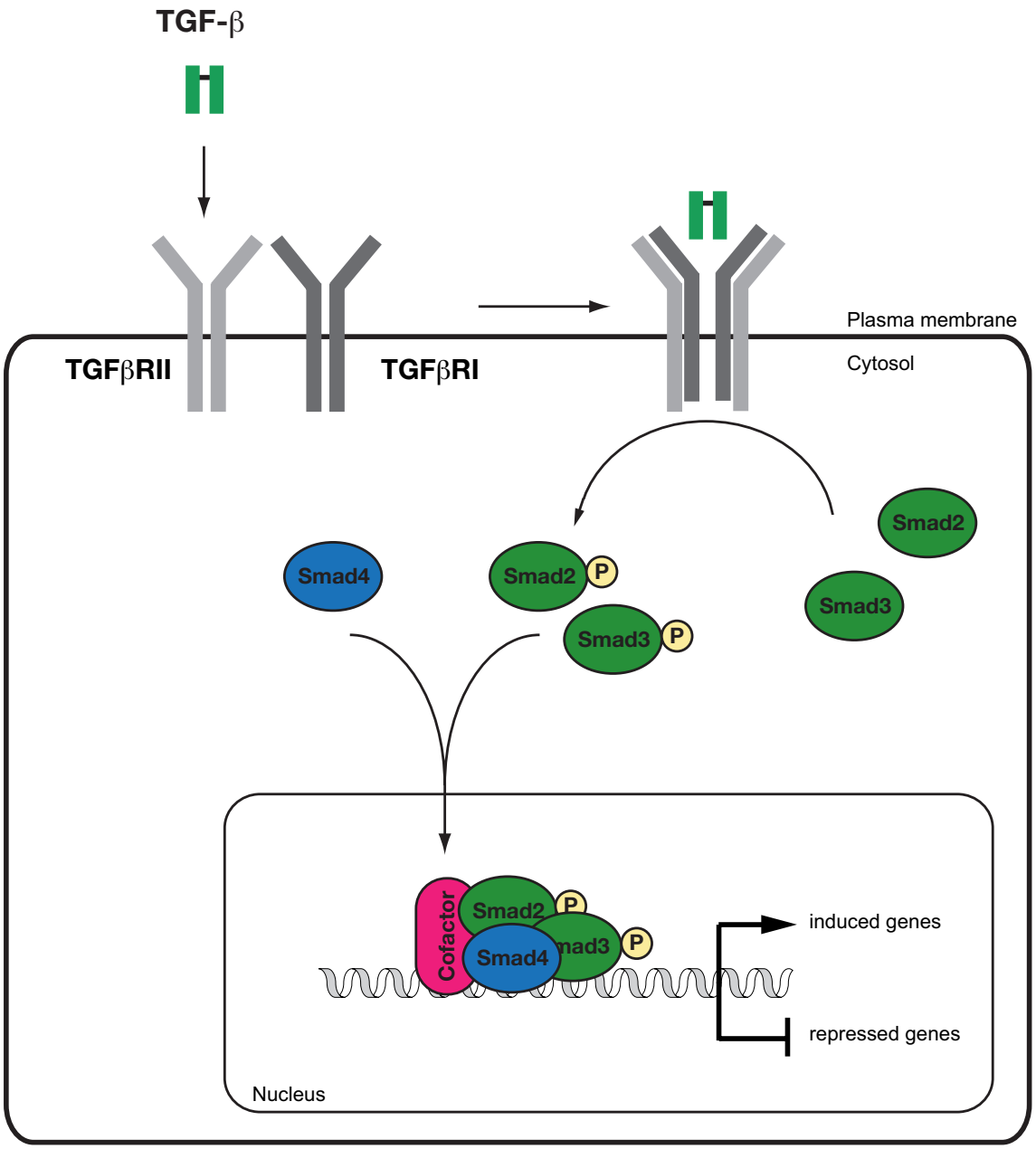

Figure 4. Schematic representation of the canonical TGF-β signalling pathway.
Active TGF-β signals through a tetrameric receptor complex composed of two TGFβRII and TGFβRI. TGF-β binds first to the TGFβRII dimer, which recruits and phosphorylates TGFβRI. Once phosphorylated TGFβRI is active and phosphorylates the R-Smads (Smad2/3) transcription factors. Phosphorylated Smad2/3 bind Smad4, allowing nuclear translocation of the heteromeric complex. In the nucleus, Smad complexes act in association with diverse cofactors to induce or repress the transcription of numerous genes.

The activated receptor complex than activate transcription factors of the Smad family. In mammals, there are five receptor-associated Smads or R-Smads (Smad1, 2, 3, 5 and 8), one common Smad or co-Smad (Smad4) and two inhibitory Smads or I-Smads (Smads 6 and 7). Activated TGFβRI recruits and phosphorylates R-Smads on C-terminal serines (Massague *et al.* 2005, Itoh and ten Dijke 2007). Once phosphorylated, Smads form a trimeric structure with the co-Smad, Smad4. Trimeric complexes comprising Smad 2, 3 and 4 recognise a consensus DNA sequence (5'-AGAC-3'), albeit with low affinity (Zawel *et al.* 1998). Smad transcription factors thus require association with other factors to increase their DNA binding and ensure full transcriptional activity. Depending on the physiological context, the Smad trimeric complexes will associate with different co-activators or co-repressors and thus up or downregulate expression of hundreds of genes that depend on nature of the associated co-factors (Xu *et al.* 2012).

In addition to the canonical TGF-β pathway, TGF-β also triggers Smad-independent signalling pathways, including MAP kinases, PI3 kinase and Rho GTPases (Travis and Sheppard 2014).

Several mechanisms modulate the intensity and the duration of TGF-β signals. One important negative feedback relies on induction of I-Smads, which are recruited to TGFβRI and prevent Smad2/3 binding and activation. I-Smads also promote TGFβRI inactivation and degradation through recruitment of phosphatases and E3-Ubiquitin ligases known as Smurfs (Smad Ubiquitination Regulatory Factors). Other inhibitory pathways inactivated R-Smads by dephosphorylation or by ubiquitination-induced degradation (Itoh and ten Dijke 2007, Xu *et al.* 2012, Travis and Sheppard 2014).

2.4. TGF-β functions in the immune system

TGF-β exerts plethora of effects on different cell types, both in physiological contexts and in diseases. We will here focus only on the role of TGF-β in the immune system. TGF-β can inhibit the proliferation, induce the apoptosis and modulate the cell differentiation of immune cells (Travis and Sheppard 2014).

TGF-β plays a crucial role in the maintenance of peripheral tolerance, as best illustrated by the severe autoimmune phenotype of *Tgfb1*$^{-/-}$ mice (Shull *et al.* 1992, Kulkarni *et al.* 1993). These mice die less than three weeks after birth of a wasting syndrome accompanied by a multifocal

inflammatory cell infiltration of tissues, leading to organ failure. TGF-β1 is thus a potent immunosuppressive molecule. However, TGF-β can be made by and act upon many immune cell types meaning that the severe phenotype observed in those mice can involve many different players. Therefore, several mouse models were developed to identify the main TGF-β producers and targets.

2.4.1. TGF-β production by T cells

Several mouse models in which the *Tgfb1* gene is deleted in T cells revealed that T cells are important but not exclusive TGF-β1 producers *in vivo*. In 2007, Li and co-workers observed that *CD4-Cre* x *Tgfb1*$^{fl/n}$ mice develop early lethal immune pathology in multiple organs associated with enhanced T cell proliferation and activation (Li *et al.* 2007). In 2011, the same group reported the phenotype of *OX40-Cre* x *Tgfb1*$^{fl/n}$ mice. In these mice, Tgfb1 production is abrogated in a subset of T cells only, namely in Tregs and activated T cells rather than in all naïve T cells like in the CD4-Cre model. This time, they observed milder signs of autoimmunity, with a less severe wasting disease and colitis beginning only at around 5 months of age. The authors concluded that TGF-β1 produced by Tregs and/or activated CD4$^+$ T cells is required to protect mice from the development of an inflammatory disorder in aging mice (Gutcher *et al.* 2011). This suggests that in this latter model other sources of TGF-β1 such as CD8$^+$ T cells or naïve CD4$^+$ T cells contribute to the maintenance of tolerance.

Finally, *Foxp3-Cre* x *Tgfb1*$^{fl/n}$ mice which carry a *Tgfb1* deletion in Tregs only, are healthy mice and show no sign of autoimmunity. They do show increased frequencies of Foxp3$^+$ Tregs in the periphery, indicating that Treg-derived TGF-β1 is required for the inhibition of Treg proliferation, but not for the maintenance of peripheral tolerance (Gutcher *et al.* 2011). In the same line, *Tgfb1*$^{-/-}$ Tregs were shown to be as protective as WT CD4$^+$CD25$^+$ Tregs against colitis in a T cell tranfer model (Fahlen *et al.* 2005). Since the suppressive activity of Tregs in this model was still inhibited by an anti-TGF-β1 antibody, it was suggested that Treg function relied on TGF-β activation even though the source of latent TGF-β was probably not the Tregs themselves (Fahlen *et al.* 2005). These results seem to indicate that TGF-β1 produced by Tregs themselves is not required for Treg suppressive function and immune homeostasis *in vivo*. Contradictory results in adoptive transfer model suggest in contrast that Treg suppression is dependent on TGF-β1 production. Mamura *et al*. transferred *Tgfb1*$^{-/-}$ splenocytes depleted of Tregs into *Rag2*$^{-/-}$ recipients. This induced autoimmune inflammatory disease in the recipient mice, which could be suppressed by co-transfer of *Tgfb1*$^{+/+}$ Tregs, but not or only partially by *Tgfb*$^{-/-}$ Tregs (Mamura *et al.* 2004). Similarly, Li *et al.* showed

that lymphopenic hosts are protected from the development of colitis induced by the transfer of naïve CD4$^+$ T cells if WT Tregs, but not *Tgfb1*$^{-/-}$ Tregs, are co-transferred (Li *et al.* 2007). Theses results demonstrated an essential role for Treg-cell-produced TGF-β1 in inhibiting colitis in adoptive transfer models, and led to the conclusion that TGF-β1 production by Tregs themselves is important for their suppressive activity. Further work will thus be needed to clarify those contradictory results.

2.4.2. T cells as targets of TGF-β in the immune system

To determine which cells are targeted by TGF-β1 *in vivo*, *Tgfb1*$^{-/-}$ mice were crossed with mice lacking MHC class II molecules and thus CD4$^+$ T cells (Letterio *et al.* 1996). These mice were substantially protected from inflammation, indicating that CD4$^+$ T cells are important mediators of the phenotype of *Tgfb1*$^{-/-}$ mice, and are probable important targets of TGF-β1 *in vivo*. To address the importance of T cells as major direct targets for TGF-β *in vivo*, Gorelik and Flavell generated mice expressing a dominant-negative (dn) TGFβRII specifically in T cells. These mice developed excessive T cell activation and age-related wasting disorder associated with multiorgan inflammation and development of autoantibodies (Gorelik and Flavell 2000). The disease was similar but not as severe as the one observed in *Tgfb1*$^{-/-}$ animals. This could be explained by incomplete inhibition of TGF-β signalling in cells expressing the dnTGFβRII. Subsequently therefore, three studies examined the effect of a conditional deletion of *Tgfbr2* or *Tgfbr1* genes in T cells using CD4-Cre mice. Complete abolition of TGF-β signalling in T cells results in a phenotype akin to the *Tgfb1*$^{-/-}$ mice (Li *et al.* 2006, Marie *et al.* 2006, Liu *et al.* 2008). These models definitely demonstrate a central role for TGF-β signalling in T cells for the maintenance of tolerance. More recently however, Zhang and Bevan questioned this interpretation based on their results obtained with *dLck-Cre* x *Tgfbr2*$^{fl/fl}$ mice. In this model, TGF-β signalling is abolished much later in developing T cells than in CD4-Cre mice. Adult *dLck-Cre* x *Tgfbr2*$^{fl/fl}$ mice showed no signs of autoimmunity, except when lymphopenia was induced (Zhang and Bevan 2012). Yet more recently, conditional deletion of *Tgfbr2* was obtained by crossing to *OX40-Cre* mice. This OX40-Cre promoter restricts deletion to activated peripheral T cells. In this case, autoimmunity was observed indicating that activated T cells are indeed kept in check by TGF-β in periphery (Ishigame *et al.* 2013).

The importance of TGF-β signalling in Tregs was also closely analysed since TGF-β signals are crucial for Treg differentiation. An absence of Tregs in *Tgfb1*$^{-/-}$ mice could explain the

autoimmunity phenotype. Analysis of thymic Foxp3$^+$ population in *Tgfb1*$^{-/-}$, in *CD4-dnTgfbr2* and *CD4-Cre* x *Tgfbr2*$^{fl/fl}$ mice revealed that this subset of CD4$^+$T cells had normal (or increased) frequencies (Fahlen *et al.* 2005, Li *et al.* 2006, Marie *et al.* 2006). However, in spleens and lymph nodes, Tregs were found to be markedly reduced. Theses results suggested that, while TGF-β signalling is not required for Foxp3$^+$ Treg development in the thymus, it is on the contrary essential for the maintenance of this subset in the periphery (Li *et al.* 2006, Marie *et al.* 2006). However, subsequent studies have questioned these observations, and found out that TGF-β was supporting the early stages of Foxp3$^+$ Tregs thymic development, notably by antagonising thymic negative selection (Liu *et al.* 2008, Ouyang *et al.* 2010).

2.4.3. Other roles of TGF-β in T cell differentiation and function

In addition to its role in maintaining tolerance by restricting T cell activation, TGF-β was shown to directly impair Th1 and Th2 differentiation by suppressing the lineage-defining transcription factors T-bet and GATA-3, respectively (Li *et al.* 2006, Marie *et al.* 2006). However, the molecular mechanism behind this effect is still unclear.

In vitro studies revealed that TGF-β diminished the CD8$^+$T cell cytolytic activity by decreasing the production of cytolysis effector molecules (Thomas and Massague 2005). Since activated Cytotoxic T Lymphocytes (CTLs) are predominantly responsible for antigen-specific clearance of tumor cells, this role of TGF-β is of particular interest in the context of our research. The inhibitory effects of TGF-β on CTLs was also shown *in vivo* by studies revealing that dnTGFβRII expression in CD8$^+$T cells or treatment with a soluble TGFβRII potentiate immune responses against transplantable tumors (Gorelik and Flavell 2001, Thomas and Massague 2005). Recently, the group of M. Li showed, that TGF-β negatively regulates CD8$^+$ T cell responses by using a TRansgenic Adenocarcinoma of Mouse Prostate (TRAMP) mice model (Donkor *et al.* 2011). Despite profound T cell infiltration into prostate tumours that develop in those mice, T cells cannot control tumour development. However, TRAMP mice with dnTGFβRII expressing CD8$^+$ T cells exhibit tumor protection, confirming that TGF-β signals in CD8$^+$ T cells inhibit their function (Donkor *et al.* 2011, Oh and Li 2013). To determine the importance of Treg-derived TGF-β in the inhibition of CD8$^+$ T cell activity, the same group crossed TRAMP with *Foxp3-Cre* x *Tgfb1*$^{fl/n}$ mice. Those mice develop tumours similarly as the TRAMP mice, suggesting that Tregs are not the main source of TGF-β1 involved in CD8$^+$ T cell inhibition in these tumours (Donkor *et al.* 2011). As stated above, one can not exclude that tumour infiltrating-Tregs activate latent TGF-β produced by other cell types (T

cells and tumor cells) to exert suppressive function on infiltrating $CD8^+$ T cells. The authors additionally showed by crossing *CD4-Cre* x *Tgfb1*$^{fl/n}$ with TRAMP mice, that T cell-derived TGF-β1 is crucial for anti-tumour response inhibition since tumour development is inhibited in those mice.

When acting in combination with pro-inflammatory cytokines like IL-6, IL-1β, and IL-21, TGF-β can also induce Th17 differentiation *in vitro*. Th17 cells are pro-inflammatory T cells characterised by the production of IL-17 and the expression of the transcription factor RORγT. *In vivo*, mice lacking TGFβRII on T cells have reduced numbers of Th17 cells. These findings highlight a fundamental role for TGF-β in Th17 differentiation and thus in enhancing pro-inflammatory T cell responses (Mangan *et al.* 2006, Veldhoen *et al.* 2006, Travis and Sheppard 2014).

Finally, *in vitro* stimulation of murine peripheral $CD4^+CD25^-$ naive T cells in the presence of TGF-β1 converts T cells into $CD4^+CD25^+Foxp3^+$ T cells. Those cells are called induced Tregs (iTregs) as opposed to thymic derived Tregs (tTregs). In mice, iTregs were shown to display immunosuppressive functions *in vivo* and *in vitro*. Distinction between iTregs and tTregs is however not clear in humans (Luo and Li 2013).

As stated earlier, work by our group showed that human Tregs, but not other T cell types, produce active form of TGF-β. We also showed that human Tregs suppress the proliferation of the T cells *in vitro* through this production of active TGF-β. Although the role played by Treg-derived TGF-β in suppression by murine Tregs is not clear yet, TGF-β production may be important for suppression by human Tregs. The mechanisms by which human Tregs activate latent TGF-β, and as for that of murine Tregs were not previously known. The following chapter describes how transmembrane protein GARP was shown to play a role in this process.

3. GARP, a transmembrane protein expressed by activated human Tregs

3.1. Structure of GARP (or LRRC32)

Glycoprotein A Repetition Predominant (GARP), also called Leucine Rich Repeat Containing Protein 32 (LRRC32), is a type I transmembrane protein of 662 amino acids. Its cytoplasmic portion comprises only 15 amino acids. Its large extracellular portion contains 20 leucine rich repeats (LRR), which are structural motifs of 20-30 amino acids with a characteristic repetitive sequence rich in leucines (Chan *et al.* 2011).

Proteins containing in their primary structure tandems of two or more LRR motifs form a superfamily of proteins found in all life forms, from viruses to eukaryotes (Kajava 1998, Bella *et al.* 2008). This family includes intracellular, extracellular and transmembrane proteins that contribute to various biological processes such as cell adhesion and signalling, platelet aggregation, neuronal development and immune responses (Bell *et al.* 2003, Bella *et al.* 2008). Although their functions are diverse, LRR-containing proteins share structural similarities. The first crystal structure of an LRR protein was published in 1993 by Kobe and Deisenhofer using porcine ribonuclease inhibitor. They observed that the arrangements of α-helices and β-strands in each individual LRR resulted in a horseshoe-like tridimensional structure (Kobe and Deisenhofer 1993, Kobe and Deisenhofer 1995). The structures of all LRR proteins studied since then revealed that proteins with LRR architecture form curved solenoid structures (Kobe and Kajava 2001, Bella *et al.* 2008). The major function of this horseshoe architecture may be to provide frameworks for stable protein-protein interactions but only few direct structural information are available (Kobe and Kajava 2001). Toll-like Receptors (TLRs) are an example of LRR-containing protein in the innate immune system. Extracellular domains of TLRs bind a variety of Pathogen-Associated Molecular Patterns (PAMPs) from bacteria, viruses and fungi. Although the structure of TLR extracellular domains have not been solved, their structure could be predicted according to the similarities between their amino acid sequences with those of known LRR structures. TLRs were suggested to have horseshoe-shaped structures providing large binding surfaces to allow PAMP recognition (Bell *et al.* 2003).

Wang and colleagues studied the structure of soluble GARP by electron microscopy analysis. They observed that GARP displays as expected a horseshoe shape (Wang *et al.* 2012).

3.2. GARP expression pattern and regulation

As assessed by Northern Blot, *GARP* mRNA is found in many human tissues including placenta, lung, kidney, and to a lesser extent heart, liver, skeletal muscle and pancreas (Ollendorff *et al.* 1994). Wang *et al.* were the first to show preferential *GARP* expression in Tregs. Our group confirmed this in Treg clones, which express 100-fold more *GARP* mRNA than Th clones. *GARP* levels increase rapidly upon TCR stimulation in Treg clones, and return to resting levels after 24 hours (Stockis *et al.* 2009b). Despite high mRNA levels in resting Tregs, GARP protein was only detected in activated Tregs and was completely absent in stimulated or resting Th cells (Stockis *et al.* 2009b). Emilie Gauthy in our lab showed that GARP expression is regulated post-transcriptionally by miRNAs in T cells (Gauthy *et al.* 2013). Zhou and co-workers also reported that microRNA *miR143-3p* targets *GARP* mRNA in $CD4^+CD25^+$ human T cells (Zhou *et al.* 2013).

GARP protein is detected on the surface of human Tregs 24 to 72 hours after TCR stimulation (Wang *et al.* 2008, Probst-Kepper *et al.* 2009, Tran *et al.* 2009, Stockis *et al.* 2009b). It was therefore proposed that GARP could play a role in Treg function.

3.3. Functions of GARP in Tregs

Soon after its identification as a Treg-specific surface protein, GARP was shown to serve as a presenting receptor for latent TGF-β1 at the cell surface (Tran *et al.* 2009, Stockis *et al.* 2009b). Tregs, but not other T cells, were known to bind latent TGF-β on their surface upon TCR stimulation. Our group, and others, reported that ectopic expression of GARP in non-Treg T cells conferred the ability to present latent TGF-β at the cell surface (Tran *et al.* 2009, Stockis *et al.* 2009b). Conversely, silencing of *GARP* in human Tregs reduced surface latent TGF-β1 at their surface (Tran *et al.* 2009). Interactions between GARP and latent TGF-β1 were shown by co-immunoprecipitation in Treg cells, and confirmed in pull-down experiments with recombinant soluble GARP and latent TGF-β1 (Stockis *et al.* 2009b). The structural view of this complex obtained by electron microscopy analysis of soluble GARP and pro-TGF-β1 revealed that GARP and TGF-β1 are closely associated (Wang *et al.* 2012).

Recently, T. Springer's group and our group showed that GARP is covalently bound to latent TGF-β1 through disulphide linkage (Wang *et al.* 2012, Gauthy *et al.* 2013). Disulphide bounds involve

Cys33 in the LAP monomers and Cys211 and Cys350 in the GARP molecule. GARP therefore binds the same Cys33 as LTBPs. It is of note that Tregs do not appear to express LTBPs (our unpublished data). But in transfected cells, GARP outcompetes LTBP for latent TGF-β1 binding (Wang *et al.* 2012). It was suggested that GARP could function as an anchor for latent TGF-β at the surface of Tregs, in a manner similar to LTBPs anchoring latent TGF-β in the extracellular matrix (Wang *et al.* 2012).

Using human Th cells transduced with GARP, we also showed that GARP favors the cleavage of pro-TGF-β1 by FURIN (Gauthy *et al.* 2013). No increase in FURIN expression or activity was detected upon transduction of GARP, and no interaction between GARP and FURIN could be evidenced. The mechanism by which GARP increases latent TGF-β production by increasing cleavage of its precursor remains therefore unknown. As a consequence of increased latent TGF-β production in the presence of GARP, we also observed increased secretion of the latent cytokine in GARP-transduced T cells (Gauthy *et al.* 2013). Interestingly, we found that latent TGF-β that is secreted by GARP-expressing T cells is secreted as complexes in which it is still disulphide linked to GARP. Secretion of GARP/latent TGF-β complexes most likely occurs through shedding of surface GARP/TGF-β1 complex. Release of GARP/latent TGF-β1 complexes was also observed in stimulated human Tregs which naturally express GARP. The majority of latent TGF-β secreted by Tregs is associated to GARP. Secretion of GARP/latent TGF-β complexes however, seems to be restricted to T lymphocytes: 293T cells transiently transfected with GARP and TGF-β1 do not release complexes in their supernatants although they do present GARP and latent TGF-β1 at their surface (Gauthy *et al.* 2013). We still don't know which protease, if any, mediates this T cell-specific release of soluble GARP/TGF-β1 complexes.

Based on the above, it appears that GARP controls processing and secretion of latent TGF-β by human Tregs. But does it contribute in any way to the regulation of the immune suppressive function of these cells? This question was addressed by several groups. Wang *et al.* and Tran *et al.* showed that silencing GARP with siRNAs in human Tregs led to a moderate but significant decrease of their suppressive activity *in vitro* (Tran *et al.* 2009, Wang *et al.* 2009). These data are in line with the hypothesis that GARP, through presentation of latent TGF-β at the surface of Tregs may contribute to Treg suppression. In contradiction with these findings however is the data provided by the group of E. Shevach using Tregs from *CD4-Cre x Garp*$^{fl/fl}$ mice (Edwards *et al.* 2013). These mice develop normally to adulthood, without any signs of autoimmunity. This

indicates that Garp expression in mouse Tregs *in vivo* is not required for immune homeostasis and the maintenance of tolerance. In addition, Garp-deficient Tregs were as competent as WT Tregs in suppressing the proliferation of target cells *in vitro* (Edwards *et al.* 2013). Therefore, whether or not Garp contributes to the immune suppressive activity of murine Tregs remains an open question.

Our group addressed the question of the role of GARP in suppression by human Tregs. We know that suppression by human Treg clones is cell-cell contact dependent, and that it is also dependent on the production of active TGF-β by the Treg cells (Stockis *et al.* 2009a). Thus, we hypothesise that GARP, through presentation of latent TGF-β at the Treg surface, may be required for the activation of the cytokine by Tregs, and hence required for the immune suppressive function of these cells. To test this hypothesis, we generated a series of monoclonal antibodies (mAbs) against human GARP (Cuende *et al.* manuscript submitted). Two of 31 anti-GARP mAbs were able to block the production of active TGF-β1 by human Tregs. Interestingly, we could show that the two blocking anti-GARP mAbs recognise an epitope that requires GARP amino acids 137-139 in GARP/latent TGF-β complexes. All other non-blocking anti-GARP mAbs recognise other epitopes. In addition to blocking active TGF-β production by human Tregs, the two blocking anti-GARP mAbs also inhibit partially the ability of Tregs to suppress the proliferation of target cells *in vitro*. Finally, we tested the blocking mAbs *in vivo*. Because our anti-human GARP mAbs do not cross-react against mouse Garp, we had to resort to a model of xenogeneic graft-versus-host disease (xGVHD) in (*NOD/Scid/Il2rg*$^{-/-}$) NSG mice. NSG mice are profoundly immuno-incompetent. Transfer of human PBMCs in NSG mice leads to efficient engraftment and proliferation of human T cells. A few weeks after transfer, severe xGVHD develops, due to the activity of human T cells against murine tissues. xGVHD can be delayed if autologous Tregs are co-transferred with the PBMCs. Tregs suppress xenogeneic T cell activity in this model. This provided us with the opportunity to test the ability of blocking anti-human GARP to inhibit human Treg function *in vivo* (Cuende *et al.* manuscript submitted). We could clearly demonstrate that blocking anti-GARP mAbs aggravated xGVHD in mice grafted with PBMCs and Tregs. Importantly, non-blocking anti-GARP mAbs had no effect. These results show that GARP is required for active TGF-β production by human Tregs and contributes to the immune suppressive function of these cells *in vivo*.

To summarise the above, we propose the following roles of GARP in the production of TGF-β by human Tregs (Figure 5). After the removal of the signal peptide in the ER, newly synthesised TGF-β1 monomers associate to form the pro-TGF-β1 dimer. Pro-TGF-β1 associates to GARP through

the formation of disulphide bounds between LAP and GARP. Co-immunoprecipitation experiments indicate indeed that pro-TGF-β is already bond to GARP in Tregs. This association favours cleavage of pro-TGF-β1 by Furin, leading to the formation of latent TGF-β1, which remains associated to GARP. GARP/latent TGF-β1 complexes translocate to the Treg surface. There, GARP/latent TGF-β1 complexes can be shed from the membrane through an unknown mechanism and secreted in the Treg supernatant. The function of soluble GARP/latent TGF-β1 complexes secreted by Tregs is not known. In response to TCR stimulation, latent TGF-β1 bound to GARP at the Treg surface can be activated. Active TGF-β1 production by Tregs acts in both autocrine manner on Tregs themselves, but also in a paracrine manner on neighbouring T cells. The paracrine activity of active TGF-β1 produced by human Tregs contributes to their immune suppressive function. This can be blocked with anti-GARP mAbs (Cuende *et al.* manuscript submitted).

It must be noted that GARP is not required for pro-TGF-β1 cleavage and latent TGF-β1 secretion. This occurs also in cells that do not express GARP. Forced expression of GARP in non-Treg cells (293T cells or Th lymphocytes) increases pro-TGF-β1 cleavage and is sufficient to induce the presentation of GARP/latent TGF-β1 complexes on the cell surface. Interestingly, secretion of soluble GARP/latent TGF-β1 complexes occurs only when GARP is in T cells, either in Tregs or in Th cells transduced with GARP. 293T cells transfected with GARP do not secrete GARP/latent TGF-β1 complexes. This appears to represent a T-cell specific mechanism, although we do not know the function of these secreted complexes. But more importantly, forced expression of GARP in non-Treg cells is not sufficient to induce active TGF-β1 production. This is true in 293T cells, but also in Th cells.

We hypothesised therefore that production of active TGF-β1 in Tregs, requires not only GARP, but also other yet unknown protein partners expressed in Tregs but not in Th cells. The aim of my thesis work was to identify proteins associated with GARP that contribute to the activation of latent TGF-β1 in human Tregs. To achieve our objectives, we used two different approaches to identify Treg proteins interacting with GARP. In the next chapter, I will present an overview of the multiple techniques available to study protein-protein interactions.

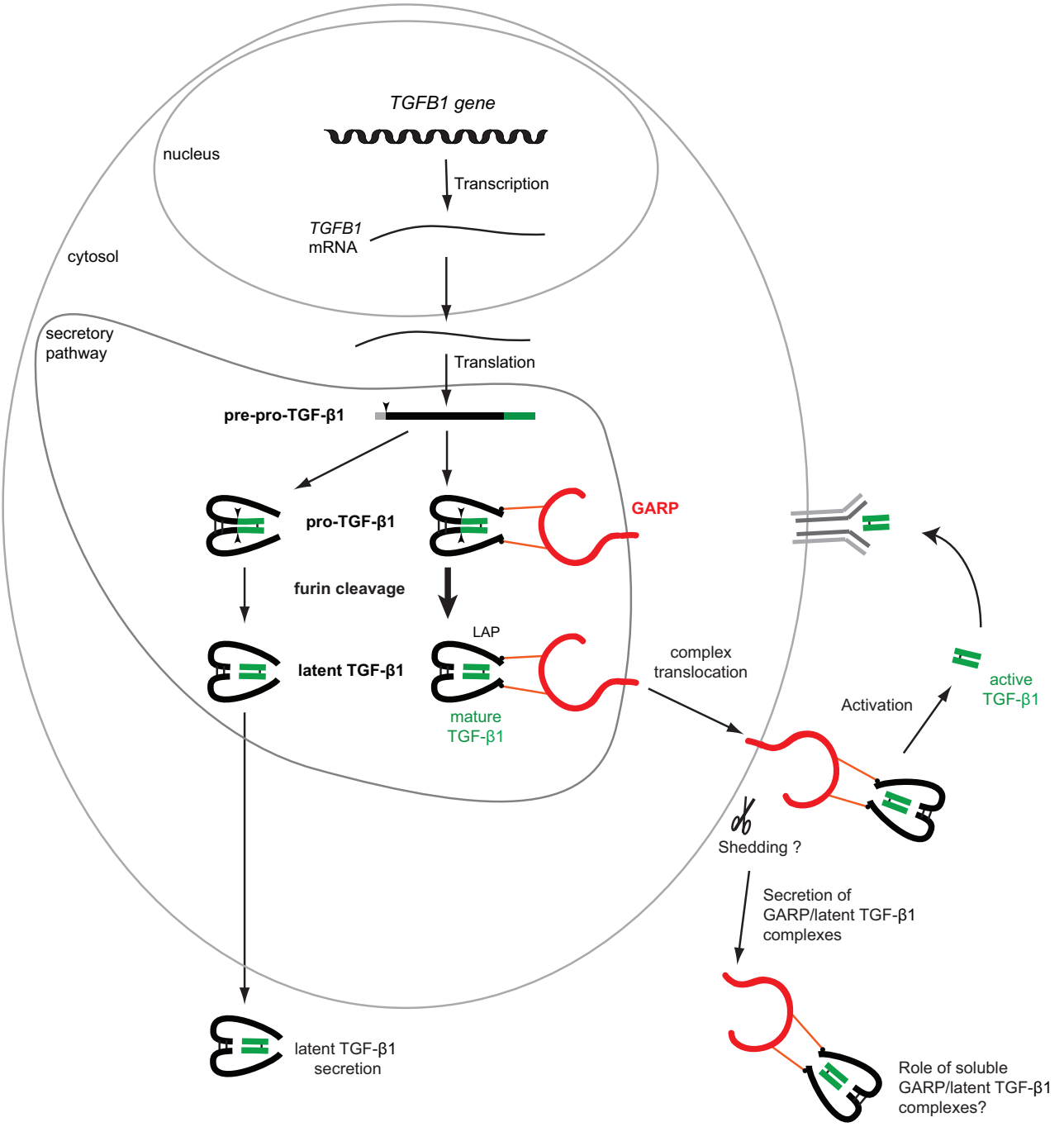

Figure 5. Schematic representation of TGF-β1 production in Tregs.
TGF-β1 is synthesised as a pre-pro-protein. In the ER, the signal peptide is cleaved and two molecules associate to form the pro-TGF-β1 dimer. A minority of pro-TGF-β1 is cleaved by furin and secreted as latent TGF-β1 free of GARP, while majority of pro-TGF-β1 disulphide links GARP in the ER. This association favours cleavage of pro-TGF-β1 by Furin leading to the formation of latent TGF-β1, which remains associated to GARP. GARP/latent TGF-β1 complexes translocate to the surface of Tregs where they are shedded (unknown mechanism). Upon TCR stimulation, latent TGF-β1 can be activated. Active TGF-β1 released from the LAP can act on the Treg itself (autocrine signalling) or on neighbouring cells (paracrine signalling).

4. How to study protein-protein interactions?

Protein-protein interactions (PPIs) are crucial for cellular function and dysfunction and large efforts are therefore invested in their identification and analysis. Over the past 25 years, many techniques to study PPI were developed that can be applied to a large variety of proteins with different structures and functions (Auerbach *et al.* 2002, Suter *et al.* 2006, Suter *et al.* 2008). A given PPI is characterised by the type of bonds occurring between the protein partners, and thus by its stability. Strong interactions occurring between proteins associated through a covalent bond are called «stable» PPIs. However, «stable» interactions represent a minority of PPIs. Examples of stable interaction include the association between two pro-TGF-β molecules through disulphide links, or the association of GARP with LAP. The majority of PPIs are transient interactions between partners that are not covalently associated. These interactions are called «labile» PPIs (Westermarck *et al.* 2013, Ngounou Wetie *et al.* 2014). Dimerisation between phosphorylated SMAD2 and SMAD3 and complex formation with SMAD4 in response to TGF-β signals are examples of «labile» interactions. Notably, some PPIs result from a combination of stable and labile interactions. This is the case for example GARP and latent TGF-β complexes in which GARP and LAP are linked through disulphide bonds and in which mature TGF-β dimers are non-covalently associated to LAP dimers (Wang *et al.* 2012, Gauthy *et al.* 2013). According to the stability of the interactions, some techniques are more appropriate than others.

Because of the wide variety of systems that have been developed in the past decades, it is difficult to present an exhaustive list of the techniques. It is important to note that a large field of research on PPI is based on biophysical techniques (X-Ray crystallography or Nuclear Magnetic Resonance), and «in-silico» methods. These techniques will not be described here but are reviewed in Wetie *et al.* and Rao *et al.* (Ngounou Wetie *et al.* 2014, Rao *et al.* 2014). Different classifications of the PPI techniques have been used in the literature. I will distinguish «in-cell» methods, which detect interactions in an intact cell system (yeasts or mammalian cells), from biochemical methods, which detect interactions in cell-free systems or after cell lysis.

4.1 PPI detection by «in-cell» methods

4.1.1 Yeast Two-Hybrid systems

(i) Classical Y2H. The Yeast Two-Hybrid (Y2H) system was originally described by Fields and Song in 1989 (Fields and Song 1989). They developed a genetic system to detect PPIs in a cellular setting taking advantage of the yeast transcriptional machinery. Their approach was based on the finding that the GAL4 transcription factor (TF) can be separated into two domains: the DNA Binding Domain (DBD) and the Activation Domain (AD). These two fragments do not associate spontaneously and are inactive when expressed individually in the absence of other in yeast. To test an interaction between two proteins, the first protein, called the bait, is fused to the DBD, and the second protein, called the prey, is fused to the AD. When those two hybrid proteins are expressed in yeast, DBD and AD are brought closely to form an active TF only if bait and prey interact with each other (Figure 6a). To measure transcriptional activity, reporter genes under the control of an Upstream Activating Sequence (UAS) are inserted in the yeast genome. These reporter genes, are transcribed only if the prey interacts with the bait that is bound to the UAS through the DBD. Originally, Fields and Song used a lacZ reporter gene allowing to use β-galactosidase activity as a sensor of the interaction between two proteins tested. Now, auxotrophic genes (HIS3, ADE2...) are used as reporters. They allow yeasts in which an interaction between bait and prey occurs to grow on selective media.

The Y2H system is suitable to test the existence of interactions between two known proteins, but it can also also serve to screen cDNA libraries to identify new partners of a bait of interest. Yeasts in which an interaction occurs grow on minimal media and the preys expressed in those yeasts can be identified by DNA sequencing. Y2H has been widely used during the last decades. For example, many studies used the Y2H system to identify proteins interacting with the SMADs. The identification of SMAD partners with this method allowed for a better understanding of the TGF-β signalling pathway and its regulation in various cell types (Liberati *et al.* 1999, Liberati *et al.* 2001, Komuro *et al.* 2004, Long *et al.* 2004).

The Y2H technique is a powerful tool for PPI study. It has the advantages to be cheap, rapid and easy to implement. Moreover, it is very sensitive and suited to test labile interactions. However, this method faces several limitations. One first obvious limitation is that baits, fused to DBD, must

not activate transcription on their own. This would indeed lead to activation of the reporter genes and thus make the selection of yeasts impossible. Some «tricks» have been set-up to avoid excessive auto-activation of reporter genes (Auerbach *et al.* 2002). When using *HIS3* reporter gene, growth medium can be supplemented with 3-aminotriazole, a competitive inhibitor of the *HIS3* gene product. This has been shown to lower background due to the self-activation of the bait-DBD fusion. Another trick is to clone the bait-DBD coding sequence downstream of a weaker promoter to decrease the number of copies of the fusion protein. A second major limitation is that bait and prey fusion proteins need to be present, and correctly folded in nucleus of yeasts to induce

transcriptional activity. This means that the classical Y2H method is not applicable to the analysis of proteins with hydrophobic sequences, typically found in transmembrane proteins for example. To circumvent this problem, membrane proteins can be expressed as truncated forms, to facilitate targeting to the nucleus. A third problem is that some interactions between two partner proteins require the presence of additional partner(s). When studying non-yeast proteins, this can be problematic as the fusion proteins are not expressed in the presence of other proteins that may be essential for the interaction to occur. Although some tricks exist to circumvent these limitations, these are not always successful. Therefore, alternative approaches have been developed (Table 1).

(ii) RNA polymerase III based system. Transcription of most of the genes coding for proteins is ensured by the RNA polymerase II enzyme (pol II). Only a few genes are transcribed with RNA polymerase III (pol III). Among these is gene *SNR6*. This gene codes for an essential small nuclear RNA (snRNA) and its transcription is induced by the binding of Transcription Factor III C (TFIIIC) on an upstream DNA sequence called «B-Block». Marsolier and colleagues showed that when B-Block is replaced by a GAL4 UAS, a truncated version of TFIIIC (t-138) associated to GAL4 DBD can recruit pol III and induce *SNR6* transcription (Marsolier *et al.* 1997). This finding led to the development of a two-hybrid system in which the bait is fused to t138 protein fragment and the prey is fused to GAL4 DBD. Interaction between bait and prey, induces pol III recruitment to the snr6 reporter, transcription and production of the snRNA and yeast survival (Figure 6b). This system has the advantage to detect interactions with reporter genes that are synthesised using pol III, therefore allowing to use transcription factors as baits, as long as these function by recruiting pol II. It was used in 2001 to screen for new BRCA1 partners in a cDNA library from mouse embryonic cDNA. The authors took advantage of a yeast reporter strain bearing a mutation in the endogenous *SNR6*

gene, making yeasts sensitive to temperature (ts snRNA yeasts). At 37°C, only yeasts transcribing the reporter WT *SNR6* gene are able to grow (Petrascheck *et al.* 2001).

(iii) Yeast three-hybrid. To test PPI in which more than two protein partners are involved, a system referred to as the Yeast Three Hybrid system (Y3H) was developed (Figure 6c). This system was first designed to study protein-RNA interactions (Sengupta *et al.* 1999, Zhang *et al.* 1999). However it was extended to the study of additional protein partners and to small molecules. In the case of additional proteins mediating an interaction between bait and prey, yeasts are transformed with an additional vector coding for a third «non-fusion» protein (Drees 1999). Of course, the identity of the «intermediate» partner must be known *a priori* in this system.

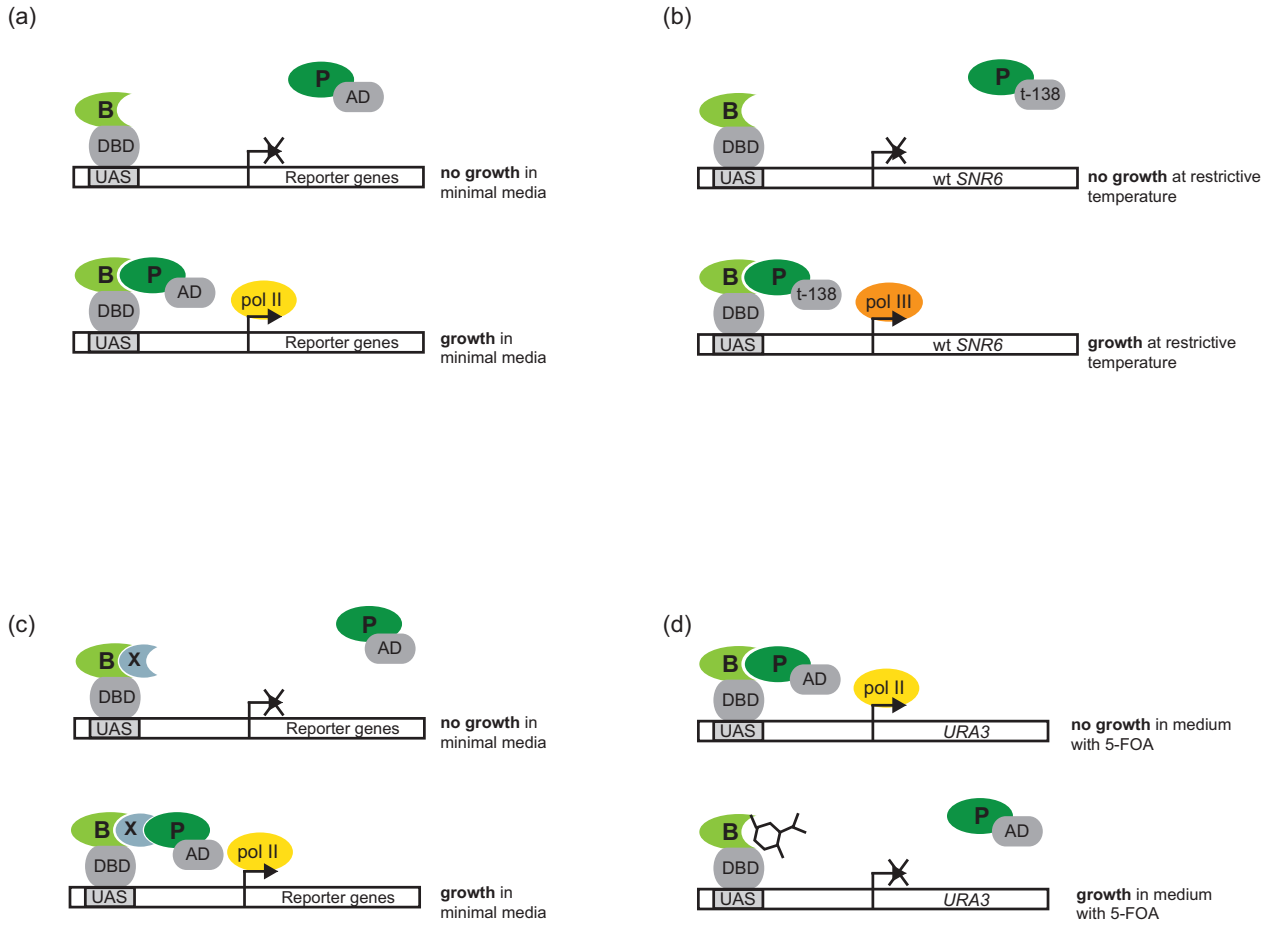

Figure 6. Yeast two-hybrid systems detecting PPI in the nuclei of yeasts.

(a) Classical yeast two hybrid (Y2H). The bait (B) is fused to the DNA Binding Domain (DBD) of GAL4 while the prey (P) is fused to the activation domain (AD) of GAL4. Interaction between Bait and Prey allows polymerase II (pol II) recruitment and gene transcription resulting in yeast growth on minimal media. **(b)** RNA-pol III based system. The bait is fused to GAL4 DBD and prey is fused to t-138, a portion of TFIIIC allowing the recruitment of pol III. Yeasts in which SNR6 is produced survive at 36°C. **(c)** Yeast three-hybrid system (Y3H). Adaptation of the Y2H system to allow interaction between bait and prey mediated by a third partner (X). **(d)** Reverse two-hybrid. Application of the Y2H to identify molecules disrupting interactions. Yeast in which interaction is prevented grow on a medium supplemented with 5-FOA.

Table 1. Yeast and mammalian two-hybrid systems.

	Technique	Short name	Interaction Reporter	Suitable bait(s)	Reference
YEASTS	**classical two-hybrid**	**Y2H**	reporter gene transcription, growth in minimal media	non-transcriptional activators, proteins able to enter nucleus	Fields and Song 1989
	RNA Polymerase III-based system	**pol III system**	growth at restrictive temperature	transcriptional activators recruiting pol II, proteins able to enter nucleus	Marsolier *et al.* 1997, Petrascheck *et al.* 2001
	SOS/Ras recruitment system	**SRS-RRS**	growth at restrictive temperature	transcriptional activators, cytosolic proteins, membrane protein (reverse)	Aronheim *et al.* 1997, Broder *et al.* 1998, Hubsman *et al.* 2001
	Split-Ubitquitin system - rUra3	**-**	Ura3/5-FOA system	nuclear, cytosolic and membrane proteins	Johnsson and Varshavsky 1994, Dünnwald *et al.* 1999
	Split-Ubitquitin system - transcription	**MYTH**	reporter gene transcription, growth in minimal media	membrane proteins	Johnsson and Varshavsky 1994, Stagljar *et al.* 1998
	Cytosolic Y2H	**cyto-Y2H**	reporter gene transcription, growth in minimal media	transcriptional activators, cytosolic proteins	Mockli *et al.* 2007
	Yeast three-hybrid	**Y3H**	reporter gene transcription, growth in minimal media	non-transcriptional activators, proteins able to enter nucleus	SenGupta *et al.* 1996
	Reverse Yeast two-hybrid	**Reverse Y2H**	Ura3/5-FOA system	non-transcriptional activators, proteins able to enter nucleus	Vidal *et al.* 1994
	Screening for interactions between extracellular proteins	**SCINEX-P**	reporter gene transcription, growth in minimal media	Extracellular and membrane proteins	Urech *et al.* 2003
MAMMALIAN	**two-hybrid "*sensu-stricto*"**	**M2H**	reporter gene transcription (luciferase)	non-transcriptional activators, proteins able to enter nucleus	Luo *et al.* 1997
	Protein-fragment Complementation Assays	**PCA**	protein activity reconstitution (enzyme or fluorescence)	nuclear, cytosolic and membrane proteins	Rossi 1997, Galarneau 2002; Wehrman 2002, Pelletier 1998; Remy 1999, Remy 2006, Paulmurugan 2002, 2003; Stefan 2007, Kerpolla 2008 Wilson 2004
	Mammalian Protein-Protein Interaction Trap	**MAPPIT**	reporter gene transcription (luciferase)	cytosolic baits	Eyckerman *et al.* 2001
	Fluorescence/Bioluminescence Resonance Energy Transfer systems	**FRET-BRET**	fluorescent signal	nuclear, cytosolic and membrane proteins	Boute *et al.* 2002, Piston and Kremers 2007

(iv) Reverse two-hybrid system. This is an application of the Y2H system to identify molecules that interrupt PPI. The group of Marc Vidal used the *URA3* reporter to allow negative selection (Vidal *et al.* 1996). In yeasts in which bait and prey interacts, the *URA3* gene is transcribed. In the presence of 5-FOA (5-Fluoroorotic acid), the URA3 enzyme modifies 5-FOA into a toxic product and yeast die. However, when the bait/prey interaction is disrupted by a protein or another molecule, the *URA3* reporter is not transcribed and yeast grow as 5-FOA remains in its non-toxic form (Figure 6d). This technique allows to screen for small molecules, supplemented in the yeast growth medium, or for proteins introduced by transformation that disrupt interaction. This application is particularly interesting for the identification of new drugs targeting PPIs.

(v) The SOS and Ras Recruitment Systems. The SOS Recruitment System (SRS) and the Ras Recruiment System (RRS) were developed in the late 90s by Aronheim and colleagues (Aronheim *et al.* 1997, Broder *et al.* 1998). In the Ras pathway, binding of growth factors induces receptors dimerisation, trans-phosphorylation on tyrosine residues and recruitment via adaptors, of a Guanyl Exchanging Factor (GEF) that activates membrane-anchored Ras by exchanging GDP by GTP. Activated Ras triggers a signalling cascade, which results in cell growth. In yeast, Ras is activated by a GEF called cdc25. The SRS and RRS exploits a yeast strain harbouring a temperature sensitive (ts) *cdc25-2* allele; the *cdc25-2* yeast strain is able to grow at 25°C but not at 36°C. The authors found that the human GEF called SOS (hSOS) can rescue yeast harbouring a ts cdc25, allowing them to grow at 36°C. To exert its function, hSOS needs to be located at the plasma membrane. This system allows to test for interactions between a cytosolic bait fused to hSOS, and a membrane-anchored prey. If the bait interacts with the prey, hSOS is recruited at the cell membrane and yeasts grow at 36°C (Figure 7a).

With this system, interaction occurs at the cell membrane and targeting to the nucleus is avoided. Moreover, the sensor of PPI is not the transcription of reporter genes but the activation of signalling pathway in the cytosol. Thus, SRS is suited to test interactions with baits that are transcriptional activators. Originally, the SRS system was used for the identification of interactions in the AP-1 family of transcription factors (Aronheim *et al.* 1997). Apart from this advantage, the technique has the limitation that baits must be cytosolic proteins: with membrane baits, hSOS will be located at the membrane in the absence of interaction with a prey. In addition, when prey cDNA libraries are screened, mammalian Ras (mRas) or GEF represent very frequent false positives.

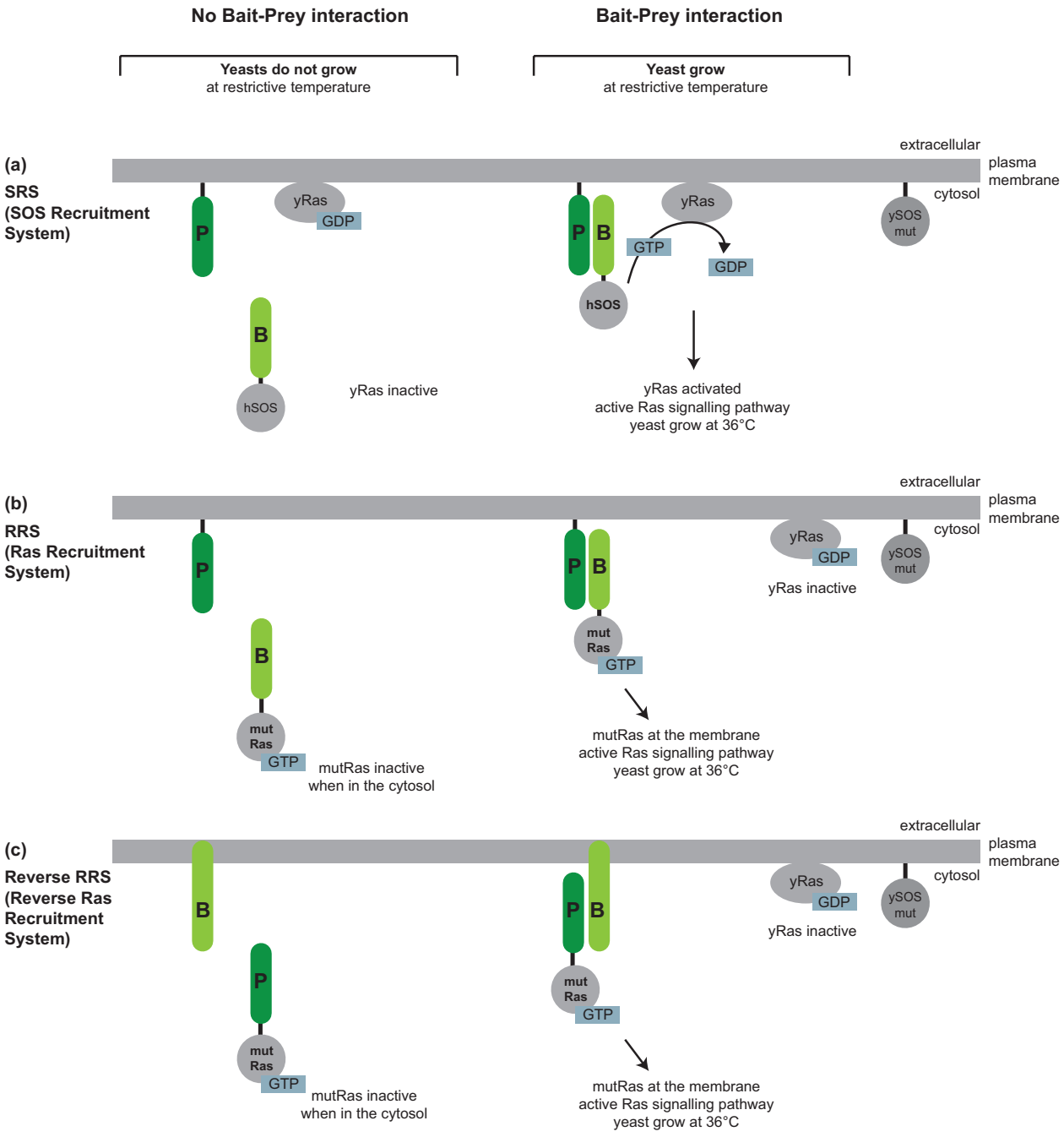

Figure 7. Ras pathway-based yeast two-hybrid systems.

These systems use a cdc25-2 mutant yeast unable to activate the Ras pathway at 36°C. **(a)** SOS-Recruitment System (SRS). The bait (B) fused to hSOS is recruited to the cell membrane through its interaction with the membrane anchored prey (P). At the cell membrane it exchanges GDP to GTP on yeast Ras (yRas) and activates yeast growth at 36°C **(b)** Ras Recruitment system (RRS). Constitutively active mutant mutRas, bypassing the need to activate yRas, is recruited at the cell membrane through interaction with anchored preys. Yeast are allowed to grow at 36°C. **(c)** Reverse Ras Recruitment system (reverse RRS). The prey is fused to mutRas and recruited at the cell membrane through interaction with the integral membrane bait. Yeast are allowed to grow at 36°C.

To improve their system, the inventors suggested to express a mammalian GTPase-activating protein (mGAP) that removes GTP from mRas but not from yeast Ras (Aronheim 1997). This allowed to decrease the number of false positives identified by SRS but not significantly enough in many instances.

The authors then designed an alternative technique called the RRS (Broder *et al.* 1998). In this system, the bait is fused to a mutated Ras (mutRas) that is constitutively active and prevented to anchor at the cell membrane. This «soluble mutRas» substitutes yRas (yeast Ras), but requires membrane localisation to function (Figure 7b). As for the SRS, yeast in which bait and prey are expressed grow only if mutRas is recruited at the cellular membrane through its interaction with a membrane-anchored prey.

To be suited for the study of interaction with a bait that is an integral membrane protein, the group of Aronheim proposed a «reverse RRS». In this reverse system, the bait is a membrane protein and the preys are fused to the mutRas (Hubsman *et al.* 2001, Kohler and Muller 2003). This is thus suited for the use of membrane baits but means that preys have to interact with the cytosolic portion of the membrane bait to be detected (Figure 7c).

(vi) Split-Ubiquitin System. This technique was first described by Johnsson and Varshavsky in 1994 (Johnsson and Varshavsky 1994). The ubiquitins are small proteins that are attached to other proteins by ubiquitin ligases. They target proteins to which they bind for degradation by the proteasome. During degradation, ubiquitins are detached from proteins to which they were transferred by Ubiquitin Specific Proteases (UBPs) and recycled. Johnsson and Varshavsky found that ubiquitins can be split into two fragments, the C-terminal part (CUB) and the N-terminal part (NUB), and that these fragments alone are not recognised by UBPs (Johnsson and Varshavsky 1994). Spontaneous reassembly of CUB and NUB can occur if the two fragments are expressed together, but a mutation in NUB prevents this spontaneous reassembly. Mutated NUB is called NUBG. The authors then developed a two-hybrid system in which the bait is fused to CUB and the prey is fused to NUBG. If bait and prey interact when expressed in yeast, CUB and NUBG are brought in close proximity, and reform a native-like ubiquitin that is recognised by yeast UBPs. To report this interaction in yeast, different systems have been designed and include the rUra3 and the transactivation systems.

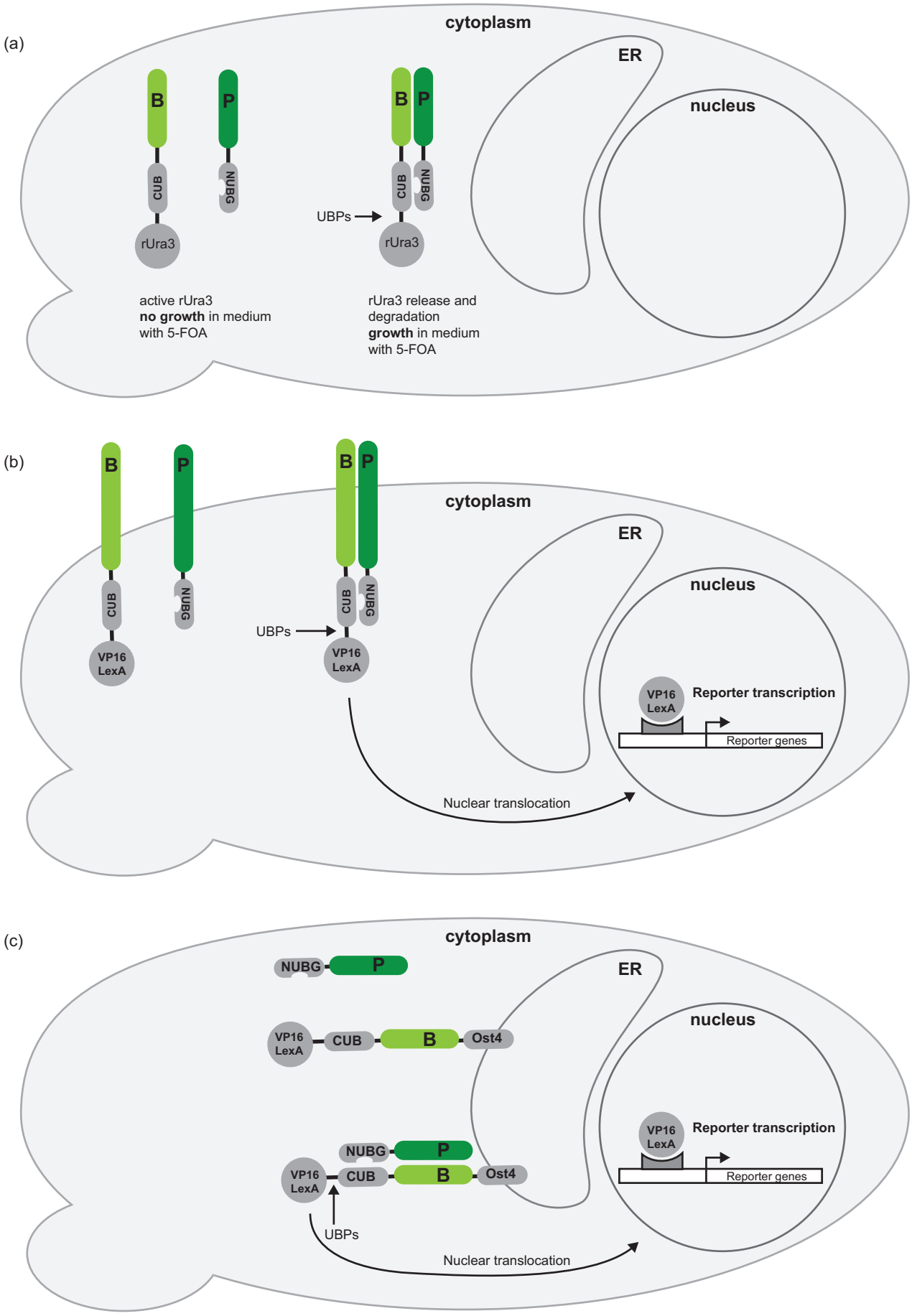
(a)
cytoplasm
ER
nucleus
B
P
CUB
NUBG
rUra3
UBPs
active rUra3
no growth in medium
with 5-FOA
rUra3 release and
degradation
growth in medium
with 5-FOA
(b)
VP16
LexA
Reporter transcription
Reporter genes
Nuclear translocation
(c)
Ost4

Figure 8. Split-ubiquitin systems in yeast.

Two fragments of ubiquitin (CUB and NUBG) are fused to Bait (B) and prey (P) respectively. When CUB and NUBG are brought in close proximity due to a bait-prey interaction, yeasts UBiquitin specific Proteases (UBPs) cleave a peptide link between the reformed ubiquitin and the reporter molecule. **(a)** When rUra3 is used as a reporter system, interaction induces release of a modified Ura3 enzyme (rUra3), which is rapidly degraded upon release from the bait. Bait-prey interaction is reported by ability to grow in the presence of 5-FOA. **(b)** Membrane Yeast Two-Hybrid (MYTH) system uses LexA-VP16 hybrid TF to report interaction using reporter genes in the yeast genome. Bait-prey interaction occurs at the cell membrane where the TF is retained. **(c)** Cytosolic Y2H. Modified split ubiquitin system with LexA-VP16 as a reporter adapted for to test interactions between cytosolic proteins. Bait is retained at the ER membrane through a fusion to Ost4 ER membrane protein.

In the rUra3 system, the bait is fused to CUB which is itself fused to the rUra3 protein (Bait-CUB-rUra3). The Ura3 protein is an enzyme that converts 5-FOA into a toxic metabolite. The rUra3 protein is a modified unstable Ura3 enzyme. When rUra3 protein is linked to CUB, it is stabilised, 5-FOA supplemented in the medium is degraded into its toxic metabolites, and yeasts do not grow (Dunnwald *et al.* 1999, Dirnberger *et al.* 2008). When a bait-prey interaction occurs, the CUB-NUBG are recognised by UBPs, which cleave the link between CUB and rUra3. Release of rUra3 from CUB results in rapid degradation of the enzyme, allowing yeasts to grow on media containing with 5-FOA (Figure 8a). Here again, as the reporter of PPI is not based on gene transcription, the interaction between bait and prey can occur anywhere in the cell and is thus applicable to almost all protein types, *i.e.* cytosolic, nuclear and membrane proteins.

In the transactivation system, the sensor of PPI is the transcription of reporter genes. The bait is a membrane protein and is fused to CUB, itself fused to a LexA-VP16 hybrid transcription factor (Stagljar 1998). Due to its association with a membrane protein, the hybrid TF cannot reach the nucleus and bind to DNA and is thus unable to exert transcriptional activity. When bait and prey interact, the UBPs cleave the link between CUB and LexA-VP16, allowing the TF to migrate to the nucleus and activate the transcription of reporter genes (Figure 8b). Like for the classical Y2H, auxotrophic markers and LacZ reporter can be used. For this system to work, CUB and NUBG have to be both in the cytosol. The topology of bait and prey and the extremity to which CUB and NUBG are fused is therefore of fundamental importance. This system has been termed «Membrane Yeast Two-Hybrid» (MYTH) because it is, compared to other Y2H systems, the only system that can test interactions between two membrane proteins (Johnsson and Varshavsky 1994).

Derived from this technique, an adapted Cytosolic Y2H (CytoY2H) was designed in 2007 (Figure 8c). The authors propose to fuse the bait on one side to Ost4, a yeast membrane protein from the ER, and on the other side to CUB-LexA-VP16 (Mockli *et al.* 2007). This allows to use the split-ubiquitin system with baits that are not membrane proteins. Interestingly, baits with transcriptional activity can also be used in cytoY2H.

(vii) Screening for interactions between extracellular proteins. Among all the two-hybrid techniques described, none is suited to identify interactions involving extracellular proteins. However, this group of proteins are particularly interesting since they are easily accessible targets in therapeutics. Proteins that are on the cell surface or secreted in the extracellular space are conducted through the secretory pathway (ER and Golgi apparatus), which provides an environment allowing correct folding, disulphide bond formation and glycosylation of the proteins (Wright *et al.* 2010). It is thus necessary, when addressing interaction between these proteins, to be in a context close to their natural setting. Only one Y2H system was fitted for the study of secreted proteins: the SCreening for INteractions between EXtracellular Proteins (SCINEX-P), designed by Urech *et al.* in 2003 (Urech *et al.* 2003). This assay takes advantage of the yeast Unfolded Protein Response (UPR). Upon accumulation of misfolded proteins in the ER, a type I transmembrane yeast protein called Ire1p homodimerises in its N-terminal luminal part. This induces association of the C-terminal, cytosolic parts of Ire1p, and triggers a signalling cascade leading to Hac1p transcriptional activator synthesis. Hac1p binds to the Unfolded Protein Response Elements (UPRE) in the promoter of genes coding for chaperone proteins. To study PPI, one bait and one prey, located in the ER, are fused to mutated cytosolic parts of Ire1p (Figure 10). These mutants complement each other to reach nearly 100% of WT activity but are inactive as monomers. If bait and prey interact in the ER, the Ire1p mutated fragments dimerise and induce UPR. For this assay, the yeast strain must harbour an inactivating mutation in the endogenous *Ire1* gene, and an UPRE must be inserted in the promoter region of the reporter genes (Urech *et al.* 2003, Pollock *et al.* 2004).

Overall, the yeast two-hybrid techniques described above allow the detection of binary interactions between two candidate proteins. It is suited to assay transiently, weakly interacting partners. The corollary to the high sensitivity of these techniques is that when used in screening, they lead to large proportions of false positives. Moreover, due to the expression of proteins in an artificial system, the Y2H systems can fail to detect some interactions that occur in a natural setting. To validate an interaction between two proteins, the use of alternative techniques might be required.

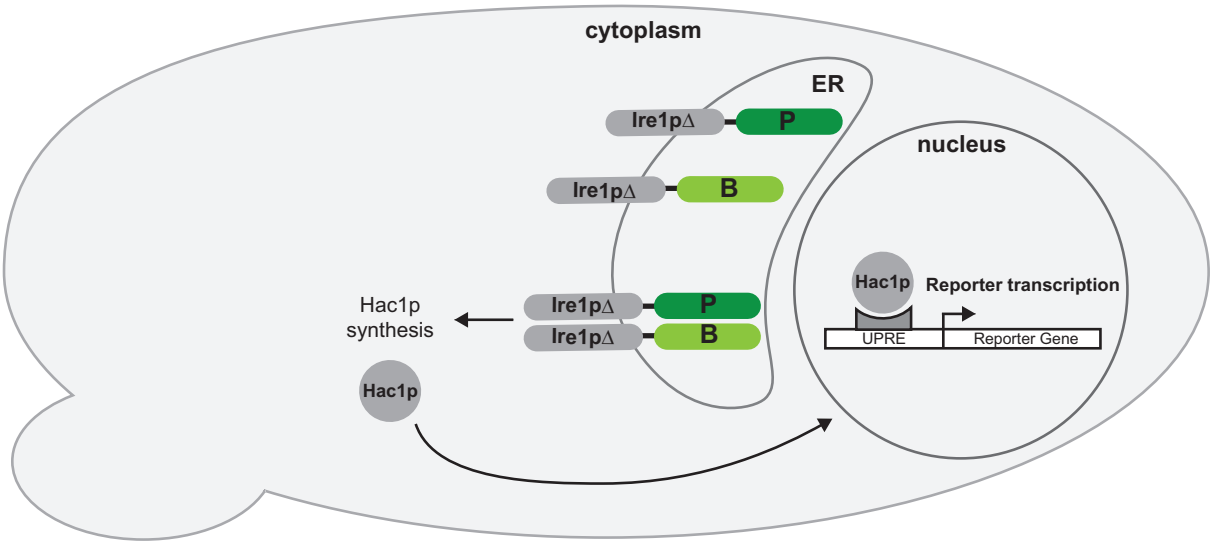

Figure 9. SCreening for INteractions between EXtracellular Proteins (SCINEX-P).
Upon accumulation of misfolded proteins in the ER, yeast Ire1p proteins dimerise and induces a signalling cascade leading to Hac1p transcriptional activator synthesis. Hac1p binds to the Unfolded Protein Response Elements (UPRE) in the promoter of genes coding for chaperone proteins. In this system, Bait (B) and prey (P) are fused to mutated Irep1 proteins so that dimerisation of Irep1 is dependent on association between bait and prey. Once Irep1 mutated proteins are in close proximity, signalling cascade is active, Hac1p is induced and can bind to UPRE sequences to induce activation or reporter genes.

4.1.2 Mammalian Two-Hybrid systems

A diverse series of mammalian two-hybrids have emerged over the past decades (Table 1). These systems were developed to evaluate interactions between proteins in a cellular system that is similar to the native cellular context of the assayed proteins. This is of particular interest for a subset of PPI in which adaptor molecules or protein modifications are required for an interaction. Additionally, the mammalian two-hybrids offer the possibility to determine spatio-temporal information about protein interactions. Indeed, PPI are dynamic, they occur in some cell compartments, at certain moments or under certain stimuli. Therefore the use of mammalian two-hybrid is better suited than the yeast systems (Remy and Michnick 1999, Remy and Michnick 2007, Lievens *et al.* 2009).

(i) Mammalian two-hybrid «sensu stricto». The original Y2H system was adapted in mammalian cells and called the Mammalian two-hybrid (M2H). Like in yeast, two hybrid proteins, a bait-DBD and a prey-AD, are expressed in mammalian cells and bait/prey interactions are reported with a gene under the control of the reconstituted TF (Figure 10). This system, as for the original Y2H, detects protein interactions within the nucleus (Luo *et al.* 1997). Classically, the DBD of GAL4 and the AD of the VP16 protein are used. This system was for instance used to shed light on the

interaction between Smads (Smad 2 and 4) and CREB binding protein (CBP) upon TGF-β treatment (Topper *et al.* 1998). In this paper, the authors transfected bait and prey in Bovine Aortic Endothelial cells and reported the interaction using a luciferase reporter gene.

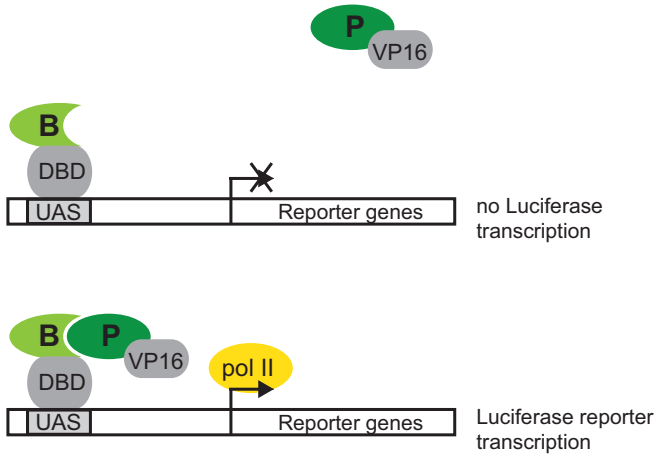

Figure 10. Mammalian two-hybrid systems *sensu-stricto* (M2H).
Bait (B) and prey (P) are fused to DBD of GAL4 and VP16 transactivator factor respectively. If bait and prey interact, reporter genes (often luciferase) is transcribed and activity is measured by light emission derived from luciferase substrate degradation. Interaction is detected in the nuclei or mammalian cells.

(ii) Protein-fragment Complementation Assay. A large family of two-hybrid systems in mammalian cells have been termed Protein-fragment Complementation Assay (PCA) (Remy and Michnick 2007, Kerppola 2008). In theses systems, bait and prey are fused to fragments of a reporter molecule. Alone, each fragment of the reporter has no activity. However, if bait and prey interact, the moieties of the reporter are brought in close proximity and the native molecule is reconstituted. The reporter molecule activity is recovered and generates a quantifiable signal (Figure 11). Different reporter molecules have been used including β-galactosidase (Rossi *et al.* 1997), β-lactamase (Galarneau *et al.* 2002, Wehrman *et al.* 2002), Dihydrofolate reductase (Pelletier *et al.* 1998, Remy and Michnick 1999), several luciferases (Paulmurugan *et al.* 2002, Paulmurugan and Gambhir 2003, Remy and Michnick 2006, Stefan *et al.* 2007) and fluorescent proteins (Wilson *et al.* 2004, Kerppola 2008).

Interaction with PCA is reported with an enzymatic activity or fluorescence of the reporter molecule, meaning that the interaction can take place anywhere in the cell, and that this technique is suited to a wide variety of bait and preys. PCAs can be applied for pairwise interaction assays but, even though it is possible, theses techniques are less amenable than Y2H screens for high throughput analysis and identification of new interacting partners (Remy and Michnick 2007, Lievens *et al.* 2009).

PCAs, with β-lactamase and Yellow Fluorescent Protein (YFP) as reporters, were used to shed light on a cross-talk between the insulin (PKB/Akt) and the TGF-β (Smad) signalling pathways. It was shown that Smad3 directly binds to PKB and that this binding is modulated by insulin and TGF-β (Remy *et al.* 2004). The advantage of using fluorescent reporters compared to enzymatic ones is that the signals emitted by the PPI can be tracked in the cell and localized in cell compartments. Moreover, compared to other complementation assays, addition of chromogenic or fluorogenic substrates is avoided since fluorescence emission is intrinsic to the complemented molecules. The PCAs using fluorescent molecules (YFP, GFP...) have been termed Bimolecular Fluorescent Complementation (BiFC) (Kerppola 2008).

Another interesting application of the PCA is the detection of PPI occurring in living animals. This was set up by Luker and colleagues in 2004 using firefly luciferase complementation and the described interaction between the Rapamycin Binding domain (FRB) of mammalian target of rapamycin (mTor) and the FK506-binding protein 12 (FKBP). They fused FRB to the N-terminal part of firefly luciferase and FKBP to the C-terminal part of firefly luciferase (Luker *et al.* 2004). Here, to validate the *in vivo* model of PCA, 293T cells expressing the two fusion proteins were injected in mice. After rapamycin injection, cells in which FRB and FKBP interact are detected via emission of bioluminescent signals. The possibilities emerging with such techniques are of huge interest especially in drug therapy development (Michnick *et al.* 2007).

A particular PCA called «split-TEV» takes advantage of a protease produced by the Tobacco Etch Virus (TEV). This system works as other PCA in the sense that the complementation of two fragments of the TEV protease leads to the reconstitution of an active enzyme. However, the activity of the protease is measured by the cleavage of a «TEV recognition sequence» and the subsequent release of a reporter protein. The reporter protein can be a TF or a luciferase enzyme. This means that this system is irreversible and that the interaction will be reported, even if two

proteins interact transiently and the TEV protease is reconstituted even for a short period of time (Wehr *et al.* 2008). This technique has not yet been used for library screenings but it has been applied recently to the identification of siRNAs inhibiting a signalling pathway in Drosophila (Suter *et al.* 2008, Wehr *et al.* 2013).

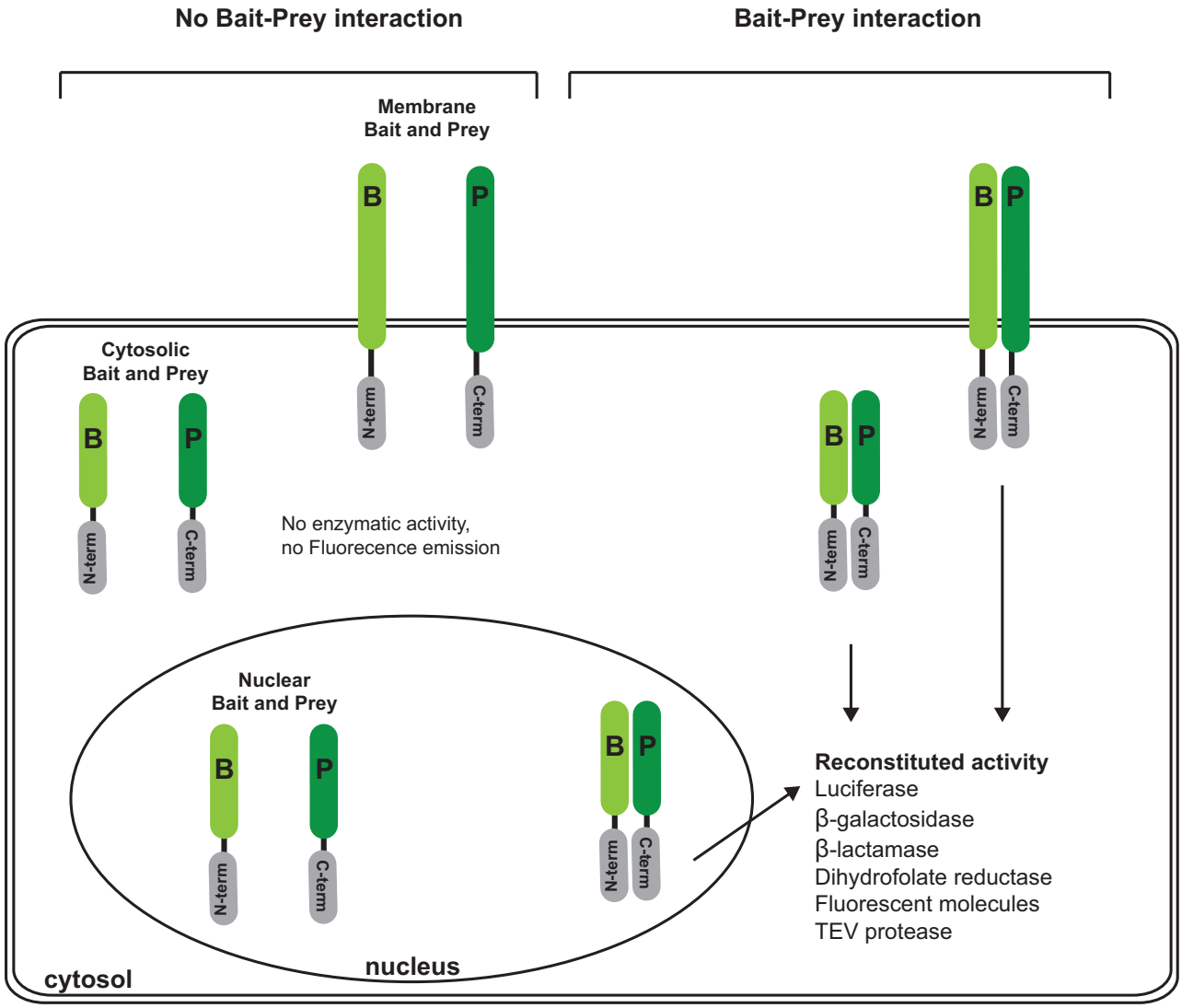

Figure 11. Mammalian Protein-fragment Complementation Assays (PCAs).
Bait (B) and prey (P) are fused to N-terminal and C-terminal fragments of reporter molecule respectively. If Bait and prey interact, reporter activity is reconstituted. Many different reporter molecules are used: Luciferase, β-galactosidase, β-lactamase, dihydrofolate reductase, fluorescent molecules, TEV protease. Interactions can take place in any cell compartment.

(iii) Mammalian protein-protein interaction trap. The MAmmalian PPI trap (MAPPIT) system was designed by the group of Tavernier in 2001 (Eyckerman *et al.* 2001). They exploited the well described ligand-induced JAnus Kinase - Signal Transducer and Activator of Transcription (JAK-STAT) signalling pathway to study PPI. A wide variety of cytokines activate the JAK-STAT pathway upon binding to their receptor. In response to ligand binding, receptors dimerise, and a conformational change activates the associated JAKs. Once activated, JAKs phosphorylate each other as well as the cytoplasmic domain of the receptor, and this enables STAT recruitment. STATs are phosphorylated by JAKs, dimerise, and translocate in the nucleus to activate gene transcription (Staerk and Constantinescu 2012). In this PPI assay system, a bait is linked to a cytokine receptor harbouring a mutation preventing STAT binding. The prey is linked to the cytoplasmic portion of another cytokine receptor chain (gp130) containing an intact STAT recruitment site. The interaction between bait and prey leads to signalling upon cognate ligand binding since the gp130-prey fusion complements the defect of the mutated receptor (Figure 12). Reporter genes such as luciferase are under the control of a STAT-dependent promoter to report interaction. This technique is very sensitive because one bait-prey interaction results in multiple activated STAT molecules, and this amplifies the reporter signal. An important feature of the assay is also its dependence on ligand binding, which allows to control the condition in which the interaction is tested. This system is only compatible with baits and preys that are cytoplasmic or nuclear proteins. A Reverse MAPPIT system was also proposed to detect proteins interrupting PPI. In this system, the bait is fused to an intact receptor and the prey is fused to an inhibitor of the signalling such as a phosphatase. If bait and prey interact, the reporter gene is silent, but if a molecule interrupts this interaction, the reporter gene is induced (Lievens *et al.* 2012).

(iv) Resonance energy transfer systems. Another particular class of mammalian two-hybrids are the Fluorescence Resonance Energy Transfer (FRET) and the Bioluminescence Resonance Energy Transfer (BRET) systems. FRET and BRET are based on the same principle. Proteins are fused to small molecules between which energy transfer occurs only if the proteins of interest are in close proximity. One molecule, termed the «donor», transfers energy to a second molecule termed the «acceptor», which in turn emits fluorescence. The PPI is thus measured at the emission wavelength of the acceptor molecule (Figure 13). In FRET, the partners tested are both fused to fluorescent proteins with overlapping emission and excitation spectra. In BRET, the donor molecule is a luciferase protein and the acceptor is a fluorescent protein (often YFP). These techniques allow real-time visualisation of interactions. The use of BRET compared to FRET has the advantage to avoid

photobleaching problems and thus to follow interaction for longer time-lapse. However, subcellular resolution is better with FRET. Those techniques are very powerful but also heavy to set up and require technical expertise (Boute *et al.* 2002, Piston and Kremers 2007, Lievens *et al.* 2009).

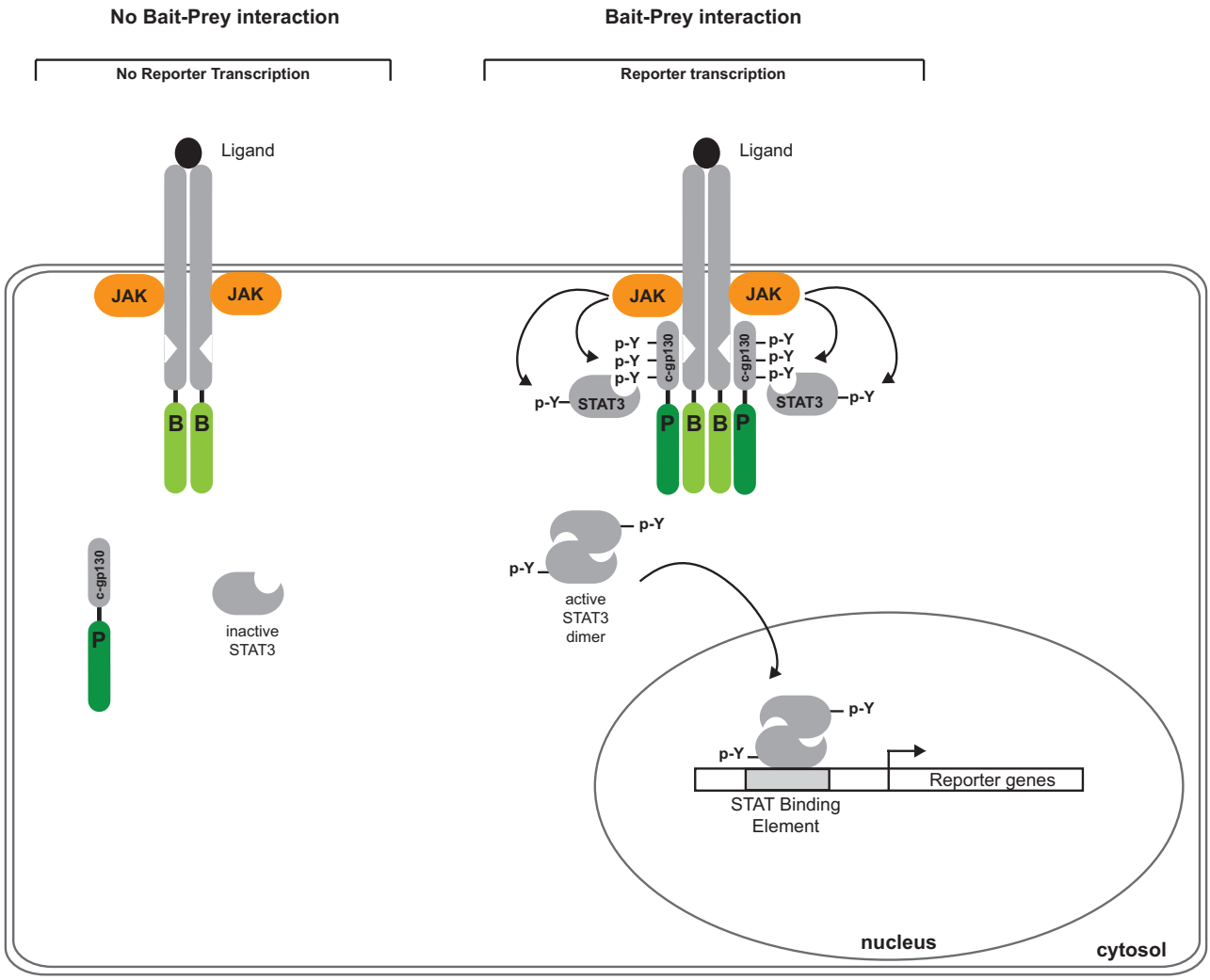

Figure 12. Mammalian protein-protein interaction trap (MAPPIT).
Bait (B) is fused to the cytosolic portion of a mutated receptor unable to induce JAK/STAT signalling pathway. Prey (P) is fused to an intact cytosolic portion of a receptor. Bait prey interaction allows STAT recruitment by the receptor and phosphorylation by the JAKs. Once phosphorylated, STATs dimerize and translocate in the nucleus where they bind to STAT binding elements and induces reporter gene transcription.

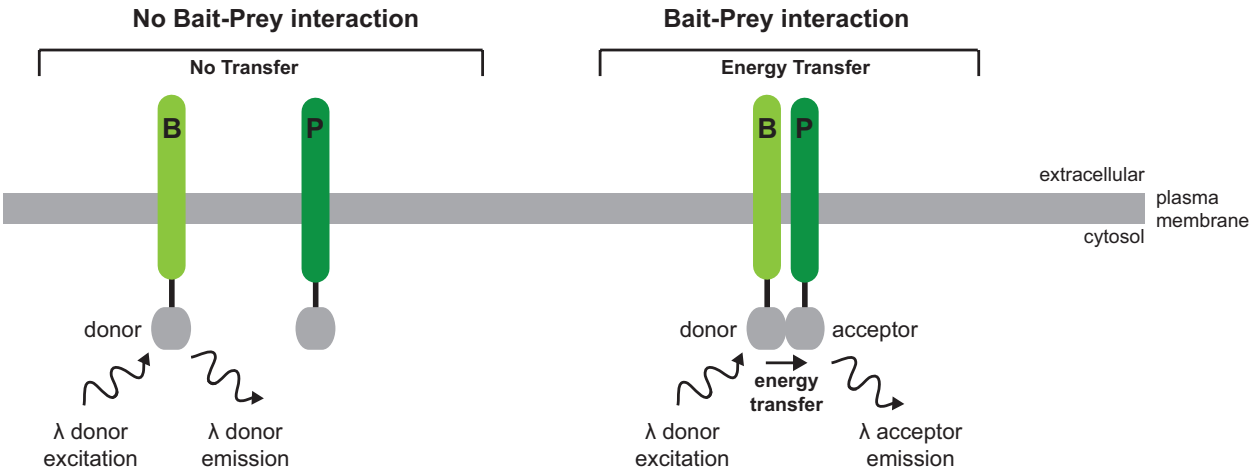

Figure 13. Fluorescence/bioluminescence resonance energy transfer systems (FRET-BRET).
FRET and BRET are based on the same principle of energy transfer between a donor and an acceptor molecule. In FRET, bait (B) and prey (P) are fused to fluorescent molecules. In BRET, bait and prey are fused to a luciferase and a fluorescent molecule respectively. If bait and prey interact, the donor molecule that is excited transfers its energy to the acceptor molecule, which in turn emits at his own wavelength. FRET and BRET are reported when a signal is detected at the acceptor molecule wavelength.

4.1.3 Proximity Ligation Assay

Proximity ligation assay (PLA) was developed by Söderberg and colleagues and allow to detect PPI of endogenous proteins in cells or tissue sections (Soderberg *et al.* 2008). The PLA technique works as follows: each of the two proteins to assay for interaction are detected with a specific primary antibody. The two primary antibodies must be derived from different species (*e.g.* mouse and rabbit) or from different isotypes (*e.g.* mouse IgG1 and mouse IgG2a). In a second step, secondary antibodies, conjugated to oligonucleotides, recognise the primary antibodies according to their species isotype. Two «connector oligonucleotides» are used to ligate the antibody oligonucleotides but this requires proximity between the two secondary antibodies, and thus interaction of the assayed proteins. If the proteins indeed interact, a proper DNA ligation product is formed and can serve as a template for rolling-circle amplification. This generates a long single-stranded DNA molecule that can be detected by fluorescent probes (Figure 14).

PLA has the advantage to reveal an interaction between endogenous proteins (not fusion proteins resulting from transfection). PLA can be performed on cells in suspension and then detected by FACS, but it is most frequently achieved on cells and tissue sections and detected by fluorescence microscopy (*in-situ* PLA). When detecting PLA signals by fluorescence microscopy, each pair of interacting protein is represented by an individual fluorescent dot that can be localised in the cell.

When detecting PLA signals by FACS, the information about the localisation of the interaction is lost but allows a more quantitative evaluation of the interactions. The obvious limitation of the PLA technique is the availability of antibodies targeting the assayed proteins and the possibility to use these antibodies in combination.

In-situ PLA was applied to the study of the kinetics of SMAD2/SMAD4 and SMAD3/SMAD4 complex translocation in the nucleus after TGF-β treatment (Zieba *et al.* 2012). Sundqvist and co-workers used PLA to highlight the interaction of AP-1 family transcription factors with the SMADs. This technique is suited for surface proteins as well as cytosolic and nuclear proteins.

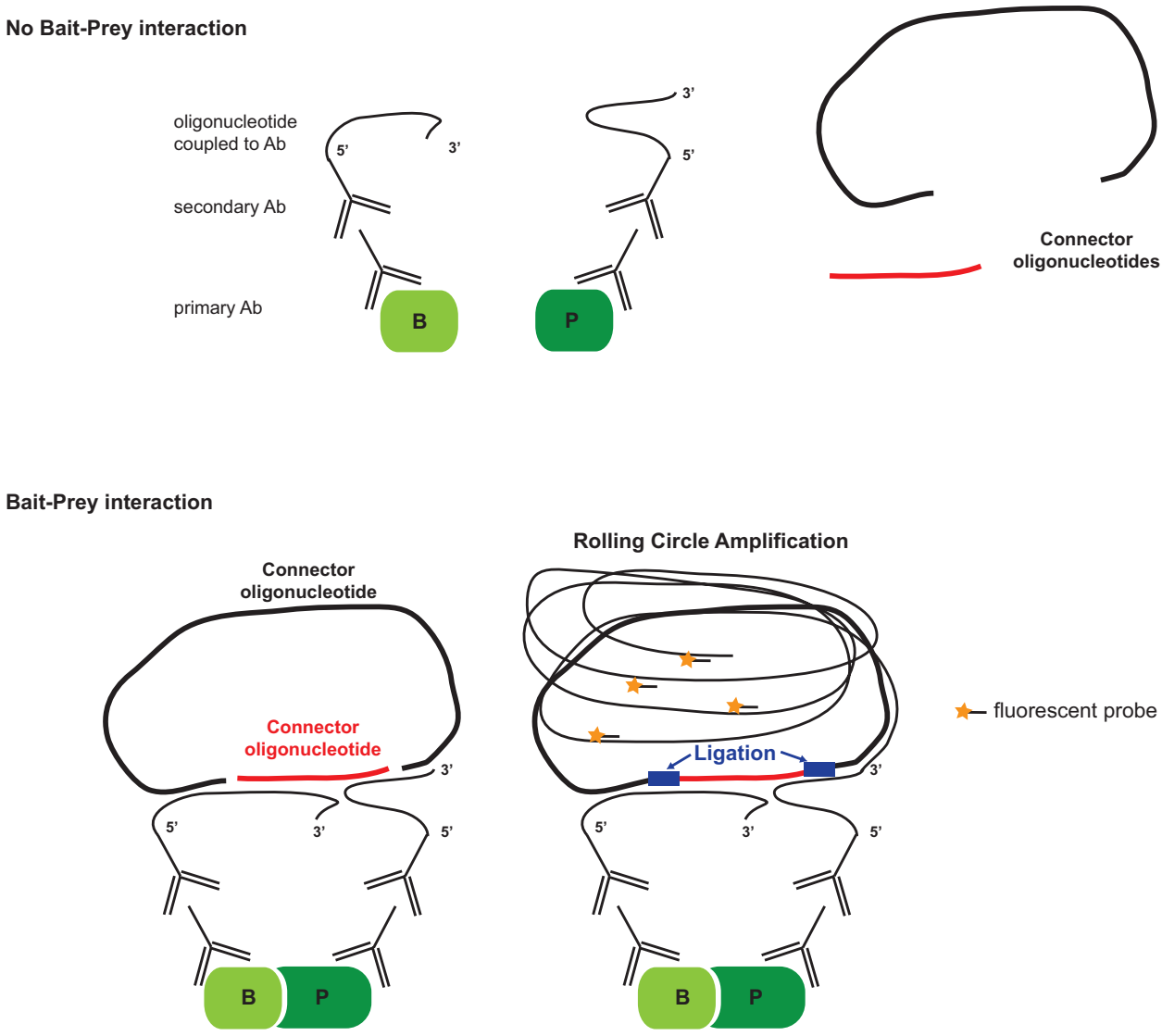

Figure 14. Proximity ligation assay (PLA).
Bait (B) and prey (P) are recognised by specific antibodies derived from different animal species. Those primary antibodies are then recognised by species secondary antibodies conjugated to oligonucleotides. If bait and prey interact, oligonucleotides bound to the antibodies are close enough to be linked through connector oligonucleotides which are then ligated. The so-formed template is amplified by rolling circle amplification. DNA is then detected by fluorescent probes.

4.2 PPI detection by biochemical methods

Simultaneous to the development of yeast and mammalian two-hybrid systems, several biochemical techniques were developed to assess PPI.

4.2.1. Co-immunoprecipitation and co-Affinity Purification

Co-ImmunoPrecipitation (co-IP) and co-Affinity Purification (co-AP) are frequently used to test interactions and are based on the same principle (Monti *et al.* 2005). In co-IP, a target protein is purified from a whole cell extract using a specific antibody (Figure 15a). The proteins interacting with the target remain attached to the latter. Complexes of antibody-target-interacting protein are fixed on a solid surface (beads or columns) through binding of the antibody. Several washing steps are applied to remove proteins attached non-specifically. Molecules remaining after washing are eluted using denaturing agents (SDS), extreme pH or high salt concentrations. The eluted proteins are then either analysed either by Western Blot (WB), if one searches for a particular interacting protein, or identified by techniques like Mass Spectrometry (Phizicky and Fields 1995).

Co-IP between endogenous proteins is a highly reliable technique to confirm an interaction between two partners. However, successful co-IP with endogenous interacting proteins are not so easy to obtain. First, this method is not convenient for labile interactions since it is not sensitive and weak interactants are generally lost during the washing steps. To increase the sensitivity of co-IP, cross-linking of proteins, which implies the chemically-mediated establishment of covalent bounds between closely interacting proteins, may be used to favour the detection of weak interactions. However this may lead to the detection of false positive interactions. Second, the availability of antibodies able to purify the native target proteins without disrupting its interactions is often a limiting step.

Co-AP is similar to co-IP and can serve as alternative when no antibody targeting the protein of interest is available. Here, isolation of the target protein is performed via a «tag» linked to the target protein (Figure 15b). The tags are chosen based on their ability to bind with high affinity to molecules that can serve for the purification step. Many different tags that have been designed, and classically include Glutathione-S-Transferase (GST-tag), Strep-tag, Calmodulin Binding Protein (CBP), or epitopes recognised by monoclonal antibodies (Monti *et al.* 2005). When expressed in cells, the tagged target protein binds to its partner(s). After cell lysis, the target is purified by

binding to molecule that has a high affinity for the tag (for the examples cited above, this would correspond to glutathione, biotin, calmodulin or a specific monoclonal antibody, respectively) .

Since co-IP and co-AP both involve cell lysis, these systems give no information about the localisation of the PPI in the cell. Subcellular fractionation can be performed before target isolation to give an indication of its location. To isolate partners of surface proteins, another strategy is to incubate whole cells in the presence of the antibody recognising a surface target. The antibody is first allowed to bind to its antigen, and cells are lysed in a second step. Antibodies are then immobilised on a solid surface as for a classical IP. This way, only surface interactants are isolated.

The challenges of co-IP and co-AP methods are mostly related to the high proportion of contaminants that are not specifically bound to the target. To address this issue, the group of Seraphin developed the so-called Tandem Affinity Purification or TAP method (Puig *et al.* 2001, Xu *et al.* 2010). Two epitope tags separated by a TEV cleavage site are fused to a target bait. This allows to purify the bait with the associated preys on the basis of a first tag. Upon cleavage by TEV protease, the second tag is revealed and used for a subsequent purification step. TAP methods allow purification of large protein complexes and leads to lower background compared to the co-AP technique. Classically, the two epitope tags used are protein A and CBP (Figure 16). TAP has been successfully applied in many prokaryotic and eukaryotic cell lines (Xu *et al.* 2010).

When looking for new interacting partners of a target protein, different strategies may be used. Proteins co-purified or co-immunoprecipitated with the protein of interest are often identified by Mass Spectrometry (MS). These techniques are then termed IP-MS or AP-MS. This usually leads to the identification of numerous potential interactants, and confirmation with other techniques is needed to exclude false positives.

Compared to two-hybrid techniques, the interaction detected by IP- or AP-MS does not require to be a direct interaction. Indeed, if the interaction between multiple partners is strong enough, and resists to all steps of the procedure, different members of the complex may be identified at once.

A very interesting study comparing data collected from AP-MS and Y2H screenings was performed by the group of Vidal in 2008 (Yu *et al.* 2008). Comparing the data from several publications, they concluded that AP-MS and Y2H screenings lead to data that are equal in quality, but the identified

interactions are different in nature. Their computational analysis led to the conclusion that AP-MS mostly reveals interactions between large and stable complexes from a same pathway, while Y2H identifies mostly binary interactions enriched in transient and «condition-specific» interactions. This suggest that Y2H screens allow to link several pathways or identify transient TF associations for example, which would fail to be identified with AP-MS methods. Overall, this study confirmed that both approaches are complementary and a combination is needed to complete the understanding of a complex PPI network.

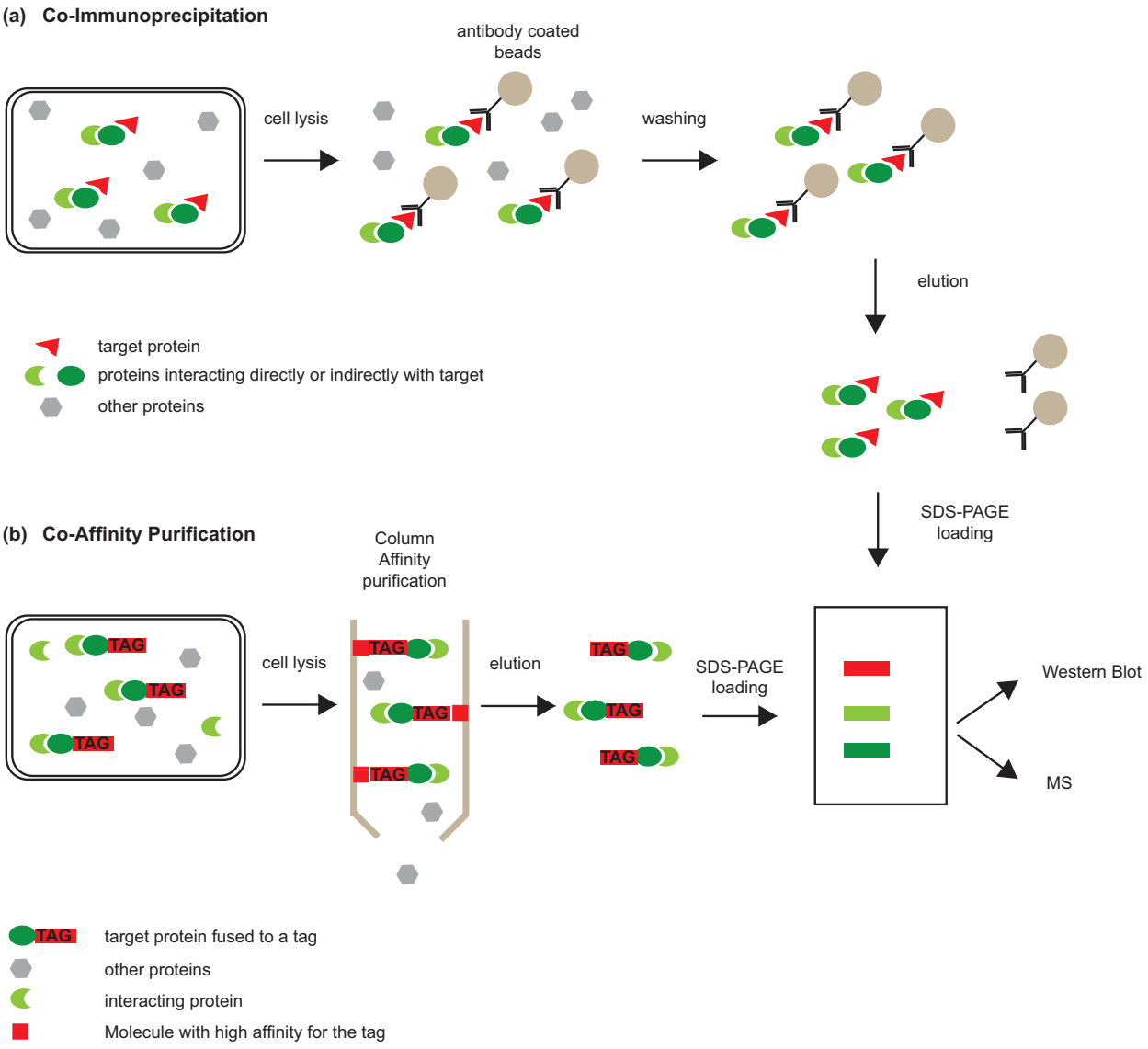

Figure 15. Co-Immunoprecipitation (co-IP) (a) and co-Affinity Purification (co-AP) (b).
Protein complexes (endogenous proteins for co-IP and fusion protein for co-AP) are isolated from cell extracts using target specific antibody coated beads (a) or affinity Tag column purification (b). Upon removal of non-specific binders, protein target and partners are eluted and loaded on SDS-PAGE gel. Proteins co-IPd or co-APd can be identified by Western Blot or screened by MS.

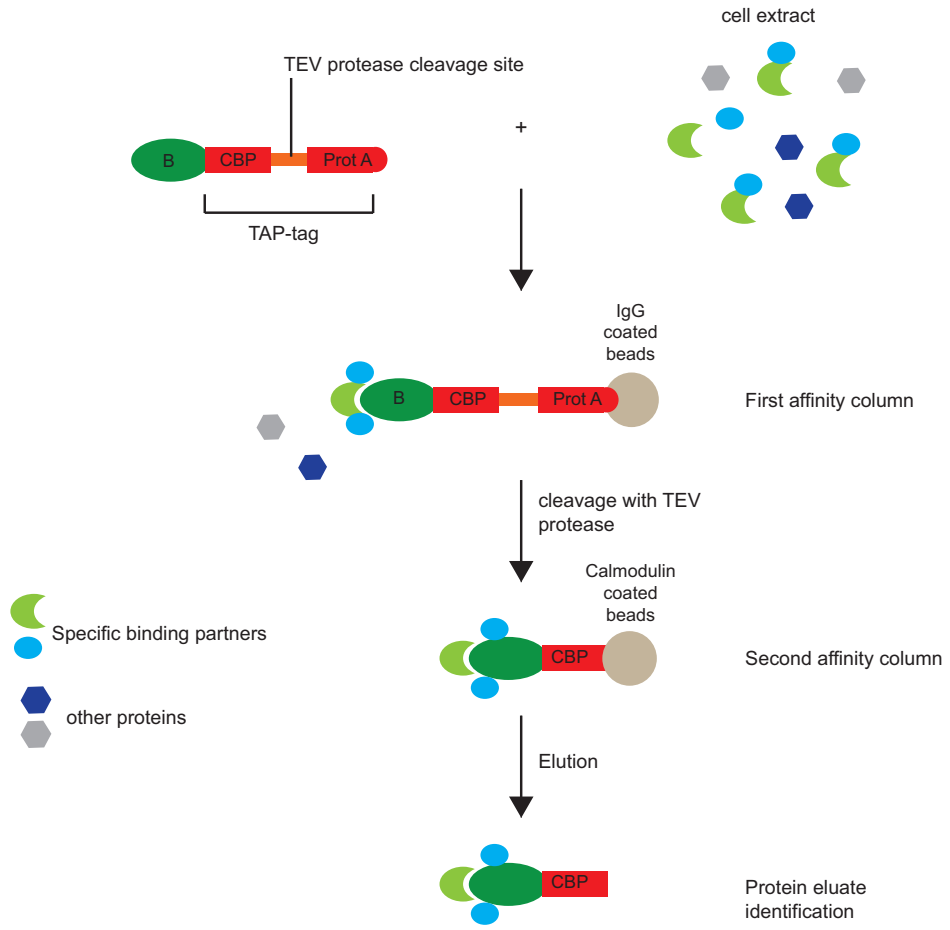

Figure 16. Tandem Affinity Purification (TAP).
The tandem affinity purification (TAP) tag consists of three components: a calmodulin-binding peptide (CBP), a tobacco etch virus (TEV) protease cleavage site and Protein A as an immunoglobulin G (IgG)-binding domain. Cells are transfected to produce TAP-tagged protein(s). Extracts are then prepared under mild conditions and TAP is carried out to conserve protein complexes integrity. The first column consists of IgG coated beads. TEV protease cleaves the immobilized multiprotein complexes. Another round of binding is carried out on a second column that consists of calmodulin coated beads. The complex is then eluted and the proteins co-purified are analysed by MS.

4.2.2. Luminescence-based mammalian interactome mapping

The luminescence-based mammalian interactome mapping or «LUMIER» is a technique combining two-hybrid and co-IP techniques that is developed to assay dynamic PPI in a high-throughput setting (Barrios-Rodiles *et al.* 2005). In LUMIER, two hybrid proteins are coexpressed in mammalian cells: one contains a Flag tag to enable IP and the other is fused to a Renilla Luciferase (RL) reporter. Interaction between the two partners is determined by detecting luciferase activity on immunoprecipitates using an antibody against the Flag tag (Figure 17). This assay was performed in a large scale analysis by the group of J. Wrana in the TGF-β signalling pathway, which relies mostly on dynamic PPI occurring upon residue phosphorylation. They coexpressed in

293T cells RL fused to known members of the TGF-β pathway and 518 flag-tagged preys. Cells were treated or not with TGF-β. Because of the huge amount of data provided by this assay, authors automated the detection of RL activity for each Flag precipitate. Their results highlighted links between the TGF-β pathway to other networks including the p21-activated kinase and Occludin (Barrios-Rodiles *et al.* 2005). This system reveals dynamic, novel or validated, interactions induced by extracellular signals.

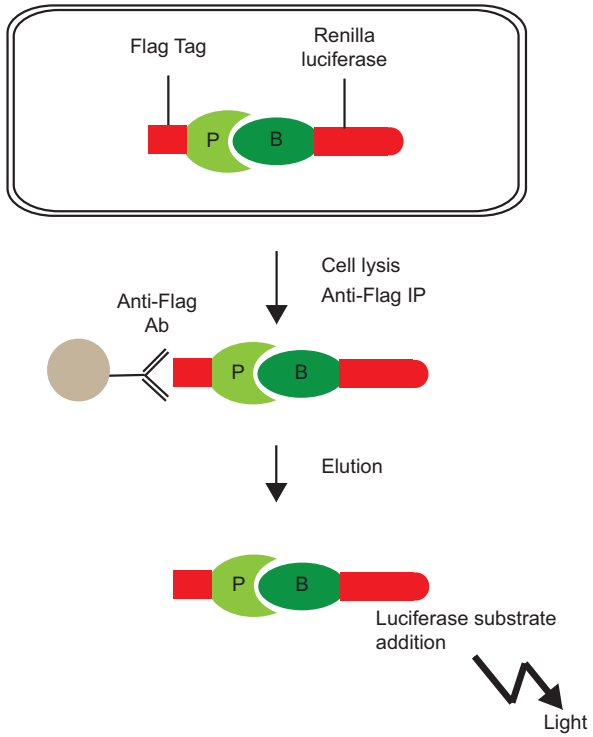

Figure 17. Luminescence-based Mammalian interactome mapping (LUMIER).
Renilla Luciferase-tagged bait is coexpressed in mammalian cells with Flag-tagged preys. Interaction between bait and prey is determined by luciferase activity in anti-Flag immunoprecipitates.

4.2.3. Overlay or Far Western

A technique called Far Western Blotting (or Far Overlay) is a method derived from classical WB to identify PPI. Prey proteins from a cell lysate are separated by PolyAcrylamide Gel Electrophoresis (PAGE) and then transferred on a membrane and probed («overlaid») with a bait (Figure 18). The bait bound to its prey(s) is detected with a specific antibody and revealed as an usual WB (Edmondson and Dent 2001). In this assay, the bait-prey interaction must be a direct interaction to be detected. To ensure proper detection of bait/prey interaction, the preys should be in their native conformation. Therefore, prey proteins can be separated in native gels or renatured after transfer on

the membrane. Moreover, to facilitate bait detection and avoid antibody requirement, baits may be fused to tags or linked to biotin or radioactive labels. Preys detected by Far Western then used to be identified by MS.

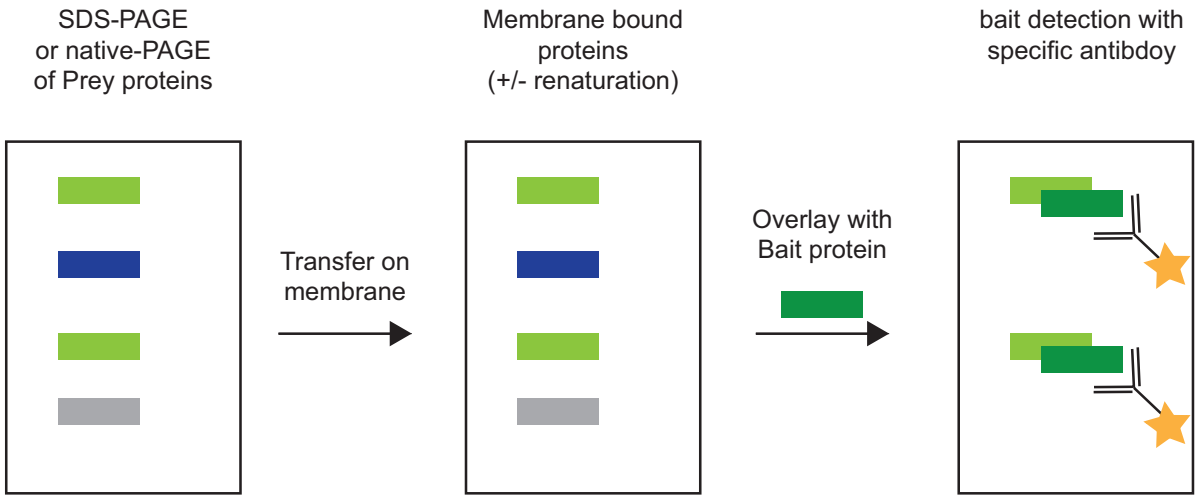

Figure 18. Far Western of (Far Overlay).
Prey proteins are first separated by PAGE and then transferred on membrane (PVDF or nitrocellulose). Membranes are «overlaid» with bait protein which is allowed to bind to its prey(s). Upon washing of the membrane, the bait that remains bound to the membrane is detected with a specific antibody like in classical WB. Prey proteins may be identified by their size, and sequenced by MS.

4.2.4. Phage Display

The phage display technique was first described in 1985 by G. Smith as a technique to identify and clone a gene for which an antibody is available (Smith 1985). The screening system uses phages (frequently bacteriophage M13) «displaying» at their surface prey proteins through a fusion with a phage coat protein (frequently pIII coat protein). Viruses presenting proteins at their surface are bound to bait proteins immobilised on a solid support. Upon washings only viruses that display preys interacting with the bait remain attached to the solid support. Bound phages are eluted and amplified in E. coli. They are then used for repeated cycles of selection and amplification, which are necessary to amplify specifically bound phages over non-specific binders (Sundell and Ivarsson 2014). The cDNA coding for the isolated preys is then identified by sequencing (Figure 19). As for Y2H and Far Western, this technique allows identification of direct interactions between two proteins and is not suited for large protein complexes. However, it is a powerful screening approach

that has been used for the identification of PPI, and widely applied for antibody screenings (Chan *et al.* 2014, Sundell and Ivarsson 2014). Recently, our group derived mAbs targeting GARP using phage cDNA libraries encoding immunoglobulin Fab fragments as preys, and GARP/TGF-β complexes as baits. This technique allowed to derive a mAb recognising the GARP/TGF-β complex and inhibiting Treg function *in vitro* and *in vivo* (Cuende *et al.* manuscript submitted).

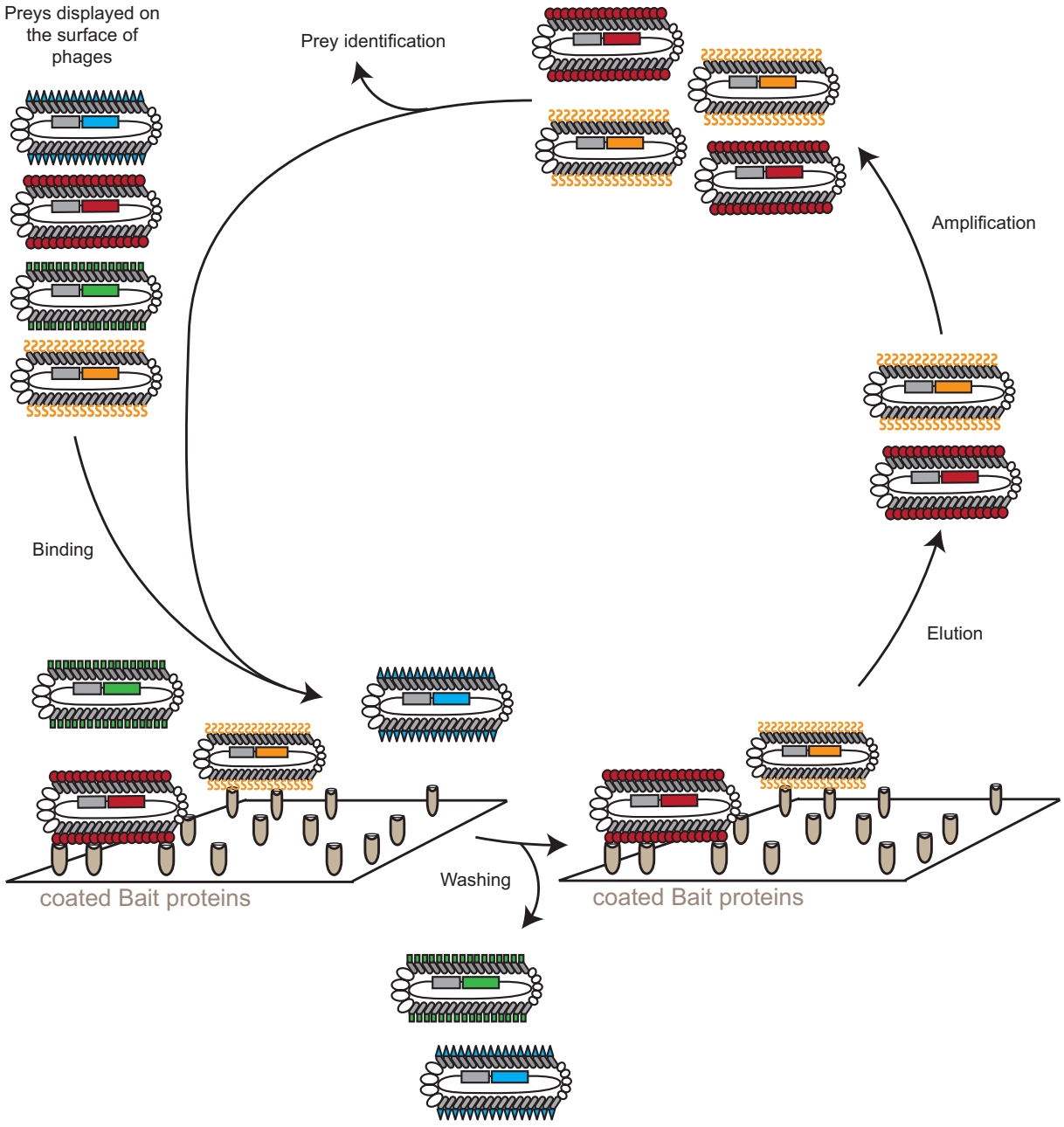

Figure 19. Phage display technique.
Adapted from Sundell and Ivarsson 2014.

4.3 The Interactome

Two-hybrid screenings as well as biochemical methods have generated over the past decades an endless amount of data concerning PPI in many different organisms. The ongoing challenging work of researchers is to design interaction networks called «interactomes» which are a representation of each binary interaction occurring in a given organism, cell type or pathway. Interactome approaches aim to provide an understanding of the complex protein interplays and help to predict the effects of drug therapies (Lievens *et al.* 2009).

Each member of an interaction network is characterised by its degree of connectivity. Nodes that are highly connected to other proteins are called «hubs». Yu *et al.* observed a striking difference in the interactomes provided by AP-MS and Y2H (Yu *et al.* 2008). The AP-MS reveals interactomes with a majority of «party-hubs» which are considered static interacting partners of a specific biological process. In contrast, Y2H methods reveals a majority of «date-hubs» which corresponds to dynamically interacting proteins. A representation of these interactomes are shown in Figure 20 .

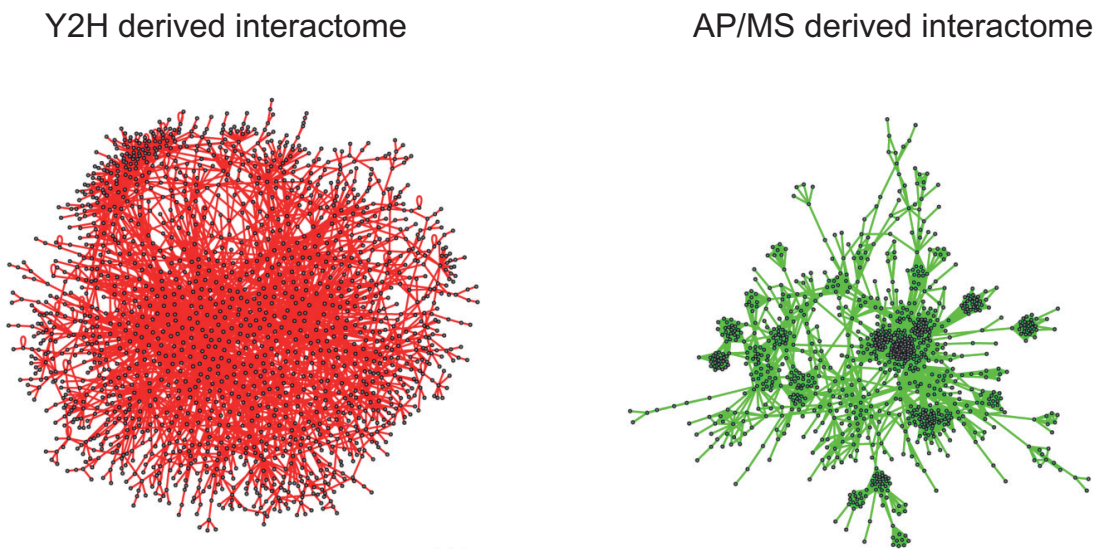

Figure 20. Adapted from Yu *et al.* 2008.
Network analysis of Y2H (left) and AP/MS (right) datasets.

Aim of the study

Production of active TGF-β1 is one important mechanism used by human Tregs to suppress other immune cells. The steps leading to this production are not yet fully elucidated. We know that TCR stimulation induces latent TGF-β1 presentation on the surface of Tregs, and this occurs through binding to GARP. Blocking anti-GARP antibodies inhibit active TGF-β1 production by TCR-stimulated Tregs, demonstrating that GARP is required for TGF-β1 activation in these cells. However, Th lymphocytes transduced with GARP present latent TGF-β1 at their surface but are unable to produce the active cytokine. Thus, although GARP is required for TGF-β1 activation by Tregs, it is not sufficient. We hypothesised that additional proteins present in Tregs but not in Th cells associate with GARP/TGF-β1 complexes and contribute to TGF-β1 activation.

The objectives of this work were to identify new protein partners of GARP that are expressed by stimulated Tregs, but not by Th cells, and that regulate TGF-β1 production in Tregs. We used two approaches to identify new GARP partners:

(i) a yeast two-hybrid system known as «split-ubiquitin» or «Membrane Yeast Two-Hybrid» (MYTH), using GARP as a bait to screen a prey cDNA library prepared from TCR-stimulated Tregs,
(ii) an IP-MS approach, in which we immunoprecipitated GARP from stimulated Treg cell lysates and attempted to identify co-immunoprecipitated proteins by MS.

Isolated protein candidates are then evaluated for their role in the regulation of TGF-β1 production by human Tregs.

Results

1. LAPTM4B interacts with GARP and decreases TGF-β1 production in human regulatory T cells

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Manuscript submitted to PlosOne

Abstract

Production of active TGF-β1 is one mechanism by which human regulatory T cells (Tregs) suppress immune responses. This production is regulated by GARP, a transmembrane protein present on stimulated Tregs, but not on other T lymphocytes (Th and CTLs). GARP forms disulfide bonds with proTGF-β1, favors its cleavage into latent inactive TGF-β1, induces the secretion and surface presentation of GARP/latent TGF-β1 complexes, and is required for the activation of the cytokine. Forced expression of GARP in non-Treg cells, however, is not sufficient to induce TGF-β1 activation, suggesting that additional Treg-specific protein(s) associated with GARP/TGF-β1 complexes regulate TGF-β1 production in Tregs. We searched for such proteins by yeast two-hybrid, using GARP as a bait to screen a human Treg cDNA library. Here we identified LAPTM4B (Lysosomal-Associated Transmembrane Protein 4B), which interacts with GARP in mammalian cells and is expressed at higher levels in Tregs than in Th cells. LAPTM4B decreases cleavage of proTGF-β1, secretion of soluble latent TGF-β1 and surface presentation of GARP/TGF-β1 complexes by Tregs, but does not contribute to TGF-β1 activation. Thus LAPTM4B binds to GARP and decreases TGF-β1 production in human Tregs. It may play a role in the control of immune responses by decreasing Treg immunosuppression.

Introduction

Regulatory T lymphocytes (Tregs) are a subset of $CD4^+$ T cells that maintain immune tolerance by suppressing auto-reactive T cells (Sakaguchi *et al.* 2010, Josefowicz *et al.* 2012). Their development and function requires transcription factor FOXP3, as illustrated by the severe autoimmune syndrome that affects mice and humans carrying a mutated *FOXP3* gene (Bennett *et al.* 2001, Brunkow *et al.* 2001, Wildin *et al.* 2001). *Foxp3* expression is a specific marker of Tregs in mice. This is not true in humans, where non-regulatory $CD4^+$ or $CD8^+$ T lymphocytes transiently express *FOXP3* upon T cell receptor stimulation (Sakaguchi *et al.* 2010). Stable *FOXP3* expression, a hallmark of Tregs in mice and humans, is ensured by the demethylation of a conserved non-coding region of gene *FOXP3*, called *FOXP3i1*, TSDR or CNS2 (Baron *et al.* 2007, Floess *et al.* 2007, Polansky *et al.* 2008, Stockis *et al.* 2009a, Zheng *et al.* 2010). Demethylated *FOXP3i1* can serve to identify and quantify Tregs in human blood or cell samples (Wieczorek *et al.* 2009, de Vries *et al.* 2011, Gauthy *et al.* 2013).

Depending on the context or the cell type to suppress, Tregs use various mechanisms of immune suppression. One mechanism implies the production of the potent immunosuppressive cytokine TGF-β1 (Vignali *et al.* 2008, Josefowicz *et al.* 2012). Its production by Tregs is regulated by GARP, a surface protein expressed on stimulated Tregs but no other T lymphocytes (Wang *et al.* 2008, Tran *et al.* 2009, Stockis *et al.* 2009b, Gauthy *et al.* 2013). TGF-β1 is synthesized in all cell types as a homodimeric proTGF-β1 precursor (Figure 1) (ten Dijke and Arthur 2007, Travis and Sheppard 2014). FURIN cleaves proTGF-β1 to generate a C-terminal fragment, or mature TGF-β1, which remains non-covalently bound to the N-terminal fragment known as LAP (Latency-Associated Peptide). This complex, called latent TGF-β1, is inactive because LAP prevents mature TGF-β1 from binding to its receptor. Latent TGF-β1 is secreted by most cell types as a soluble form. In the secretory pathway of stimulated human Tregs, GARP forms disulfide bonds with the proTGF-β1 precursor and favors its FURIN-dependent cleavage into latent TGF-β1 (Wang *et al.* 2012, Gauthy *et al.* 2013). GARP/latent TGF-β1 complexes are then presented on the Treg surface (Tran *et al.* 2009, Stockis *et al.* 2009b, Gauthy *et al.* 2013). Stimulated Tregs release mature TGF-β1 from surface GARP/latent TGF-β1 complexes, a process called “latent TGF-β1 activation”. Binding of active TGF-β1 to its receptor leads to autocrine and paracrine signaling followed by phosphorylation of SMAD transcription factors (Stockis *et al.* 2009a). We have recently produced GARP monoclonal antibodies that are able to block TGF-β1 activation by Tregs, thus GARP is necessary for this activation (Cuende *et al.* manuscript submitted). But it is not sufficient because

Th lymphocytes and 293T cells transfected with GARP present GARP/latent TGF-β1 complexes on their surface but do not produce active TGF-β1 (Stockis *et al.* 2009b). This suggests that at least one additional protein expressed in Tregs, but not in Th lymphocytes or 293T cells, cooperates with GARP to activate latent TGF-β1.

Here, we sought to identify proteins that bind to GARP, are expressed at higher levels in Tregs than in Th cells, and contribute to the regulation of TGF-β1 production by human Tregs. We identified Lysosomal-Associated Transmembrane 4B (LAPTM4B), and showed that LAPTM4B is a negative regulator of TGF-β1 production in human Tregs.

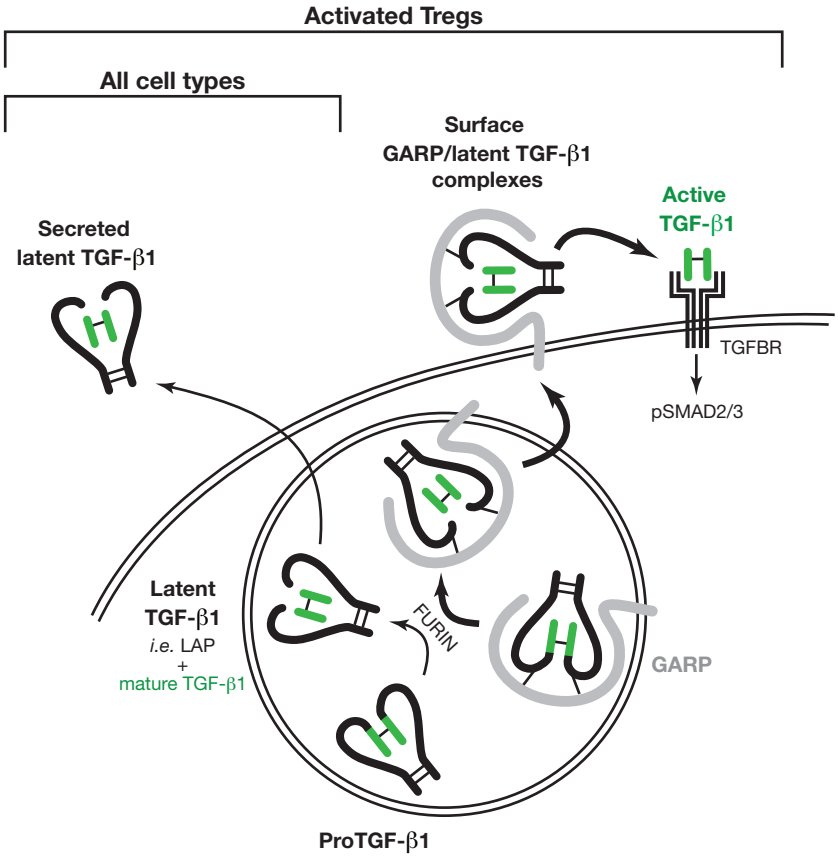


Figure 1. GARP and production of TGF-β1.
Production and processing of TGF-β1 in cells expressing GARP (Tregs) or not (other cell types). Double curves represent cell membranes, with the secretory pathway as a circle. In latent TGF-β1, the Latency-Associated Peptide (LAP) is represented as thick black lines, and the mature TGF-β1 as green lines. Thin black lines represent disuflide bonds. Although not shown in this figure, in Tregs latent TGF-β1 can also be secreted as a soluble form in which it is covalently associated to GARP (Gauthy *et al.* 2013).

Results

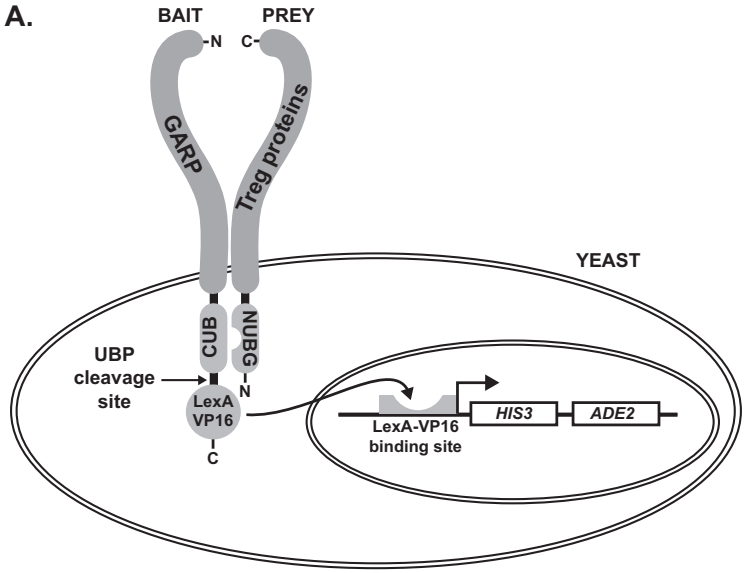
Identification of GARP binding proteins in a yeast two-hybrid system

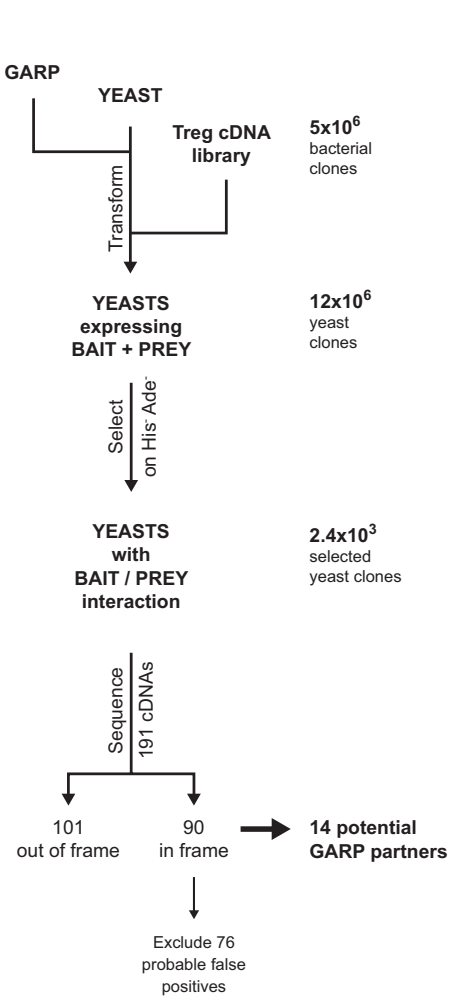
To identify proteins binding to GARP in human Tregs, we used the yeast two-hybrid system known as "split-ubiquitin". As shown in Figure 2A, the bait is a transmembrane protein fused to the C-terminal moiety of Ubiquitin (CUB) and to the LexA-VP16 hybrid transcription factor. Due to the fusion, LexA-VP16 is retained in the cytoplasm and cannot exert transcriptional activity. The prey cDNA library encodes proteins fused to the N-terminal moiety of Ubiquitin (NUBG), which harbors a mutation that prevents spontaneous association with CUB. In yeasts transfected with bait and prey, reconstitution of a functional Ubiquitin by reassembly of CUB and NUBG occurs only if bait and prey interact. Reconstituted Ubiquitin is recognized by Ubiquitin specific proteases (UBPs) that will cleave off LexA-VP16 from the bait, allowing its migration to the nucleus, transcription of *HIS3* and *ADE2* reporter genes and growth of yeast on media lacking histidine and adenine (Figure 2A). It must be noted that productive bait/prey interactions occur only if CUB and NUBG are both located in the cytosol. Thus, when NUBG is fused to the N-terminal extremity of preys, only cytosolic proteins or type II membrane proteins can be identified using this approach. Here, we used GARP-CUB-LexA-VP16 as a bait and a prey cDNA library constructed from 3 different human Treg clones stimulated during 24 hours with anti-CD3 and anti-CD28 antibodies (Stockis *et al.* 2009a).

We transformed the Treg cDNA library in yeasts expressing GARP-CUB-LexA-VP16 (Figure 2B). Selection on HIS^- ADE^- medium yielded 2371 yeast clones in which the bait interacted with a prey. We sequenced prey cDNAs from 191 selected clones. Of these, 101 encoded out of frame fusions or contained non-coding genomic DNA, whereas 90 encoded proteins fused in frame with the NUBG coding sequence. The list of these cDNAs was compared to that of cDNAs frequently isolated in unrelated split-ubiquitin screens (Dualsystems and our unpublished data) to exclude 45 cDNAs, which were likely to be false positives. We further excluded 31 other cDNAs encoding proteins involved in protein translation or post-translational modifications, or proteins that do not have a predicted N-terminus in the cytosol. Altogether, this left us with 14 cDNAs encoding proteins that potentially interact with GARP.

We examined the expression profiles of the corresponding mRNAs in stimulated human Treg and Th clones, using available expression microarray data (Figure 2C, (Stockis *et al.* 2009a)). Two mRNAs, *LAPTM4B* and *CD9*, were expressed at >7-fold higher levels in Treg *versus* Th clones

(Figure 1C). As CD9 expression in Tregs was low and could not be confirmed to be higher than in Th by RT-qPCR, we focused our analyses on LAPTM4B.





C.

Potential GARP partners		Mean expression in Treg clones	Mean expression in Th clones
LAPTM4B	**lysosomal-associated transmembrane protein 4B**	53	7
CD9	tetraspanin 29	1	0
C18orf32	chromosome 18 open reading frame 32	*nd*	*nd*
DPH3	DPH3 homolog isoform 1	*nd*	*nd*
CCDC127	coiled-coil domain-containing protein 127	*nd*	*nd*
TRAM1	translocating chain-associated membrane protein 1	51	54
HSP90	heast shock protein HSP 90-alpha	166	185
HTATIP2	oxydoreductase HIV-1 Tat interactive protein 2	20	15
NPM1	nucleaophosmin	179	183
PIN4	peptidyl-prolyl cis-trans isomérase NIMA-interacting 4 isoform 1	9	5
PIGP	phosphatidylinositol glycan anchor biosynthesis, class P	17	14
PPIA	peptidylprolyl isomérase A (cyclophilin A)	290	315
TPI1	triosephosphate isomérase 1	74	71
KIAA0368	proteasome-associated protein ECM29 homolog	8	7

Figure 2. Identification of potential GARP binding partners using a two-hybrid split-ubiquitin system in yeast.
A. Schematic representation of the yeast two-hybrid «split-ubiquitin» system.
B. Screening of a Treg cDNA library to identify GARP binding partners using the split-ubiquitin system in yeast.
C. List of 14 potential GARP partners and their expression levels in TCR-stimulated human Treg and Th clones (means of 3 or 2 clones, respectively) as measured with Affymetrix expression microarrays (Stockis *et al.* 2009a).

Two mRNA variants of LAPTM4B are expressed in Tregs

According to public databases, transmembrane protein LAPTM4B is encoded by a gene reported to comprise 7 exons: E1a and E2 to E7. The mRNA variant isolated from our prey cDNA library contained an alternative first exon, which we named E1b (Figure 3).

To determine which *LAPTM4B* mRNA variants are expressed in human Tregs, we analyzed stimulated Treg clones by RT-PCR. Sense primer "A1", located immediately upstream of the described start codon in the RefSeq sequence, combined to antisense primer "R" did not yield a PCR amplification product, indicating that the RefSeq *LAPTM4B* mRNA is not expressed in human Tregs (Figure 3 and data not shown). Primer "A2", straddling a described alternative start codon (Shao *et al.* 2003), and "R" amplified a product of 677 bp, indicating that an mRNA variant that we will refer to as "*Variant a*" (*Va*) is expressed in human Tregs. The precise 5' extremity of *Va* is not known. Finally, primers "B" and "R" yielded a product of 608 bp, indicating that human Tregs express a second mRNA variant that we named "*Variant b*" (*Vb*). We used 5'RACE to identify the 5' extremity of *Vb* (Figure 3).

LAPTM4B variants have multiple AUG start codons in frame with the longest open reading frame, and thus potentially encode several LAPTM4B isoforms, which differ only by the length of their N terminus (Figure 3). Two isoforms were previously described (Shao *et al.* 2003): LAPTM4B$_{iso35}$ (35 kDa) is encoded by the RefSeq variant only, whereas LAPTM4B$_{iso24}$ (24 kDa) is encoded by RefSeq and *Va* variants. *LAPTM4B Vb* can encode a previously unknown 20 kDa isoform, named LAPTM4B$_{iso20}$, which lacks the first 66 amino-acids of LAPTM4B$_{iso24}$. RefSeq and *Va* variants also encode LAPTM4B$_{iso20}$. Because Tregs express *Va* and *Vb* but not the RefSeq variant, we hypothesized that LAPTM4B$_{iso24}$ and LAPTM4B$_{iso20,}$ but not LAPTM4B$_{iso35}$, are present in Tregs. We could not verify this hypothesis due to the lack of appropriate antibodies.

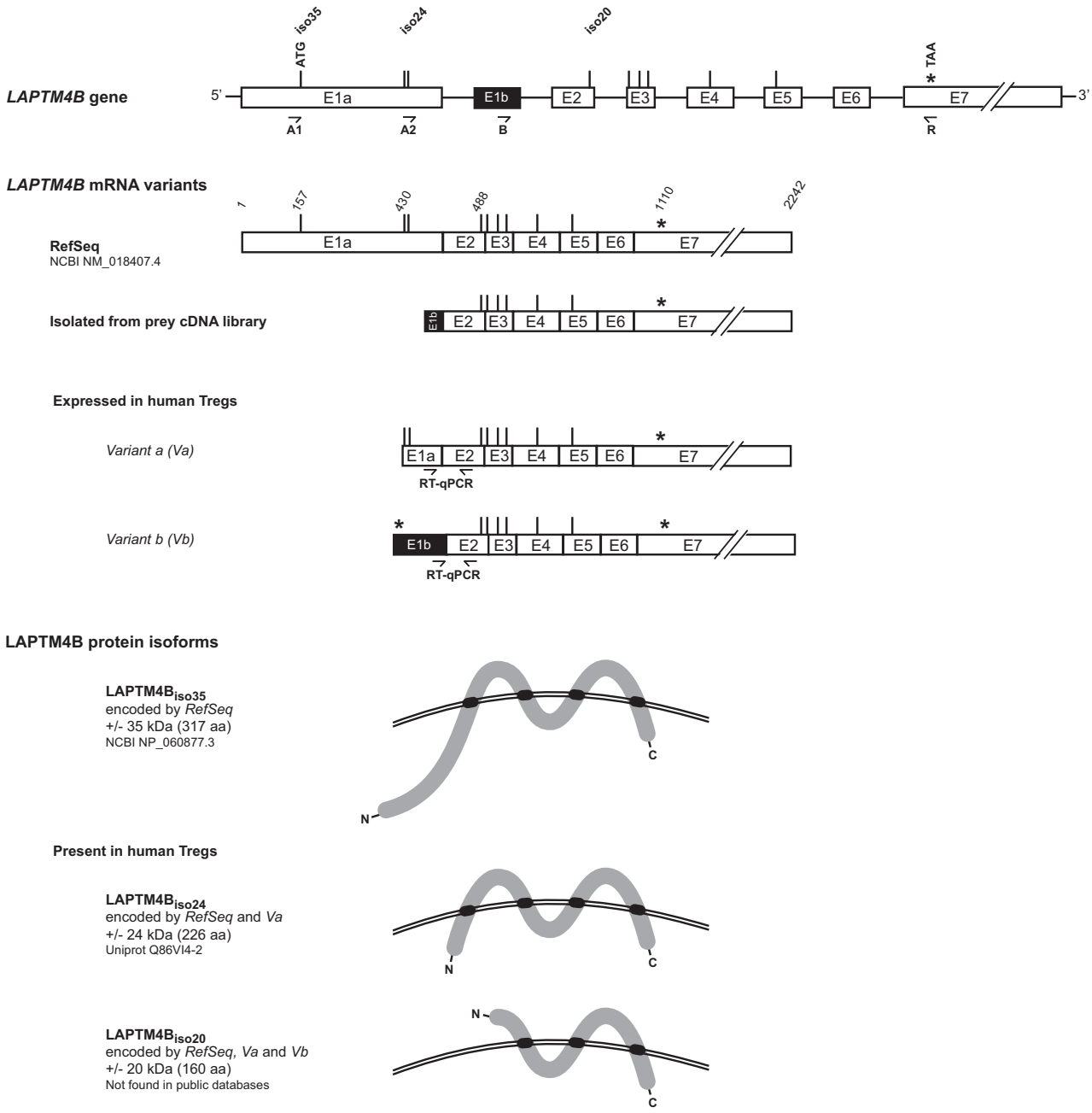


Figure 3. *LAPTM4B* mRNA variants expressed in human Tregs encode two protein isoforms that differ at their N-terminus.

Schematic representations of *LAPTM4B* gene, mRNA variants and proteins isoforms. Exons are represented as boxes, introns as thin horizontal lines. All start and stop codons in frame with the reported start of the RefSeq mRNA are indicated by vertical lines and by *, respectively. Primers used for RT-PCR are indicated by arrows. LAPTM4B protein isoforms are represented as thick grey lines, with transmembrane domains highlighted in black. Double curved lines represent the plasma membrane. Start codons for the translation of the various protein isoforms are indicated above the representation of the gene.

Va and *Vb* mRNA variants of *LAPTM4B* are expressed at higher levels in Tregs than in Th cells

We used RT-qPCR to measure expression levels of *LAPTM4B Va* and *Vb* mRNAs in resting or stimulated Treg and Th clones, using primers illustrated in Figure 3. As shown in Figure 4, *Va* is expressed at higher levels than *Vb* in both types of T cells. Expression of *Va* was significantly higher in Treg than in Th clones, both at rest and after stimulation (on average 1,9-fold higher at rest, and 2,7-fold higher after stimulation). Expression of *Vb* was detected in stimulated Treg clones only (>3 copies/10^5 EF-1 copies in 5 out of 7 stimulated Treg clones).

Altogether, *LAPTM4B Va* and *Vb* variants are expressed at higher levels in stimulated human Tregs than in resting Tregs and in resting or stimulated Th cells.

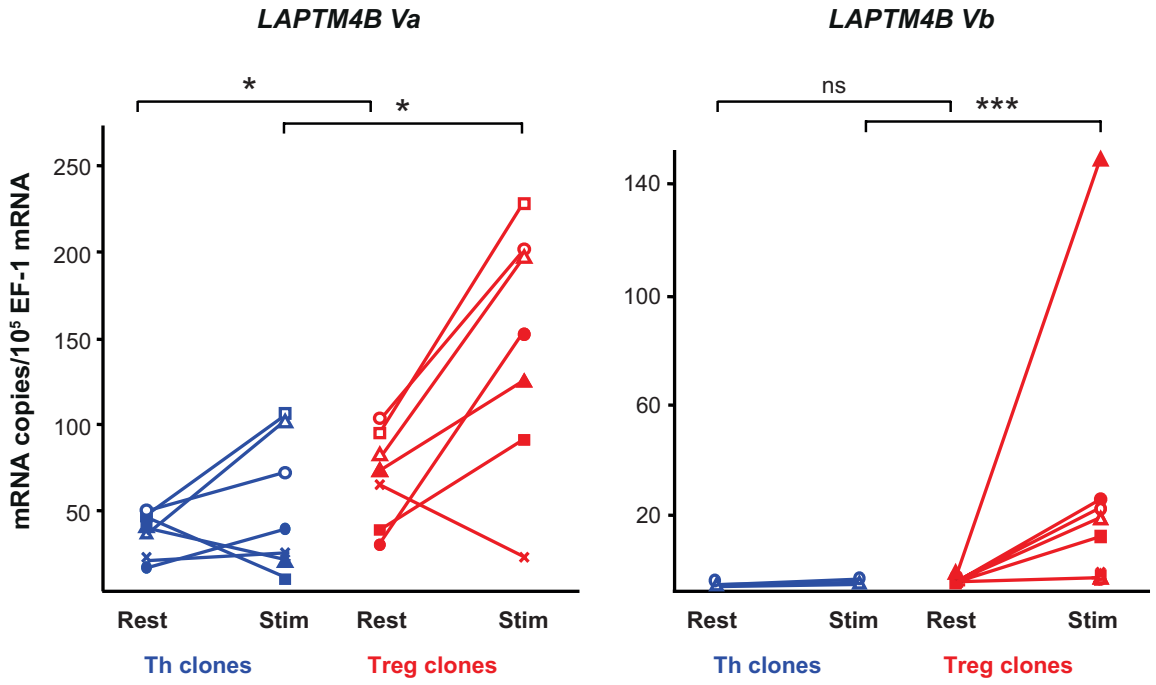


Figure 4. *LAPTM4B Va* and *Vb* are expressed at higher levels in human Tregs by comparison to Th cells. Expression of the indicated *LAPTM4B* mRNA variants was measured by RT-qPCR in 7 Th clones (blue lines) and 7 Treg clones (red lines), at rest or 24 hours after stimulation with anti-CD3 and anti-CD28 antibodies. * pvalue < 0,05; *** pvalue < 0,001 by Mann-Withney analysis.

Confirmation of the interaction between GARP and LAPTM4B in mammalian cells

To test if GARP and LAPTM4B interact in mammalian cells, we used a protein complementation assay in which two distinct, inactive fragments of humanized Gaussia Luciferase (hGLuc1 and hGLuc2) are fused to the C-terminus of candidate proteins and expressed in 293T cells (Remy and Michnick 2006). Luciferase activity is recovered only if candidate proteins interact with each other. We co-transfected constructs encoding hGLuc1 fused to N-terminally HA-tagged GARP, and hGLuc2 fused to HA-tagged LAPTM4B. Two *LAPMT4B* constructs were tested, encoding only LAPTM4B$_{iso20}$, or both LAPTM4B$_{iso20}$ and LAPTM4B$_{iso24}$ (*i.e.* LAPTM4B$_{iso20/24}$). In the latter construct, only LAPTM4B$_{iso24}$ is tagged with HA. All HA-tagged proteins were expressed at similar levels as determined by Western Blot (Figure 5, top panel). High luciferase activity was detected in cells co-expressing GARP and LAPTM4B$_{iso24/20}$, or GARP and LAPTM4B$_{iso20}$ (Figure 5, bottom panel). As expected, no or very low activity was detected in cells expressing any protein alone, or in cells co-expressing GLuc fragments fused to GARP and EPOR, or to LAPTM4B and EPOR. EPOR was taken here as a negative control.

These results show that GARP and LAPTM4B isoforms interact in mammalian cells, and that the first 66 amino acids of LAPTM4B$_{iso24}$ (Figure 2) are not required for this interaction.

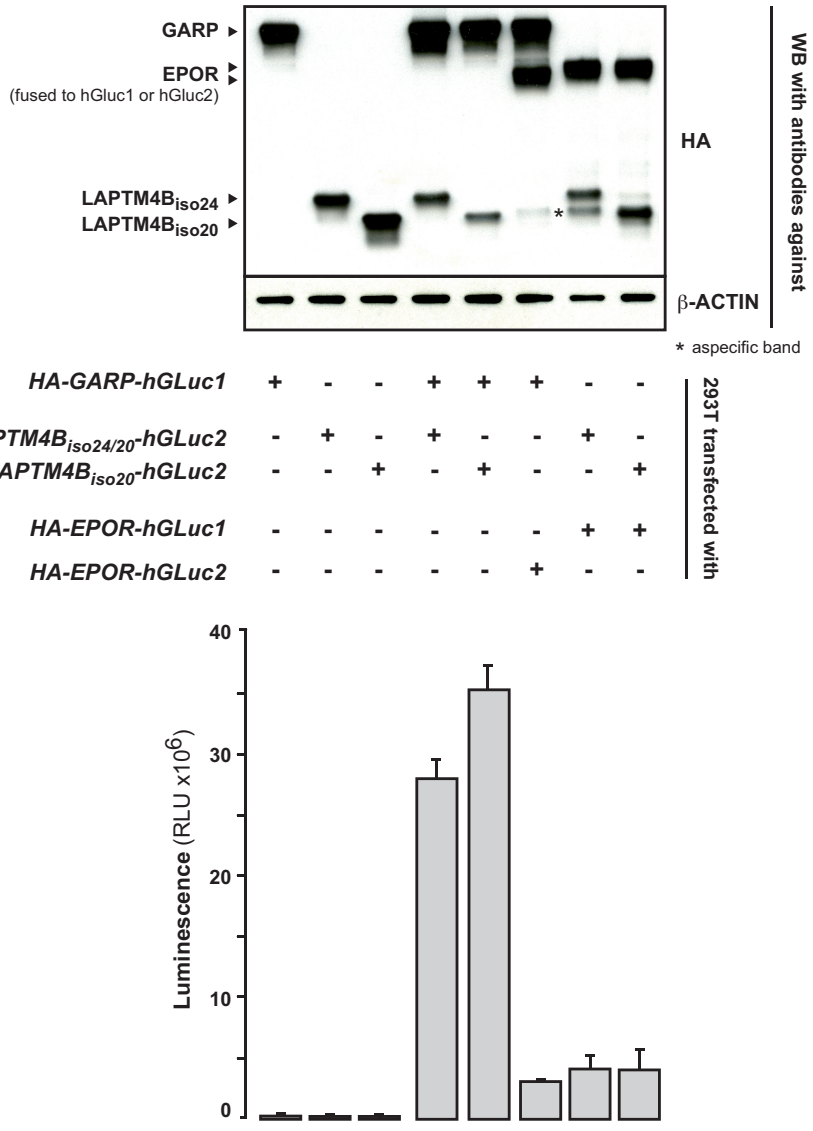


Figure 5. GARP interacts with LAPTM4B in mammalian cells.
293T cells were transfected with constructs encoding GARP, LAPTM4B or negative control EPOR, fused to hGLuc1 or hGLuc2 as indicated. The top panel shows Western Blot analysis of transfected cells. Bar graphs in the bottom panel show luciferase activity in live cells 24 hours after transfection. One experiment representative of three independent experiments is shown.

Overexpression of LAPTM4B in 293T cells decreases cleavage of proTGF-β1, secretion of latent TGF-β1 and surface presentation of GARP-latent TGF-β1 complexes

We used constructs encoding both LAPTM4B$_{iso24}$ and LAPTM4B$_{iso20}$ to evaluate whether LAPTM4B influences the regulation of TGF-β1 production by GARP in transfected 293T cells.

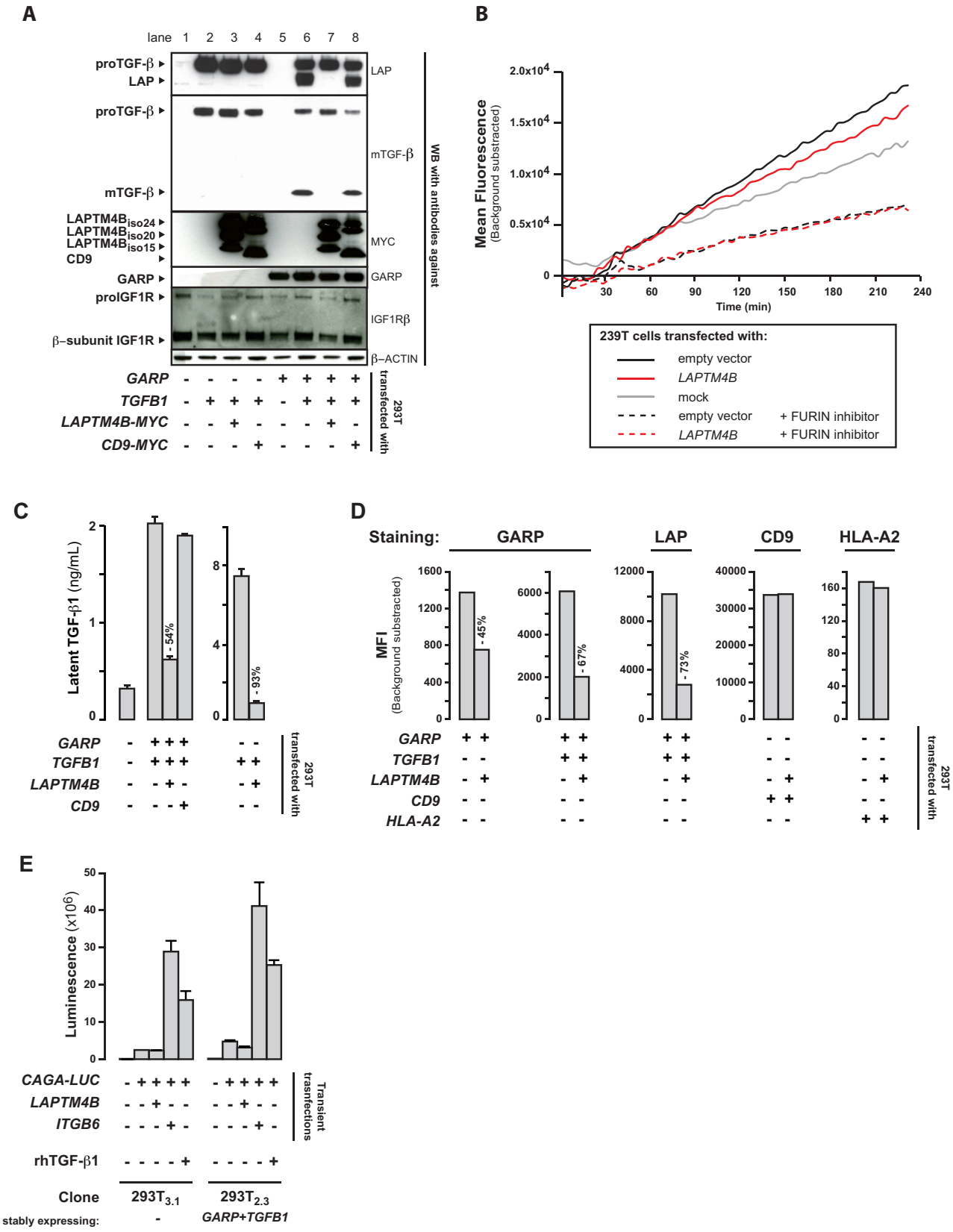
First, we examined by Western Blot whether LAPMT4B affects the cleavage of proTGF-β1 into latent TGF-β1 (Figure 6A). In cells transfected with *TGFB1* alone (lane 2), proTGF-β1 is abundant but LAP or mature TGF-β1 (mTGF-β1) are not detected, indicating that cleavage of the precursor does not occur at high levels. Co-transfection of *TGFB1* with *LAPTM4B* (lane 3) does not increase cleavage. In contrast and as expected, co-transfection of *TGFB1* with *GARP* (lane 6) induces cleavage as evidenced by the detection of abundant LAP and mature TGF-β1. Co-transfection of LAPTM4B (lane 7) abolished this GARP-induced cleavage, an effect that was not observed upon co-transfection of *CD9* (lane 8), taken here as a negative control. As proTGF-β1 cleavage depends on FURIN, decreased proTGF-β1 cleavage in the presence of LAPTM4B could result from a direct effect on FURIN activity. We examined whether the cleavage of endogenous proIGF1R, another known substrate of FURIN, into its β-subunit was also reduced in 293T cells transfected with *LAPTM4B*, but this was not the case (Figure 6A). We also measured FURIN activity on an exogenous fluorogenic substrate, and the transfection of *LAPTM4B* did not decrease FURIN activity in this assay neither (Figure 6B). We conclude that LAPTM4B reduces the cleavage of proTGF-β1 in the presence of GARP, without reducing overall FURIN activity.

We next examined whether this decreased cleavage of proTGF-β1 resulted in a reduction of latent TGF-β1 secretion. We indeed found 54% less latent TGF-β1 in the supernatants of 293T cells transfected with *GARP*, *TGFB1* and *LAPTM4B*, by comparison to cells transfected with *GARP* and *TGFB1* only (Figure 6C). This effect was not observed with a negative control (*CD9*). Interestingly, the reduction of latent TGF-β1 secretion by LAPTM4B was also observed in the absence of GARP, indicating that the regulatory effect of LAPTM4B on latent TGF-β1 secretion is not dependent on its interaction with GARP (Figure 6C). Incidentally and as expected, secretion of latent TGF-β1 was lower in the presence of GARP because GARP tethers latent TGF-β1 at the 293T cell surface (Wang *et al.* 2012, Gauthy *et al.* 2013).

We then used flow cytometry to examine whether LAPTM4B influenced surface levels of GARP or GARP/latent TGF-β1 complexes on transfected 293T cells. GARP levels were reduced by 45% upon co-transfection with *LAPTM4B* (Figure 6D). In cells transfected with *GARP* and *TGFB1*, co-transfection of *LAPMT4B* reduced surface GARP by 67% and surface LAP by 73%. Surface levels

of unrelated proteins such as HLA-A2 or CD9 were not affected by co-expression of LAPTM4B. Thus, LAPTM4B reduces surface levels of GARP and GARP/latent TGF-β1 complexes.

Finally, we tested whether LAPTM4B regulates latent TGF-β1 activation (Figure 6E). We used a reporter assay in which the luciferase gene is under the control of a *CAGA* promoter activated by SMAD2/3 transcription factors in response to TGF-β1 signals (Dennler *et al.* 1998). We transiently co-transfected the *CAGA-LUC* reporter with *LAPTM4B* in clones of 293T cells stably expressing or not *GARP* and *TGFB1*. Co-transfection of LAPTM4B did not increase luciferase activity above background (*i.e.* transfection of reporter alone), indicating that LAPTM4B expression does not activate latent TGF-β1. This was true also in a clone stably expressing GARP, which is not sufficient to induce active TGF-β1 production (Figure 6E). As expected, high luciferase activity was induced by transfection of integrin β6, or by addition of recombinant active TGF-β1, both taken here as a positive controls.We conclude that in transfected 293T cells LAPTM4B decreases the cleavage of proTGF-β1, the secretion of latent TGF-β1 and the surface presentation of GARP/latent TGF-β1 complexes, but does not activate latent TGF-β1 in cooperation with GARP. Thus LAPTM4B appears to be a negative regulator of TGF-β1 production in these cells.


A
lane 1 2 3 4 5 6 7 8
proTGF-β
LAP
proTGF-β
mTGF-β
mTGF-β
LAPTM4Biso24
LAPTM4Biso20
LAPTM4Biso15
CD9
GARP
proIGF1R
β–subunit IGF1R
LAP
mTGF-β
MYC
GARP
IGF1Rβ
β–ACTIN
WB with antibodies against
GARP - - - - + + + +
TGFB1 - + + + - + + +
LAPTM4B-MYC - - + - - - + -
CD9-MYC - - - + - - - +
293T transfected with
B
Mean Fluorescence
(Background substracted)
2.0x10^4
1.5x10^4
1.0x10^4
0.5x10^4
0
30 60 90 120 150 180 210 240
Time (min)
239T cells transfected with:
empty vector
LAPTM4B
mock
empty vector + FURIN inhibitor
LAPTM4B + FURIN inhibitor
C
Latent TGF-β1 (ng/mL)
2
1
0
8
4
0
- 54%
- 93%
GARP - + + + - -
TGFB1 - + + + + +
LAPTM4B - - + - - +
CD9 - - - + - -
293T transfected with
D
Staining: GARP LAP CD9 HLA-A2
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1600
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CD9 - - - - - - + + - - - -
HLA-A2 - - - - - - - - + + - -
293T transfected with
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Luminescence (x10^6)
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CAGA-LUC - + + + + - + + + +
LAPTM4B - - + - - - - + - -
ITGB6 - - - + - - - - + -
Transient transfections
rhTGF-β1 - - - - + - - - - +
Clone 293T3.1 293T2.3
stably expressing: - GARP+TGFB1

Figure 6. LAPTM4B decreases cleavage of proTGF-β1, surface presentation of GARP/TGF-β1 complexes, and secretion of latent TGF-β1.

A. Cell lysates of transfected 293T cells were analysed by Western Blot.

B. Specific FURIN activity was measured in cell lysates 24 hours after transfection, by capturing FURIN on immobilized anti-FURIN antibody, then incubating captured proteins with the fluorogenic substrate pERTKR-AMC. Graphs show mean fluorescence intensity (MFI) at the indicated time after addition of the substrate. The FURIN inhibitor Dec-RVKR-CMK was added to some conditions to verify the specificity of the assay.

C. Concentration of latent TGF-β1 in the acid-treated supernatants was measured by ELISA.

D. Surface levels of GARP, LAP, CD9 and HLA-A2 in transiently transfected 293T were assessed by FACS.

E. Clones of 293T cells stably expressing or not human GARP and human TGF-β1 were transfected with LAPTM4B or ITGB6 together with CAGA-LUC reporter. Luciferase activity was measured 24 hours after transfection. Recombinant human (rh) TGF-β1 was added during 6 hours at 4 ng/mL in the positive control condition, as indicated.

For A, C, D and E, 293T cells were transfected with *TGFB1* construct coding for the full length pre-proTGF-β1 that is processed in 293T like in other cell types.

LAPTM4B silencing in human polyclonal Tregs increases surface presentation of GARP/TGF-β1 complexes and secretion of latent TGF-β1

We next wished to evaluate whether LAPTM4B plays a role in human Tregs. Polyclonal Tregs were obtained by a short *in vitro* amplification of blood $CD4^+CD25^+CD127^{lo}$ cells. They contained 47 % of cells with demethylated *FOXP3i1*. They were transfected with siRNAs and stimulated or not through their TCR to induce TGF-β1 activation. *siLAPMT4B* reduced the expression of *LAPTM4B Va* and *Vb* by ≥85% (Figure 7A). It increased by 41-53% the secretion of latent TGF-β1 by resting and stimulated Tregs (Figure 7C), and by >75% the surface presentation of GARP and latent TGF-β1 on stimulated Tregs (Figure 7D). As expected, *siLAPMT4B* did not reduce the activation of TGF-β1 by stimulated Tregs (Figure 7B).

These results confirm that LAPTM4B is a negative regulator of TGF-β1 production in human Tregs.

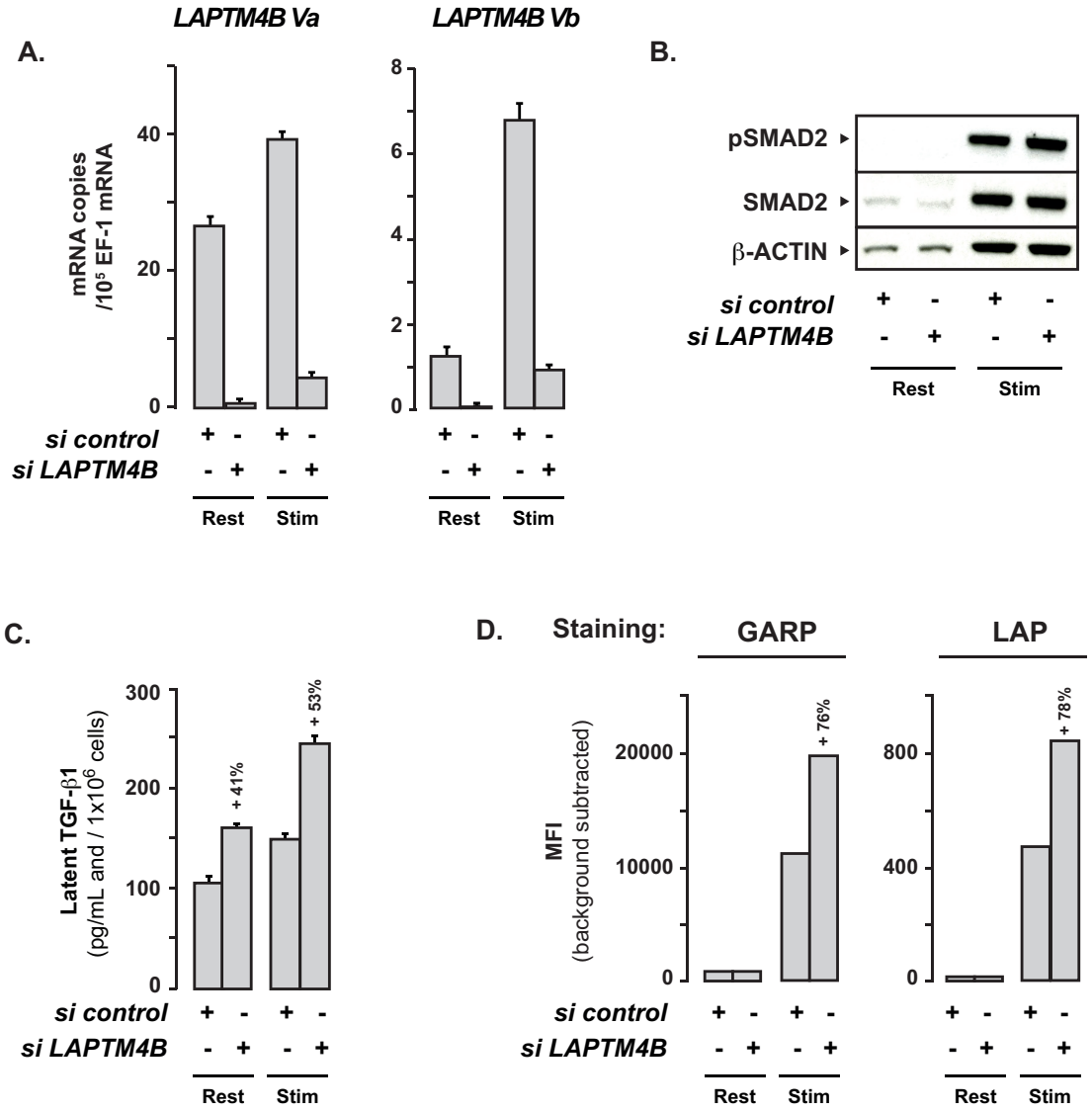

Figure 7. LAPTM4B decreases GARP surface levels and TGF-β secretion in polyclonal Tregs.
Polyclonal human Tregs were transfected with the indicated siRNAs, left resting or stimulated with anti-CD3/CD28 coated beads, and collected 78 hours after transfection. One experiment representative of three independent experiments is shown.

A. RT-qPCR analysis of *LAPTM4B Va* and *Vb* expression.

B. Western Blot analysis of cell lysates with anti-pSMAD2, anti-total SMAD2 and anti-β-ACTIN antibodies. Detection of pSMAD2 indicates active TGF-β production.

C. ELISA on acid-treated supernatants to measure total TGF-β secretion. As no active TGF-β is detected in this assay, total TGF-β corresponds to latent TGF-β.

D. Surface staining of GARP and LAP on stimulated cells was assessed by FACS using anti-GARP and anti-LAP antibodies respectively.

Discussion

LAPTM4B belongs to a family of 3 glycoproteins with several transmembrane domains. The two other members of the family are LAPTM4A and LAPTM5, with 46% and 23% sequence identities with LAPTM4B, respectively. Little is known about the physiological function of LAPTM4B. In tumors, it appears to play an oncogenic role: high LAPTM4B levels are associated to poor prognosis in many types of cancers (Zhou *et al.* 2007, Yang *et al.* 2008, Zhou *et al.* 2008, Yang *et al.* 2010, Yang *et al.* 2010, Yin *et al.* 2011, Kang *et al.* 2012, Zhang *et al.* 2012, Tang *et al.* 2014, Zhang *et al.* 2014), and *in vitro* and *in vivo* analyses of cells in which *LAPTM4B* was overexpressed or silenced indicate that LAPTM4B favours proliferation, migration, invasion, tumorigenesis and metastasis (Liu *et al.* 2009, Yang *et al.* 2010). LAPTM4B may also contribute to chemotherapy resistance in breast cancer (Li *et al.* 2010).

Our results are the first to describe a function for LAPTM4B in the immune system: the decrease of latent TGF-β1 production, secretion and surface presentation in Tregs. Downregulation of surface presentation appears to result from a direct effect of LAPTM4B on surface GARP levels, as we show that LAPTM4B interacts with GARP, a receptor for latent TGF-β1, and reduces surface GARP levels in transfected 293T cells in the absence of TGF-β1. Another LAPTM family member was reported to interact and to downregulate surface receptors on immune cells. Murine Laptm5 binds to proteins of the T and B cell antigenic receptor complexes (TCR and BCR, respectively), and surface levels of TCR and BCR were higher on activated *Laptm5*$^{-/-}$ lymphocytes than in activated wild-type lymphocytes (Ouchida *et al.* 2008, Ouchida *et al.* 2010). C-terminal polyproline-tyrosine (PY) motifs target Laptm5 to lysosomes and are required for the Laptm5-mediated downmodulation of surface TCR levels, suggesting a mechanism by which Laptm5 promotes lysosomal targeting and degradation of receptors to which it is bound (Pak *et al.* 2006, Ouchida *et al.* 2008). PY motifs are also present in the C-terminus of LAPTM4B (Ouchida *et al.* 2008, Milkereit and Rotin 2011). It is therefore possible that LAPTM4B downregulates GARP surface levels in human Tregs through a similar mechanism. However, we observed in transfected cells that GARP and LAPTM4B co-localize mostly in the median Golgi, and that the localization of GARP was not modified in the presence of LAPTM4B (unpublished data). It is worth noting that the downregulation of latent TGF-β1 secretion by LAPTM4B may occur through another mechanism, independent from GARP, as it was also observed in transfected 293T cells in the absence of GARP.

Two isoforms of LAPTM4B, LAPTM4B$_{iso35}$ and LAPTM4B$_{iso24,}$ were described previously (Shao *et al.* 2003). Here we identify in stimulated human Tregs an mRNA variant coding for a third, shorter isoform LAPTM4B$_{iso20}$. Tregs also contain LAPTM4B$_{iso24,}$ but not LAPTM4B$_{iso35}$. We showed that LAPTM4B$_{iso20}$ interacts with GARP, indicating that the 66 N-terminal amino-acids of LAPTM4B$_{iso24}$ are not required for the interaction. We could not determine whether LAPTM4B$_{iso24}$ also interacts with GARP, as all our constructs encoding LAPTM4B$_{iso24}$ encode also LAPTM4B$_{iso20}$. We do not know whether LAPTM4B$_{iso24}$ or LAPTM4B$_{iso20}$, or both regulate GARP and TGF-β1 production in Tregs, because our siRNA approach silenced all these *LAPTM4B* mRNA variants.

We report that *LAPTM4B* expression is higher in human Tregs than in Th cells. Overexpression of *LAPTM4B* in human or mouse Tregs, as well as in non-Treg cells transduced with *FOXP3,* was observed in published expression microarray data sets (Herman *et al.* 2004, Ocklenburg *et al.* 2006, Williams and Rudensky 2007), suggesting that gene *LAPTM4B* might be transcriptionally regulated by FOXP3.

In conclusion, we uncovered an inhibitory mechanism of GARP and TGF-β1 production mediated by LAPTM4B in human Tregs. Mechanisms of immunosuppression by human Tregs include the production of active TGF-β1, released from GARP/latent TGF-β1 complexes at the Treg surface (Tran *et al.* 2009, Stockis *et al.* 2009b). Tregs are potent inhibitors of immune responses, and excessive or insufficient Treg function is implicated in various human diseases. Pharmacological inhibition of LAPTM4B could increase TGF-β1 production, and hence Treg function, in patients suffering from autoimmune disease or undergoing allograft rejection.

Materials and Methods

Ethics statement

Experiments with human cells were approved by our Institution's ethics committee (Commission d'Ethique Biomédicale Hospitalo-Facultaire de l'Université catholique de Louvain), under registration number B403201110966. Written informed consent for the use of blood samples was not always obtained, in accordance with the Belgian law of 19 December 2008 which states that, in the absence of written opposition by the patient, consent is considered given for residual body material. This applies to blood samples from hemochromatosis patients. No patient opposed the use of blood samples. Data obtained from blood samples were analyzed anonymously.

Split-ubiquitin system in yeast

We used the split-ubiquitin system from Dualsystems Biotech AG, according to the manufacturer's instructions. Briefly, *GARP* ORF was cloned into *Sfi* I sites of bait plasmid pBT3-SUC. The resulting plasmid was used to transform and select GARP expressing NMY51 yeasts. A prey cDNA library was synthesized in pPR3N plasmid, starting from 2 µg of total RNA isolated from 3 different human Treg clones stimulated during 24 hours with anti-CD3 and anti-CD28, as described (Stockis *et al.* 2009a). The Treg cDNA library contained $5{,}2 \times 10^6$ independent bacterial colonies, which were collected and used to purify plasmid DNA with the PureLink Plasmid Maxiprep kit (Life Technologies). Library plasmid DNA (28 µg) was transformed in GARP-expressing yeasts. An aliquot of transformed yeasts were selected on SD Leu^-Trp^- medium to assess transformation efficiency. The remaining yeasts were selected on SD $Leu^-Trp^-His^-Ade^-$ to isolate clones transformed with potential GARP interactants.

RT-PCR and RT-qPCR analyses of *LAPTM4B* expression in human Tregs

Total RNA was extracted, reverse transcribed and submitted to PCR or qPCR as previously described (Stockis *et al.* 2009a). qPCR amplifications were done with ABI Prism 7300 Real Time PCR System (Applied Biosystems) under standard conditions: 95°C for 10 min, 45 cycles of 95°C for 15 s, and 60°C for 1 min.

Primer sequences (5' to 3'):

A1: ATTAACAAGGATCCGCGATGACGTCACGGACTCGG

A2: ATTAACAAGGATCCGCGATGAAGATGGTCGCGCCC

B: GCTCTATGGTGCCTGGGCCA

R: AACTGATTCTCGAGCGCAGACACGTAAGGTGGCGG

qPCR for LAPTM4B Va :

Sens: ACCATCCTGCTCGGCGTCTG ;

Anti-sens : CGGATCAGCCAGGGCACTCAAT

qPCR for LAPTM4B Vb :

Sens: GCTCTATGGTGCCTGGGCCA ;

Anti-sens: CGGATCAGCCAGGGCACTCAAT ;

FAM-TAMRA probe : GTACCACAGCATTGATGATCTCATTCCCC

T cell electroporation

Human polyclonal cell population enriched in Tregs ($CD4^+CD25^+CD127^{lo}$ cells) were isolated from hemochromatosis donors as previously described (Gauthy *et al.* 2013). Expanded Tregs (10^7 cells) were electroporated using the "Unstimulated Human T Cells 4D-Nucleofector solution" , mixed with siRNAs (5 µM of control siRNA #4390843 or siLAPTM4B #4392420 from Ambion) and electroporated in a 4D-Nucleofector instrument (Lonza). Immediately after transfection, cells were re-stimulated with anti-CD3/CD28 coated beads (Dynabeads Human T-Activator CD3/CD28, Life Technologies) in IMDM supplemented with 10% human serum, L-arginine, L-asparagine, L-glutamine, β-mercaptoethanol ($5x10^{-5}$M), methyl-tryptophane (200 µM). Analyses were performed 78 hours after transfection.

Cell transfections

293T cells or a 293T cell clone stably expressing human GARP and human TGF-β1 were transiently transfected with plasmids indicated in the figures using the TransIT-LT1 transfection Reagent (Mirus Bio). Cells were analysed 24 hours after transfection. Luciferase activity was measured with the Britelite Plus Reporter Gene Assay System (Perkin Elmer).

Western Blot

Cells were lysed in Laemmli buffer supplemented with 5% β-mercaptoethanol as previously described (Stockis *et al.* 2009a) and submitted to SDS-PAGE and Western Blot with the following primary antibodies, as indicated in the figures: anti-GARP (Enzo Life Sciences #ALX-804-867), anti-TGF-β1 (BD Pharmingen #555052), biotinylated anti-LAP (R&D Systems #BAF246), anti-Myc (ROCHE # 11-667-149-001), anti-PSMAD2 (Cell Signaling #3108), anti-SMAD2 (Cell Signaling #3122), anti-IGF1Rβ (Cell Signaling #3027), anti-HA (Eurogentec #MMS-101R) or anti-β-ACTIN (Sigma #A5441).

FACS analysis

Cells were stained with mouse monoclonal biotinylated anti-GARP antibody (clone MHGARP6, manuscript submitted) followed by streptavidin coupled to PE (BD Pharmingen #554061), anti-LAP antibody coupled to APC (R&D Systems #27232), anti-CD9 antibody coupled to PE (BioLegend #312105), anti-HLA-A2 antibody coupled to FITC (BioLegend #343304). Data were collected on a FACS LSR Fortessa (BD Biosciences) and analyzed with the FlowJo software (Tree Star).

Latent TGF-β1 concentrations in supernatants

Acidified supernatants were analyzed by ELISA according to the manufacturer's instructions (Human TGF-β1 duoset, R&D Systems).

Fluorogenic assay for FURIN-specific activity

Assays were performed as described by Bourne *et al.* (Bourne and Grainger 2011). Briefly, transfected 293T cells were lysed in 5x lysis buffer (500 mM HEPES pH 7.0, 2,5% Triton X-100, 5 mM calcium chloride, 5 mM ß-mercaptoethanol). Lysates containing $3.3x10^5$ cell equivalents were seeded in black opaque 96 well plates pre-coated with a goat polyclonal anti-FURIN antibody (R&D Systems #AF1503). Where indicated, the FURIN Inhibitor I Dec-RVKR-CMK (Calbiochem) was added at a final concentration of 50 µM. The FURIN fluorogenic peptide substrate pERTKR-AMC (R&D Systems) was added at a final concentration of 100 µM. Fluorescence intensities were measured in duplicate wells every 3 min after addition of substrate, on a Victor X2 plate reader (Perkin Elmer), with excitation at 380 nm and emission at 460 nm.

Gaussia Luciferase complementation assay

Protein complementation assays (PCAs) were performed as described by Remy and Micknick (Remy and Michnick 2006). Briefly, 293T cells were transfected with Lipofectamine 2000 Transfection Reagent in triplicate wells (96-well plates) with 0,05 µg of each plasmid (Life Technologies). Medium was changed to DMEM without phenol red (Life technologies) and native coelenterazine (Nano technologies #303) was added on live cells 24 hours after transfection. Luciferase activities were measured on live cells in a VICTOR X Light device (Perkin Elmer).

Acknowledgments

We thank Suzanne Depelchin for excellent editorial help, Nicolas Dauguet for FACS sortings and Maria Panagiotakopoulos and Stéphanie D'Hondt for technical assistance. We acknowledge Vitalina Gryshkova from Ludwig Institute for Cancer Research Ltd (Brussels, Belgium) for kindly sharing complementation assay vectors and technical help. We also thank A. Moustakas for kindly providing the TGF-β signalling reporter construct. We are grateful to Donatienne Tyteca for the help with immunofluorescence analysis and confocal microscopy.

2. Identification of proteins interacting with GARP in human Tregs by immunoprecipitation followed by mass spectrometry

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* These authors contributed equally to the work.

Abstract

Human regulatory T lymphocytes (Tregs) are a subset of T lymphocytes that inhibit immune responses by producing the immunosuppressive cytokine TGF-β1. Production of active TGF-β1 is a highly regulated process. In human Tregs it is controlled by GARP, a surface protein that is not expressed on other T lymphocytes (Th cells or CTLs). GARP interacts with the latent inactive form of TGF-β1, and is required for activation of the cytokine close to the Treg surface. Lentiviral-mediated expression of GARP in Th cells is not sufficient to induce TGF-β1 activation, and we thus hypothesized that additional Treg proteins interacting with GARP/TGF-β1 complexes are required for this activation. To identify such proteins, we analyzed proteins co-immunoprecipitated with GARP in human Tregs by tandem mass spectrometry. We identified proteins candidates encoded by 34 different genes. Of these, 7 were found expressed at higher levels in Tregs than in Th cells by RT-qPCR. We are in the process of cloning the 7 candidates to test their ability to activate TGF-β1 in a luciferase reporter assay. At the present time, 4 candidates were tested but none was found to activate TGF-β1 in this system.

Introduction

Human regulatory T cells (Tregs) are a subset of $CD4^+$ T lymphocytes with immunosuppressive functions. They prevent autoimmune pathology by inhibiting auto-reactive T cells, but they also play detrimental roles in cancer patients by inhibiting anti-tumor immune responses (Sakaguchi *et al.* 2010, Josefowicz *et al.* 2012). Pharmacological targeting of Treg-mediated immunosuppression could be beneficial in several human pathologies. Multiple mechanisms of Treg suppression have been described in mice, where Tregs can be unambiguously identified based on the expression of transcription factor Foxp3. Which mechanisms play a role in humans is more difficult to study, notably because FOXP3 is also expressed in activated non-regulatory T cells, precluding the use of this protein marker to identify Tregs in humans (Sakaguchi *et al.* 2010). Stable FOXP3 expression, in contrast, is a hallmark of Tregs in both humans and mice. It is ensured by demethylation of a regulatory region in the *FOXP3* gene called *FOXP3i1*, CNS2 or TSDR (Baron *et al.* 2007, Floess *et al.* 2007, Polansky *et al.* 2008, Stockis *et al.* 2009a, Zheng *et al.* 2010).

Using clones of human Tregs characterized by a demethylated *FOXP3i1*, we showed that human Tregs, but not other types of T cells (Th cells or CTLs) produce the active form of TGF-β1, a cytokine with well-known immunosuppressive functions (Stockis *et al.* 2009a, Oh and Li 2013). We also showed that TGF-β1 production by human Tregs mediates their immune suppressive function *in vitro* and *in vivo* (Cuende *et al.* manuscript submitted, Stockis *et al.* 2009a). Targeting TGF-β1 production by human Tregs could therefore serve to modulate their function in patients with autoimmune disease or cancer.

Most cells produce the latent inactive form of TGF-β1, whereas production of the active cytokine is highly regulated and restricted to a few cell types. In Tregs, active TGF-β1 production is regulated by GARP, a transmembrane protein that is not expressed by other types of T cells (Cuende *et al.* manuscript submitted, Tran *et al.* 2009, Stockis *et al.* 2009b). GARP covalently binds the homodimeric TGF-β1 precursor called proTGF-β1, and favors its cleavage by FURIN into an N-terminal fragment called LAP (Latency Associated Peptide) and a C-terminal fragment corresponding to mature TGF-β1 (Wang *et al.* 2012, Gauthy *et al.* 2013). LAP and mature TGF-β1 remain non-covalently associated in a so-called latent TGF-β1 complex, in which the mature cytokine is prevented from binding to its receptor by LAP. To exert activity, mature TGF-β1 must be released from LAP, a process referred to as “TGF-β activation” (Travis and Sheppard 2014). GARP presents latent TGF-β1 at the Treg surface. Using blocking anti-GARP antibodies, we recently showed that GARP is also required for TGF-β1 activation by Tregs (Cuende *et al.*

manuscript submitted). However, lentiviral-mediated expression of GARP in Th cells is not sufficient to induce TGF-β activation (Stockis *et al.* 2009b). We hypothesized that additional Treg protein(s) interacting with GARP/latent TGF-β1 complexes cooperate with GARP to activate TGF-β1. In a previous attempt to identify such proteins, we screened a Treg cDNA library by yeast two-hybrid with GARP as a bait. This lead to the identification of LAPTM4B, which turned out to be a negative regulator of TGF-β1 production in Tregs (Huygens at al., manuscript submitted). Here, we used an alternative approach, based on the immunoprecipitation (IP) of GARP from stimulated human Treg clones, followed by the identification of co-immunoprecipitated proteins by Mass Spectrometry (MS). We report the identification of 7 candidates that are expressed at higher levels in Tregs than Th cells. We are currently testing which candidates cooperate with GARP to activate TGF-β1. Such proteins could represent new pharmacological targets to achieve inhibition of Treg function in patients suffering form cancer or chronic infections.

Results

Identification of proteins interacting with GARP by IP-MS in stimulated human Tregs.

We used a monoclonal anti-GARP antibody that recognizes GARP/latent TGF-β1 complexes (Cuende *et al.* manuscript submitted) to immunoprecipitate (IP) proteins from TCR-stimulated human Tregs. We used two types of human Tregs: a Treg clone (*i.e.* a pure population of cells with demethylated *FOXP3i1*, (Stockis *et al.* 2009a)), and polyclonal Tregs obtained by a short amplification of $CD4^+CD25^{hi}CD127^{lo}$ cells isolated from the blood of patient I. The polyclonal Treg population contained 56% of cells with demethylated *FOXP3i1*. Treg and control Th cells (blood $CD4^+CD25^-CD127^{hi}$ cells) were stimulated during 24 hours with anti-CD3 and anti-CD28 antibodies prior to IP. We performed 3 independent anti-GARP IPs on Treg lysates (test IPs A, B and C), and 3 negative-control IPs using an isotype control on Treg lysates or an anti-GARP antibody on polyclonal Th cells which do not express GARP (control IPs D, E and F). Immunoprecipitated proteins were separated by SDS-PAGE under reducing conditions, and gels were revealed by silver staining (Figure 1A). No band visible in test IPs but not in control IPs could be identified. We thus submitted the total immunoprecipitated products to analysis by tandem mass spectrometry (MS). For this, we cut out 21 contiguous horizontal fragments in each of the 6 lanes of the polyacrylamide gels (Figure 1A), and submitted proteins contained in each fragment to in-gel trypsin digestion, then to tandem MS. The peptides identified by MS were matched to a human protein database. This lead to the identification of 2092 proteins in the 3 test IPs (Figure 1B). We discarded from this list proteins also found in the negative control IPs (1641 proteins), and proteins with a predicted molecular weight (MW) at least 3x lower than the average MW expected in the gel fragment in which the peptide was identified. This left us with a list of 431 potential GARP partners. We choose to focus our analysis on proteins encoded by genes expressed at higher levels in Treg than in Th clones. We thus examined previously reported expression microarray data obtained from Treg and Th clones (Stockis *et al.* 2009a). Expression data was not available for 133 candidates, due to the absence of corresponding probes on the microarray chips. Of the 298 remaining candidates, 35 were encoded by genes with a detectable expression level in Tregs that was ≥ 1,5x higher than in Th clones. As expected, GARP was present among these 35 proteins. Altogether, this left us with a list of 34 candidate proteins (Figure 1B and Table 1).

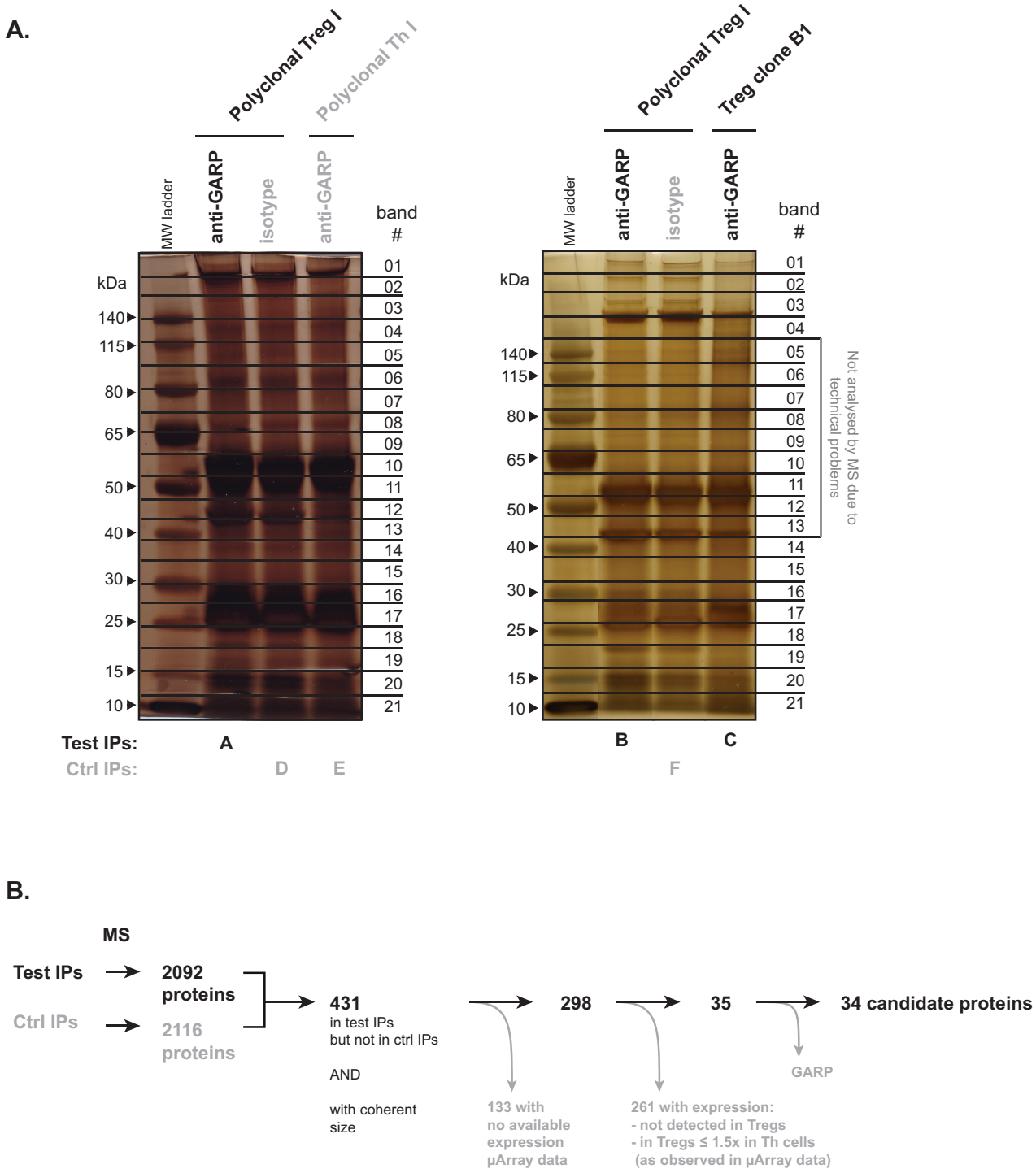


Figure 1. Identification of proteins interacting with GARP in Tregs by IP-MS

A. Treg clone B1 and polyclonal Tregs or Th cells from patients I were stimulated for 24 hours with anti-CD3 and anti-CD28 antibodies. Cell lysates were immunoprecipitated with the indicated antibodies. IP products were submitted to SDS-PAGE and proteins were revealed by silver-staining. Each lane was cut into 21 horizontal bands, as indicated, and proteins in each band were submitted to MS analysis.

B. Strategy to select candidates identified by MS.

Table 1. Potential GARP partners identified by IP-MS

	Proteins from which peptides identified by MS are derived			Characteristics of peptide(s) identified by MS			Expression of the corresponding genes					
	Uniprot protein name	UniProt IDs	Predicted MW (kDa)	Peptide length (aa)	Identified in		Gene ID	In expression µArrays			by RT-qPCR	
					IP product ID	Band #		Mean level in 3 stimulated Treg clones**	Mean level in 2 stimulated Th clones***	Ratio Treg / Th	Ratio in stimulated polyclonal cells (Treg / Th)	Ratio in stimulated clones (Treg / Th)
1	Leucine-rich repeat-containing protein 32 (GARP)	Q14392	72	13	A	07	*LRRC32*	11,7	0,4	28,8	58,5	9,9
2	Isoform 2 of TBC1 domain family member 8	O95759	103	10	A	07	*TBC1D8*	2,9	0,3	11,4	89,9	234,8
3	Microtubule-associated serine/threonine-protein kinase 4	H7C1I9	180	9	C	20	*MAST4*	6,3	0,8	7,6	3,2	4,4
4	Rho guanine nucleotide exchange factor 3	H7C4P0	26	10	A	10	*ARHGEF3*	17,2	3,5	4,9	1,6	2,7
5	Nuclear factor of-activated T-cells, cytoplasmic 1	K7EQ04	8	20	C	15	*NFATC1*	3,1	1,5	4,4	1,6	6,9
6	NACHT, LRR and PYD domains-containing protein 1	I3L2G5	118	7, 7	B, C	03, 15	*NLRP1*	2,3	0,6	3,6	2,1	3,6
7	Leptin receptor	P48357	132	19, 19	A, C	03, 14	*LEPR*	1,1	0,3	3,6	4,9	1,3
8	Integrin alpha-M	P11215	127	17	B	04	*ITGAM*	13,4	4,2	3,2	2,5	11,9
9	Phospholipase D3	Q8IV08	55	10	C	03	*PLD3*	2,8	1,0	2,9	2,5	2,3
10	Isoform 2 of Ras-related protein Rab-5B	P61020	19	14	C	17	*RAB5B*	6,6	2,8	2,4	1,6	0,8
11	Inositol 1,4,5-trisphosphate receptor type 2	Q14571	308	19	B	04	*ITPR2*	4,4	1,9	2,3	2,0	2,2
12	Nesprin-1	Q5JV20, H0Y325	191	11, 20	A, C	13, 17, 20	*SYNE1*	4,9	2,2	2,2	1,9	2,4
13	Isoform 5 of Glycogen debranching enzyme	P35573	173	21	A	16	*AGL*	15,8	7,4	2,1	2,2	1,0
14	Isoform 5 of Filamin-B	O75369	230	12	B	02	*FLNB*	2,6	1,2	2,1	1,5	1,0
15	WD repeat-containing protein 7	K7ELZ4	11	16	B	17	*WDR7*	10,4	5,1	2,0	1,7	1,5
16	Pericentriolar material 1 protein	A6NNN6	81	16	B	21	*PCM1*	3,0	1,5	2,0	nd*	nd
17	Inositol 1,4,5-trisphosphate receptor type 3	Q14573	304	26	A	17	*ITPR3*	3,4	1,7	2,0	0,9	0,9
18	Isoform 3 of Protein EFR3 homolog A	Q14156	88	13	A	09	*EFR3A*	13,2	6,8	1,9	1,5	1,1
19	Spatacsin	H0YLK7	66	10	B	18	*SPG11*	26,5	13,7	1,9	2,4	1,9
20	Protein sel-1 homolog 3	Q68CR1	129	14	A	05	*KIAA0746*	29,1	15,6	1,9	1,2	1,4
21	Tubulin alpha-1A chain	F8VRZ4	12	9	B	18	*TUBA1A*	99,6	52,1	1,9	nd	nd
22	Solute carrier family 23 member 2	B4DJZ1	58	20	C	14	*SLC23A2*	2,2	1,2	1,7	0,8	1,0
23	Isoform 3 of Probable helicase senataxin	Q7Z333	299	20	A	14	*SETX*	9,4	5,4	1,7	1,6	0,9
24	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	E9PLY0, H0Y390	120	11, 12	B, C	01, 14	*MACF1*	6,5	3,7	1,7	nd	nd
25	Isoform 2 of Structural maintenance of chromosomes flexible hinge domain-containing protein 1	A6NHR9	216	5	B	04	*SMCHD1*	12,4	7,2	1,7	1,2	2,2
26	Nuclear receptor corepressor 1	E7EVU5	63	20	A	20	*NCOR1*	6,2	3,6	1,7	1,4	1,1
27	Centrosome-associated protein 350	H0Y6Q4	37	10	A	17	*CEP350*	11,8	6,9	1,7	nd	nd
28	Myeloid zinc finger 1	P28698	82	12	A	12	*MZF1*	2,2	1,3	1,7	1,7	2,0
29	E3 ubiquitin-protein ligase UBR4	B4DPF6	80	17	B	02	*UBR4*	14,2	8,6	1,7	2,5	1,5
30	Tribbles homolog 3	B0QYQ2	30	13	C	20	*TRIB3*	4,5	2,8	1,6	1,3	0,6
31	SNF-related serine/threonine-protein kinase	E7EUC4	61	18	A	01	*SNRK*	15,7	10,0	1,6	1,5	3,4
32	Serine/threonine-protein kinase N1	Q16512	104	10	B	17	*PKN1*	3,8	2,5	1,6	1,4	0,9
33	Isoform 2 of E3 UFM1-protein ligase 1	O94874	82	23	B	20	*KIAA0776*	9,4	6,1	1,6	1,3	1,1
34	DNA-binding protein SATB1	C9JLL5	13	13	C	17	*SATB1*	52,3	34,0	1,5	0,8	1,2
35	Isoform 2 of Uncharacterized protein C11orf71	Q6IPW1	16	10	B	17	*C11orf71*	2,1	1,4	1,5	nd	nd
							EEF1A1	368,2	369,3	1,0	2,0	0,7
							ACTB	362,5	362,5	1,0	1,6	0,5
							B2M	387,7	381,5	1,0	2,8	1,9

* nd : not determined

** Treg A1, B1, B3 clones (Stockis et al. 2009a)

*** Th A1, B1 clones (Stockis et al. 2009a)

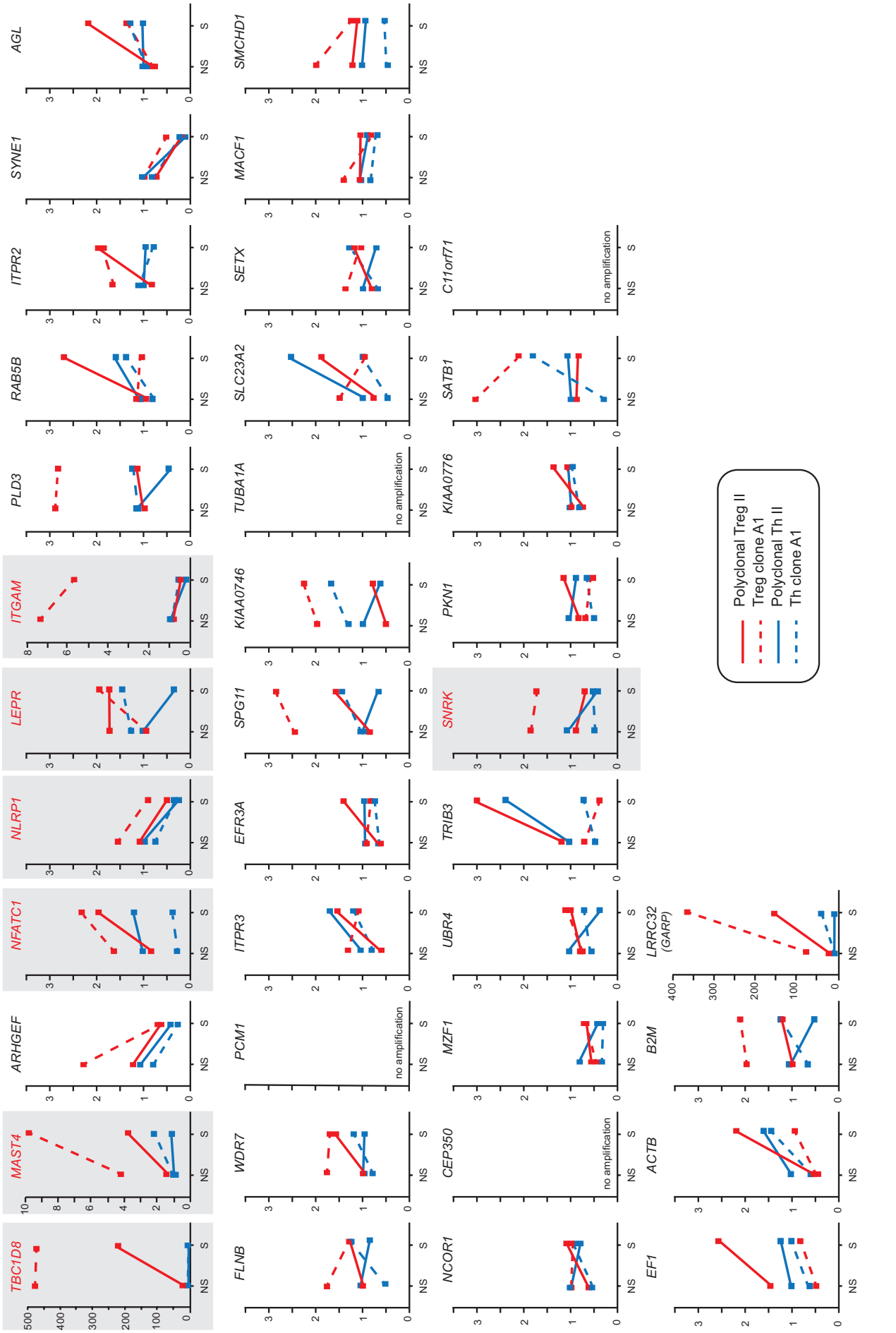
Expression of candidates in Tregs and Th cells by RT-qPCR analysis

We next measured expression levels of the candidates by RT-qPCR, to verify the microarray data and extend our analysis to additional human Treg and Th cell samples. Here, we tested both T cell clones and polyclonal populations. Polyclonal Treg and Th populations were derived from patient II, as described above. The polyclonal Treg population from patient II contained 68% of cells with demethylated *FOXP3i1*. Expression levels of the various candidates were measured in resting cells or 24 hours after stimulation of their TCR (Figure 2 and Table 1). The expression of 4 candidate genes could not be examined because no specific PCR products were amplified with the primers that we had selected. Among the 30 remaining candidates, 7 showed a ≥ 3x higher expression in the stimulated Treg clone than in the Th clone, or in stimulated Treg polyclonal cells than in autologous Th polyclonal cells (candidates highlighted by grey boxes in Figure 2 and Table 1). Expression levels of 3 housekeeping genes were also examined as controls, and did not show comparable increases in Treg cell samples.

Analysis of the ability of candidates to activate TGF-β1 in 293T cells

To test if any of the 7 candidates is able to activate TGF-β1 in the presence of GARP, we resorted to a luciferase reporter assay in a 293T cell clone stably expressing GARP and TGF-β1. In this assay, cells are transiently co-transfected with a plasmid encoding one candidate together with a plasmid containing the luciferase gene under the control of a *CAGA* promoter, which is activated by SMAD2/3 transcription factors in response to TGF-β1 signals (Dennler *et al.* 1998). So far, we succeeded in cloning and testing 4 candidates (LEPR, ITGAM, SNRK, and NLRP1), but none was able to induce luciferase activity above levels observed in cells transfected with the reporter alone, or co-transfected with PLD3 encoding vector, taken here as a negative control (Figure 3). As expected, co-transfection of integrin β6, a known activator of TGF-β1 in epithelial cells, or addition of rhTGF-β1 induced high luciferase activity (Munger *et al.* 1999).

Figure 2. Expression of genes encoding potential GARP partners in Treg and Th cells.
RNA was isolated from the indicated Treg and Th cell populations at rest (NS: no stimulation), and 24 hours after stimulation with anti-CD3 and anti-CD28 antibodies (S: stimulated). Sets of primers specific for the indicated genes were used to amplify reverse-transcribed RNA in real-time qPCR. Graphs show expression levels relative to expression in the non-stimulated polyclonal Th II cells ($2^{\Delta Ct}$ method). Relative expression levels of GARP and of three housekeeping genes (EF1, βACTIN and β2-MICROGLOBULIN) are shown at the bottom for comparison.


AGL
SYNE1
ITPR2
RAB5B
PLD3
SMCHD1
MACF1
SETX
SLC23A2
TUBA1A
C11orf71
SATB1
KIAA0776
PKN1
SNRK
ITGAM
LEPR
NLRP1
NFATC1
ARHGEF
KIAA0746
SPG11
EFR3A
ITPR3
PCM1
TRIB3
UBR4
MZF1
LRRC32 (GARP)
B2M
ACTB
EF1
TBC1D8
MAST4
WDR7
CEP350
FLNB
NCOR1
no amplification
NS
S
Polyclonal Treg II
Treg clone A1
Polyclonal Th II
Th clone A1

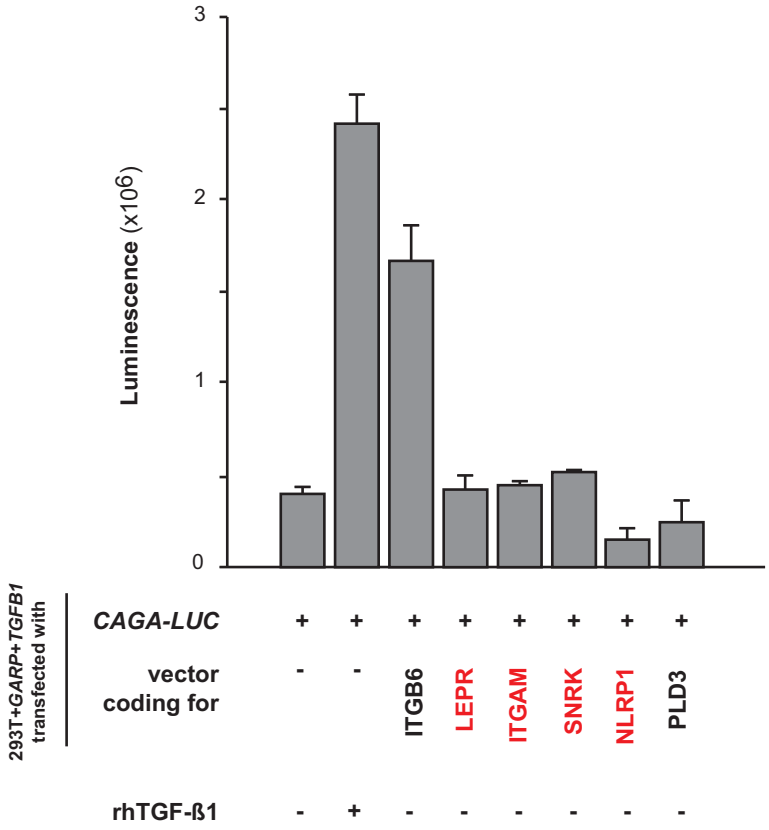


Figure 3. Luciferase reporter assay to measure TGF-β1 activation in a 293T cell clone stably expressing GARP and TGF-β1.

A 293T cell clone stably expressing GARP and TGF-β1 was transiently co-transfected with a CAGA-LUC reporter and a plasmid encoding a candidate protein, a negative control (PLD3) or ITGβ6. Luciferase activity was measured 24 hours after transfection. In one control condition, recombinant human (rh) TGF-β1 (4 ng/mL) was added for 24 hours.

Discussion

We attempted to identify Treg proteins implicated in TGF-β1 activation by IP followed by MS analysis. This lead to the identification of a plethora of proteins (>2000), most of which are probable false positives. The list of potential candidates was narrowed down first by eliminating proteins also identified in negative control IPs, and then using a criteria based on higher expression of the encoding genes in Tregs by comparison to Th cells. This left us with a list of 7 new potential GARP interactants overexpressed in Tregs. These candidates are currently being assessed for their ability to activate TGF-β1 in 293T cells stably expressing GARP. We already tested 4 candidates (LEPR, ITGAM, SNRK, and NLRP1), but none was able to activate TGF-β1.

One of these four candidates is ITGAM (integrin αM or CD11b). We had considered this candidate particularly promising because other integrins were previously shown to activate TGF-β1 in cells such as dendritic cells or fibroblasts (Travis and Sheppard 2014). Our results in the 293T reporter assay were thus very disappointing. However, it must be noted that integrins are heterodimers comprising an α chain and a β chain. Therefore, the inability of ITGAM to activate TGF-β1 in transfected 293T cells may be due to the absence of the appropriate β chain. ITGAM is known to pair with ITGB2 (integrin β2 or CD18), forming the so-called Mac-1 heterodimeric integrin (Abram and Lowell 2009). We thus tested ITGAM in the 293T cell reporter assay, this time co-transfecting ITGB2 with ITGAM. But we observed no TGF-β1 activation in this assay neither (data not shown).

We are now left with only three candidates to test in the 293T reporter assay (TBC1D8, MAST4 and NFATC1). All three show very striking overexpression in stimulated Treg cells by comparison to Th cells, and this is particularly the case for TBC1D8. To date, the cloning of these candidates has remained challenging, but we hope to obtain vectors to test them very soon. TBC1D8 is a member of the TBC1 domain containing family of proteins that have been described to function as GTPase-activating proteins (GAPs) for Rab GTPases. A putative transmembrane domain was described in TBC1D8 sequence, suggesting that the protein could span cell membranes (Yonekura *et al.* 1999, Barr and Lambright 2010). However no experimental data are available to support this hypothesis. MAST4 is a member of the Microtubule-Associated serine/threonine-protein kinase (MAST) family of proteins, which are cytosolic kinases. MAST4 protein function has not been well characterised. Interestingly, it was shown to associate with SMAD1 suggesting a role in signalling induced by TGF-β family of proteins (Colland *et al.* 2004). Finally, NFATC1 is a Ca2+-regulated transcription factor from the Nuclear Factor of activated T-cells protein (NFAT) family that regulates transcription of numerous genes in T cells. These three proteins constitute very interesting candidates by their expression profile and functions.

Materials and Methods

Polyclonal cell sortings and short-term TCR stimulation

Polyclonal T cells were sorted as described previously (Gauthy *et al.* 2013). For protein identifications, polyclonal $CD4^+$ (Th) and $CD4^+CD25^+CD127^-$ (Treg) cells were stimulated with anti-CD3 and anti-CD28 as previously (Stockis *et al.* 2009b). For qPCR analysis polyclonal $CD4^+$ and $CD4^+CD25^+CD127^-$ cells were incubated with Dynabeads® CD3/CD28 at a 1 cell to 1 bead ratio. All stimulations were performed in IMDM supplemented with 10% human serum, L-arginine, L-asparagine, L-glutamine, β-mercaptoethanol ($5x10^{-5}$M), methyl-tryptophane (200 µM).

Immunoprecipitation and SDS-PAGE gel

Cell lysates were incubated with 40 µg/ml of MHG-6 anti-GARP antibody (in house antibody, (Cuende *et al.* manuscript submitted)), or isotype IgG2a control antibody. Lysate-antibody mixtures were incubated with Protein-G coated on magnetic beads (Dynabeads, Invitrogen). Beads were washed of unbound proteins using a Dynal magnet (Invitrogen). Immunoprecipitates were eluted from beads by boiling in Laemmli sample buffer supplemented with 5% β-mercaptoethanol (BioRad) and loaded on SDS-PAGE gel (4-12%, Bis-Tris Life Technologies).

Silver staining and MS

SDS-PAGE gels were silver stained using SilverQuest kit (Life Technologies). Gels were fragmented and each piece was destained using the SilverQuest destainer reagents. Proteins were submitted to in-gel trypsin digestion. Peptides were analysed by capillary LC-tandem mass spectrometry in a LTQ XL ion trap mass spectrometer (ThermoScientific) fitted with microelectrospray probe. The data were analysed with the ProteomeDiscoverer software (ThermoScientific), and the proteins were identified with SEQUEST against a target-decoy nonredundant human protein database obtained from NCBI. The False Discovery Rate was below 5%.

RT-qPCR SYBR

Total RNA was extracted and reverse transcribed using the SuperScript Vilo cDNA synthesis kit (Life Technologies). qPCRs were done using Takyon SYBR or Probe Mastermix Kit (Eurogentec) and amplifications were performed on ABI Prism 7300 or Step One Plus Real Time PCR Systems (Applied Biosystems)under standard conditions: 94°C for 3 min, 45 cycles of 94°C for 10 s, and 60°C for 1 min.

qPCR primers for candidates used are, 5’ to 3’ Forward, Reverse :

LEPR: GTTCCTGGGCACAAGGACTTA, ACCACATGTCACTGATGCTGT ;

NFATC1: AAAACTGACCGGGACCTGTG, GAACGGGGCTGGTTATCCTC ;

NLRP1: CTGCCTGCAAACTCATACGC, CCCTCAGTTCCTGCCTCATC;

SNRK: CGGATCGAGACGCCATTGTA, CAGGATCCTTTCAGCCAGCA;

PKN1: GGACAATGAGAGGCATGAGGT, AATGACAGGGTTGCGGAAGG ;

KIAA0746: ACTTTCTGGGAACCTTTCTGCT, ATGGTCTTGGAGGGGGATCG;

ITPR2: TGGGGTTAGTGGATGACAGATG, TAGGGCACACCTTGAAAAGGC;

ITPR3: AACAAGACCGACTACACGGG, CCCTCATTGCTGACAAGGGA;

NCOR1: GAAAATCCAGAAGACAGCGAAG, GTGTTTCCTCGAGAAGTAGCAT;

UBR4: CGACCAGTGGAACTGTCCTG, ATGATTGGGCCAACGGCAAA;

RAB5B: TGTCAAAGGGCAGTTCCATGA, AGACAAACGGACTGGGTGAG;

PLD3: CTGCGTGACAACCATACCCA, ACACGGGCATATGGGATTCG;

TRIB3: CCACGCGGAACGACGG, GTCATCCAACTCCAACCGCT;

SLC23A2: GGGCTCGTCCTTCCAAGTTA, GCCCCCTACAAACATAGCAGT;

SPG11: TGCCCGCATCCAACTGAAAT, GCCTTCAGCAGCAGTTGTTT;

WDR7: TGGATGTGTCCGCTGTTCTG, TCAGAGGCAACCCCATTGTG ;

STEX: GTCACCATCACACGAGCCAA, CAGCTGATTCCAATGCTGGTT;

MZF1: GAAGCTGGGGGCATCTTCTC, GTGGAGGTGAGGGCTTACAC;

SATB1: CAGCAGGAAATGAAGCGTGC, GTAACAGCTCGCACAACCATC;

KIAA0776: ACACATACAAGATGCCCCTGA, AATACTGAACGTACCACCTCG;

EFR3A: AGTCGCTAGGTGGAAGTGGA, CGGTGTCCACAATGCTTTTTCT;

SMCHD1: TCGGGGAATGGTATTTGGAGC, GTTTGCACACAGCAGAACGA;

AGL: GCCCGGAGACTACTGCAAAG, GGAAGTCCTTTCCAAGGGGA;

FLNB: CGCCTCACTGTTATGAGCCT, TCTTGCCTTTTGCGCCATTC;

ARGHEF: AGGGGCATTCAGCAACAATG, TGTAGCGAGTGGGTCTGACT;

SYNE1: AGCCAGATGGCTGAACATCA, TCCTCTGCGAGAGAGGACTG;

MACF1: GAAAAGCCAGGATTCGGTGC, TTTCTTCTGAACCCGGTCCC;

TBC1D8: CAGAACGTGCTTCGAGTCGT, GCTCCCAGTAACAGCTCATCA;

MAST4: ATGGTGAGGCGGAGCAAGAA, TTCGGCTGGTGTGGAGTAAA;

ITGAM: GTCCAGCTTCAGGGATCCAG, TAGTCGCACTGGTAGAGGCT.

For *ACTB, B2M, EF1* and *GARP* taqman was performed. qPCRs primers used for *GARP*, *EF1* are described (Stockis *et al.* 2009a). qPCR primers, 5' to 3':

ACTB: Forward: ATTGCCGACAGGATGCAGAA,

Reverse: GTCATACTCCTGCTTGCTGA,

Probe: TCAAGATCATTGCTCCTCCTGAGC;

B2M: Forward: GGAGGCTATCCAGCGTACT,

Reverse: GACCAGTCCTTGCTGAAAGACA,

Probe: GCTATGTGTCTGGGTTTCATCCATCCG.

Transient transfections

A 293T cell clone stably expressing GARP and TGF-β1 was transiently transfected with plasmids indicated in the figures using Lipofectamine 2000 (Life Technologies). Cells were analysed 24 hours after transfection. Luciferase activity was measured with the Britelite Plus Reporter Gene Assay System (Perkin Elmer).

Discussion and Perspectives

1. Two distinct approaches to identify GARP partners in human Tregs

Human Tregs but not other T cells activate TGF-β upon TCR stimulation. Although TGF-β production is an important immunosuppressive mechanism elaborated by Tregs, the crucial step of TGF-β activation is not fully understood. Tregs, and not other Th cells, present latent TGF-β at their surface through binding to a transmembrane protein called GARP. Surface presentation of latent TGF-β is required for TGF-β activation by Tregs: antibodies against the GARP/latent TGF-β complexes inhibit active TGF-β production by Tregs (Cuende *et al.* manuscript submitted). When transduced with GARP, Th cells acquire the ability to present latent TGF-β at their cell surface but are still unable to activate TGF-β (Stockis *et al.* 2009b). Therefore, we hypothesised that one or more additional Treg protein(s) interact with the GARP/latent TGF-β complexes to allow TGF-β activation. We decided to look for proteins that interact with GARP. To achieve this, we used two different techniques. First, we used the MYTH system in yeasts with GARP as a bait and a Treg cDNA library as preys. Second, we used an IP-GARP on Treg cell lysates followed by MS analysis.

As for all Y2H systems, the MYTH system is suited to search for labile or weak interactions between two proteins. In contrast, IP-MS approach is more adapted to identify strong interactions between two or more partners. These systems are thus complementary and allow to cover a large panel of possible interactions. Notably, the GARP partners that we identified with each method did not overlap.

The MYTH system is designed to test interactions using transmembrane proteins as baits. Since TGF-β activated by Tregs has paracrine actions on neighbouring T cells but is not soluble, we assume that TGF-β activation occurs close to the cell surface of Tregs and that it is likely that the crucial partner involved in TGF-β activation is also located at the cell membrane, together with the GARP/TGF-β complex. Therefore, the MYTH system, out of all Y2H systems, is the most adequate to identify this type of preys.

The first prey cDNA library that we used was designed in such way that NUBG is fused at the N-terminus of preys. Screening of this library allowed for the identification of preys that are either membrane protein with a cytosolic N-terminal extremity (type II membrane proteins) or cytosolic proteins. It did therefore not cover all possible interacting proteins. This system led to the

identification of 14 potential GARP interactants. One of these, namely LAPTM4B, displays higher expression in Tregs than in Th cells at mRNA levels and could therefore be an interesting partner of GARP (see below). We also constructed prey cDNA libraries in which the NUBG was fused at the C-terminus of the preys. This was expected to allow the identification of type I membrane proteins interacting with GARP. However, screening of this library lead to too many false positives, and we did not succeed in identifying any candidates with this approach (data not shown).

The main limitation of the MYTH approach is that it detects binary interactions between bait and prey. However, the interaction between GARP and a partner in Tregs could necessitate the presence of TGF-β associated to GARP. We also tried to improve the efficiency of the screening by expressing human TGF-β in yeasts, postulating that interesting GARP partners could bind GARP better in the presence of TGF-β. We succeeded in expressing human TGF-β in yeast, and could show that it was properly processed into latent TGF-β (*i.e.* cleaved into LAP and mature TGF-β). Unfortunately, expression of TGF-β was toxic in yeast, leading to slow growth and inefficient library transformation. Thus we could not efficiently screen prey cDNA library screen in these conditions.

The alternative IP-MS approach has the advantage to detect interactions with endogenous GARP in human Tregs *i.e.* unmodified. However the technique is limited by the strength of the interactions between GARP and its partner(s), and by the affinity of the antibody. The anti-GARP antibody used for IP has been derived in house (Cuende *et al.* manuscript submitted). It binds both free GARP and GARP bound to latent TGF-β. This antibody is not a blocking antibody: it does not inhibit TGF-β activation by Tregs, and therefore is not expected to disrupt interactions with proteins essential for TGF-β activation. The IP-MS approach allowed the identification of 34 potential GARP partners with 7 of them displaying higher expression in Tregs compared to Th cells (see below).

Since cell-cell contact is required to allow TGF-β signalling in a Treg target, one possibility is that the essential partner leading to TGF-β activation is presented by the target cell and not by the Treg itself. Such activation could be termed «trans» activation, by contrast to «cis» activation achieved by a protein on the Treg cell itself (Figure 1). The group of T. Springer, described a «trans» activation of TGF-β bound to GARP triggered by integrins β6 and β8 expressed on cells that do not express GARP (Wang *et al.* 2012). This was done in transfected 293T cells expressing GARP and

TGF-β mixed in culture with 293T cells transfected with ITGβ6 or ITGβ8 and co-cultured with a reporter cell line expressing luciferase in response to TGF-β signals. In this experiment, the authors observed indeed luciferase activity indicating that integrins can lead to activation of latent TGF-β presented by GARP in trans. The MYTH system is unable to detect «trans» interactants because reporting of the interaction require bait and prey to be located in the same yeast. The IP-MS approach, however, could lead to the identification of such partners, but will require a strong interaction between the partners to be conserved along the lysis and washing processes.

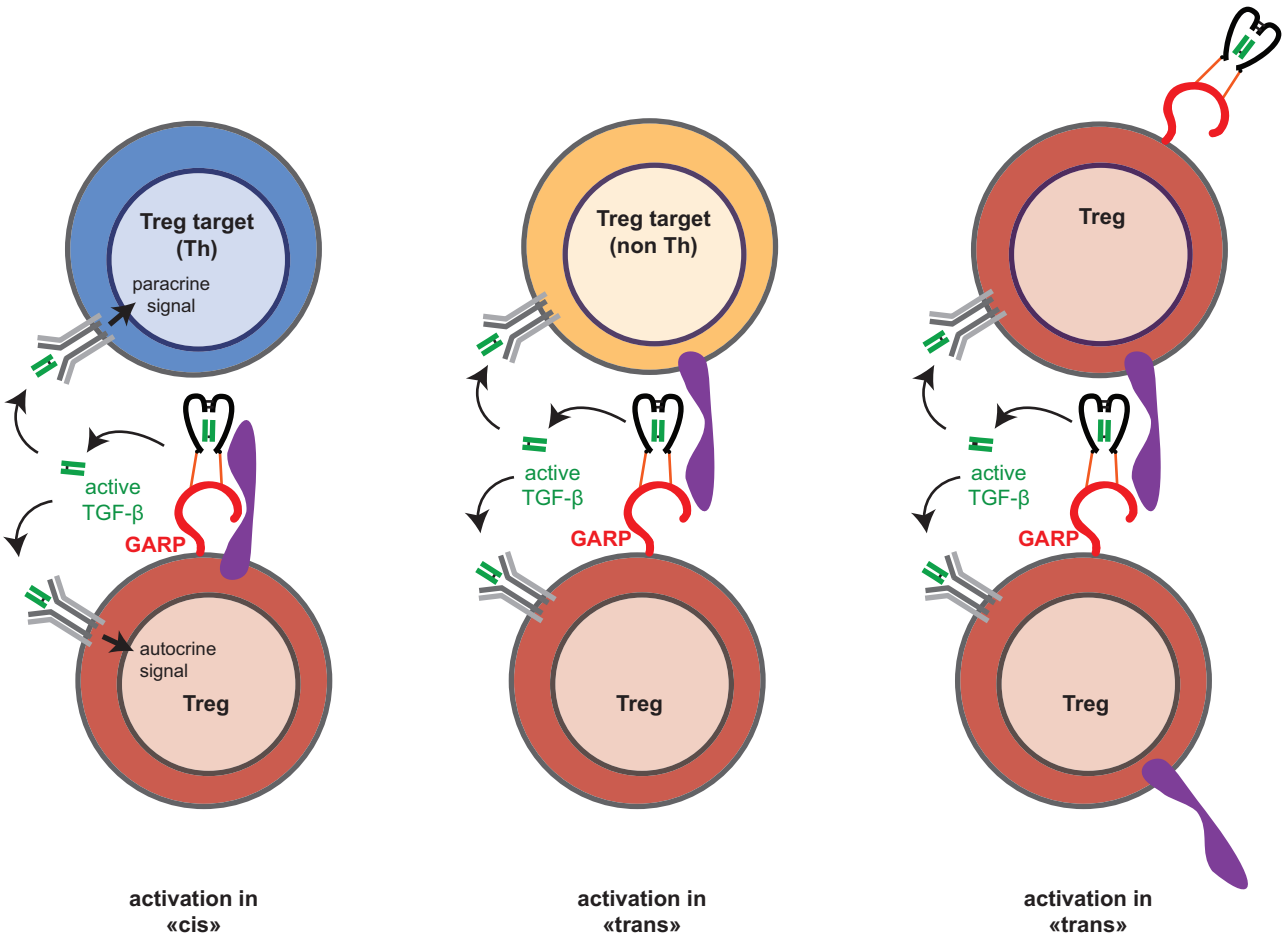


Figure 1. Representation of «cis» and «trans» models for TGF-β activation by Tregs.
Latent TGF-β1 is presented at the surface of Tregs through binding to GARP. Activation may occur in «cis» : Treg proteins may interact with GARP/TGF-β complex in the Treg itself, leading to TGF-β activation. Active TGF-β can then act in an autocrine or paracrine manner. Alternatively, TGF-β activation may occur in «trans» meaning that the protein required for TGF-β activation is located on the surface of the cell facing the Treg (another cell type or a Treg).

A class of proteins that represents interesting GARP/TGF-β potential partners are secreted proteins. They could be involved in latent TGF-β activation or in shedding of GARP/TGF-β complexes. However, secreted proteins cannot be identified in the MYTH approach. A different Y2H system specifically designed for the identification of extracellular proteins does exist: the SCINEX-P system, detecting interactions in the ER (Urech *et al.* 2003). We could have used this technique to identify GARP/TGF-β partners. Yet, secreted proteins could have been identified with the IP-MS system if they were retained at the cell membrane via the interaction with GARP. However, none of the candidates identified by IP-MS were extracellular proteins. To identify secreted proteins interacting with GARP, we also attempted to fuse preys with a transmembrane domain to retain the extracellular proteins at the cell membrane. We first assayed this system by fusing TGF-β with a transmembrane domain. However, we did not succeed in expressing TGF-β in fusion with transmembrane domain in yeast and did not further used this approach.

Altogether, our two approaches to identify GARP partners led to identification of a few interesting candidates that include membrane and cytosolic proteins. Unfortunately, we haven't succeeded yet in showing that any of them is implicated in the TGF-β activation process. Below we discuss further these interesting candidates.

2. Seven candidates identified by IP-MS

The IP-MS approach identified a short-list of 7 potential GARP partners with higher mRNA expression in Tregs than in Th. Their interaction with GARP will have to be confirmed by an alternative technique, using for example the Gaussia luciferase complementation assay that we successfully used to confirm interaction with LAPTM4B. But instead of first verifying interaction for these candidates, we chose to start by screening which ones could be implicated in TGF-β activation, using a reporter assay in 293T cells. Four candidates, NLRP1, LEPR, ITGAM, and SNRK, did not induce TGF-β activation in this assay. Three candidates, namely TBC1D8, MAST4, and NFATC1 have not been tested yet in this assay, and are thus still potentially interesting candidates.

TBC1D8, or TBC1 domain family member 8, is a 1140 amino acid protein, member of the TBC1 domain-containing family of proteins. This family includes proteins containing a Tre-2/Bub2/Cdc16 (TBC) domain. This domain is a conserved protein motif that consists of approximately 200 amino acids and is thought to function as a Rab-GAP domain (Barr and Lambright 2010, Fukuda 2011). More than 40 distinct TBC domain-containing proteins have been identified and over 60 Rab proteins are found in humans. Not all Rab proteins have been studied but the current view is that Rab proteins are involved in vesicle trafficking processes (Barr and Lambright 2010). The GAP activity and specificity of most TBC proteins have never been determined (Fukuda 2011). *TBC1D8* gene is highly conserved and found in many species including human, chimpanzee, rhesus monkey, mouse, rat, chicken and zebrafish. No function for this gene has been identified in the immune system. However, it was suggested to be important for endothelial cell proliferation and angiogenesis, and was therefore initially called Vascular Rab-GAP/TBC1 domain containing protein (VRP) (Yonekura *et al.* 1999). It was proposed that TBC1D8 contains a putative transmembrane domain, but this was never confirmed experimentally (Yonekura *et al.* 1999). The topology and subcellular location of TBC1D8 remain to be studied.

MAST4, or Microtubule-associated Serine/Threonine kinase 4, is a member of the MAST family of cytosolic kinases that comprises 5 members: MAST 1 to 4, and the MAST-like protein. The functions of these proteins have not been well characterised. Interestingly though, a Y2H system identified MAST4 as a partner for SMAD1, suggesting a role for this protein in signalling pathway with members of the TGF-β family (Colland *et al.* 2004).

NFATC1, or Nuclear Factor of activated T-cells cytoplasmic 1 protein, is a Ca^{2+}-regulated transcription factor from the NFAT family. NFAT transcription factors regulate several processes in vertebrates, including the development and function of the innate and adaptive immune system (Vihma *et al.* 2008, Zanoni and Granucci 2012). NFAT proteins are located in the cytosol in an inactive phosphorylated state. Upon dephosphorylation by calcineurin, NFAT proteins are activated and translocate to the nucleus where they associate with other factors to modulate gene transcription. In T cells, NFAT induce and repress the transcription of many genes, resulting in modulation of T cell activation, proliferation and differentiation (Zanoni and Granucci 2012).

These three candidates indicate a potential role for GARP in signalling pathways. The possibility that GARP induces intracellular signals has not been studied yet but cannot be excluded. The intracellular portion of GARP is very small (15 amino acids), suggesting that, signalling through GARP would require the recruitment of adaptor molecules. We tested potential signalling through GARP by comparing Th cells transduced or not with GARP, and exposed or not to latent TGF-β, and then analysed p-JNK, p-ERK and p-p38 by WB. No signs of differential intracellular signalling due to GARP expression was detected using antibodies against theses molecules. Of course, signalling through GARP could occur through many other pathways. If interaction with TBC1D8, MAST4 or NFATC1 are confirmed, this line of research could be further explored.

3. LAPTM4B, a candidate identified by MYTH

3.1. Regulation of LAPTM4B expression

Using the MYTH approach with GARP as a bait and a Treg cDNA library as prey, we identified a new partner of GARP called LAPTM4B. Transcription of the *LAPTM4B* gene yields at least two mRNA variants in human Tregs: *Va* and *Vb*. Expression of *Va* and *Vb* is higher in Tregs than in Th clones after TCR stimulation. We also examined *Va* and *Vb* expression in human tissues (data not shown). We observed that while *Va* is ubiquitously expressed, *Vb* is only detected at high levels in stimulated Treg clones or polyclonal Tregs. Low level expression of *Vb* was also detected in some tissues including muscle and bone marrow.

How the *LAPTM4B* gene is regulated at a transcriptional level has not been extensively studied. The group of Zhang investigated this question in human breast cancer cells (Zhang *et al.* 2013). They revealed that the first exon of *LAPTM4B* contains conserved binding site for cAMP responsive element binding protein-1 (CREB1), and showed using electrophoretic mobility shift assay (EMSA) that CREB1 directly binds to this *LAPTM4B* regulatory region. This regulatory mechanism could also function in Tregs since CREB transcription factors were shown to control gene transcription in these cells, such as *Foxp3* transcription in murine Tregs (Kim and Leonard 2007, Ogawa *et al.* 2014).

Very interestingly, overexpression of *LAPTM4B* in human or mouse Tregs was observed in several published expression microarray data sets (Herman *et al.* 2004, Williams and Rudensky 2007). Moreover, transduction of non-Treg cells with *FOXP3* leads to *LAPTM4B* overexpression (Ocklenburg *et al.* 2006). Together, these data suggest that gene *LAPTM4B* might be transcriptionally regulated by FOXP3.

LAPTM4B variants encode different protein isoforms with truncated N-terminal portions, depending on the start codon used to initiate translation. Unfortunately, we lacked antibodies against the LAPTM4B isoforms expressed in Tregs to study protein expression.

3.2. Roles of LAPTM4B in human Tregs

We showed for the first time a function of LAPTM4B in the immune system. LAPTM4B interacts with GARP. GARP and LAPTM4B appear to associate directly, as suggested by the fact that they interact when co-expressed in yeast. However, one cannot formally exclude that the interaction between the two partners requires an additional protein that is present in mammalian cells and for which a homolog exists in yeast. Which or whether both isoforms LAPTM4B$_{iso24}$ and LAPTM4B$_{iso20}$ interact with GARP is not yet elucidated. In the Gaussia luciferase complementation assay, co-transfection of GARP with a vector encoding LAPTM4B$_{iso24}$ and LAPTM4B$_{iso20}$ leads to similar luciferase activity compared to a vector encoding LAPTM4B$_{iso20}$ only, indicating that the N-terminus of LAPTM4B$_{iso24}$ is not required for the interaction with GARP. To define which isoform(s) interact(s) with GARP, we could perform PCA using LAPTM4B constructs in which in frame ATGs are mutated, so that vectors would code for only one of the two LAPTM4B isoforms.

Overexpression analysis in 293T cells, and knock-down approach in Tregs revealed that LAPTM4B decreases GARP surface levels, cleavage of pro-TGF-β precursor, and therefore latent TGF-β secretion and surface presentation. Again, which isoform of LAPTM4B plays these roles in human Tregs is not elucidated. Indeed, all isoforms were silenced in Tregs with *siLAPTM4B*.

Altogether, we concluded that LAPTM4B is a negative regulator of TGF-β production in Tregs, acting through both GARP-dependent and GARP-independent ways.

3.3 Other described functions of LAPTM4B, and LAPTM family members

Studies of LAPTM4B functions were so far limited to the analysis of isoform LAPTM4B$_{iso35}$, which does not appear to be present in human Tregs. Several studies revealed that high expression of LAPTM4B$_{iso35}$ in human cancers is associated to poor prognosis (Zhou *et al.* 2007, Yang *et al.* 2008, Zhou *et al.* 2008, Yang *et al.* 2010, Yang *et al.* 2010, Yin *et al.* 2011, Kang *et al.* 2012, Zhang *et al.* 2012, Tang *et al.* 2014, Zhang *et al.* 2014). High LAPTM4B$_{iso35}$ expression in cancer tissues was demonstrated by immunohistochemistry using antibodies against the N-terminus of the protein. These antibodies do not bind LAPTM4B$_{iso24}$ and LAPTM4B$_{iso20}$, which lack the N-terminus found in LAPTM4B$_{iso35}$.

In cancer cells LAPTM4B$_{iso35}$ isoform was shown to increase cell proliferation, migration, and invasion *in vitro,* and to increase the metastatic potential of tumour cells *in vivo* (Liu *et al.* 2009, Yang *et al.* 2010). *LAPTM4B* gene amplification was shown to induce resistance to cancer cell chemotherapy and recurrence of breast cancer (Li *et al.* 2010).

In addition to LAPTM4B, the LAPTM family comprises two other members: LAPTM4A and LAPTM5. Analysis of the subcellular localisation of LAPTM4A and 5 proteins indicate that they are present in late endosomes and lysosomes whereas, LAPTM4B was found present at the cell membrane (Milkereit and Rotin 2011). We analysed LAPTM4B subcellular localisation in transiently transfected COS7 cells by immunofluorescence. As shown in Figure 2, LAPTM4B staining (red) co-localises with the median Golgi marker GM-130 (green). This contrasts with the results obtained by Milkereit and Rotin, although we cannot exclude the presence of LAPTM4B on the membrane in addition to the Golgi on the basis of our results.

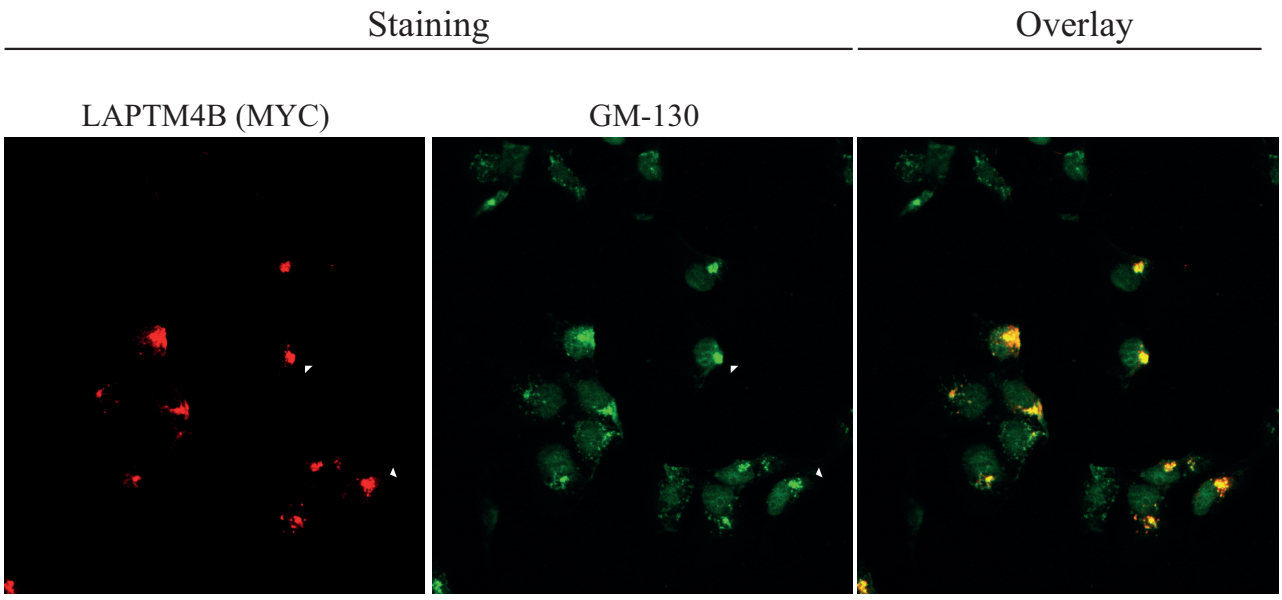


Figure 2: Subcellular localisation of LAPTM4B.
COS7 cells were transfected with a vector coding for LAPTM4B in frame with a MYC Tag. 24 hours after transfection, cells were stained with anti-MYC (red) and anti-GM-130 (green) antibodies. Two transfected cells and their GM-130 staining are indicated by arrowheads.

LAPTM proteins share conserved PY motifs in their C-terminus, which bind to WW domains in proteins with whom they associate. LAPTM4B and LAPTM5 were both shown to interact with E3 ubiquitin ligase Nedd4 (Pak *et al.* 2006, Milkereit and Rotin 2011). Interaction of LAPTM5 with Nedd4 was found to be crucial for its transport from the Golgi to the lysosomes, but this was not the case for LAPTM4B (Pak *et al.* 2006, Milkereit and Rotin 2011). Very interestingly, in a Y2H screen, LAPTM5 was also found to interact with SMURF2, an ubiquitin ligase for SMADs known to downregulate TGF-β signals (Colland *et al.* 2004, Itoh and ten Dijke 2007). We could thus speculate that several LAPTM proteins control TGF-β signalling pathway in T cells. LAPTM5 was also shown to interact with and regulate surface levels of T cell and B cell receptors (Ouchida *et al.* 2008, Ouchida *et al.* 2010). This function require PY motifs in LAPTM5, and appear to occur through targeting of the immune receptor components to the lysosomes. We could speculate that LAPTM4B also regulates GARP levels by promoting its lysosomal degradation. Mutating PY motifs in LAPTM4B could help determine whether they are important for association with GARP and regulating of its surface levels. However, our analyses as well as observations from others (Milkereit and Rotin 2011) did not reveal a lysosomal localisation of LAPTM4B, suggesting that other mechanisms are probably involved. We analysed by immunofluorescence whether the subcellular location of GARP was modified in the presence of LAPTM4B. In transfected COS7 cells, we observed that GARP and LAPTM4B co-localise mostly in the median Golgi. We assessed whether GARP subcellular location was modified by looking at the distribution of GARP staining in individual cells. As shown in Figure 3, the localisation of GARP was not modified in the presence of LAPTM4B.

3.4. LAPTM4B in Treg suppressive function

Our initial aim was to identify Treg proteins involved in TGF-β1 activation, one mechanism of the Treg immunosuppressive function. However, LAPTM4B expression did not induce TGF-β1 activation in a 293T clone stably expressing GARP and TGF-β1. In the same line, *siLAPTM4B* did not modify TGF-β1 activation in human Tregs. We did not test whether LAPTM4B silencing increases the Treg immunosuppressive function, but this could be of interest. By inducing general decrease in TGF-β1 production, LAPTM4B could dampen the Treg suppressive activity. Moreover, *Laptm5* KO in murine T cells led to an increase in TCR surface levels compared to their WT counterparts (Ouchida *et al.* 2008). Further studies will be needed to evaluate the importance of LAPTM4B in Treg function.

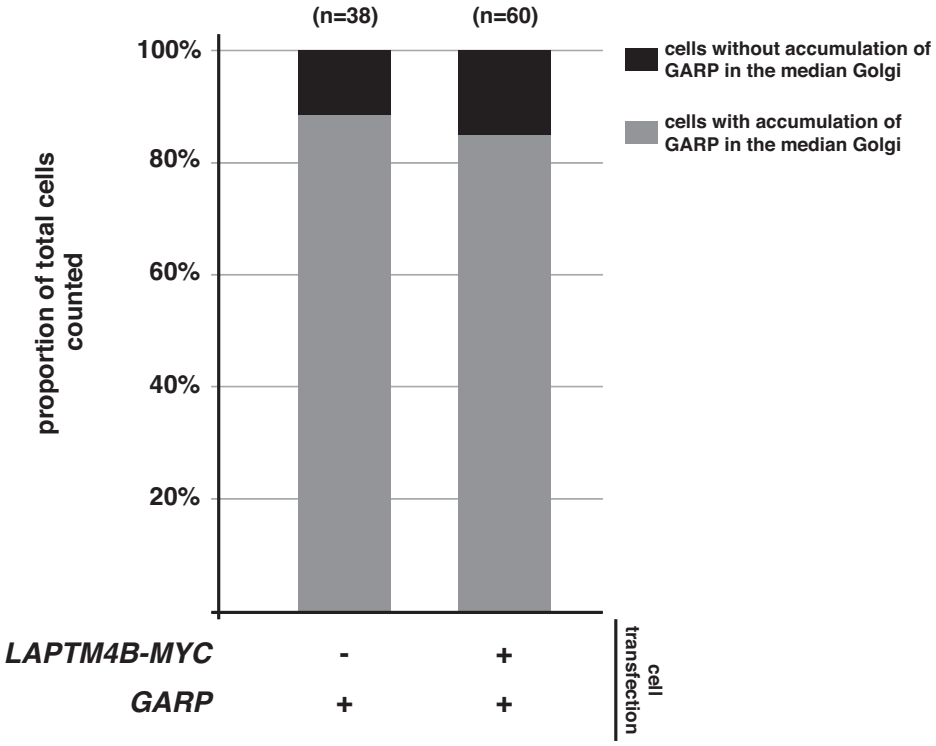


Figure 3: Subcellular localisation of GARP in the presence or absence of LAPTM4B.
COS7 cells were transiently transfected with GARP alone or in combination with LAPTM4B (MYC tagged). 24 hours after transfection, cells were stained with anti-GARP and anti-MYC antibodies. Localisation of GARP was assessed in 60 cells co-transfected by GARP and LAPTM4B and in 38 cells transfected with GARP alone. Proportion of cells showing GARP accumulation in the Median Golgi is represented by grey bar while proportion of cells showing a distribution of GARP all across the secretory pathway are annotated in black.

4. TGF-β activators in Tregs

So far, none of the tested candidates isolated by IP-MS or by Y2H appears to contribute to activation of TGF-β. To address this question, we transfected each candidate in a 293T cell clone stably expressing GARP and TGF-β, together with a luciferase reporter activated upon TGF-β signals. This 293T cell clone presents latent TGF-β at the cell surface, but does not activate the cytokine spontaneously. Transfection of known activators of TGF-β, such as ITGβ6 or ITGβ8, induces TGF-β activation in this clone. It is therefore a very sensitive system, which could be implemented for large screenings. Sara Lecomte in our group will further exploit this system to perform a high-throughput screening for TGF-β activators in human Tregs. She will co-transfect pools of a Treg cDNA library in the 293T cell clone expressing GARP and TGF-β, together with the TGF-β luciferase reporter plasmid. The objective of this project will be to identify proteins involved in TGF-β activation in Tregs. In contrast to our approach, the candidates will not be selected on the basis of an interaction with GARP.

Very recently, Edwards and co-workers suggested a role for the αvβ8 integrin in TGF-β activation from GARP/TGF-β complexes in mouse Tregs (Edwards *et al.* 2014). By RT-qPCR, they revealed that the *Itgb8* is expressed at substantially higher levels in Tregs than in non-Treg $CD4^+$ T cells. They showed that WT Tregs, but not *Itgb8* KO Tregs, promote the *in vitro* differentiation of naïve T cells into Th17 cells or iTregs, two processes known to rely on TGF-β (Edwards *et al.* 2014). Moreover, co-culture of $Garp^{-/-}/Itgb8^{+/+}$ Tregs with $Garp^{+/+}/Itgb8^{-/-}$ Tregs lead to Th17 differentiation of naïve T cells, indicating that Itgb8 can activate latent TGF-β presented by GARP in trans. However, the suppressive activity of $Itgb8^{-/-}$ Tregs *in vitro* and *in vivo* was not different from that of their WT counterparts (Edwards *et al.* 2014). This confirmed their previous observation that, the suppressive activity of Tregs in the absence of GARP remains unaltered although TGF-β activation is impaired (Edwards *et al.* 2014). Altogether, these results indicate that the release of active TGF-β from GARP/TGF-β complexes by mouse Tregs is mediated by the αvβ8 integrin, and that the activation process can occur in «trans». In our group, Julie Stockis investigated the possible implication of the αvβ8 integrin in TGF-β activation by human Tregs as well. The study of *ITGB8* mRNA expression in human Treg and Th cells revealed that its expression is higher in Tregs compared to Th cells upon TCR stimulation (Stockis *et al.* unpublished data). Theses results are in line with the observations made in murine Tregs by the group of Shevach (Edwards *et al.* 2014). Overexpressing ITGB8 integrin in our 293T cell clones

expressing GARP/TGF-β leads to TGF-β activation, as reported with the CAGA-LUC reporter. However, activation is inferior to that obtained by overexpressing ITGB6. Up to now, we have not been able to show that anti-integrin β8 blocking antibodies inhibits TGF-β activation by human Tregs. We are trying to derive our own anti-integrin β8 antibodies to further explore this analysis. This will be part of the project of Sara Lecomte in our laboratory. She will also try to derive antibodies targeting proteins that she will identify by screening the Treg cDNA library in the TGF-β-reporter assay in 293T cells. She may also attempt to obtain antibodies against TBC1D8, the only candidate protein identified by IP-MS which could be localised at the cell membrane. If any of these antibodies block TGF-β activation by Tregs *in vitro*, they will be tested for their ability to inhibit the immune suppressive function of Tregs *in vivo*. The ultimate objective would consist in obtaining new therapeutic tools to boost immune responses in cancer or chronic infections.

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