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Lysosomal-associated Transmembrane Protein 4B (LAPTM4B) Decreases Transforming Growth Factor β1 (TGF-β1) Production in Human Regulatory T Cells*

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Background: TGF-β1 produced by regulatory T lymphocytes suppresses excessive immune responses.
Results: Lysosomal-associated transmembrane protein 4B (LAPTM4B) was found to inhibit TGF-β1 production in Tregs.
Conclusion: LAPTM4B, known to exert oncogenic functions in tumor cells, also plays a role in the immune system.
Significance: LAPTM4B may represent a new therapeutic target to modulate immunosuppression by Tregs.

Production of active TGF-β1 is one mechanism by which human regulatory T cells (Tregs) suppress immune responses. This production is regulated by glycoprotein A repetitions predominant (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 activation of the cytokine in Tregs. We explored whether 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 assay, using GARP as a bait to screen a human Treg cDNA library. We identified lysosomal-associated transmembrane protein 4B (LAPTM4B), 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. Therefore, LAPTM4B binds to GARP and is a negative regulator of TGF-β1 production in human Tregs. It may play a role in the control of immune responses by decreasing Treg immunosuppression.

Regulatory T lymphocytes (Tregs)[4] are a subset of $CD4^+$ T cells that maintain immune tolerance by suppressing autoreactive T cells (1, 2). 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 (3–5). *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 (TCR) stimulation (2). 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 (6–10). Demethylated *FOXP3i1* can serve to identify and quantify Tregs in human blood or cell samples (11–13).

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 (1, 14). Its production by Tregs is regulated by GARP, a surface protein expressed on stimulated Tregs but no other T lymphocytes (11, 15–17). TGF-β1 is synthesized in all cell types as a homodimeric proTGF-β1 precursor (Fig. 1) (18, 19). 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 latency-associated peptide (LAP). 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 (11, 20). GARP·latent TGF-β1 complexes are then presented on the Treg surface (11, 15, 16). Stimulated Tregs release mature TGF-β1 from surface GARP·latent TGF-β1 complexes, a process called "latent TGF-β1 activation." This allows binding of active TGF-β1 to its receptor, leading to autocrine and paracrine signaling followed by phosphorylation of SMAD transcription factors (8). GARP is necessary for TGF-β1 activation by Tregs because some anti-GARP monoclonal antibodies are

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[4] The abbreviations used are: Treg, regulatory T cell; GARP, glycoprotein A repetitions predominant; LAP, latency-associated peptide; Th, T helper lymphocyte; CTL, cytotoxic T lymphocyte; TSDR, Treg-specific demethylated region; TCR, T cell receptor; EPOR, erythropoietin receptor; BCR, B cell receptor; RT-qPCR, quantitative RT-PCR; CUB, C-terminal moiety of ubiquitin; NUBG, N-terminal moiety of ubiquitin.

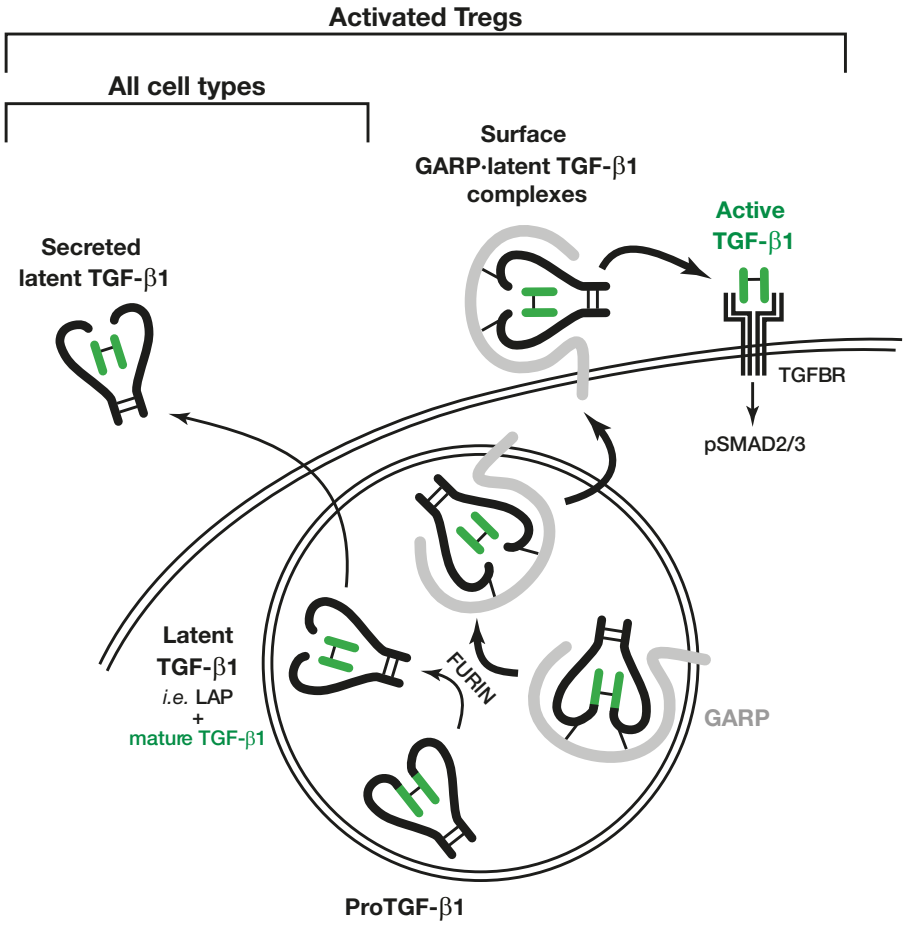


FIGURE 1. **Production and processing of TGF-β1 in cells expressing (Tregs) or not expressing (other cell types) GARP.** *Double curves* represent cell membranes, with the secretory pathway shown as a *circle*. In latent TGF-β1, the LAP is represented as *thick black lines*, and the mature TGF-β1 as *green lines*. *Thin black lines* represent disulfide 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 with GARP (11).

able to block this process (21). GARP is therefore a regulator of TGF-β1 production by human Tregs.

We postulated that additional proteins expressed in Tregs but not in other T lymphocytes cooperate with GARP to regulate TGF-β1 production. Here we sought to identify proteins that bind to GARP and are expressed at higher levels in Tregs than in Th cells to identify new regulators of TGF-β1 production in human Tregs. As a source of human Tregs, we used Treg clones described previously, *i.e.* pure populations of cells with a demethylated *FOXP3i1* allele, or blood $CD4^+CD25^+CD127^{lo}$ cells shortly expanded *in vitro*, *i.e.* polyclonal populations enriched in cells with a demethylated *FOXP3i1* allele. Both cell populations are suppressive *in vitro*, express FOXP3, and, upon TCR stimulation, induce GARP expression and produce active TGF-β1 (8, 11, 15, 22–24). On the basis of their suppressive activity and mRNA, protein, and epigenetic profile, we termed these human cells "Tregs" (Treg clones or polyclonal Tregs), as proposed by Abbas *et al.* (25).

Experimental Procedures

Split-ubiquitin System in Yeast—We used the split-ubiquitin system (Dualsystems Biotech AG) according to the instructions of the manufacturer. Briefly, the *GARP* ORF was cloned into SfiI sites of the bait plasmid pBT3-SUC. The resulting plasmid was used to transform and select GARP-expressing NMY51 yeasts. A prey cDNA library was synthesized in the pPR3N plasmid, starting from 2 μg of total RNA isolated from three different human Treg clones stimulated during 24 h with anti-CD3 and anti-CD28, as described previously (8). The Treg cDNA library contained 5.2×10^6 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 synthetic defined Leu^-Trp^- medium to assess transformation efficiency. The remaining yeasts were selected on synthetic defined $Leu^-Trp^-His^-Ade^-$ medium 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 described previously (8). qPCR amplifications were done with the 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.

The sequences of primers used to amplify the LAPTM4B mRNA variants shown in Fig. 3 (5′ to 3′) were as follows: primer

A1, ATTAACAAGGATCCGCGATGACGTCACGGACTCGG; primer A2, ATTAACAAGGATCCGCGATGAAGATGGTCGCGCCC; primer B, GCTCTATGGTGCCTGGGCCA; and primer R, AACTGATTCTCGAGCGCAGACACGTAAGGTGGCGG. The primers used for RT-qPCR analysis of LAPTM4B Va expression (5′ to 3′) were as follows: sense, ACCATCCTGCTCGGCGTCTG; antisense, CGGATCAGCCAGGGCACTCAAT. The primers used for RT-qPCR analysis of LAPTM4B Vb expression (5′ to 3′) were as follows: sense, GCTCTATGGTGCCTGGGCCA; antisense, CGGATCAGCCAGGGCACTCAAT; and FAM-TAMRA TaqMan probe, GTACCACAGCATTGATGATCTCATTCCCC.

Electroporation of siRNA in Tregs—Human polyclonal cell populations enriched in Tregs ($CD4^+CD25^+CD127^{lo}$ cells) were isolated and expanded from hemochromatosis donors as described previously (11). Expanded Tregs were mixed with siRNAs (3–80 pmol/10^6 cells, as indicated in the figure legends) and electroporated using unstimulated human T cells 4D-Nucleofector solution and a 4D-Nucleofector instrument (Lonza). Immediately after transfection, cells were restimulated with anti-CD3/CD28-coated beads (Dynabeads human T-activator CD3/CD28, Life Technologies) in Iscove's modified Dulbecco's medium supplemented with 10% human serum, L-arginine, L-asparagine, L-glutamine, β-mercaptoethanol (5×10^{-5} M), and methyl-tryptophan (200 μM).

The sequences of siRNAs (Silencer Select siRNAs from Life Technologies, 5′ to 3′) were as follows: si*LAPTM4B #1* (catalog no. S30812), GGAUCAGUAUAACUUUUCAtt (sense) and UGAAAAGUUAUACUGAUCCgg (antisense); si*LAPTM4B #2* (catalog no. s502845, UCAAUGCUGUGGUACUGUUtt (sense) and AACAGUACCACAGCAUUGAtg (antisense).

Cell Transfections—293T cells or a 293T cell clone stably expressing human GARP and human TGF-β1 were transiently transfected with the plasmids indicated in the figures using TransIT-LT1 transfection reagent (Mirus Bio). Cells were analyzed 24 h after transfection. Luciferase activity was measured with the Britelite Plus reporter gene assay system (PerkinElmer Life Sciences).

Western Blot Analysis—Cells were lysed in Laemmli buffer supplemented with 5% β-mercaptoethanol as described previously (8) and submitted to SDS-PAGE and Western blot analysis with the following primary antibodies, as indicated in the figures: anti-GARP (Enzo Life Sciences, catalog no. ALX-804-867), anti-TGF-β1 (BD Biosciences, catalog no. 555052), biotinylated anti-LAP (R&D Systems, catalog no. BAF246), anti-Myc (Roche, catalog no. 11-667-149-001), anti-PSMAD2 (Cell Signaling Technology, catalog no. 3108), anti-SMAD2 (Cell Signaling Technology, catalog no. 3122), anti-IGF1Rβ (Cell Signaling Technology, catalog no. 3027), anti-HA (Eurogentec, catalog no. MMS-101R), or anti-β-Actin (Sigma, catalog no. A5441).

FACS Analyses—Cells were stained with mouse monoclonal biotinylated anti-GARP antibody (clone MHG-6 (21)) followed by streptavidin coupled to phycoerythrin (BD Biosciences, catalog no. 554061), anti-LAP antibody coupled to allophycocyanin (R&D Systems, catalog no. 27232), anti-CD9 antibody coupled to phycoerythrin (BioLegend, catalog no. 312105), or anti-HLA-A2 antibody coupled to FITC (BioLegend, catalog no. 343304). Data were collected on a FACS LSR Fortessa (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Latent TGF-β1 Concentrations in Supernatants—Supernatants treated with acid or left untreated were analyzed by ELISA according to the instructions of the manufacturer (human TGF-β1 Duoset, R&D Systems).

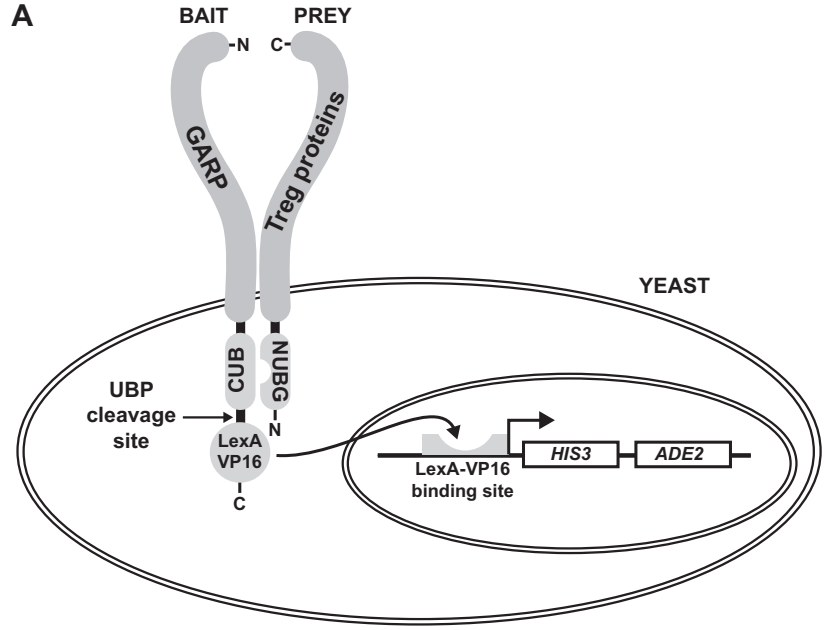
Fluorogenic Assay for FURIN-specific Activity—Assays were performed as described by Bourne and Grainger (26). Briefly, transfected 293T cells were lysed in 5× lysis buffer (500 mM HEPES (pH 7.0), 2.5% Triton X-100, 5 mM calcium chloride, and 5 mM β-mercaptoethanol). Lysates containing 3.3×10^5 cell equivalents were seeded in black opaque 96-well plates precoated with a goat polyclonal anti-FURIN antibody (R&D Systems, catalog no. AF1503). Where indicated, the FURIN inhibitor I Dec-RVKR-CMK (decanoyl-Arg-Val-Lys-Arg-chloromethylketone; 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 the addition of substrate on a Victor X2 plate reader (PerkinElmer Life Sciences), with excitation at 380 nm and emission at 460 nm.

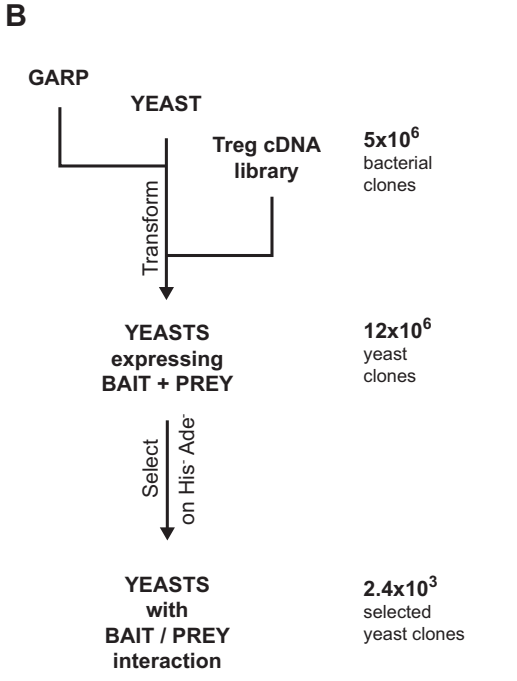
Gaussia Luciferase Complementation Assay—Protein complementation assays were performed as described by Remy and Michnick (27). Briefly, 293T cells were transfected with Lipofectamine 2000 transfection reagent (Life Technologies) in triplicate wells (96-well plates) with 0.05 μg of each plasmid. The medium was changed to DMEM without phenol red (Life Technologies), and native coelenterazine (Nano Technologies, catalog no. 303) was added to live cells 24 h after transfection. Luciferase activities were measured on live cells in a Victor X Light device (PerkinElmer Life Sciences).

Ethics Statement—Experiments with human cells were approved by the ethics committee of our institution's (Commission d'Ethique Biomédicale Hospitalo-Facultaire de l'Université catholique de Louvain) under registration number B403201110966.

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 Fig. 2*A*, the bait is a transmembrane protein fused to the C-terminal moiety of Ubiquitin (CUB) and to the LexA-VP16 hybrid transcription factor. Because of 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 when bait and prey interact. Reconstituted Ubiquitin is recognized by Ubiquitin-specific proteases that 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 (Fig. 2*A*). It must be noted that productive bait/prey interactions occur only when CUB and NUBG are





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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*, 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 three or two clones, respectively), as measured with Affymetrix expression microarrays (8). *nd*, not determined.

both located in the cytosol. Therefore, when NUBG is fused to the N-terminal extremity of prey, 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 three different human Treg clones stimulated for 24 h with anti-CD3 and anti-CD28 antibodies (8).

We transformed the Treg cDNA library in yeasts expressing GARP-CUB-LexA-VP16 (Fig. 2*B*). 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 with that of cDNAs frequently isolated in unrelated split-ubiquitin screens (Dualsystems)[5] to exclude 45 cDNAs that were likely to be false positives. We further excluded 31 other cDNAs encoding proteins involved in protein translation or posttranslational modifications or proteins that do not have a predicted N terminus in the cytosol. This left us with 14 cDNAs encoding proteins that potentially interact with GARP.

[5] C. Huygens, S. Liénart, O. Dedobbeleer, J. Stockis, E. Gauthy, P. G. Coulie, and S. Lucas, unpublished data.

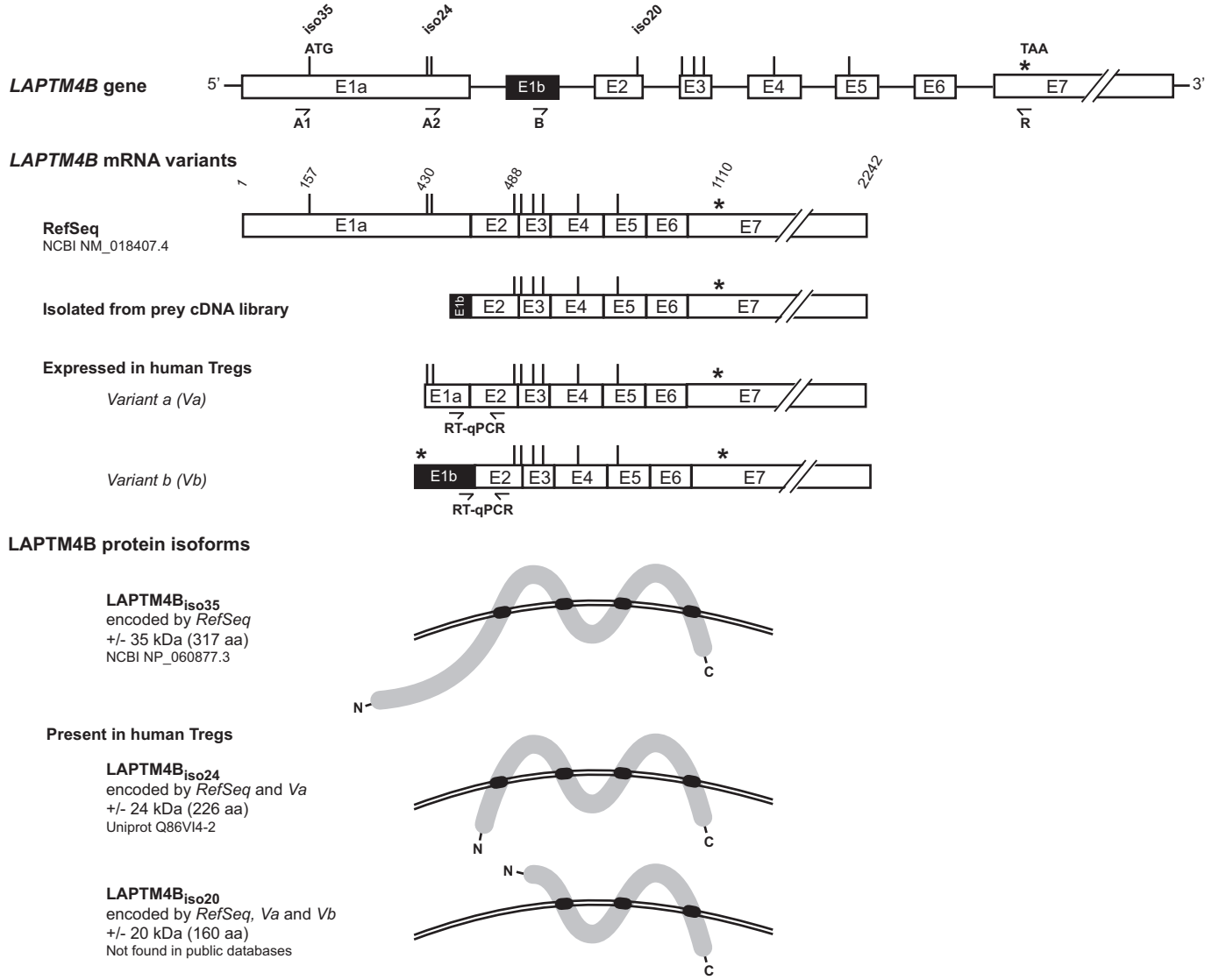


FIGURE 3. **LAPTM4B mRNA variants expressed in human Tregs encode two protein isoforms that differ at their N termini.** Shown are schematics of the LAPTM4B gene, mRNA variants, and proteins isoforms. Exons are represented as *boxes* and introns as *thin horizontal lines*. All start and stop codons in-frame with the reported start of the RefSeq mRNA are indicated by *vertical lines* and *asterisks*, respectively. Primers used for RT-PCR are indicated by *arrows*. LAPTM4B protein isoforms are represented as *thick gray 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.

We examined the expression profiles of the corresponding mRNAs in stimulated human Treg and Th clones using available expression microarray data (8). Two mRNAs, *LAPTM4B* and *CD9*, were expressed at >7-fold higher levels in Treg *versus* Th clones (Fig. 2*C*). Because 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.

Two mRNA Variants of LAPTM4B Are Expressed in Tregs—According to public databases, the transmembrane protein LAPTM4B is encoded by a gene reported to comprise seven exons: E1a and E2-E7. The mRNA variant isolated from our prey cDNA library contained an alternative first exon, which we named E1b (Fig. 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 (Fig. 3 and data not shown). Primer A2, straddling a described alternative start codon (28), 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. Sequencing confirmed that *Va* does not contain exon E1b. 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 we named "*Variant b*" (*Vb*). We used 5′ rapid amplification of cDNA ends to identify the 5′ extremity of *Vb* (Fig. 3).

LAPTM4B variants have multiple AUG codons in-frame with the longest open reading frame. Although translation of most eukaryotic mRNA initiates at the first AUG, an alternative mechanism, termed leaky scanning, can result in translation initiation at more distal AUG codons and has been reported to

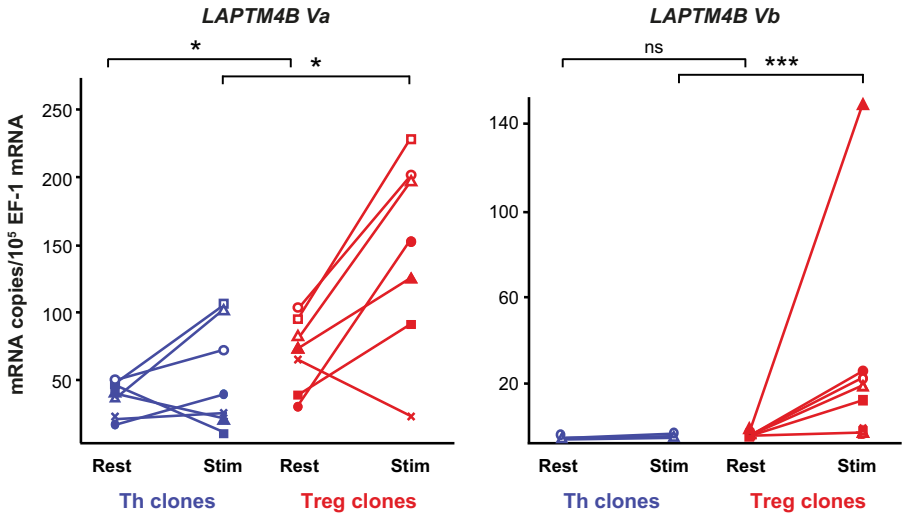


FIGURE 4. **LAPTM4B *Va* and *Vb* are expressed at higher levels in human Tregs compared with Th cells.** Expression of the indicated *LAPTM4B* mRNA variants was measured by RT-qPCR in seven Th clones (*blue lines*) and seven Treg clones (*red lines*) at rest or 24 h after stimulation with anti-CD3 and anti-CD28 antibodies. *, $p < 0,05$; ***, $p < 0,001$; *ns*, not significant; Mann-Whitney analysis.

lead to the production of at least two LAPTM4B isoforms (28). *LAPTM4B* variants therefore potentially encode several LAPTM4B isoforms that differ only by the length of their N termini (Fig. 3). $LAPTM4B_{iso35}$ (35 kDa) is encoded by the RefSeq variant only, whereas $LAPTM4B_{iso24}$ (24 kDa) is encoded by RefSeq and *Va*. *LAPTM4B Vb* can encode a previously unknown 20-kDa isoform, $LAPTM4B_{iso20}$, that lacks the first 66 amino acids of $LAPTM4B_{iso24}$. RefSeq and *Va* also encode $LAPTM4B_{iso20}$. Because Tregs express *Va* and *Vb* but not RefSeq, we hypothesized that $LAPTM4B_{iso24}$ and $LAPTM4B_{iso20}$, but not $LAPTM4B_{iso35}$, are present in Tregs. We could not verify this hypothesis because of the lack of appropriate antibodies.

The Va and Vb mRNA of LAPTM4B Are Expressed at Higher Levels in Tregs Than in Th Cells—We used RT-qPCR to measure the expression levels of *LAPTM4B Va* and *Vb* mRNAs in resting or stimulated Treg and Th clones using the primers illustrated in Fig. 3. As shown in Fig. 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 (more than three copies/10^5 EF-1 copies in five of seven stimulated Treg clones). Together, *LAPTM4B Va* and *Vb* are expressed at higher levels in stimulated human Tregs than in resting Tregs and in resting or stimulated Th cells.

Confirmation of the Interaction between GARP and LAPTM4B in Mammalian Cells—To test whether 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 (27). Luciferase activity is recovered only when candidate proteins interact with each other. We cotransfected constructs encoding hGLuc1 fused to N-terminally HA-tagged GARP and hGLuc2 fused to HA-tagged LAPTM4B. Two *LAPTM4B* 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 analysis (Fig. 5, *top panel*). High luciferase activity was detected in cells coexpressing GARP and $LAPTM4B_{iso24/20}$ or GARP and $LAPTM4B_{iso20}$ (Fig. 5, *bottom panel*). As expected, no or very low activity was detected in cells expressing any protein alone or in cells coexpressing GLuc fragments fused to GARP and EPOR or to LAPTM4B and EPOR. EPOR was taken as a negative control. These results show that GARP and LAPTM4B isoforms interact in mammalian cells and that the first 66 amino acids of $LAPTM4B_{iso24}$ (Fig. 3) are not required for this interaction.

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 analysis, whether LAPTM4B affects the cleavage of proTGF-β1 into latent TGF-β1 (Fig. 6*A*). In cells transfected with *TGFB1* alone (Fig. 6*A*, *lane 2*), proTGF-β1 is abundant, but LAP or mature TGF-β1 are not detected, indicating that cleavage of the precursor does not occur at high levels. Cotransfection of *TGFB1* with *LAPTM4B* (Fig. 6*A*, *lane 3*) does not increase cleavage. In contrast and as expected, cotransfection of *TGFB1* with *GARP* (Fig. 6*A*, *lane 6*) induces cleavage, as evidenced by the detection of abundant LAP and mature TGF-β1. Cotransfection of LAPTM4B (Fig. 6*A*, *lane 7*) abolished this GARP-induced cleavage, an effect that was not observed upon cotransfection of *CD9* (Fig. 6*A*, *lane 8*), taken here as a negative control.

Because 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 (Fig.

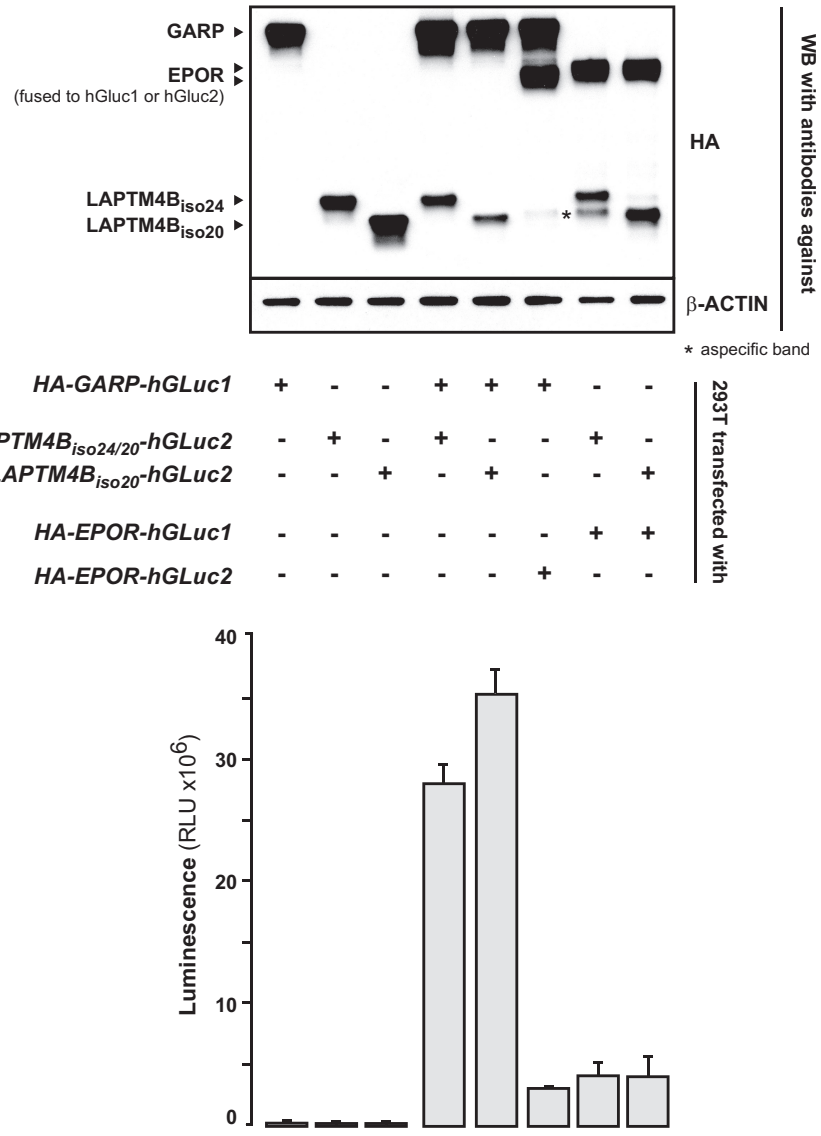


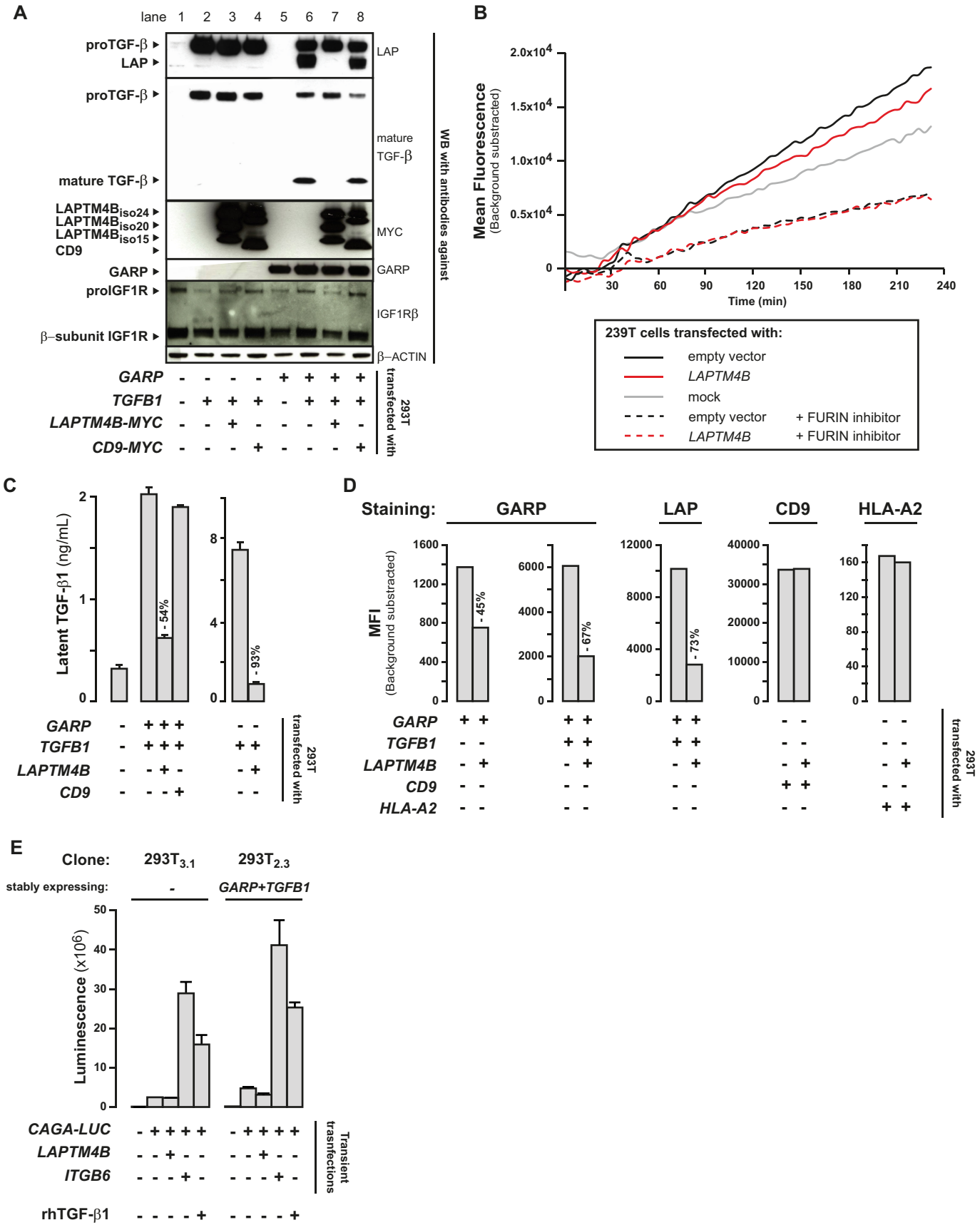
FIGURE 5. **GARP interacts with LAPTM4B in mammalian cells.** 293T cells were transfected with constructs encoding GARP, LAPTM4B, or negative control EPOR fused to hGLuc1 or hGLuc2 as indicated. *Top panel*, Western blot analysis of transfected cells. *Bottom panel*, luciferase activity in live cells 24 h after transfection. One experiment representative of three independent experiments is shown. *WB*, Western blot; *RLU*, relative light units.

6*A*). We also measured FURIN activity on an exogenous fluorogenic substrate, and the transfection of *LAPTM4B* did not decrease FURIN activity in this assay either (Fig. 6*B*). 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 pro-TGF-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* compared with cells transfected with *GARP* and *TGFB1* only (Fig. 6*C*). 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 (Fig. 6*C*). 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 (11, 20).

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 cotransfection with *LAPTM4B* (Fig. 6*D*). In cells transfected with *GARP* and *TGFB1*, cotransfection of *LAPTM4B* 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 coexpression of LAPTM4B. Therefore, LAPTM4B reduces surface levels of GARP and GARP·latent TGF-β1 complexes.

Finally, we tested whether LAPTM4B regulates latent TGF-β1 activation (Fig. 6*E*). 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 (29). We transiently cotransfected the *CAGA-LUC* reporter with *LAPTM4B* in clones of 293T cells stably expressing or not expressing *GARP* and *TGFB1*. Cotransfection of LAPTM4B did not increase luciferase activity above back-


A
lane 1 2 3 4 5 6 7 8
proTGF-β
LAP
LAP
proTGF-β
mature TGF-β
mature TGF-β
LAPTM4B iso24
LAPTM4B iso20
LAPTM4B iso15
CD9
MYC
GARP
GARP
proIGF1R
IGF1Rβ
β–subunit IGF1R
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
MFI (Background substracted)
1600
1200
800
400
0
6000
4000
2000
0
12000
8000
4000
0
40000
30000
20000
10000
0
160
120
80
40
0
- 45%
- 67%
- 73%
GARP + + + + + + - - - -
TGFB1 - - + + + + - - - -
LAPTM4B - + - + - + - + - +
CD9 - - - - - - + + - -
HLA-A2 - - - - - - - - + +
293T transfected with
E
Clone: 293T3.1 293T2.3
stably expressing: - GARP+TGFB1
Luminescence (x10^6)
50
40
30
20
10
0
CAGA-LUC - + + + + - + + + +
LAPTM4B - - + - - - - + - -
ITGB6 - - - + - - - - + -
Transient trasnfections
rhTGF-β1 - - - - + - - - - +

ground (*i.e.* transfection of the 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 (Fig. 6*E*). As expected, high luciferase activity was induced by transfection of integrin β6, a known activator of TGF-β1 (30), or by addition of recombinant active TGF-β1, both used 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. Therefore, LAPTM4B appears to be a negative regulator of TGF-β1 production in these cells.

LAPTM4B Silencing in Human Polyclonal Tregs Increases the 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 42–58% of cells with demethylated *FOXP3i1*. They were transfected with siRNAs and stimulated through their TCR to induce TGF-β1 activation or left unstimulated. *siLAPTM4B #1* reduced the expression of *LAPTM4B Va* and *Vb* by >85%. It increased by 41–53% the secretion of latent TGF-β1 by resting and stimulated Tregs and by ±100% the surface presentation of GARP and latent TGF-β1 on stimulated Tregs (Fig. 7*A*). Increases in surface GARP and latent TGF-β1 were detected with amounts of *siLAPTM4B #1* as low as 16 pmol/10^6 cells (Fig. 7*B*) and were also observed with *siLAPTM4*B *#2*, which targets *LAPTM4B* at a site different from that targeted by *siLAPTM4B #1* (Fig. 7*C*). Neither of the two *siLAPTM4B* modified surface levels of CD4, used here as an unrelated negative control (Fig. 7*C*). As expected, *siLAPTM4B* did not reduce the activation of TGF-β1 by stimulated Tregs (Fig. 7*D*). These results confirm that LAPTM4B is a negative regulator of TGF-β1 production in human Tregs.

Discussion

LAPTM4B belongs to a family of three glycoproteins with four or five 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 with poor prognosis in many types of cancers (31–41), and *in vitro* and *in vivo* analyses of cells in which *LAPTM4B* was overexpressed or silenced indicate that LAPTM4B favors proliferation, migration, invasion, tumorigenesis, and metastasis (42, 43). Two mechanisms by which LAPTM4B plays oncogenic roles have recently been identified. Both imply facilitation of the prosurvival functions of the EGF receptor in cancer cells. In the presence of EGF, LAPTM4B enhances signaling by blocking the lysosomal degradation of activated EGF receptor (44), and in the absence of EGF, it interacts with inactive EGF receptor in the endosomes to initiate cell-protective autophagy (45).

Our results are the first to describe a function for LAPTM4B in the immune system. It decreases the production, secretion, and surface presentation of latent TGF-β1 by Tregs. Down-regulation of surface presentation appears to result from a direct effect of LAPTM4B on surface GARP levels because 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 has been reported to interact and to down-regulate 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 (46, 47). C-terminal polyproline-tyrosine (PY) motifs target Laptm5 to lysosomes and are required for the Laptm5-mediated down-modulation of surface TCR levels, suggesting a mechanism by which Laptm5 promotes lysosomal targeting and degradation of receptors to which it is bound (47, 48). PY motifs are also present in the C terminus of LAPTM4B (47, 49). It is therefore possible that LAPTM4B down-regulates GARP surface levels in human Tregs through a similar mechanism. However, we observed in transfected cells that GARP and LAPTM4B colocalize mostly in the median Golgi and that the localization of GARP was not modified in the presence of LAPTM4B (unpublished observations).[6] It is worth noting that the down-regulation of latent TGF-β1 secretion by LAPTM4B may occur through another mechanism, independent from GARP, because it was also observed in transfected 293T cells in the absence of GARP.

Two isoforms of LAPTM4B, $LAPTM4B_{iso35}$ and $LAPTM4B_{iso24}$, have been described previously (28). 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

[6] C. Huygens, D. Tyteca, and S. Lucas, unpublished observations.

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 293T cells transfected as indicated were analyzed by Western blot. *B*, specific FURIN activity was measured in cell lysates 24 h after transfection by capturing FURIN on immobilized anti-FURIN antibody and 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 under 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 expressing human GARP and human TGF-β1 were transfected with *LAPTM4B* or *ITGB6* together with the CAGA-LUC reporter. Luciferase activity was measured 24 h after transfection. Recombinant human (*rh*) TGF-β1 was added during 6 h at 4 ng/ml under the positive control condition, as indicated. The *TGFB1* construct used for transfections contains the full-length *TGFB1* ORF coding for the pre-proTGF-β1 precursor, which is processed in 293T cells like in all other cell types.

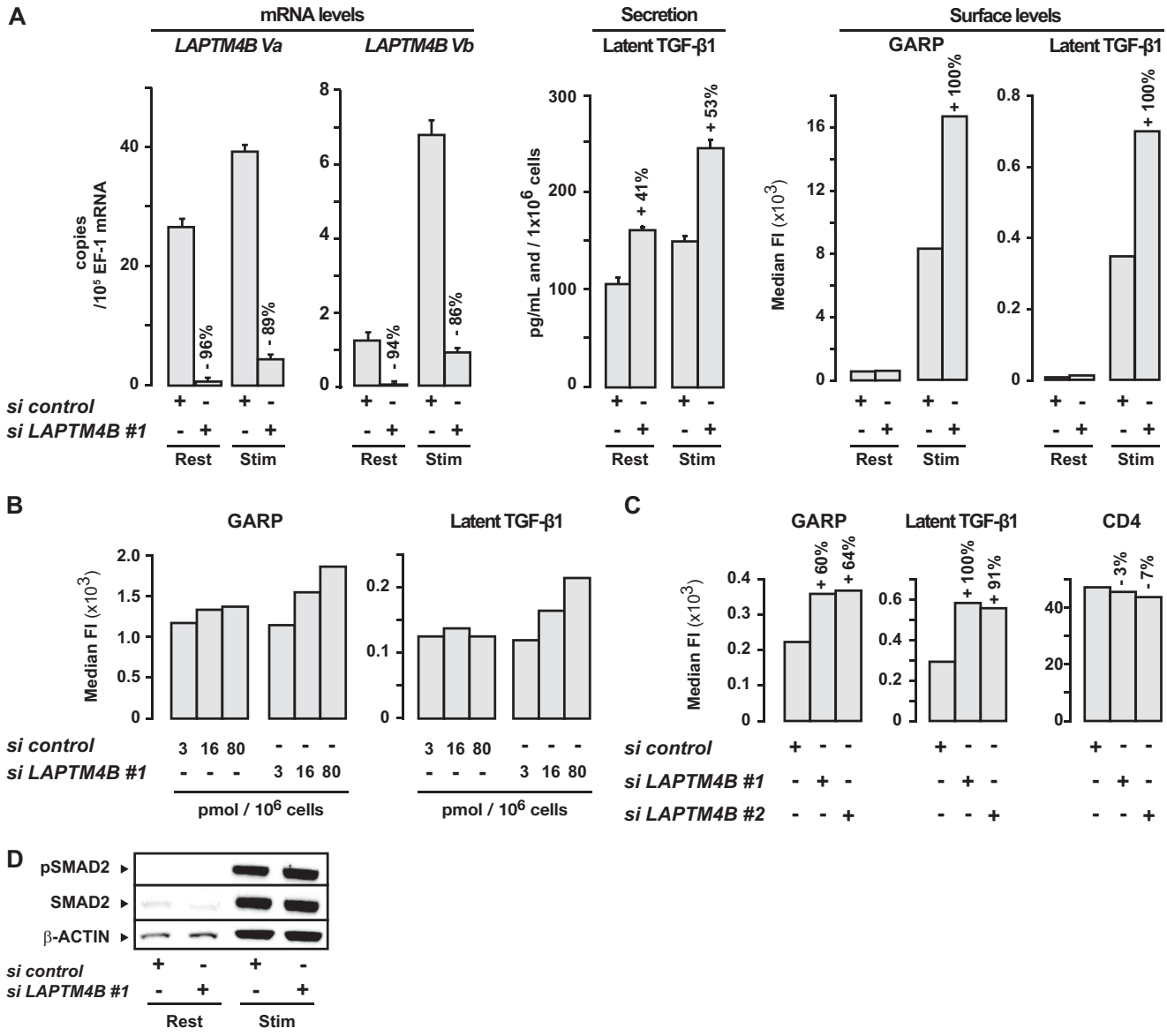


FIGURE 7. **LAPTM4B decreases GARP surface levels and TGF-β1 secretion in human Tregs.** *A*, polyclonal human Tregs were transfected with the indicated siRNAs (50 pmol/10^6 cells) and then left resting (*Rest*) or stimulated (*Stim*) with anti-CD3/CD28-coated beads for 3 days. Levels of *LAPTM4B Va* and *Vb* mRNAs were measured by RT-qPCR. Amounts of total and active TGF-β1 were measured by ELISA in supernatants treated with acid or left untreated. Because no active TGF-β1 was detected in non-acidified supernatants, total TGF-β1 in acidified supernatants corresponded to latent TGF-β1. Surface levels of GARP and latent TGF-β1 were measured by flow cytometry with anti-GARP and anti-LAP antibodies, respectively. The percent increase or decrease compared with the control siRNA is indicated above the *bar graphs*. One experiment representative of three is shown. *B*, as *A*, with the indicated amounts of siRNAs. *FI*, fluorescence intensity. *C*, as *A*, with 80 pmol/10^6 cells siRNAs and cells stimulated during 4 days. *D*, Western blot analysis of cells shown in *A* with anti-pSMAD2, anti-total SMAD2, and anti-β-ACTIN antibodies. Detection of pSMAD2 indicates active TGF-β1 production.

for the interaction. We could not determine whether $LAPTM4B_{iso24}$ also interacts with GARP because all of our constructs encoding $LAPTM4B_{iso24}$ also encode $LAPTM4B_{iso20}$. 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 of these *LAPTM4B* mRNA variants.

We report that *LAPTM4B* expression is increased upon TCR activation of human Tregs and that it is higher in Tregs than in Th cells. Overexpression of *LAPTM4B* in human or mouse Tregs as well as in non-Tregs transduced with *FOXP3* was observed in published expression microarray data sets (50–52), suggesting that gene *LAPTM4B* might be transcriptionally regulated by FOXP3. Up-regulation of *LAPTM4B* expression in Tregs after TCR stimulation may represent a negative feedback mechanism that eventually shuts down production of TGF-β1 to ensure return to a resting state.

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 (15, 16). Tregs are potent inhibitors of immune responses, and excessive or insufficient Treg function is implicated in various human

diseases. Pharmacological inhibition of LAPTM4B could sustain or increase TGF-β1 production and, therefore, Treg function, in patients suffering from autoimmune disease or allograft rejection.

Author Contributions—C. H. and S. Lucas designed the study. C. H., S. Liénart, O. D., J. S., and E. G. performed the experiments. C. H., S. Lucas, and P. G. C. wrote the paper. All authors analyzed the results and approved the final version of the manuscript.

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Note Added in Proof—As was brought to our attention by Dr Z. Li, we wish to mention that GARP was also shown to interact with GP96, a protein controlling the folding of several immune-related proteins in the ER (Zhang, Y., Wu, B. X., Metelli, A., Thaxton, J. E., Hong, F., Rachidi, S., Ansa-Addo, E., Sun, S., Vasu, C., Yang, Y., Liu, B., and Li, Z. (2015) GP96 is a GARP chaperone and controls regulatory T cell functions. *J. Clin. Invest.* **125,** 859–869).

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