TSC/mTOR Signalling Controls Thymocyte Development and Proliferation/Differentiation of T Lymphocytes and Nerve Cells

Thesis

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TSC/mTOR signalling controls thymocyte development and proliferation/differentiation of T lymphocytes and nerve cells

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To Michela

"The teacher who is indeed wise does not bid you to enter the house of his wisdom but rather leads you to the threshold of your mind."

Kahlil Gibran

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Student declaration

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4) Romana Tomasoni, Veronica Basso, and Anna Mondino. 'T-lineage restricted Tsc1 deletion unbalances mTOR/Akt/FoxO signalling hindering thymic T cell development and mature T cell homeostasis'. In preparation

Abstract

This thesis is focused on the role of the Tuberous Sclerosis Complex (TSC)/mammalian Target of Rapamycin (mTOR) pathway in the proliferation, survival and differentiation of T cells and PC12 nerve cells. TSC and mTOR play important roles in these cellular processes in different cell models. We wondered which could be the contribution of these pathways in T cells and in particular in thymic development and in another cellular model of differentiation like the PC12 cells.

In the first part of the thesis the results of the studies on the differentiation of mature T cells are presented. The involvement of mTORC1 and mTORC2 was analysed in these processes by pharmacological inhibition with Rapamycin. The mTOR complexes were found to affect cytokines expression by influencing DNA methylation of their promoters.

The role of the TSC pathway in thymic development was then evaluated. TSC1 was genetically inactivated early on during thymic development. It was possible to observe an increase in proliferation and cell death of TSC1 deleted cells. The increased proliferation was due to increased mTORC1 signalling, while the increased cell death results from increased FoxO activity, a consequence of defective mTORC2/Akt signalling.

The last part of the thesis focuses on the study of the TSC pathway in PC12 cells, a model of neuronal differentiation. The TSC complex plays a critical role in balancing the processes of proliferation and neurosecretory functions in these cells by its ability to affect the β-catenin and REST pathways. The signalling loop that connects these three central players can alternatively control these important processes.

1. General introduction

The interest of Dr. Mondino's lab is to define signalling pathways controlling cell growth, proliferation, differentiation and survival in T lymphocytes. This thesis reports results from the study of several of these events, relative to the activity of Tuberous Sclerosis Complex (TSC) and the mammalian Target of Rapamycin (mTOR), able to coordinate cell growth and cell metabolism, and by that the potential of T cells and nerve cells to proliferate, differentiate and survive. The thesis has been organized as follows: a general overview of TSC and mTOR and of the upstream and downstream signalling events implicated in cell growth, proliferation, differentiation and survival and three distinct chapters dealing with the role of TSC/mTOR in $CD4^+$ T cell differentiation, in T cell development and in nerve cell functions.

1.1 TSC

Tuberous Sclerosis Complex (TSC) and the mammalian Target of Rapamycin (mTOR) are important regulators of cell growth and proliferation. They can integrate many different signals such as nutrients, the energy status of the cell and growth factors and can orchestrate diverse processes such as autophagy, ribosome biogenesis and metabolism. Deregulation of these complexes can lead to serious diseases, such as cancer, diabetes or degenerative syndromes, involving many different systems and organs.

Tuberous Sclerosis Complex (TSC) is an autosomal dominant genetic disease with formation of hamartomas in multiple organ systems with an incidence of 1 in 6000 births, arising from mutations in either hamartin (TSC1) or tuberin (TSC2). Mutations in both TSC1 or TSC2 range from nonsense, missense, insertion and deletion mutations, while large genomic deletions are more common for the TSC2 gene [1] . TSC1 and TSC2 form a complex in cells [2, 3], involved in many different cellular mechanisms such as cell size, cell cycle, cellular proliferation and cell death [4-7] . This is due to critical positioning of the TSC1/TSC2 complex at the crossroad of many different signalling pathways

integrating the energy status of the cell with nutritional availability and extracellular growth factor signalling. Binding of TSC1 to TSC2 stabilizes TSC2 by preventing its ubiquitin-mediated degradation [8, 9]. TSC1 and TSC2 are both regulated by phosphorylation.

TSC1 is phosphorylated at multiple Thr and Ser sites by Cyclin-Dependent Kinase 1 (CDK1) during the G_2/M phase of the cell cycle [10] in HEK293 cells. CDK1 is active in late cell cycle phases and it favours entry into mitosis when it is bound to Cyclin B1 (review by Nigg E. A. [11]). CKD1 phosphrylates TSC1 at three residues (Thr417, Ser584 and Thr1047) that lie within the TSC1-TSC2 interaction domain. Hamartin phosphorylation by CDK1 decreases the inhibition of p70S6K [10], suggesting that regulation of TSC1/TSC2 during G_2/M could be important for the integration of cell division with activities regulated by p70S6K such as cell size, protein synthesis and growth factor signalling.

TSC1 can also be negatively regulated through IKKβ-mediated Ser phosphorylation [12]. IKKβ is a major downstream kinase in the TNFα signalling pathway. TNFα is an important proinflammatory cytokine that is thought to be a potential mediator involved in different human disease such as cancers [13]. Lee et al. [12] demonstrated that TNFα activates the mTOR pathway through IKKβ. In MCF-7 and MDA-MB-453 cells, IKKβ, in fact, phosphorylates TSC1 at Ser487 and Ser511 and thus suppresses its function and consequently impairs the antiangiogenic function of TSC1 [12]. The results from Lee et al. [12] hint at a possible mechanism linking the TNFα and mTOR signalling pathways and at their connection to TNFα-mediated human tumour angiogenesis.

Also TSC2 is the substrate for many upstream kinases. It can be phosphorylated by the AMP-activated protein Kinase (AMPK) [14]. AMPK is one of the most important energy sensors in cells. AMPK role is quite conserved during evolution. The primary role

of the AMPK orthologue in yeast is in the response to glucose starvation [15]. Also in cells from mammals [16] and *Drosophila* [17] AMPK can be activated by glucose starvation. AMPK senses the AMP/ATP ratio of the cell. Decrease in ATP levels and increase in AMP levels activate AMPK [15]. Inoki et al. [14] demonstrated that in HEK293 cells, under energy starvation conditions, AMPK phosphorylates TSC2 in Thr1227 and Ser1345 and in this way enhances TSC2 function. This phosphorylation is required for translation regulation and for the control of cell size following energy deprivation. The authors also showed that AMPK mediated phosphorylation of TSC2 can protect cells from apoptosis induced by energy deprivation [14].

Also, Glycogen Synthase Kinase 3β (GSK3β) can phosphorylate TSC2 [18]. GSK3β is inhibited following activation of the Wnt pathway. The Wnt family is important for many different cellular functions such as cell growth, proliferation, polarity, differentiation and development [19, 20]. Deregulation of different components of the Wnt signalling pathway has also been involved in benign colorectal adenomas and cancers [21, 22]. Activation of the Wnt pathway inhibits GSK3β, this leads to decreased phosphorylation and thus stabilization of β-catenin. β-catenin can then translocate to the nucleus and form a complex with the DNA binding protein T Cell Factor (TCF) [21, 23] inducing the transcription of different genes such as Cyclin D and VGF [24, 25]. Inoki et al. [18] for the first time reported a role for the Wnt signalling in protein translation and not only in the regulation of gene transcription. The authors, in fact, demonstrated that Wnt activates the mTOR pathway and increases cell size through inhibition of GSK3β. In the different mammalian cells analysed (HEK293 and LEF), GSK3β phosphorylates TSC2 on Ser1337 and Ser1341 and activates it. The inhibition of GSK3β thus leads to inhibition of TSC2 and subsequent activation of mTORC1. By the use of Rapamycin, an inhibitor of mTOR, the authors further demonstrated that activation of mTOR by Wnt is important for mammary tumour cells growth linking the TSC2/mTOR pathway to tumorigenesis induced

by hyperactivation of the Wnt pathway.

Erk is another kinase that can phosphorylate TSC2 [26]. The Raf-MEK1/2-Erk1/2 signaling pathway is important in the regulation of differentiation and cell growth. This pathway can be activated by growth factors, inflammatory cytokines or physical stress and has as targets nuclear transcription factors, cytoplasmic translation initiation factors and members of the Bcl-2 family [27]. Ma et al. [26] showed that Erk associates with TSC2 and phosphorylates it on Ser664 in 293 cells. Erk phosphorylation leads to the disruption of the TSC1/TSC2 complex and thus to inhibition of TSC2 activity. As a consequence of ERK-mediated TSC2 phopshorylation, the TSC1/TSC2 complex was inactivated, contributing to deregulation of mTOR signalling, cell proliferation and oncogenic transformation. Accordinly, by expressing a form of TSC2 that cannot be phosphorylated by Erk in tumour cells with constitutive activation of Erk the authors observed that this particular form of TSC2 could block tumorigenecity *in vivo*, suggesting that the Ras/MAPK pathway could affect mTOR signalling through TSC and thus contribute to tumour progression [26].

The other key kinase in the regulation of TSC2 is Akt. Three isoforms of Akt have been identified: Akt1, Akt2 and Akt3. All of them are activated by growth factors and insulin through PI3K and are inhibited by the tumour suppressor PTEN [28]. Akt controls cellular functions such as cell survival, cell growth, differentiation, angiogenesis and glucose metabolism [28-30]. Akt has been shown to phosphorylate and thus regulate TSC2. Akt can phosphorylate TSC2 at Ser924 and Thr1518 [31] in *Drosophila.* The phosphorylation of TSC2 inhibits the stability of the TSC1/TSC2 complex favouring in this way cell growth in *Drosophila melanogaster* [31]. In NIH 3T3 and HEK 293 cells Manning et al. [32] demonstrated that Ser939 and Thr1462 are the phosphorylation sites phosphorylated by Akt on TSC2. These sites align with the phosphorylation sites found by Potter et al. to be target of Akt in *Drosophila* TSC2 [31]. TSC2 mutants that cannot be phosphorylated in these sites inhibits insulin-stimulated S6K1 phosphorylation and

activity, underlying the importance of Akt control of TSC2 in cell growth [32]. Akt inhibition of TSC2 activity results in increased phosphorylation of two mTORC1 targets: S6K and 4E-BP1[33]. In HeLa cells Akt phosphorylation of TSC2 results also in the degradation of the cyclin-dependent kinase inhibitor p27 and thus favours cell proliferation [34].

The different signalling pathways described above that influence TSC1/TSC2 activity are activated in many cellular processes of key importance for the life of diverse kind of cells. In particular these pathways are quite active during different stages of T cell development and activity. To date, however, there are no reports in the literature that link TSC1/TSC2 phosphorylation and activity to any of these processes in T cells.

The TSC complex thus, being at the point of convergence of key regulators of many cellular functions, controls a widespread array of signalling molecules. Among these is the mammalian Target Of Rapamycin (mTOR). The TSC2 protein acts as a GTPase activating protein toward Ras homolog enriched in brain (Rheb), a GTPase of the Ras family. When GTP-bound, Rheb activates mTOR Complex 1 (mTORC1) by preventing its association with the endogenous inhibitor FKBP38 [35, 36]. By stimulating the conversion of active Rheb-GTP to the inactive form, Rheb-GDP, the TSC1/TSC2 complex inhibits mTORC1, and signalling downstream to mTORC1 [37]. The TSC1/TSC2 complex also controls activation of mTOR Complex 2 (mTORC2) [38]. In addition to the mTOR pathway, the TSC1/TSC2 complex also directly repress β-catenin activity [39-41] thus impacting on proliferation and survival [42].

Positioning of the complex both downstream and upstream several signalling pathways accounts for TSC being a multisystem disorder [43] characterized by the development of benign tumours and hamartomas in the brain, heart and kidney [44]. Dermatological abnormalities, such as hypomelanotic macules, are also evident in pediatric patients [45]. Another clinical manifestation of TSC is the lymphangioleiomyomatosis (LAM). This disease can be sporadic or associated with TSC. LAM is characterized by the

development of lung tumours with abnormal smooth muscle cell growth, cystic destruction of the lungs and loss of pulmonary function [46-50]. The central nervous system might also be affected by mutations in either TSC1 or TSC2. The hallmarks are cortical tubers and subependymal nodules [51-53]. The affected subjects can exhibit cognitive defects, epilepsy, autism and attention-deficit hyperactive disorder [51, 54].

1.2 mTOR

The Target of Rapamycin (TOR) is a serine-threonine kinase first identified in yeast as results of efforts aimed at understanding the mechanism of action of the macrolide Rapamycin (Rapa). In yeast two genes have been described TOR1 and TOR2, which function has been linked to cellular growth [55]. mTOR is the mammalian homolog of TOR. mTOR plays critical roles in cell metabolism, growth, proliferation and survival and in many physiological and pathological processes such as angiogenesis, adipogenesis, innate and adaptive immune responses, tumour formation or insulin resistance. Accordingly inhibitors of mTOR and mTOR-dependent pathways are under evaluation for the treatment of human diseases such as cancer, type 2 diabetes and neurofibromatosis type 1.

mTOR is a 289-kDa protein that is part of the Phospho-Inositide 3-Kinase (PI3K)-related Kinase (PIKK) family [56]. Other members of this family include ATM, ATR, NA-PK and hSMG1. These proteins are characterized by a C-terminal protein kinase domain similar to the lipid kinase PI3K. The C-terminal end of mTOR contains several important elements, including the kinase catalytic domain (KIN). The KIN domain also contains a small region that is probably a site of phosphoregulation called the Negative Regulatory Domain (NRD) or 'Repressor Domain' [57, 58]. This domain contains sites of phosphorylation that are conserved in other kinases with similar structure. In this region there are key sites of phosphorylation for the activity of mTOR, in particular threonine 2446, serine 2448 and serine 2481. These sites of phosphorylation are associated with

increased mTOR activity. Serine 2448, in particular, is a target of Akt as well as p70S6 Kinase (S6K) [59-62]. Other sites of phosphorylation are substrates of the downstream effectors of mTOR itself. In this way they make possible mechanisms for feedback regulation [57, 58]. Finally, adjacent to the KIN domain is the FKBP12–Rapamycin Binding domain (FRB), the site of inhibitory interaction between Rapa and mTOR.

mTOR can form at least two different complexes. mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [63, 64]. mTORC1 is a homodimer [65-68] composed by mTOR itself and by other four different proteins: the scaffolding protein regulatory-associated protein of mTOR (raptor) as well as mammalian Lethal with Sec13 protein 8 (mLST8), the Proline-Rich Akt Substrate 40 kDa (PRAS40), and DEP-domain-containing mTOR-interacting protein (Deptor). The interaction between mTOR and its binding partners is thought to be mediated by the many heat domains in the mTOR N-terminal. Raptor is important for mTORC1 assembly and because it recruits some of mTORC1 substrates such as 4E-BP1 [69, 70]. Raptor has also been shown to be important for the subcellular localization of mTORC1 and as a sensor of the amino acids levels [71]. The role of mLST8 is not clear. Its deletion, in fact, has no consequences on mTORC1 activity [72]. Both PRAS40 and Deptor are substrates of mTORC1. When they are in the dephosphorylated state they seem to repress mTORC1 activity. After activation mTOR phosphorylates both of them leading to their dissociation from the complex and thus increasing the activity of the complex itself [73-78]. PRAS40 is also a target of Akt. The phosphorylation of PRAS40 by Akt blocks the PRAS40 inhibition of mTORC1 [77]. Main substrates of mTORC1 are S6 Kinase 1 (S6K1) and 4E-BP1, important for mRNA translation.

The central component of mTORC2 is Rictor (RAPTOR-independent companion of mTOR). mTOR can bind only to Rictor or to Raptor, so the binding with one of these prevents binding with the other [63, 79]. Also mTORC2 is a multimer like mTORC1 [80]. Also like mTORC1 it can bind to mLST8 and to Deptor [63, 73, 79]. Proteins specific of

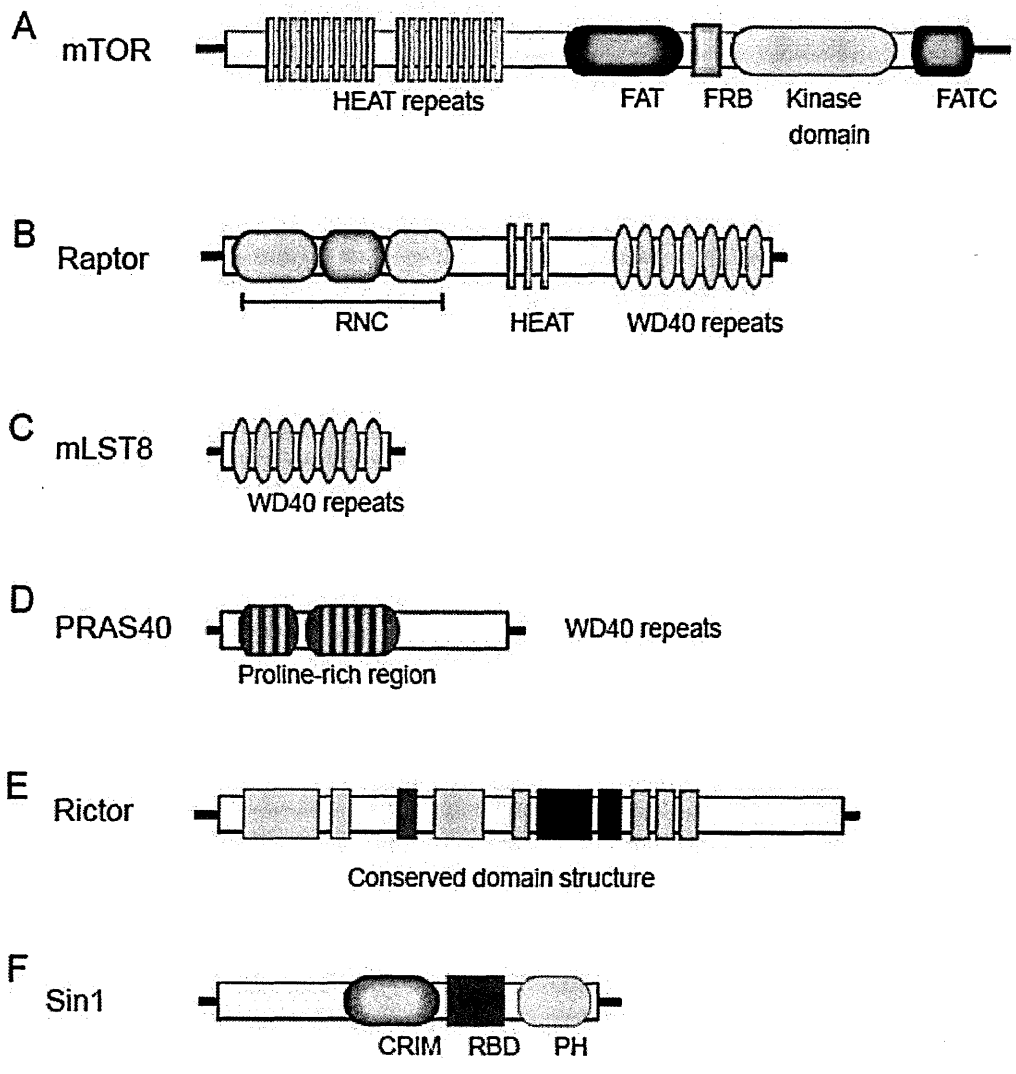

Fig I. mTOR domains. Schematic representation of the different domains of proteins belonging to the mTOR complexes. HEAT: a protein interaction structure of two tandem anti-parallel αhelices found in *h*untingtin, *e*longation factor 3, PR65/*A* and *T*OR; FAT: a domain structure shared by *F*RAP, *A*TM and *T*RRAP, all of which are PIKK family members; FRB: *F*KBP12/*r*apamycin *b*inding domain; FATC: *FAT C*-terminus; RNC: *R*aptor *N*-terminal *c*onserved domain; WD40: about *40* amino acids with conserved *W* and *D* forming four anti-parallel beta strands; CRIM: *c*onserved *r*egion *i*n the *m*iddle; RBD: *R*as *b*inding *d*omain. Yang Q., Cell Research, 2007 [81]

mTORC2 instead are mammalian stress-activated protein kinase interacting protein (mSIN1) and protein observed with Rictor-1 (Protor-1, also known as PRR5). Rictor is essential for mTORC2 catalytic activity [79]. Probably it is important for the recruitment of mTORC2 targets to the complex in a way similar to what Raptor does for mTORC1. Rictor and mSIN1 stabilize each other and they may thus be important for the integrity of

mTORC2 [82, 83]. mSIN1 however probably has other roles beside the structural one of helping in keeping the integrity of mTORC2. mSIN1, in fact, has regions homologous to the Ras-binding domain and a Pleckstrin Homology (PH)-like domain that could be functional [84]. Protor-1 binds Rictor. This protein is not essential for mTORC2 activity [76]. While mLST8 is not essential for mTORC1 activity, it is of fundamental importance for mTORC2 function [72]. In a similar way to what happens with mTORC1, Deptor binds to the FAT domain of mTOR and it inhibits also mTORC2 [73]. Main substrates of mTORC2 are Akt, Serum- and Glucocorticoid-induced protein Kinase 1 (SGK1) and Protein Kinase Cα (PKCα), important for cell proliferation and survival and for regulation of cytoskeletal organization.

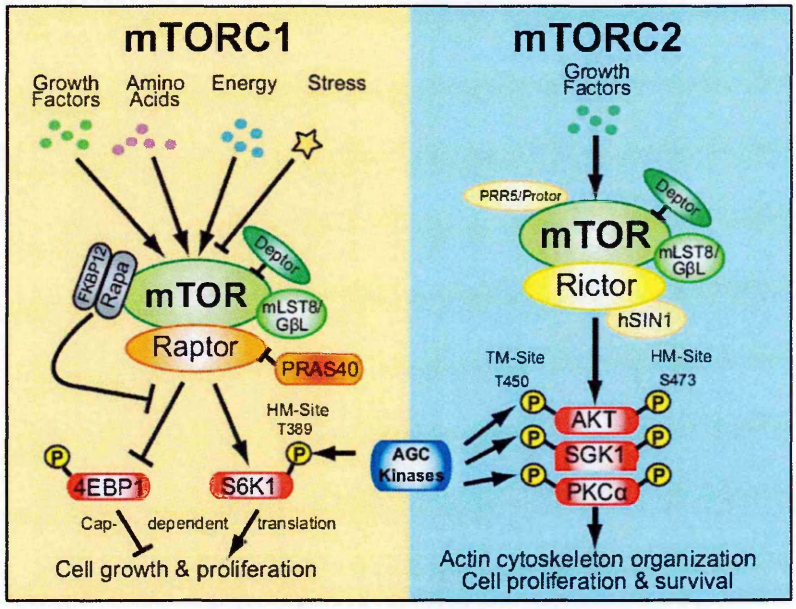

Fig II. mTOR complex components. Schematic of the different proteins of the mTOR complexes. Foster K.G., The Journal of Biological Chemistry, 2010 [85]

Both mTORC1 and mTORC2 were reported to be sensitive to Rapamycin (Rapa), although to different extents, and with different kinetics. Rapa is a macrolide produced by Streptomyces hygroscopicus, a soil bacterium found on Easter Island [86]. For years Rapa

has been considered a specific inhibitor of mTORC1, via its binding to FKNP12. When Rapa binds to the FKNP12 protein it forms a drug-receptor complex that can interact with the FKBP12-Rapamycin Binding (FRB) domain of mTOR. In this way it can directly inhibit mTORC1 function (reviewed by Guertin D. A. and Sabatini D. M. [87]). Prolonged treatment with Rapa, however, in certain cell types can inhibit also mTORC2. In this case Rapa-FKBP12 cannot directly bind to mTORC2. Rapa however can bind to free mTOR and prevent its binding to Rictor and assembly of the mTOR complex 2 [88]. Recent reports highlighted the fact that while mTORC1 is sensitive to very low doses of the drug (100-500 pM), mTORC2 can be inhibited only at higher doses (5 nM-1 uM) [89]. Recent reports also showed mTORC1 functions resistant to Rapa. By using a cap-dependent translation reporter vector Choo et al. [90] demonstrated that Rapa exhibits differential regulation of S6Ks and 4E-BP1 in a cell specific manner. While Rapa could potently inhibit S6K activity during the whole duration of the treatment (24 hours) in HEK293 and MEFs cells, the authors observed an initial inhibition of 4E-BP1 phosphorylation that recovered over time and this recovery in phosphorylation correlated with reinitiation of cap-dependent translation. Catalytic inhibitors of mTOR could prevent Rapa-resistant re-phosphorylation of 4E-BP1 [90].

Thoreen et al. [91] demonstrated that Torin1, a highly potent inhibitor of mTOR that can directly inhibit both mTORC1 and mTORC2, is able to impair cell growth and proliferation to a greater degree that Rapa, revealing Rapa-resistant functions of mTOR. Torin1 treatment of MEFs, in fact, completely inhibited proliferation and caused a G_1/S cell cycle arrest compared to the reduction in the proliferation rate of Rapa treated cells. Torin1 decreased also cell size to a greater degree than Rapa. These effects were not due to Torin1 inhibition of mTORC2 as Torin1 continued to dramatically suppress proliferation and reduce cell size also in Rictor KO MEFs. The authors also showed that mTORC1 regulation of 4E-BP1 phosphorylation and binding to eIF-4E reveals rapamycin-resistant functions [91] by looking at MEFs treated with Rapa or Torin1. By using other two TOR

inhibitors: PP242 and PP30, Feldman et al. [92] demonstrated that these inhibitors affect proliferation of primary myotubes more completely than Rapa. While PP242 does not have an evident effect on actin stress fibers in NIH 3T3 cells, the authors show that it can inhibit proliferation of primary MEFs more completely than Rapa. Also 4E-BP1 phosphorylation and Cap-Dependent translation in primary MEFs are more strongly inhibited by PP242 than Rapa, revealing mTORC1 Rapa-independent activity. By using SIN KO MEFs the authors demonstrated that the effect observed with administration of PP242 was due to its inhibitory activity on mTORC1 [92]. The phosphorylation pathways addressed by these different groups may vary among different cell types. Rapa has been shown, in fact, to cause substantial decrease in overall protein translation in some cell types such as NIH 3T3 cells [93] while having little effect in other cells such as HEK293 [94].

The use of Rapa has helped the understanding of some key functions of mTOR in T cells. It was found to be a poor antibiotic and instead to have potent immunosuppressive activities. It was initially thought to exert this activity by inhibiting IL-2-induced proliferation of T cells [95], and by that induce T cell unresponsiveness [96]. This is in contrast to Cyclosporine A (CSA), which inhibits calcineurin and TCR signalling, and overall T cell activation [97]. Results generated by Sara Colombetti, a previous PhD student in the lab, and by others, showed that Rapa does not block T cell proliferation, but only delays initial G_1 to S cell cycle transition. Most recent studies have provided evidence that Rapa, rather than being an immunosuppressant, exerts immunomodulatory activity, by preventing effector cell differentiation, and favouring the generation of regulatory T cell [98-103].

1.3 Signalling events controlling TSC/mTOR

Many different signals have been identified as regulators of the TSC/mTOR pathway. Among these growth factors, amino acids, glucose, energy status and many forms of stress (e.g. osmotic stress, DNA damage). All of these signals can cooperate or

antagonize each other. The result of the integration of all of these inputs is the precise activation of TSC/mTORC1. The regulation of mTORC2 is not so well known, it seems that only growth factors can directly regulate the complex.

The regulation of mTORC1 may be mainly through the direct regulation of proteins of the complex, such as PRAS40, or through the modification of Rheb, the GTPase that when bound to GTP can directly interact and activate mTORC1 [35].

1.3.1 Growth factors

Several growth factors such as insulin and IGF-1 promote anabolic process such as translation, lipid biosynthesis and nutrient storage, through activation of mTORC1. After insulin binds to its receptor, it activates phosphoinositide 3-kinase (PI3K) through Insulin Receptor Substrate 1 (IRS1). PI3K then is able to generate phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P_3) by direct phosphorylation of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) [104].

In lymphocytes PI3K activation has been demonstrated to follow antigen-receptor engagement [105]. Naïve T cells get activated when they encounter a cognate antigen presented by major histocompatibility (MHC) complexes on the surface of antigen-presenting cells (APCs). This leads to engagement of different signal transduction proteins that control processes such as proliferation. Among these signaling pathways the PI3Ks are of fundamental importance [106]. Costello et al. [107], by using a fusion protein made of the PKB PH domain (that binds PIP_3) and GFP, demonstrated that naïve T cells produce PIP_3 within seconds after the encounter with APCs loaded with their cognate peptide. PIP_3 production was sustained for several hours and occurred both inside and outside the T cell-APC contact zone. Different molecules downstream the TCR have been shown to be able to regulate PI3K activity. Among these components of the TCR itself such as TCR ζ [108] and different CD3 chains (γ, δ, ε) [109, 110]. Also adapter molecules like Linker for Activation of T cells (LAT) [111], T cell receptor interacting molecule (TRIM) [112] and

Shc [113] and also the small GTPase Rac [114] have all been shown to activate PI3K. The Lck kinase as well has been demonstrated to recruit PI3K to the CD4 coreceptor [115].

Activation of PI3K was also linked to costimulation by CD28 [116-118] and also to signals triggered by cytokines such as IL2 [119, 120] and IL15 [121]. IL2 and IL15, however, can activate the PI3K pathway with different kinetics. As shown by Cornish et al. [121] regulation of S6 phosphorylation by IL2 and IL15 is similar during an early 6-hour time point, but during prolonged exposure to the cytokines only IL-2 stimulated CD8+ T cells were able to maintain high levels of S6 phosphorylation [121].

PtdIns(3,4,5)P_3 is bound both by 3-phosphoinositide-dependent protein kinase 1 (PDK1) and by Akt through their pleckstrin homology (PH) domain. The binding of PDK1 to PtdIns(3,4,5)P_3 its not essential for its catalytic activity. However it favours its recruitment to the plasma membrane where it can interact with Akt and phosphorylate this protein on the residue Thr308. In addition to Thr308 by the way of PDK1, Akt also requires phosphorylation in Ser473 by mTORC2 [122] for full activation. Active Akt can then phosphorylate TSC2, inhibit it and thus lead to the activation of mTORC1.

Growth factors can phosphorylate mTORC1 also through mechanisms not involving TSC. Akt, for example, can directly phosphorylate PRAS40, the component of mTOR complex 1. PRAS40 inhibits the function of mTORC1. Following phosphorylation by Akt it does not exert any more this inhibitory function, thus Akt activity results in activation of mTORC1 [74-78, 123]. Salmond et al. [124] demonstrated that also in CD8+ T cells Akt can control mTOR activity. Administration of Wortmannin, in fact, can decrease S6 phosphorylation in these cells. We obtained similar results in CD4+ purified T cells. Treatment of cells with wortmannin, in fact, reduced S6 phosphorylation (data not shown).

Following activation by growth factors, mTORC1 is able to turn off the activation signal by a negative feedback loop, mediated by S6K1, one of the mTORC1 main targets. S6K1 can phosphorylate IRS1. This prevents its binding to the insulin receptor, and thus

terminates insulin-mediated PI3K activation. Zhang et al. [125] demonstrated that in MEFs S6K1 can inhibit the activity also of other growth factor receptors independent on IRS1, suggesting that probably it has other targets besides it [125]. Tzatsos A. [126] showed that in HEK293 cells mTORC1 can also directly interact with IRS1 through Raptor and prevent IRS1 association to PI3K [126]. The feedback loop was initially revealed following Rapa treatment or in the presence of constitutive mTORC1 activation. Harrington et al. [127] demonstrated that in MEFs Rapa treatment and thus mTOR inhibition leads to increased PI3K signalling, evaluated by Akt phosphorylation, due to increased IRS-1 function. In the case of hyperactivation of mTORC1, due to loss of TSC2, the PI3K signalling was found to be suppressed, because of lack of activation by IRS-1, found to be a substrate of S6K [127]. This feedback pathway is active also in different cellular models. Tremblay et al. [128] demonstrated the presence of the feedback loop in L6 muscle cells. The authors showed that incubation of these cells with amino acids leads to time-dependent deactivation of IRS1/PI3K activity. In particular after 30 min of stimulation with insulin Tremblay et al. observed a strong decrease of the activity of this pathway compared to the maximal activation at 5 min of stimulation. This deactivation was associated with a concomitant increase in the phosphorylation of p70S6K. Treatment with Rapa, instead, maintained PI3K maximally active for up to 30 min [128]. Tremblay et al. [129] demonstrated the presence of the feedback loop also in 3T3-L1 and human adipocytes. In particular they showed that insulin stimulated the mTOR pathway assessed by increase in p70S6K1 phosphorylation. Treatment with Rapa increased insulin-stimulated glucose transport. They authors showed also that treatment with Rapa prevented the relocalization of IRS1 from the low-density membranes to the cytosol in response to insulin [129]. Martin et al. [130] showed the presence of the loop in vascular smooth muscle cells while Tremblay et al. [131] confirmed the presence of this feedback loop in many different cellular models such as L6 muscle cells, 3T3-L1 adipocytes and Fao hepatoma cells. The presence of the loop has been demonstrated. It is thus possible that this mechanism of

regulation is present also in other cellular models such as lymphocytes, cells in which the PI3K and mTOR pathways plays key roles in different cellular processes.

1.3.2 Glucose and energy

TSC/mTOR have also been shown to be under the control of glucose levels. Glucose deprivation leads to decreased ATP levels and inhibition of mTORC1 via a mechanism involving AMPK and TSC2 [14, 132]. This is due to the fact that a high ratio of AMP/ATP signals to TSC2 through the 5' AMP-activated protein kinase (AMPK) [14]. Activated AMPK decreases processes that are particularly demanding from an energetic point of view and increases processes that lead to generation of ATP such as fatty acid oxidation. Once AMPK is activated it can hinder cell growth and the ATP consumption resulting from this process [133]. AMPK is activated when phosphorylated by its upstream kinase LKB1 [134]. AMPK, when active, phosphorylates TSC2 leading to its activation. This in turn suppresses mTORC1 activity [14, 135, 136]. AMPK can also directly phosphorylate raptor and lead to its sequestration by 14-3-3, a protein that by binding to specific phosphopeptides of its targets can regulate the activity of its binding partners. The net result is again inhibition of the kinase activity of mTORC1 [137].

1.3.3 Oxygen

Oxygen levels also impacts on TSC/mTOR signalling events. Hypoxia, a situation characterized by reduced levels of oxygen, has been shown to lead to inhibition of mTORC1. Hypoxia indeed can decrease ATP levels and thus activate AMPK. This leads to activation of the TSC1/TSC2 complex and thus mTORC1 inhibition. Hypoxia, as well as energy depletion, can also activate the Redd1/RTP801/DDIT4 pathway [138, 139]. Redd1 phosphorylates and activates TSC2 [140] by stabilizing the interaction between TSC1 and TSC2 [141, 142], and by that inhibits mTORC1 activity. Another way in which hypoxia can further suppress mTORC1 is through the promyelocytic leukemia (PML) tumour

suppressor. PML has been shown to bind to mTOR and inhibit it by sequestering it in nuclear bodies [143].

1.3.4 Amino acids

mTOR has also been described as a sensor of amino acids availability. A decrease in amino acids intake leaves the cell without substrates essential for protein synthesis. Amino acids starvation, especially of leucine, leads to a fast dephosphorylation of the mTORC1 effectors S6K1 and 4E-BP1, while re-addition of amino acids re-establishes proper levels of phosphorylation of both mTORC1 targets (reviewed by Hay N. and Sonenberg N. [57]). So different amino acids have been shown to signal to mTORC1. However it is not clear how they can do this. It seems that in this process of activation mTORC1 recruitment to lysosomal membranes, where it can bind to Rheb, is of key importance [71, 144]. On the lysosomes can be found a particular complex termed Ragulator composed of MP1, p14 and p18 [144]. This complex is a scaffold protein for Rag GTPases. Rags loading with GTP seems to be regulated by amino acids. Amino acids stimulation leads to the activation of the Ragulator that then interacts with raptor, serving as a docking site for mTORC1 and thus bringing the mTORC1 complex to the lysosomal surface. Once on the surface of the lysosomes mTORC1 can interact with Rheb-GTP [144]. Another way in which amino acids have been proposed to activate mTORC1 is through inhibition of TSC1/TSC2. Gao et al. [145] showed that loss of TSC2 makes cells resistant to amino acids withdrawal. This seems to correlate with the fact that amino acids can signal through TSC1/TSC2. Other studies propose a mechanism independent of TSC1/TSC2 and Rheb. These studies demonstrate that hVPS34 can signal directly to mTORC1 the amino acids levels of the cell without using the TSC1/TSC2-Rheb pathway [146, 147].

1.4 TSC/mTOR and cell growth and proliferation

Cell growth is the consequence of a series of processes involving macromolecules synthesis. These lead to the increase in cell size and the increase in production of substrates important for the increased energy consumption required by the growing cell. mTORC1 directly controls the synthesis of both proteins and lipids, and by that cell growth.

Protein synthesis requires high amounts of ATP and GTP and also the production of a large number of ribosomes. That's why it is quite a costly process, from the energetic point of view, for the cell. mTORC1 is of fundamental importance in regulating the translational machinery of the cell and also in translating a particular subset of messenger RNAs (mRNAs), important for cell growth and proliferation. mTORC1 phosphorylates the eukaryotic Initiation Factor 4E (eIF4E)-binding protein 1 (4E-BP1) and the p70 ribosomal S6 kinase 1 (S6K1). By phosphorylating 4E-BP1 mTORC1 precludes its binding to eIF4E. eIF4E can thus favour cap-dependent translation (reviewed by Richter J. D. and Sonenberg N. [148]). Some particular mRNAs such as Cyclin D3 and VEGF are particularly sensitive to 4E-BP1 function. It is not clear which are the specific features of these mRNAs, but probably their specific sensitivity is due to the fact that they are characterized by extensive secondary structures in the 5' untranslated regions (UTRs). These structures require unwinding by the eIF4F complex (reviewed by Ma X. M. and Blenis J. [149]). The other class of mRNAs controlled by mTORC1 is that of the 5' TOP mRNAs. These mRNAs have short 5' UTRs characterized by stretches of pyrimidines [150]. They encode for components of the translational machinery.

mTORC1 control of S6K1 contributes to increase protein synthesis as well. S6K1 belongs to the AGC family of protein kinases. It is activated when phosphorylated at two sites, a site in the C-terminal hydrophobic motif and a site in the T loop of the kinase domain. mTORC1 phosphorylates the Thr380 in the hydrophobic motif, the site on the T loop, instead, is phosphorylated by PDK1. By acting on S6K1 mTORC1 can increase mRNA biogenesis and cap-dependent translation of ribosomal proteins (reviewed by Ma

X. M. and Blenis J. [149]). The activation of S6Ks by mTORC1 leads to the subsequent activation of proteins such as S6K1 aly/REF-like target (SKAR), Programmed Cell Death 4 (PDCD4), eukaryotic Elongation Factor 2 Kinase (eEF-2K), eukaryotic initiation factor 4B (eIF4B), and ribosomal protein S6 (reviewed by Ma X. M. and Blenis J. [149]). S6K1 can directly phosphorylate the 40S ribosomal protein S6. While it was thought that this would lead to the translation of a subset of mRNAs characterized by a 5' tract of oligopyrimidine (TOP), this was recently shown not to be the case [151, 152].

mTORC1 can also promote ribosome biogenesis by acting on protein phosphatase 2A (PP2A) and the Transcription Initiation Factor IA (TIF-IA) [153]. Experiments performed in yeast and in mammalian cells have shown that Rapa can inhibit ribosome biogenesis by inhibiting the transcription of RNA polymerase I (Pol I)-dependent rRNA genes, Pol II-dependent ribosomal protein genes (RP genes), and Pol III-dependent tRNA genes and also by inhibition of the processing of 35S rRNA (reviewed by Martin D. E. and Hall M. N. [154]), and thus ribosome biogenesis.

Lipid biogenesis is also critical for cell growth. The involvement on mTOR in this process has emerged only recently. It has been shown that mTORC1 can increase the activity of Sterol Regulatory Element Binding Protein 1 (SREBP1) [155] and of Peroxisome Proliferator-Activated Receptor-γ (PPARγ) [156]. These transcription factors control the expression of genes that encode for proteins important in lipid and cholesterol homeostasis. The involvement of mTOR in cell growth has been demonstrated in different models from yeast to *Caenorhabditis elegans* and *Drosophila melanogaster* [157-159]. Inhibition of mTOR by rapamycin has further proved the involvement of mTOR in cell growth in different cells such as renal epithelial cells, pancreatic beta cells, T cells, and different kinds of tumour cells like hepatocellular carcinoma cells [160-163].

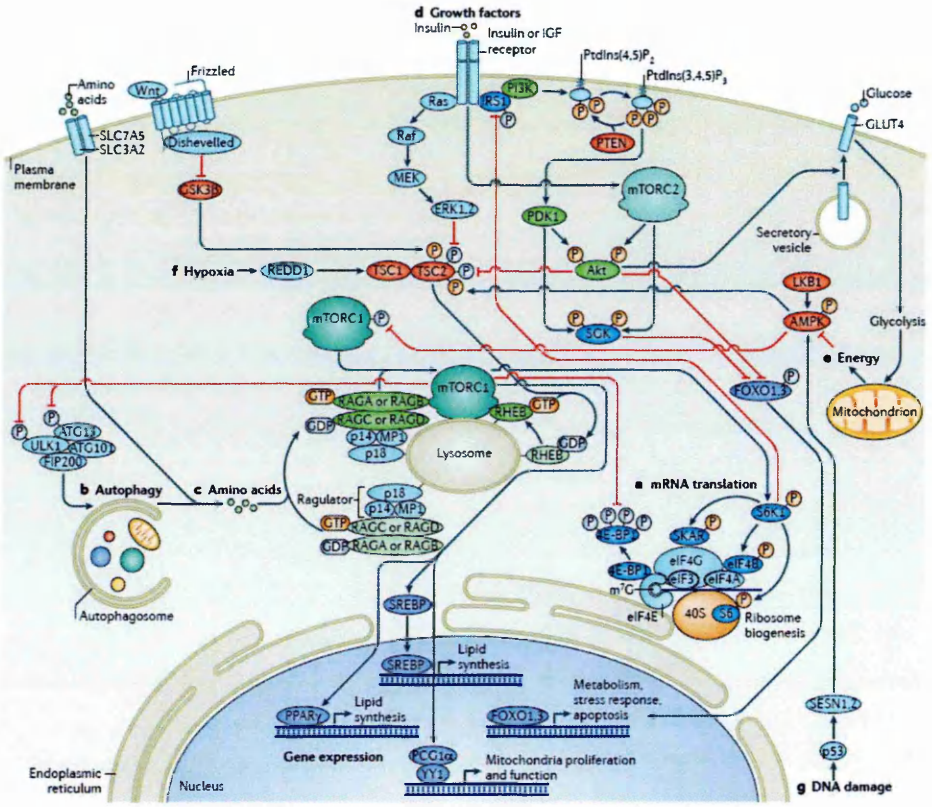

Fig III. TSC/mTOR signalling. The TSC/mTOR pathway is regulated by growth factors, energy levels and nutrients. The scheme shows the main signalling pathways that regulate and are regulated by TSC1/TSC2, mTORC1 and mTORC2. Zoncu R., Nature Reviews Molecular Biology, 2010 [164]

TSC and mTOR, being at the crossroad of many different signalling pathways integrating the energy status of the cell with nutritional availability and extracellular growth factor signalling, play a critical role in cell proliferation [4-7]. TSC1/TSC2 has been shown to play a critical role in proliferation by different groups either by over-expressing but also by down-regulating the function of the complex. The first experiments that demonstrated a role for TSC2 in cell proliferation were performed by Orimoto et al. and Jin et al. in cells coming from the Eker rat [165, 166]. This rat is characterized by a germ-line mutation in the TSC2 gene and it is inclined to the development of neoplasias in a variety of organs such as the kidney, uterus and spleen. The over-expression of TSC2 in different tumour cell lines derived from this rat [165, 166] decreases the proliferation rate

of these cells. On the other hand, decreased functional activity of the complex leads to an increase in cell proliferation, making these cells prone to undergo a transformation process. For example Soucek et al. [167] by silencing TSC2 expression in Rat1 fibroblasts made these cells enter the cell cycle and they also noticed a shortening of the G_1 phase. Other TSC2 negative cells reveal an increased rate of proliferation, as shown by fibroblast and smooth muscle cells from the Eker rat [168, 169]. Similar evidences were found also in the *gigas* mutant in *Drosophila*, another animal model of TSC2 loss. The mutant cells endoreplicate their DNA and this can be an indication that TSC2 is essential for the decision of entering the M or S phase. However the molecular mechanisms responsible for these effects have remained obscure for a long time. Even now it's not exactly clear which is the role of TSC1/TSC2 in controlling cell proliferation and whether it plays a direct or indirect role. A possible explanation of the link between TSC1/TSC2 and proliferation may be through the GTPase Rap1a. Tuberin act as GAP for this protein [170]. Yoshida et al. [171] demonstrated that Rap1 can induce DNA synthesis when microinjected into 3T3 cells. Thus a possible mechanism through which TSC2 may affect cell proliferation could be by indirectly acting on Rap1. Another interesting player affected by TSC1/TSC2 is the cyclin-dependent kinase inhibitor p27 [169]. In a recent report Lacher et al. [172] demonstrated that TSC2 control of proliferation pass through Rheb. In TSC2-deficient cells Rheb is constitutively functional, this leads to activation of AMPK through a mechanism mTORC1 independent. Short et al. [173] showed that active AMPK can decrease nuclear levels of p27. In a similar way Rheb can also control p27 activity and thus proliferation of TSC deficient cells. Nuclear p27, in fact, hinders cell cycle progression (reviewed by Assoian R. K. [174]). mTORC1 is also critical for cell proliferation both during fetal and adult life [175-178]. Although Rapa has been proposed as anti-proliferative agent, several reports now have identified Rapa-independent or resistant mechanism of proliferation. This is also the case for T cells. Indeed, Sara Colombetti in the lab demonstrated that mTOR inhibition by Rapa only delays initial cell cycle entry, and

that once the cells start to proliferate they do so via a Rapa-insensitive pathway [179].

1.5 TSC/mTOR and cell survival

TSC1/TSC2 complex also controls cell death. In a recent work carried on in *Drosophila melanogaster* Hsieh et al. [180] showed a connection between TSC/Rheb/Tor/S6k and sE2F1. The E2F family of proteins controls a variety of cellular processes involving cell cycle progression and survival [181]. Another important player of life and death that has a key role in acting together with TSC1/TSC2 in this model is Retinoblastoma (Rb). This family is composed by proteins that are other factors that control survival [181, 182]. It is now emerging a picture of the integrated signalling of these proteins in which mutations of TSC1 and Rb act together in favouring S-phase entry and apoptosis by increasing the expression of dE2F1 at the post-transcriptional level [180].

TSC1/TSC2 complex can also play a role in another kind of cell death that is ER stress-induced apoptosis. Kang et al. [183] demonstrated that TSC1/TSC2 and Rheb are key players in the Unfolded Protein Response (UPR). UPR is an important cellular process that by sensing ER stress can bring about cellular responses such as the general inhibition of protein synthesis and the transcription of stress responsive genes. In this way UPR is supposed to protect cells from harmful conditions. An excessive UPR however can induce apoptosis [184-187]. Kang et al. [183] further investigated the previously studied role of TSC1/TSC2 in UPR [188]. They demonstrated that TSC1/TSC2 is involved in a wide variety of ER stress responses and its role seems to be to protect cells from apoptosis induced by UPR. They also show that TSC1/TSC2 accomplish this by acting on Rheb/mTORC1, but quite interestingly, this effect is Rapa insensitive, showing another Rapa-independent function of mTORC1.

TSC1 and TSC2, together with mTORC1, are some of the most important proteins involved in the sensing of the energy levels of the cells. It is thus not surprising that TSC1/TSC2 plays a role also in energy deprivation-induced apoptosis. Inoki et al. [37]

demonstrate that TSC2, following phosphorylation by AMPK, is activated and leads to mTORC1 inhibition. Under stressful conditions this predisposes the cell to survive in a hostile environment. Loss of TSC function in low glucose conditions, in fact, leads to cell death. The involvement of mTORC1 in this process is of key relevance. Choo et al. [189], in fact, demonstrated that metabolic stress and apoptosis is prevented in TSC KO cells by inhibition of mTORC1 [189].

1.6 TSC/mTOR and disease

Mutation in negative regulators of mTOR, such as LKB1 and PTEN, can give rise to familial cancer syndromes. Sporadic mutations in important proteins in the mTOR pathways such as PI3K, Akt and PTEN, together with p53, are among the most common alterations in human cancer [190].

The inactivation of 4E-BP1, one of the main mTORC1 targets, plays a role in the growth of sporadic cancers. MEFs that do not express 4E-BP1 and 4E-BP2 show increased proliferation, suggesting that mTORC1 inhibition of 4E-BP1 is important for cell cycle progression [191].

Also the other mTOR complex, mTORC2, can play a role in tumorigenesis. mTORC2, in fact, activates Akt and SGK [192] and by this can lead to tumour initiation. Akt favours cell proliferation, survival and nutrient uptake in tumour cells (reviewed by Hsu P. P. and Sabatini D. M. [193]). Many different tumours result from inactivation of PTEN (the inhibitor of Akt signalling) or by activating mutations of PI3K. These kinds of tumours are heavily dependent on the pro-survival role of Akt. Inhibiting mTORC2 in these situations may be of key importance. In PTEN-deficient mice, in fact, Rictor is essential for the growth of tumour cell lines and prostate tumours [194-197].

T cells development, survival and cell metabolism needs the coordinate regulation of proliferation, survival and cell metabolism. We hypothesised a role for TSC/mTOR in

these processes and we thus investigated their possible involvement by pharmacological inhibition of the mTOR complexes or by genetic inactivation of the TSC complex in T cells. We also analysed the contribution of TSC/mTOR to PC12 nerve cell, used to model nerve cell proliferation and differentiation.

The thesis is divided in three chapters that cover these points. The first chapter focuses on the pharmacological inhibition of mTOR by Rapa in mature T cells. The role of Rapamycin inhibition of mTOR in T cells differentiation was investigated. The second chapter presents the data obtained from conditional TSC1 KO mice in which the TSC1 gene has been conditionally silenced during thymic development. Results show that the TSC complex affect T cells development and maturation by influencing proliferation and survival of these cells. The third chapter revolves around the analysis of the TSC pathway in PC12 cells. Data demonstrate TSC involvement in nerve cell proliferation and differentiation.

2. mTOR controls T cells differentiation

2.1 Introduction

2.1.1 T cell activation

mTOR has been shown to play essential roles in many different aspects of a T cell life. It is important during T-cell activation, anergy induction and maintenance, lineage commitment and other immune responses (reviewed by Mondino A. and Mueller D. L. [198]). Inhibition of mTOR activity by the way of Rapa leads to T-cell anergy [199], induction of regulatory T cells [98-103], impairment of effector T-cell generation (results discussed in this thesis and by Delgoffe G. M. et al. [103]) and enhanced memory T-cell responses to microbial pathogens [200]. mTOR is also of primary importance for T-cell trafficking *in vivo* and it can accomplish this by regulating the expression of CCR7 [201]. In T cells mTOR has mainly being described to be controlled by the PI3K-PDK-Akt-TSC pathway (reviewed by Zhong X. P. et al. [202]). This pathway can be activated by many different events such as TCR and CD28 engagement and IL-2, IL-7, IL-4, IL-12, IFN-γ and Wnt signalling [179, 203-206].

In response to TCR and CD28 engagement, CD4 naïve T cells express and secrete IL-2, and also high-affinity IL-2R. In this way they can respond to IL-2 and initiate IL-2-dependent proliferation [95, 120, 207, 208]. T cells proliferation is also controlled by TCR and CD28-dependent, IL-2 independent signalling events. The degree and length of TCR and CD28 stimulation are important for triggering exit of the G_0 stage and they are also critical for establishing how many cycles of proliferation a cell can complete [209-213]. Colombetti et al. [179] showed that prolonged TCR/CD28 activation leads to cell proliferation via an mTOR dependent mechanism that does not require IL-2. However in the presence of optimal concentrations of IL-2 T cells can proliferate in an mTOR independent manner [179].

2.1.2 T cell differentiation

Once naïve T cells are activated and induced to proliferate they can undergo a process of differentiation into effector, memory or regulatory cells. Several events are known to control helper T cell differentiation (reviewed by Bonnevier, J.L. and Mueller D. L. [214]). Engagement of the TCR and the costimulatory receptor dictates initial T cell activation and early transcription of cytokine genes [215]. Later on, helper T cell differentiation is regulated by cytokines. In particular when naïve CD4+ T cells are activated in the presence of IFN-γ and IL-12 they become Th1 effector cells, while in the presence of IL-4 they become Th2 effector cells. If they are stimulated with IL-6 and TGF-β they will become Th17 cells while when stimulated in presence of high concentrations of TGF-β they will acquire a regulatory phenotype and become Foxp3+ T cells [216].

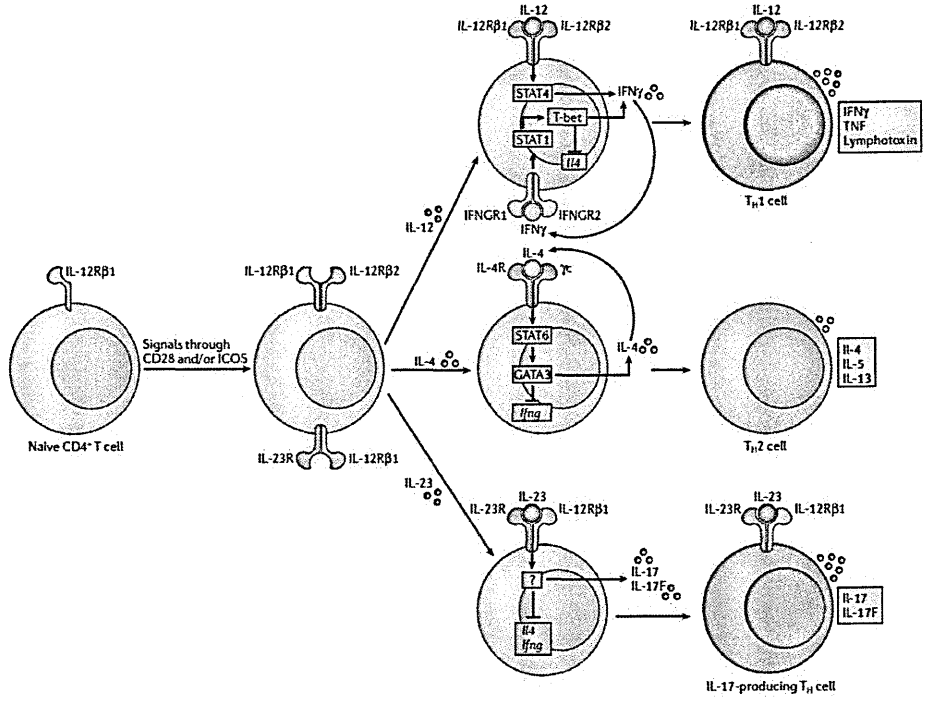

Fig IV. Th cell differentiation. Naïve CD4+ T cells are activated by Antigen-Presenting Cells (APCs) and CD28. After activation they start to produce low levels of cytokine and cytokine receptors. The differentiation towards a specific phenotype depends on the environment in which a cell is activated. Dong C., Nature Reviews Immunology, 2006 [217]

IL-4 and IL-12, through cytokine receptors and Signal Transducer and Activator of Transcription (STAT) 4 and STAT6 respectively [218], induce or repress the transcription of genes encoding key transcription factors [214, 219] needed for effector cytokine gene expression (*Ifnγ* and *Il4*). Transcription factors control cytokine gene transcription and also control chromatin landscape remodelling of lineage-specific genes (*Ifnγ* and *Il4*) [220]. For instance in the case of Th1 differentiation, TCR/CD28 as well as IL-2 driven signals induce the expression of T-bet. This transcription factor acts via autocatalytic and feed-forward mechanisms that involve signal and transcriptional networks. When activated T-bet can ensure its stabilization, its epigenetic remodelling activity and induces polarization of CD4+ T cells. Epigenetic modifications of cytokine loci mainly consist of modification of DNA and histones. Typical modifications are DNA CpG methylation, histone methylation and acetylation and DNase I HS induction. Agarwal et al. [221] were able to discover Th cell specific HSs in the *Il4* and *Ifnγ* loci. Another key epigenetic modification that can play a role in Th cell differentiation is DNA CpG methylation. Makar et al.[222] demonstrated that DNA methyltransferase 1 (Dnmt-1) binding to the *Il4* locus decreases in the process of Th2 differentiation. The events leading to transcription factors stabilization and to epigenetic remodelling of effector cytokine-encoding genes, however, remain largely to be characterized. Recent reports, published during the course of the research activity reported in this thesis, support a role for mTOR in such events. These will be reviewed and discussed, in relation to the experimental findings, in the discussion.

mTOR has also been implicated in controlling T cell trafficking. In the case of CD8+ T cells some of the surface markers such as CD44, CD62L and CCR7, useful for the identification of naïve, effector or memory T cells, have also a function in controlling T cell migration [223]. Naïve T cells express CD62L and CCR7, these molecules facilitate their trafficking between secondary lymphoid organs. After activation of the cells these receptors are down-regulated while others such as Very Late Antigen 4 (VLA4), ligands for P-selectin and E-selectin, CXC3 and CCR5 are upregulated [224, 225]. In this way

activated cells are driven to the inflamed tissues. The regulation of these proteins is controlled by the PI3K-mTOR pathway. Indeed, Sinclair et al. [201] showed that T cell activation in the presence of LY294002 and Rapa caused upregulation of KLF2, a transcription factor that controls CD62L and CCR7 expression [201].

Rapamycin controls T cells functions. The drug delays IL-2-induced G_1-S cell cycle transition by preventing Cdk2 and Cdc2 kinase activation [226] and p27Kip downregulation [95], favours the establishment of T cell unresponsiveness [96, 227] and prevents its reversal by IL-2 [179]. This drug has been indeed extensively used as immunosuppressant in solid organ as well as in pancreatic islet transplantation [228]. In mouse models, Rapa prevents graft rejection, graft versus host disease, and graft versus leukemia, but also induces an autoimmune-like syndrome when administered as single agent [229]. Dr. Mondino's lab has also reported the ability of T cells to overcome Rapa-mediated cell cycle arrest, and the existence of Rapa-insensitive lymphocyte proliferation [179], thus questioning the interpretation for the immunosuppressive activity of the drug. More recently, it was found that Rapa influences the development of CD4+ CD25+ regulatory T cells *in vitro* [99, 101, 230], T cell migration [201] and the accumulation of central memory-like T cells *in vivo* [200]. Preliminary experiments in the lab indicated that Rapa might work as an immunomodulator and impact on T cell differentiation, rather than on T cell expansion. To better define this possibility the impact of Rapa on TCR/CD28-driven T cell activation, expansion and differentiation was investigated. Results indicate that TCR/CD28 engagement evokes mTORC1 and mTORC2-dependent signalling, both sensitive to Rapa, and these are critical for proper expression of T-bet and GATA3, of *Ifnγ, Il4* and *Foxp3* genes, and impact on the control of the DNA methylation state of promoter regions of genes important for cell fate determination.

2.2 Materials and methods

2.2.1 Mice

BALB/c mice were obtained from Charles River (Calco). DO11.10 mice (H-2d) were bred and maintained in the SPF animal facility according to the Institutional Guidelines. These mice express a transgenic αβ TCR specific for the CD4-restricted chicken ovalbumin derived peptide (OVA323-339) on the majority of the CD4+ T cells. The Ethical Committee of the San Raffaele Scientific Institute approved the animal usage.

2.2.2 Primary T cell cultures

CD4+ T cells were purified by negative selection using anti-CD8 (clone KT1.9) and anti-I-Ab-d/I-E (clone B21-22) rat Abs and sheep anti-rat-coated magnetic beads (Dynal Biotech LTD., UK) to a purity of >95%. Cells were then stimulated on immobilized anti-CD3 and anti-CD28 mAb (0.5 μg/ml and 5 μg/ml) in the absence or in the presence of RAPA (100 nM) or of Recombinant Mouse TRAIL R2/TNFRSF10B Fc Chimera (50 ng/ml, R&D Systems).

After 3-7 days cells were stimulated with PMA (0.05 μg/ml) and Ionomycin (1 μg/ml) for 4 hours, of which the last 2 in the presence of Brefeldin, and intracellular cytokine staining was performed and evaluated on a BD Biosciences FACSCalibur dual-laser cytometer using Flowjow acquisition analysis software.

Cell were always kept in RPMI-1640 medium (Invitrogen) with 5% FBS until lysis. This prevented inactivation of S6 phosphorylation.

2.2.3 FACS analysis

Abs (conjugated with FITC, PE, PerCP, PB, Pecy7 or allophycocyanin) directed against the following surface markers were obtained from eBioscience, BD Pharmingen, or Caltag Laboratories and used PE and PerCP in 1/200; PB, APC and Pecy7 in 1/400 and FITC in 1/100 dilution to analyze immune cell subsets in single cell suspensions of

lymphonodes: CD4, CD8, Foxp3, T-Bet, GATA-3, Phospho-Tyr694-Stat5. Software as well as FlowJo Software (Tree Star).

2.2.4 Western blot analysis

CD4+ purified cells were stimulated on immobilized anti-CD3 and anti-CD28 mAb (0.5 μg/ml and 5 μg/ml). When indicated cells were treated with Rapa (1 or 100 nM, Calbiochem), SL0101 (3 or 30 uM, Toronto Research Chemicals Inc.) or UO126 (10 uM, Promega). T cells and thymocytes extracts were washed twice with ice-cold PBS and lysed in lysis buffer containing SDS 2% and Tris-HCl 62.5 mM pH 6.8.

Proteins were quantified by BCA assay and appropriate amounts (30 or 50 μg) were separated on standard 10-15% SDS-PAGE. For Western blotting, gels transferred to nitrocellulose filters were first blocked for 1 h with 5% nonfat dry milk in PBA, and then incubated o.n. with the primary antibody diluted in PBS with 5% Bovine Serum Albumin (BSA), washed in PBST (3-fold for 10 min), incubated for 1 h with the peroxidase-conjugated secondary antibody, washed again in PBST as described above and once in PBS. Photographic development was by chemiluminescence (ECL, Amersham Bioscience or Immobilon substrate, Millipore). Western blot bands were quantified by the ImageJ program (rsb.info.nih.gov/ij).

anti-Akt, anti-P(S473)-Akt, anti-P(T308)-Akt, anti-4E-BP1, anti-GSK3β, anti-P(Ser235/236)-S6 and anti-S6, anti-STAT1, anti-P(S727)-STAT1, anti-STAT5, anti-P(T389)-P70S6K: Cell Signalling Technology (Milan, Italy); T-bet, GATA-3 and actin, which were obtained from Santa Cruz Biotechnology Inc, and anti H3K27-me3 histone, which was obtained from Millipore. Horseradish peroxidase-conjugated goat antimouse and anti-rabbit pAbs: Bio-Rad. The BCA Protein Assay Kit from Pierce.

Antibody anti-	Company	Cat. N°
T-Bet	Santa Cruz Biotechnology	Sc-21749
STAT1	Cell Signaling	9172
H3-K27me3	abcam	ab6147
STAT5	Cell Signaling	9358
Phospho-STAT1 (Ser727)	Cell Signaling	9177
Phospho-Akt (Thr308)	Cell Signaling	2965
Phospho-Akt (Ser473)	Cell Signaling	9271
4E-BP1	Cell Signaling	9452
Phospho-S6 (Ser235/236)	Cell Signaling	2211
Phospho-p70S6K (Thr389)	Cell Signaling	9205
Actin	Santa Cruz Biotechnology	Sc-1616

2.2.5 CFSE proliferation assay.

Cells were labelled with CFSE (Molecular Probes). They were washed and resuspended in PBS at a final density of $20x10^6$/ml, were mixed with the same volume of CFSE fluorescent dye (1 μM) in PBS and incubated for 8 min at room temperature. Deacetylated CFSE was quenched by the addition of FBS.

Cells were then washed with complete medium and seeded in 24 well dishes at a density of 1 x 10^6/well in the presence of immobilized anti-CD3/CD28 mAb (0.01 μg/ml and 5 μg/ml respectively) or recombinant mouse IL-7 (0.5 ng/ml). When indicated cells were treated with Rapa (1 or 100 nM, Calbiochem). After 3-5 days of culture CFSE-labelled cells were collected, costained with anti-CD4, anti-CD8 mAb, and TOPRO-3 and analyzed by flow cytometric using a FACSCalibur.

2.2.6 Quantitative real-time RT-PCR (qPCR)

RNA was extracted with RNeasy Mini Kit (Qiagen) following manufacturer's instructions and its concentration was determined by spectrophotometry.

1-2 μg of total RNA were used to generate cDNA templates for RT-PCR, using Oligo-(dT)12-18, dNTPs and RNAsin RNAse (from Promega). Retrotranscriptase M-MLV was from Invitrogen.

q-PCR was performed using Real Time Taqman universal master mix (Applied Biosystems) according to a standard protocol, using 50 ng template cDNA. All primers were used at the final concentration of 900 nM. Values were normalized to the concentration of GAPDH mRNA. q-PCR was performed using an ABI PRISM 7700 Sequence Detection System.

Specific mRNA expression was normalized by housekeeping GAPDH expression according to theΔΔCT method. In the other cases, data were expressed as ΔΔCT fold increase over control cells.

2.2.7 Methylated DNA Immunoprecipitation (MeDIP)

DNA was sonicated to yield fragments ranging in size between 300 and 600 bp. Sonicated DNA was then used as a whole-genome reference for comparative analysis (Input DNA), or immunoprecipitated with a mouse monoclonal anti-5-methylcytidine antibody from Eurogentec (#BI-MECY-1000). The methylation status of *Ifnγ, Il4* and *foxp3* genes was analyzed by standard real time quantitative PCR by employing primers targeting proximal promoter regions (IFN-γ: left -163: AACATGCCACAAAACCATAGC; right-25: CACCTCTCTGGCTTCCAGTT; IL-4: left -226 GGTCCTCTGGAAGAGCAACA; right -96 CTTCGGTGCTGCAGATACAA; FoxP3 left -454 CCTCCAACGTCTCACAAACA; right -300 CCCCTCACCACAGAGGTAAA). with a mouse monoclonal 5-methylcytidine antibody from Eurogentec (#BI-MECY-1000). Real Time PCR was performed using the Sybr Green technique (LightCycler 480 SYBR Green I Master). Fractional methylation DNA recovery in MeDIP fractions was expressed relative to input (CTIp-CTinput=ΔCT). To compare different experiments data were expressed relative to control cells (Nil) as follows: 2–(ΔCT-ΔCTNil). Unmethylated and methylated housekeeping genes (β-actin and Xist respectively) were used as controls.

2.2.8 Statistical analysis.

The significance of the data was assessed using the two-tailed t-Test when two groups are compared, the one-tail ANOVA test with Dunns post test when three or more groups were compared and the two-way ANOVA test with Bonferroni post test when curves with different time points were compared. Data shown are means ± S.E.M. The number of experiments is specified in the Figures or Figure legends. $P<0.05$ is considered significantly different. In the Figures ****=p< 0,0001; *** = $p<0.001$; **= $p<0.01$; *= $p<0.05$.

2.3 Results

2.3.1 Rapamycin prevents differentiation of IFN-γ producing cells

To study the role of mTOR in T cell activation and differentiation, the ability of Rapa to interfere with differentiation of IFN-γ-producing cells was analysed.

Stimulation of T cells with antigen leads to proliferation of the cells that is partially sensitive to Rapa (Fig. 2.1 A) after 3 days of stimulation. After 7 days of stimulation Rapa-treated cells are not sensitive any more to Rapa-inhibition (Fig. 2.1 A) suggesting that mTOR blockade by Rapa only delays T cells proliferation. A high percentage of cells stimulated with antigen for 7 days produce IFN-γ following optimal restimulation with PMA/Ionomycin. Cells treated with Rapa, instead, fail to produce IFN-γ after PMA/Ionomycin restimulation (Fig. 2.1 B) suggesting defective T cell differentiation in the presence of Rapa. A higher percentage of cells treated with Rapa, however, produce IL-2 compared to control cells not treated with the drug (Fig. 2.1 C).

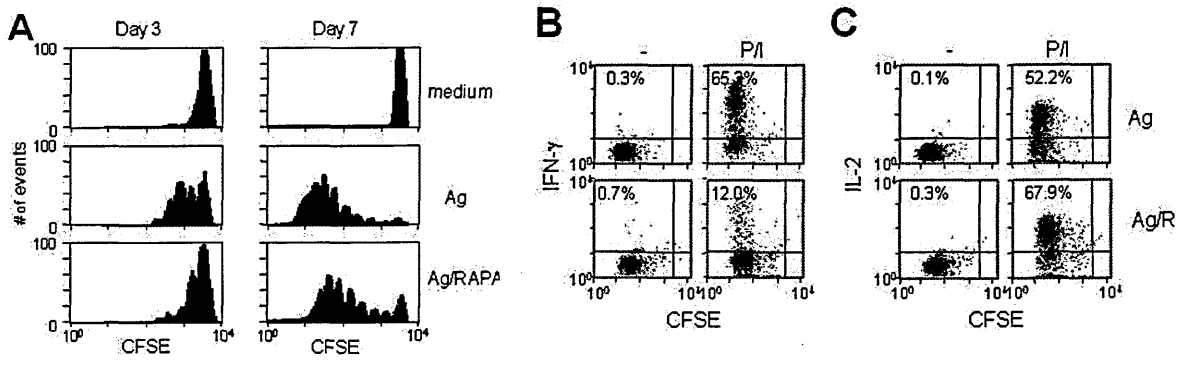

Fig 2.1 Rapa affects CD4+ T cells differentiation. A-C) CFSE labelled purified CD4+ T cells from LN of ovalbumin specific DO11.10 TCR transgenic mice were stimulated with antigen ($OVA_{323-339}$) (0.25 uM) and irradiated APC (syngeneic splenocytes) in the absence or in the presence of Rapa (R) (100 nM). After 3 and 7 days of culture cells were analyzed by flow cytometry (A). In B and C cells were stimulated as in A for 7 days and then re-stimulated for 4 hours with medium only (-) or with PMA (0.05 ug/ml) and Ionomycin (1 ug/ml) (P/I), and analysed following staining with IFN-γ (B) and IL-2 (C). In A histograms represent the CFSE profile of CD4+ cells after 3 and 7 days of culture. In B and C dot plots depict viable CD4+ cells. The percentage of cytokine producing cells is reported. Cytokines producing cells were below 2% in unstimulated cultures.

2.3.2 Activation of mTORC1 and mTORC2 signalling following TCR/CD28 stimulation.

To better understand the ability of Rapa to interfere with differentiation of IFN-γ or IL-4-producing cells the role of mTOR in T cell activation was investigated. Purified primary CD4+ T cells were analyzed after stimulation with TCR/CD28. The activity of mTORC1 and mTORC2 was evaluated by assessing the phosphorylation of p70S6K (Thr389), S6 (Ser235/236) and 4EBP-1 (mTORC1) and of Akt in ser 473 (mTORC2) [57] (Fig. 2.2). Stimulation caused the phosphorylation of all the kinases analysed, in particular of p70S6K (Thr389), S6 (Ser235/236), 4EBP-1, and Akt both in Ser 473 and in Thr 308. p70S6K (Thr389) and 4EBP-1 phosphorylation readily decreased after Rapa treatment. S6 (Ser235/236) phosphorylation instead was only partially sensitive to Rapa treatment. Also treatment of the cells for 30 min with UO126 (an inhibitor of MAPK) and with different concentrations of SL0101 (an inhibitor of RSK) partially inhibited S6 (Ser235/236) phosphorylation. Only the combination of Rapa and UO126 or Rapa and the higher concentration of SL0101 completely inhibited S6 (Ser235/236) phosphorylation (Fig. 2.2 C). This is in accordance with previous published data in which CD8+ T cells were analysed [124].

Akt phosphorylation was already detectable after 30 min but peaked by 1-2 hours of stimulation but decreased at later time points. After 1 hour of stimulation the phosphorylation of Akt in Ser 473 was higher in stimulated cells that were treated with Rapa but after 4 hours of stimulation it was lower than in the control stimulated cells (Fig. 2.2 A, B). While the initial increase of phosphorylation in Akt on ser 473 could be indicative of the existence of the mTORC1-dependent negative feedback loop, the decrease in AKT phosphorylation in ser 473 could indicate mTORC2 inhibition by Rapa [88]. To further prove TCR/CD28 effect on the mTOR pathway, the phosphorylation of different targets of mTORC1 and mTORC2 was evaluated not only during the first phases of

activation but also at later time points, cells were thus stimulated for 24 hours. This experiment demonstrated that the effect seen during acute treatment is preserved also at later time points, suggesting that the effects observed in Rapa treated cells (described in the following paragraphs) are probably due to mTORC1 and mTORC2 down-regulation rather than to compensatory mechanisms. Treatment with Rapa for 24 hours strongly inhibited Akt phosphorylation in Ser 473, the cells in culture with the drug in fact show less than 10% residual phosphorylation (Fig. 2.2 D).

These results show that both mTORC1 and mTORC2 are activated by TCR/CD28 and they are sensitive to Rapa inhibition with different kinetics.

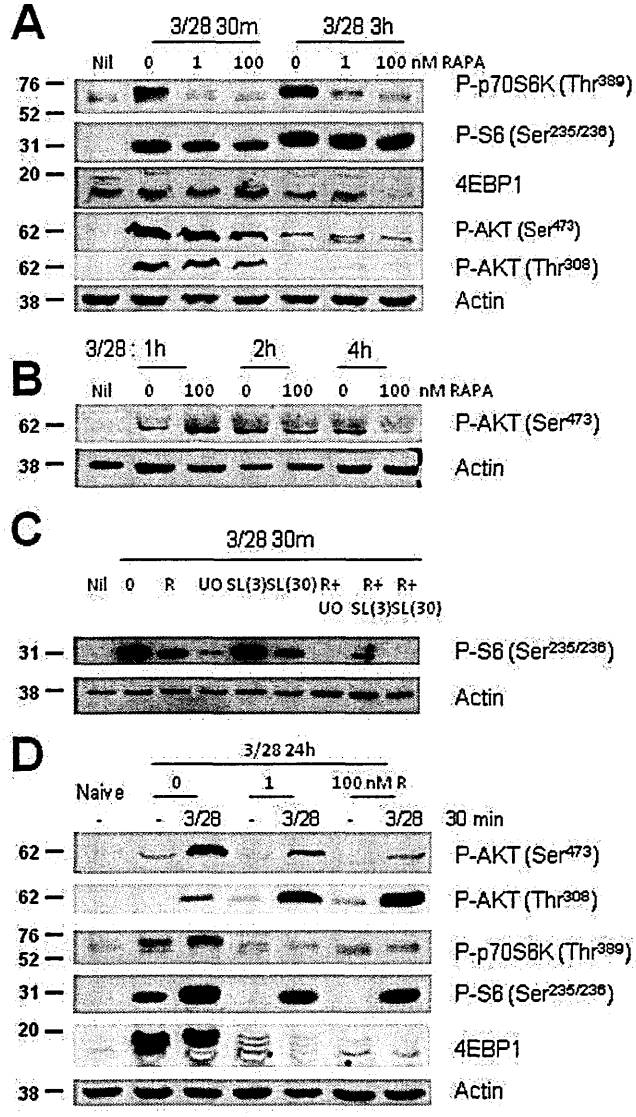

Fig 2.2 TCR/CD28 activates both mTORC1 and mTORC2 signalling. CD4+ T cells purified from lymph node pools of 5 BALB/c mice were left untreated (Nil) or stimulated

with immobilized anti-CD3 (0.5 ug/ml) and anti-CD28 (5 ug/ml) mAb for 30 min and 3 hours (A), for 1, 2, and 4 hours (B), only for 30 min (C) and for 24 hours (D). Cells were cultured in the absence (0) or in the presence of RAPA (R; 1 and 100 nM in A and D, only 100 nM in B and C), of UO126 (10 uM in C), of SL0101 (3 or 30 uM in C) or with the combination of the drugs (C). In D, cells were activated on immobilized anti-CD3/CD28 mAb for 24h (3/28 24 h) in the absence (0) or in the presence of RAPA (1 and 100 nM R), and then lysed (-) or re-stimulated with CD3/CD28 mAb for 30 min (3/28). SDS-PAGE analyses were performed as detailed in Materials and Methods 2.2.4. Phosphorylation of the mTORC1 targets 4EBP1 and p70S6K (Thr 389), of p70S6K substrate S6 (Ser 235/236), of the mTORC2 target Akt (Ser 473) and of PDK1 target Akt (Thr 308) were assessed by western blot analysis (A and D). Phosphorylation of p70S6K substrate S6 (Ser 235/236) and of the mTORC2 target Akt (Ser 473) were assessed by western blot analysis (B and C respectively). Actin is depicted as loading control, and relative migration of molecular weight markers is depicted. One of 3-5 independent experiments is depicted. Molecular weight markers are indicated in KDa.

2.3.3 Rapamycin hinders TCR/CD28-driven *Ifnγ*, *Il4* and *Foxp3* gene expression.

TCR and CD28 engagement drives transcription of *Ifnγ* and *Il4* [220]. Next we thus investigated whether Rapa would interfere with TCR/CD28-induced cytokine expression. Real-time PCR experiments showed that IL-2, IFN-γ and IL-4 mRNAs were induced in response to CD3/CD28 stimulation (Fig. 2.3 A). The peak of expression ranged between 24 and 48 hours depending on the cytokine analyzed, and expression decreased thereafter. When cells were activated in the presence of Rapa, expression of IL-2, IFN-γ and IL-4 was found to be lower within the first 24 hours of treatment (Fig. 2.3 A, B). However at later times IL-2 expression was recovered while IFN-γ and IL-4 expression was severely decreased by Rapa treatment also at later time points (Fig. 2.3 A, B).

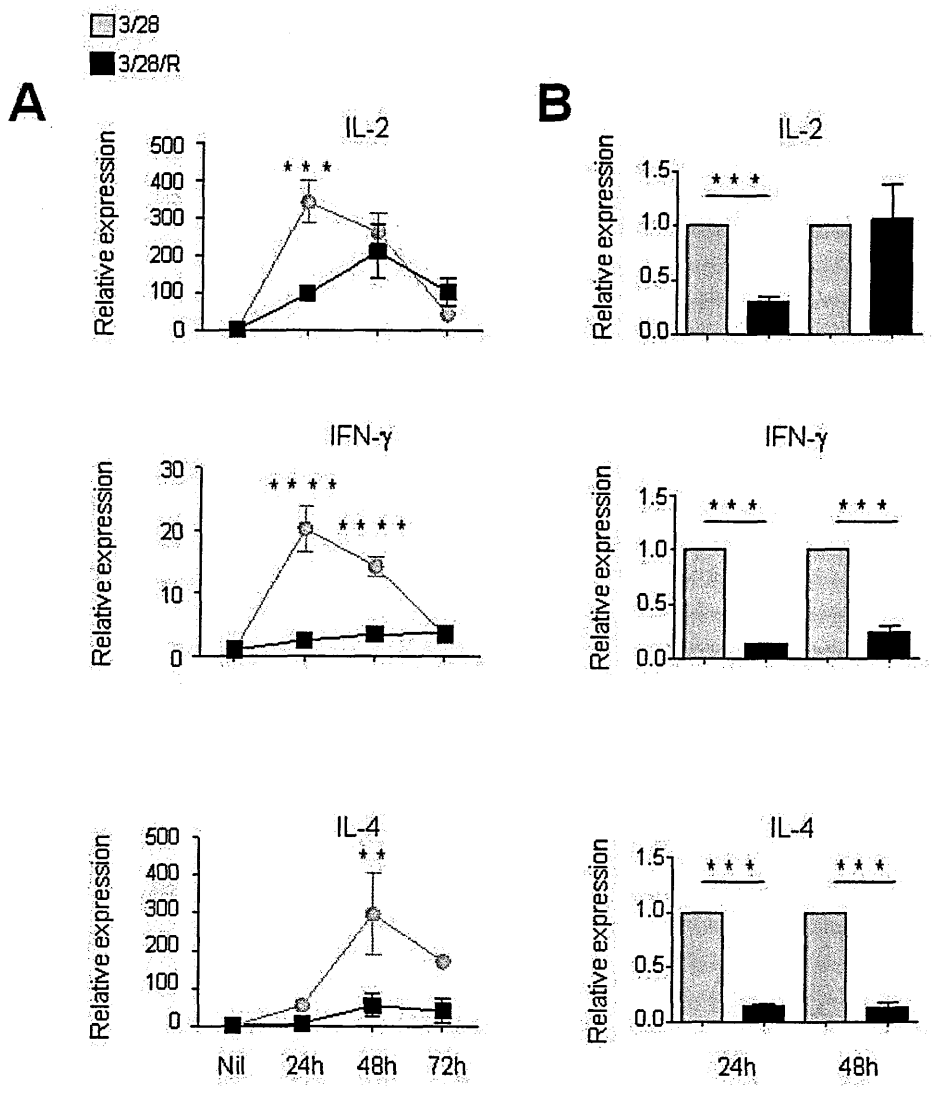

Fig 2.3 TCR/CD28 induced cytokine transcription is sensitive to Rapa treatment. A, B) CD4+ T cells were purified from lymph node pools of 5 BALB/c mice and left untreated (Nil) or stimulated with immobilized anti-CD3 (0.5 ug/ml) and anti-CD28 (5 ug/ml) mAb in the absence (3/28) or in the presence of Rapa (100 nM, 3/28/R) for 24, 48 or 72h. mRNA expression was quantified by RT-real time PCR in 5 (24/48h) and 3 (72h) independent experiments. A) Cytokine mRNAs were first normalized to the GAPDH housekeeping gene, and then expressed relatively to control unstimulated cells (Nil) according to the ΔΔCT method (±SE). Statistical significance was analysed by two-way ANOVA test with Bonferroni post test. B) mRNA levels of the indicated genes were first normalized to the GAPDH housekeeping gene, and then expressed relatively to the levels found in CD3/CD28 activated control cells. Statistical significance (two-tailed t-Test) was determined by comparing CD3/CD28-cultured control and drug-treated cells within each time point by the ΔΔCT method, using CD3/CD28 control cells as reference.

Foxp3 levels were also examined. Following stimulation with CD3/CD28 also Foxp3 mRNA increased. At early time points Foxp3 expression was not sensitive to Rapa (Fig. 2.4 A, B). However at later time points while expression of Foxp3 mRNA decreased in stimulated cells, cells that were stimulated and treated with Rapa showed sustained levels of Foxp3 mRNA (Fig. 2.4 A, B). Also Foxp3 protein levels were already detectable in resting cells but declined after 72 hours of stimulation. In the presence of Rapa, instead, these levels were sustained also at later time points (Fig. 2.4 C). These results may suggest that mTOR blockade by Rapa limits TCR/CD28-dependent T cell differentiation by inhibiting *Ifnγ* and *Il4* expression and favouring *Foxp3* expression.

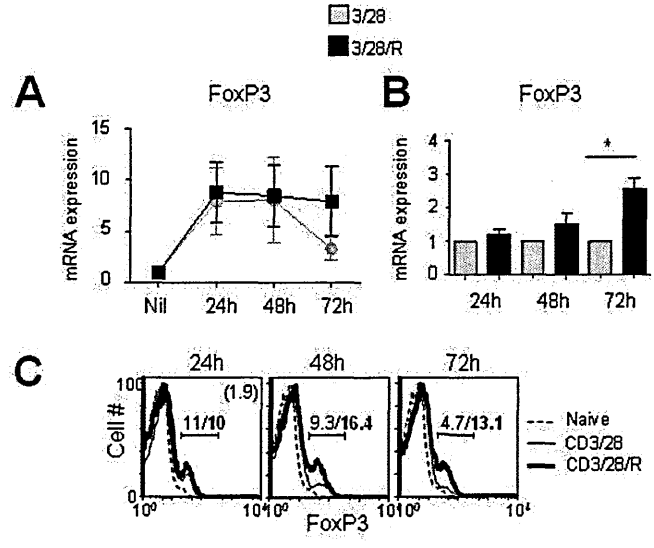

Fig 2.4 mTOR blockade favours Foxp3 expression. A-C) CD4+ T cells were purified from lymph node pools of 5 BALB/c mice and left untreated (Nil) or stimulated with immobilized anti-CD3 (0.5 ug/ml) and anti-CD28 (5 ug/ml) mAb in the absence (3/28) or in the presence of Rapa (100 nM, 3/28/R) for 24, 48 or 72h. Foxp3 mRNA expression was quantified by RT-real time PCR in 4 (24/48h) and 3 (72h) independent experiments. A) Foxp3 mRNA was first normalized to the GAPDH housekeeping gene, and then expressed relatively to control unstimulated cells (Nil) according to the ΔΔCT method (±SE). Statistical significance was analysed by two-way ANOVA test with Bonferroni post test. B) mRNA levels of Foxp3 were first normalized to the GAPDH housekeeping gene, and then expressed relatively to the levels found in CD3/CD28 activated control cells. Statistical significance (two-tailed t-Test) was determined by comparing CD3/CD28-cultured control and drug-treated cells within each time point by theΔΔCT method (±SE), using CD3/CD28 control cells as reference. C) At the indicated times cells were recovered and analyzed by flow cytometry after staining with anti-CD25 and Foxp3 or isotype control mAb. Naïve cells were used as controls (dotted line). Data are representative of two independent determinations. The frequency of naïve cells is reported in brackets. The frequencies of control and Rapa-cultured cells are indicated in plain and bold text, respectively.

2.3.4 Rapa interferes with T-bet and GATA-3 expression and function.

Following TCR/CD28 stimulation T cells starts to produce IL-2. IL-2 then initiates Th cell polarization by activating Signal Transducer and Activator of Transcription 5 (STAT5) and in this way favours the entry of the cell into the cell cycle. The activated cell can differentiate into the different lineages in response to the cytokine milieu in which it is activated. The different cytokines can induce the activation of particular lineage-specific transcription factors. For example in Th1 development IFN-γ and IL-27 induce STAT1 activation and thus the transcription factor T-bet. T-bet then can further increase the transcription of *Ifnγ*. For Th2 differentiation instead GATA-3 is the key transcription factor responsible for *Il4* expression. Thus STAT-1 and STAT-5, T-bet and GATA-3 expression was evaluated following TCR/CD28 stimulation of primary cells with or without Rapa (Fig. 2.5). Following stimulation with CD3/CD28 both STAT-1 and STAT-5 protein levels and phosphorylation were increased to similar extents in control cells and cells treated with Rapa (Fig. 2.5 A, B). In naïve cells T-bet and GATA-3 proteins and mRNA were undetectable (Fig. 2.5 C-G). Following stimulation mRNA and proteins were induced. The peak of expression was reached at 48 hours, by 72 hours the levels already started to decrease. Rapa induced a reduction in the mRNA and protein levels of both factors at the population and at the single cell level (Fig. 2.5 C-I). T-bet and GATA-3 activity was evaluated by looking at the mRNA levels of some of their important target genes. In a similar way to IFN-γ, also IL12 receptor-β chain (IL12Rβ) and the transcription co-activator Hlx-1, two important T-bet target genes [231] were not properly induced when the cells were treated with Rapa (Fig. 2.5 J). Likewise, GATA-3 target genes IL-5 and IL-13 were expressed at very low levels in Rapa treated cells (Fig. 2.5 K). These results indicate that activation in the presence of Rapa delays expression of T-bet and GATA-3 and expression of their target genes while it favours Foxp3 expression.

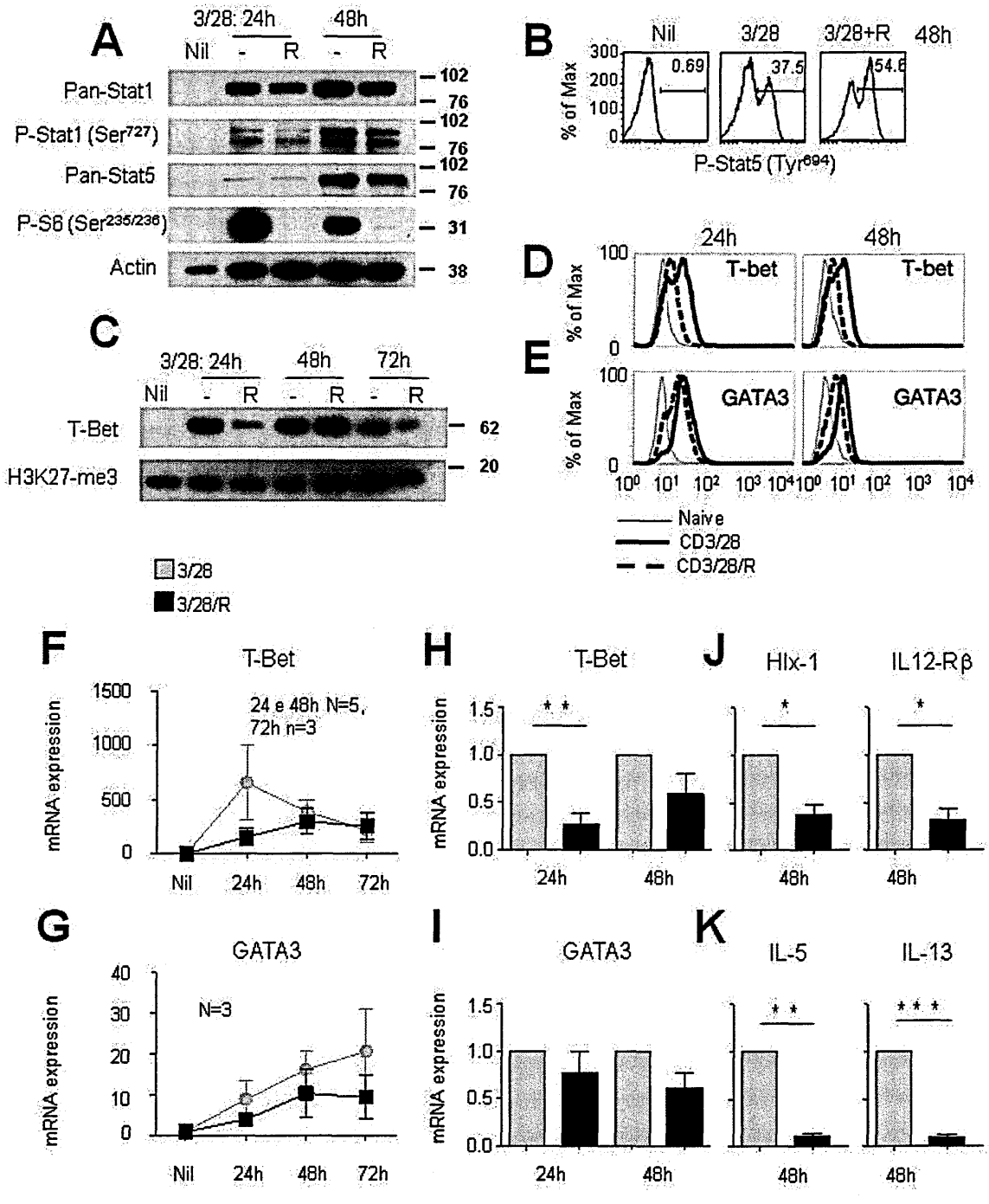

Fig 2.5 mTOR blockade hinders T-bet and GATA-3 expression and transcriptional activity. CD4+ T cells purified from BALB/c LN were left untreated (Nil) or stimulated with immobilized anti-CD3 (0.5 ug/ml) and anti-CD28 (5 ug/ml) mAb in the absence (-) or in the presence of Rapa (100 nM, R) for 24 and 48h (A, D, E, H, I), 48h (B, J, K), 24, 48 and 72h (C, F, G). Protein and mRNA levels were determined by SDS-PAGE (A, C), flow cytometry (B, D, and E) and RT-real time PCR (F-K). A, C) SDS-PAGE analyses were performed as detailed in Materials and Methods 2.2.4. Phosphorylation of STAT1 (Ser 727), total levels of STAT1, STAT5 and T-Bet were assessed by western blot analysis (A and C). Actin and histone H3 are depicted as loading controls, and relative migration of molecular weight markers is depicted. One of 3 independent experiments is depicted. Molecular weight markers are indicated in KDa. B, D, E) Cells were left untreated (Nil) or stimulated as in A and stained for intracellular Phospho-STAT-5-Tyr 694, T-Bet and

GATA-3 contents (Nil: thin line; 3/28; thick line; 3/28/R; dotted line). F-K) T-Bet (F, H), GATA-3 (G, I), Hlx-1 (J), IL12-Rβ (J), IL-5 (K) and IL-13 (K) mRNA expression was quantified by RT-real time PCR in 5 (24/48h) and 3 (72h) independent experiments. F, G) T-Bet and GATA-3 mRNA was first normalized to the GAPDH housekeeping gene, and then expressed relatively to control unstimulated cells (Nil) according to the ΔΔCT method (±SE). Statistical significance was analysed by two-way ANOVA test with Bonferroni post test. H-K) mRNA levels of the different genes were first normalized to the GAPDH housekeeping gene, and then expressed relatively to the levels found in CD3/CD28 activated control cells. Statistical significance (two-tailed t-Test) was determined by comparing CD3/CD28-cultured control and drug-treated cells within each time point by the ΔΔCT method (±SE), using CD3/CD28 control cells as reference.

2.3.5 Rapa-sensitive events control methylation of *Ifnγ*, *Il4* and *Foxp3* promoter regions.

Epigenetic modifications are important in controlling the transcription of genes involved in T cell differentiation. Gene expression is favoured by increased histone acetylation both of the *Ifnγ* and the *Il4* loci [221, 232]. DNA methylation is another important epigenetic marker that shapes the chromatin landscape of differentiating T cells [222]. T-bet is important in remodelling the *Ifnγ* locus [231]. As T-bet expression and activity is impaired by Rapa treatment, the influence of Rapa on promoter methylation of different cytokines was evaluated by MeDIP. MeDIP is a technique that requires precipitation of DNA and then recognition of the methylated CpG islands. DNA was sonicated to obtain fragments of 300-600 bp. The DNA was then immunoprecipitated with an Ab that recognized the methylated CpG islands and the proximal promoter region of the different cytokines was analyzed by real-time qPCR. As control genes β-actin and Xist were used. β-actin as a representative of a hypo-methylated gene and Xist of a gene that is fully methylated in one of the two copies (Fig. 2.6 A). After stimulation with CD3/CD28 the methylation of *Ifnγ* and *IL4* promoter regions decreased compared to the methylation of naïve unstimulated cells (Fig. 2.6 B, C). The cells treated with Rapa, instead, showed methylation profiles similar to those of naïve cells. Foxp3 promoter behaved in a completely different way. Following CD3/CD28 stimulation the methylation of Foxp3 promoter increased, but not in

the presence of Rapa (Fig. 2.6 B, C). These results suggest that mTOR blockade by Rapa could affect the methylation of the promoter regions of cytokines important in the process of T cell differentiation.

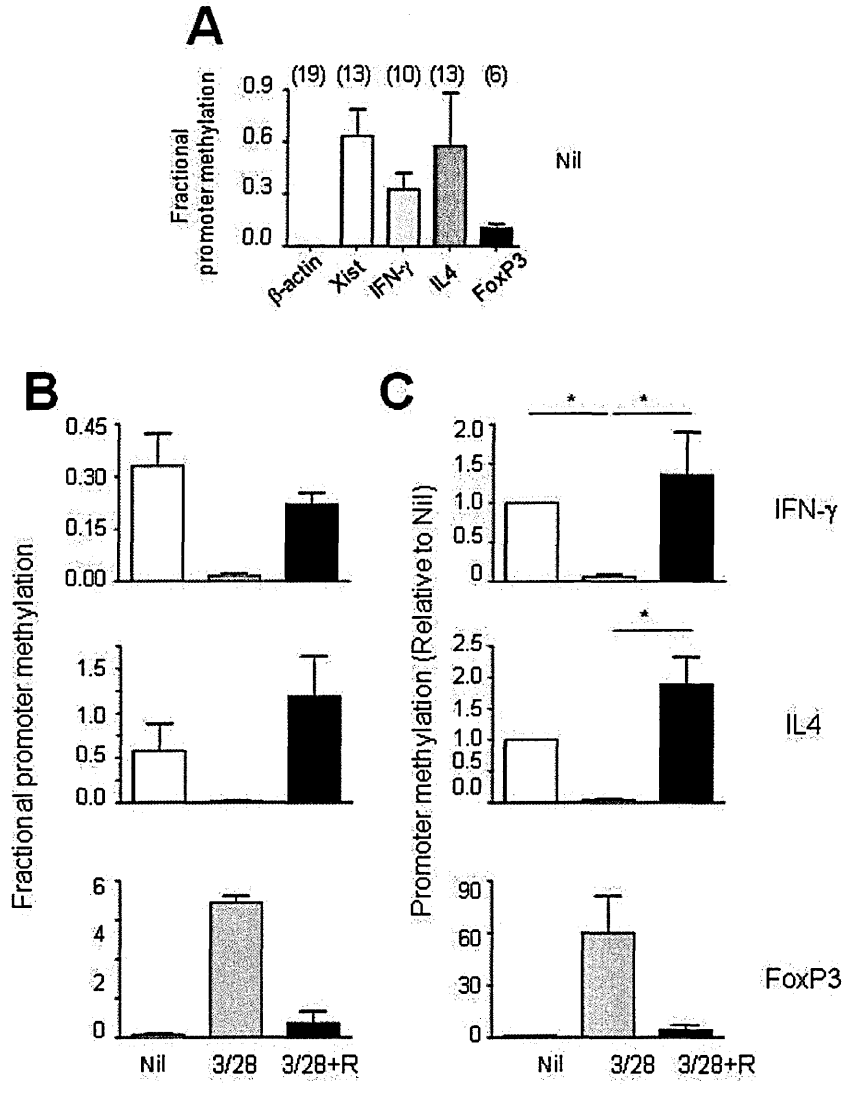

Fig 2.6 mTOR-dependent signalling controls DNA methylation. CD4+ T lymphocytes were purified from BALB/c LN and left untreated or stimulated with immobilized anti-CD3 (0.5 ug/ml) and anti-CD28 (5 ug/ml) mAb in the absence (-) or in the presence of Rapa (100 nM, R) for 48 hours. Cells extracts were analyzed by MeDIP assays with a mouse monoclonal anti-5-methylcytidine Ab (see Materials and Methods 2.2.7). A) Fractional methylation was expressed relative to input (CTIp-CTinput=ΔCT) (±SE). Numbers in brackets in A represent CpG hits in the regions of interest. B, C) DNA recovery in MeDIP fraction over input is expressed relative to that found in control cells. Changes in promoter region methylation following activation, either absolute (B) or relative to unstimulated (Nil) (C) are reported. Data are representative of 5 (IFN-γ and IL-4) and 3 (Foxp3) independent determinations. Trends depicted in the figure were found in all of the experiments. Statistical significance was analysed by one-tail ANOVA test with Dunns post test.

2.4 Discussion

Before the beginning of the experiments reported in this thesis it was known that mTOR was critical for T cell proliferation, and for the maintenance of T cell responsiveness [96, 198]. The results reported in this thesis support the idea that mTOR is also central to TCR/CD28 activation of naïve T cells and for controlling acute *Ifnγ*, *Il4* and *Foxp3* transcription, methylation of critical promoter regions and for differentiation of IFN-γ and IL-4 secreting cells in Th0 conditions. While in the process of completing these studies, this notion has been confirmed by a number of reports [89, 103, 233], which, using genetic approaches allowing inactivation of mTOR, Rictor and Raptor, provided elegant evidences for the role of mTORC1 and mTORC2 in polarization of Th1, Th2, Th17 and Treg cells in response to polarizing conditions. Thereafter will follow a discussion of the findings reported in this thesis that correlates them with these recent publications.

The process of T cell differentiation has been linked to several events. These are initiated by TCR/CD28 and IL-2/IL-2R signalling, which drives cell cycle transition, concomitantly starts *Ifnγ*, *Il4*, *Il5*, and *Il13* transcription [220] and favours histone acetylation and other epigenetic changes such as the fixation of particular DNA methylation patterns at the *Ifnγ* and the *Il4* loci [221, 222, 232, 234]. In the case of the *Ifnγ* gene, for instance, the promoter is in a "poised" state that results in low *Ifnγ* expression in naïve CD4+ T cells. After activation in Th1 conditions (IL-12 and anti-IL-4 mAb) the epigenetic landscape of the *Ifnγ* promoter changes. The most relevant epigenetic modifications during activation are the increase in CpG demethylation and progressive polarized histone acetylation patterns [215] and the acquirement of DNAse hypersensitivity in the *Ifnγ* promoter and in multiple cis-regulatory regions [235]. Chromatin modifications are brought about by different proteins, among these one of the most important is T-bet. This transcription factor can further modify the *Ifnγ* gene by leading to chromatin looping of this locus [236]. In the case of IL-4 the key transcription

factor that allows epigenetic modifications important for IL-4 transcription is GATA-3 [237].

We found that mTOR was recruited by acute TCR and CD28 engagement and that Rapa inhibited both mTORC1 and mTORC2 signalling, even if with different kinetics. Activation of naïve T cells in the presence of Rapa resulted in delayed expression of T-bet and GATA-3, and in defective demethylation of *Ifnγ* and *Il4* promoter regions. The reasons for delayed T-bet and GATA-3 expression remain to be determined. We found that STAT1 and STAT5 proteins were induced upon CD3/CD28 activation and phosphorylated to similar extents in both control and Rapa treated cells. Lee et al. [233] demonstrated that Rictor KO cells could not up-regulate T-bet and GATA-3, suggesting that mTORC2 was directing T-bet and GATA-3 expression. Accordingly these authors found that the expression of constitutively active mutants of Akt and PKC-θ, two of the mTORC2-controlled substrates could revert defective T-bet and GATA-3 expression in Rictor-deficient cells [233]. A most recent report by the Powell group, instead demonstrated that mTORC1 and mTORC2 control T-bet and GATA-3 via STAT4 and STAT6 respectively [89]. Our studies are consistent with the contribution of both mTORC1 and mTORC2 to transcription factor expression and cytokine gene transcription. Indeed while mTORC1 and cell cycle entry can be inhibited acutely and at low Rapa doses, mTORC2 and cytokine gene expression and differentiation requires higher doses and more prolonged treatments. We found that Rapa treatment hinders Akt phosphorylation in Ser473, supporting inhibition of mTORC2. Although we did not obtain convincing results on the state of PKC phosphorylation, inhibition of Akt might impede PKCθ recruitment and by that impinge on GATA-3 expression and consequently *Il4* transcription. Likewise, inhibition of mTORC1 can lead to decreased T-bet activity and thus decreased *Ifnγ* transcription. A delay in T-bet and GATA-3 expression leads to the preservation of DNA methylation at *Ifnγ* and *Il4* promoter regions. Whether delayed T-bet expression is causative of defective *Ifnγ* de-methylation and transcription, or whether defective de-methylation precedes optimal T-bet

expression and consequent binding to the DNA remains to be understood. It's important to underline that even if T-bet expression was mostly recovered after 48h of activation in the presence of Rapa, its target genes never recovered in their mRNA expression. This could be due to suboptimal binding to promoter regions that may remain methylated in the presence of Rapa.

While Rapa hindered activation-induced demethylation of *Ifnγ* and *Il4* loci, it prevented activation-induced methylation of *Foxp3* promoter region, which normally inhibits its transcription [238], and instead prolonged Foxp3 expression in the presence of Rapa. In cells treated with Rapa we observed a block in the methylation of *Foxp3* promoter, this is reflected by the higher mRNA and protein levels observed in Rapa treated cells compared to control stimulated cells.

In accordance to our data, Delgoffe et al. [103] demonstrated that CD4+ T cells that lack mTOR, and thus both mTORC1 and mTORC2, cannot differentiate into Th1, Th2 or Th17 cells [103]. The lack of differentiation was associated to the lack of up-regulation of the appropriate effector-specific transcription factors such as T-bet for Th1 cells, GATA-3 for Th2 cells and RORγt for Th17 cells [103]. mTOR deficient T cells could not differentiate thus in effector T cells, but they differentiated into Foxp3+ regulatory T cells [103]. In a recent paper the same group demonstrated that specific inactivation of mTORC1, through deletion of Rheb, prevents CD4+ T cells from becoming Th1 or Th17 cells both *in vitro* and *in vivo*. T cells lacking mTORC2, in which Rictor gene has been deleted, instead, cannot differentiate to Th2 cells [89]. Both mTORC1 and mTORC2 activity, instead, is necessary for the acquisition of a regulatory phenotype [89]. Lee et al. [233] demonstrated the role of mTORC2 in Th1 and Th2 differentiation. The authors showed that by silencing Rictor in T cells they prevented differentiation of T cells into Th1 and Th2 subsets. Deletion of Rictor had no impact on cell survival, but affected cell proliferation. They further showed that mTORC2 exerts its effect through activation of Akt and PKCθ [233]. The results from Lee et al. [233] and Delgoffe et al. [89, 103] show that

mTORC2 plays different roles in T cell differentiation. These discrepancies between the reports may be due to the fact that they make use of two different animal models. While Delgoffe et al. silence Rictor at the DP stage of thymocyte differentiation, Lee et al. use a CRE mouse under the Lck promoter, thus silencing Rictor early on during thymic development, at the DN2 stage. In our model we acutely inhibit mTORC1. The defect in IFN-γ production could reflect the data from mTORC1 deficient mice that show problems in Th1 differentiation. However we were able to demonstrate that Rapa can block mTORC2 as well, albeit with different kinetics. The defect in IL-4 expression thus, could be the result of inhibition of mTORC2.

Our results recapitulate, but only to some extent, recents reports. Delgoffe et al. [103] and Lee et al. [233] demonstrated that by genetic inactivation of mTOR and Rictor, respectively, they could prevent T cells differentiation towards Th1 and Th2 phenotypes. Our experiments performed on CD4+ purified T cells demonstrated that by pharmacological inhibition of mTOR we could not fully recapitulate the findings obtained in the conditional KO animals cited above. In particular Rapa administration affected T cell differentiation in Th0 conditions leading to a decrease in the percentage of IFN-γ and IL-4 producing cells, but Rapa treatment did not completely block differentiation of these cells, which was instead found in mTOR and Rictor deficient cells. Furthermore, in the presence of Th1 conditions (i.e. ricombinant IL-12 and anti-IL-4) Rapa did not affect differentiation of IFN-γ producing cells, in contrast with the findings obtained in KO animals [89, 233]. These discrepancies might infer the presence of Rapa-resistant functions of mTOR in primary lymphocytes.

Recent reports evidenced the importance of mTOR in the generation of effector and memory CD8+ T cells as well. The group of Rao et al. [239] demonstrated that blocking mTORC1 with Rapa leads to loss of T-bet expression, a transcription factor of key importance for the generation of effector T cells. Rapa, instead, favours sustained Eomes expression and thus differentiation towards a memory phenotype in CD8+ T cells [239].

Araki et al. [200] demonstrated that treatment with Rapa of mice infected with lymphocytic choriomeningitis virus (LCMV) led to a rise in the number and quality of antigen-specific CD8+ T memory cells [200]. Rapamycin can do this by increasing the number of memory precursors during the phase of T cell expansion. By using an RNAi system the authors show that this regulation of memory vs effector differentiation is mediated specifically by mTORC1 [200].

mTOR thus appears to be of fundamental importance for differentiation of T cells. Inhibition of mTOR leads to impaired differentiation to effector cells in CD4+ and CD8+ cells [240]. Block of mTOR, in fact, favours the conversion of cells to a regulatory phenotype or to a memory phenotype, both in CD8+ T cells [200, 239] and in CD4+ T cells. In CD4+ T cells, in particular, we demonstrated that following Rapa treatment for 5-7 days, Rapa-cultured cells showed a proliferative profile similar to untreated cells. These cells were characterized by a decreased percentage of IFN-γ and IL-4 producing populations, while IL-2 producing cells were enriched in Rapa-treated cultures, suggesting a more memory like phenotype.

Our data, although in line with those obtained with Frap, Rictor and Raptor deficient T cells, show some differences between genetic inactivation and pharmacological inhibition of mTOR. For instance, while Frap, Rictor and Rheb deficient lymphocytes failed to differentiate into IFN-γ secreting cells in Th1 polarizing conditions [89, 103, 233], cells activated in the presence of Rapa, retained both IFN-γ and IL-2 expression. After Rapa treatment we observe a partial block in IFN-γ and IL4 production and a skewing towards Foxp3 producing cells, similar to what is observed by genetic inactivation of the genes of the mTOR complexes. This discrepancy with the fact that with genetic inactivation the authors observe a complete block might be due to the failure of Rapa to fully inhibit mTORC2, or to the ability of IL-12 to control *Ifnγ* expression via mTOR-dependent Rapa-insensitive mechanisms.

Future challenge will be to identify the mechanisms by which Rapa affects TCR/CD28/IL-2 controlled methylation of cytokines promoter regions. DNA methylation occurs at proximal and distal promoter sites of genes important for immune function [241]. DNA methyltransferases (Dnmt) maintain specific patterns of DNA methylation during DNA replication. Dnmt1, Dnmt3a and b have different roles in the cell. Dnmt1 is the Dnmt important for preserving the pattern of the parental-strand methylation in the newly synthesized daughter strand during DNA replication. Dnmt3a and b, instead, are of key relevance for the *de novo* introduction of methyl groups on cytosine residues [242]. Defective Dnmt1 expression or function causes passive demethylation of specific regions and leads to transcriptional up-regulation of previously suppressed cytokine genes and to auto-immune manifestations [222, 243]. Dnmt3a is induced following TCR engagement and controls *Ifnγ* and *Il4* promoter methylation [244]. The existence of DNA demethylases is a matter of intense debate [245], however in some cases active demethylation was observed soon before DNA replication [246]. mTOR might also influence DNA methylation by controlling expression/activity of yet to be defined DNA demethylases or of histone acethylases/deacetylases/methylases, also involved in epigenetic changes. In this respect it is of interest that TOR was linked to the Esa1 histone acetylase pathway in yeast [247].

Given the role of mTOR in differentiation of both helper and cytotoxic effector cells, of Treg cells and of T central memory lymphocytes, understanding whether and how mTOR controls epigenetic modifications and by that T cell fate will be instrumental to define the mechanism of action and long-term consequences of Rapa administration *in vivo*.

3. TSC1 is critical for T cell development and homeostasis

3.1 Introduction

While studying the role of mTOR in activation and differentiation of mature T cells, we became aware of the availability of TSC1 fl/fl mice within the Institute. To define the impact of TSC signalling in developing and mature T cells we decided to generate T-lineage restricted deficient mice by crossing the TSC1 fl/fl mice to the Lck-CRE and CD4-CRE deleters. Thereafter is reported a brief review of the principal events characterizing thymic lymphocyte development.

3.1.1 T cell development

The thymus is considered the primary lymphoid organ for the generation of T cells. Its microenvironment, in fact, makes available a particular combination of different stromal cell types important for the development and selection of thymocytes that will become mature T cells (reviewed by Gill J. et al. [248]). Anatomically, it can be divided into two principal areas: the outer cortex and the inner medulla.

Development of T cells in the thymus is a strictly controlled process involving different steps. Each phase of the process is characterized by the expression of particular surface markers. During each step of differentiation, developing thymocytes acquire the expression of receptors that can signal to the cell and induce it to transit to the next step in the process.

Two different T cells population come out from thymic selection: the α-β and γ-δ T cells. They are characterized by the expression of two different T Cell Receptors (TCR). While γ-δ cells respond to unprocessed antigens, α-β cells need presentation of the antigen by Major Histocompatibility Complex (MHC) [249]. 20% of the cells exiting the thymus are γ-δ cells, the others are α-β T cells. Both of these cell types start their life in the thymus as Double Negative (DN) cells. They are defined in this way because they do not express either CD4 or CD8 coreceptors. At the earliest stages the DN cells are also negative for

CD3/TCR complex. These DN cells can be divided in 4 different subsets (DN1, DN2, DN3 and DN4) according to the expression of two different markers CD25 and CD44 (reviewed by Rothenberg E. V. and Taghon T. [250]). The earliest stage, the DN1, is characterized by cells that are CD44+ and CD25-. C-kit and Stem Cell Factor (SCF) genes have been proved important for DN1 cellularity [251]. The following step of differentiation is marked by the expression of CD25 in these cells. The passage from DN1 to DN2 cells is driven by IL-7 [252-255] signalling. The following step, the DN3, pre-T cell stage is characterized by the loss of expression of CD44. During this phase TCRγ, δ and β chain genes are rearranged and cells become fully committed to the T-cell lineage. After TCRβ chain has been successfully rearranged it is expressed on the cell surface together with a surrogate light chain, the pre-Tα (pTα). They form the pre-T cell receptor. This receptor associates with the CD3 complex and together they signal to the cell (reviewed by von Boehmer H. and Fehling H. J. [256]). Cells that cannot express a functional β chain are arrested at this point, the beta-checkpoint [257]. The signals coming from the pre-TCR lead the cell to undergo several rounds of proliferation and to express both CD4 and CD8. These surface co-receptors associate with the TCR during antigen recognition and by binding to the MHC allow an effective T cell response [258]. These co-receptors bind to the lateral face of the MHC molecules. In particular CD4 recognizes MHC class II molecules while CD8 recognizes MHC class I molecules.

This stage is referred to as Double Positive (DP) stage. At this point immature thymocytes shut off one of the two coreceptors to give rise to CD4+ or CD8+ Single Positive (SP) cells. The process involved in this maturation step is that of positive selection. This process is connected to the functional specialization of these cells. If they become CD4+ cells they will acquire helper function, otherwise if they turn into CD8+ T cells they will acquire cytotoxic functions [259].

The different developmental stages occur in distinct thymic niches. CD4-CD8- (DN) cells migrate across the cortex. CD4+CD8+ (DP) cells that are selected to become SP

cells proceed with a reverse migratory path across the cortex towards the medulla. During their migration through the cortico-medullary junction and into the medulla, the developing thymocytes interact with dendritic cells and specialized medullary Thymic Epithelial Cells (mTECs) that present self-antigens.

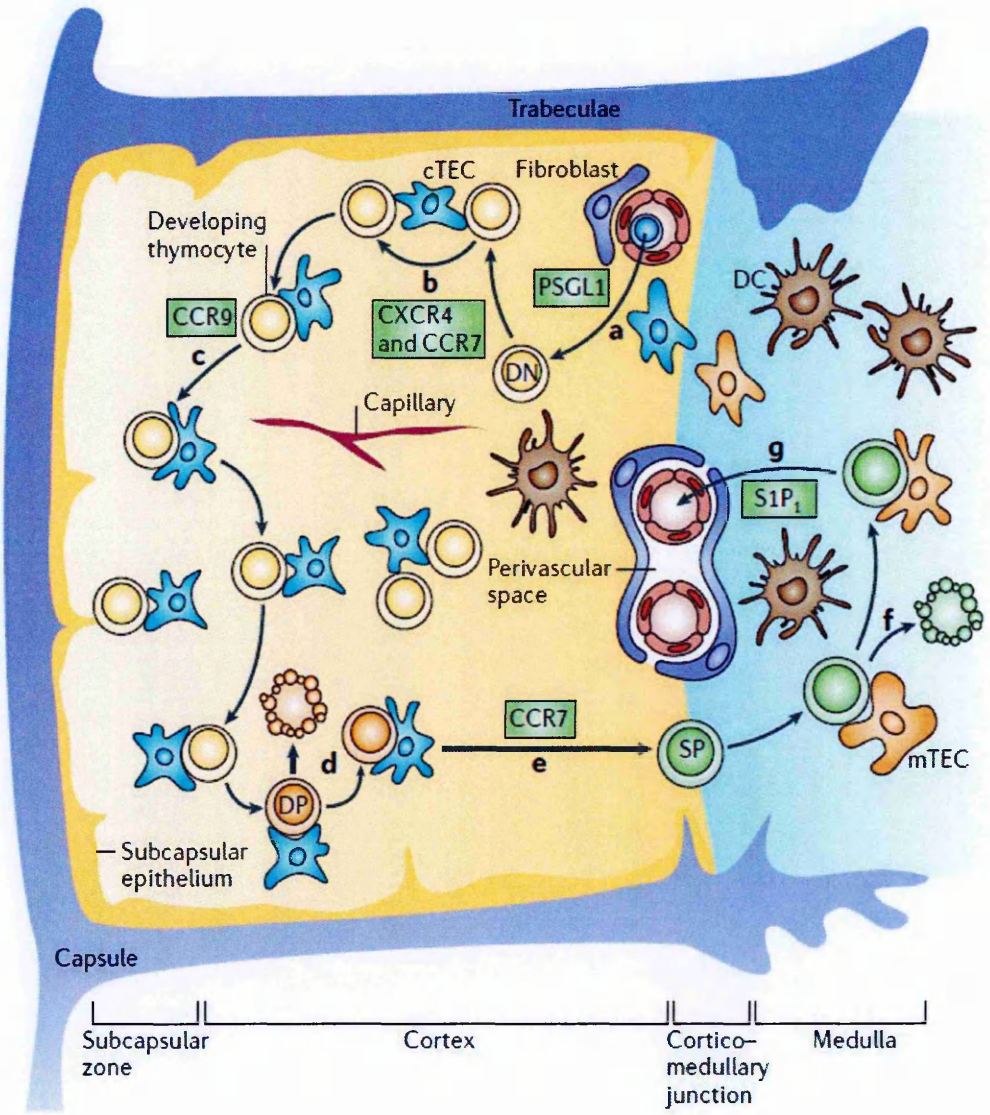

Fig V. T cell development in the thymus. Immature thymocytes migrate into the thymus through the vasculatures enriched at the cortico-medullary junction. The DN thymocytes migrate outward to the subcapsular region. In the outer cortex are generated the DP thymocytes that differentiate into SP cells. Takahama Y., Nature Reviews Immunology, 2006 [260]

3.1.2 Signalling pathways controlling thymocyte development.

During thymic development expression and function of the TCR play a key role in driving the immature thymocytes to become mature T cells (reviewed by Matthews S. A. and Cantrell D. A. [261]). Two key molecules downstream the TCR that are of fundamental importance for T cell development are the kinases Lck and Fyn (reviewed by Zamoyska R. et al. and by Salmond R. J. et al. [262, 263]). These proteins are important for TCR activation [264]. Nika et al. [264] showed that high amounts of phosphorylated Lck and Fyn are present in naïve T cells. TCR ligation did not increase basal phosphorylation of Lck and did not increase either its kinase activity. The reduction of this basal phosphorylation of Lck, instead, compromised TCR signalling initiation, underlying the importance of Lck in T cell activation [264]. Active Lck can in turn phosphorylate CD3 and Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) [265] favouring the recruitment of the ζ-chain associated protein of 70 kDa (ZAP-70) kinase to the TCR [266]. In turn ZAP-70 phosphorylates and activates the protein Linker for Activation of T cell (LAT) [267] that in turn activates downstream kinases and enzimes important for T cell development such as the PI3K/Akt and ERK/MAPK pathway. The active form of Fyn can help in the activation of the signalling pathways described above. TCR is known to activate also the PI3K pathway.

The importance of these kinases is evidenced by the fact that Lck deficient mice show an incomplete block in the maturation of the DN3 thymocytes (this function is TCR independent as it occurs when the cells do not yet have a fully developed TCR) [268] and also in the maturation of DP thymocytes. The passage from DP to SP cells, in fact, leads to the maturation of very few mature peripheral T cells in mice lacking Lck. Fyn KO mice do not show particular problems in the number and percentage of different thymocytes subsets [269, 270], suggesting possible compensatory mechanisms carried out by Lck activation. The double KO of both Lck and Fyn, instead, completely blocks development at the DN3 stage [271, 272], stressing again the importance of the two kinases for proper T cell

maturation.

Besides signalling from the TCR, another key trigger of T cell development is IL7. Alves et al. [273] by using an IL7 reporter mouse demonstrated the existence of TEC expressing high levels of IL7. IL-7 driven survival and proliferative signalling is needed by early T cell precursors positioned in the inner part of the cortex and by SP mature T cells that reside in the medulla [274, 275]. The authors found IL-7 producing cells in both the compartments that are probably responsible for nurturing thymocytes during both early and late phases of development. In human thymocytes Johnson et al. [276] demonstrated that IL7 signalling can activate the PI3K/Akt signalling pathway.

Genetic inactivation of PI3K subunits and Akt underlined the importance for this pathway in T cell development. Mice lacking the p110γ subunit of PI3K for instance show reduction by half in the size of the thymus [277]. DP cells from these mice reveal reduced survival after anti-CD3 stimulation *in vivo*. Thymocytes from p110γ KO mice are characterized by increased apoptosis following stimulation with anti-CD3 and adenosine analogues. Also mice lacking the PI3K subunits p110γδ show reduction in the size of the thymus and reduction in the DP population due to an increase in apoptosis of these cells [278].

Akt1 KO mice show spontaneous apoptosis and increased susceptibility to different apoptotic stimuli [279] and reduction in the size of the thymus, followed by accumulation of DN cells [280]. Also combined silencing of different Akt isoforms leads to defective thymic development. Thymocytes lacking both Akt1 and Akt2 show defective development after the DN3 stage and they have decreased glucose uptake. The developmental block is due to increased apoptosis in Akt1 and Akt2 deficient cells [281]. Conversely DP thymocytes that express a constitutively active form of Akt1 reveal a survival advantage [282]. Na et al. [283] demonstrated that by overexpressing a membrane-targeted constitutively active version of PKB they observed accumulation of CD4+ T cells in the spleen and lymphonodes of transgenic mice. Peripheral CD4+ T cells

could survive better following different apoptosis inducing treatments, suggesting a role for PKB in triggering survival signals. The increased number of CD4+ T cells was shown to be due to the fact that PKB can favour thymic selection of these cells [283].

Interestingly enough PTEN-deficient thymocytes are resistance to apoptosis induced by adenosine analogues, by γ irradiation and UV irradiation. These mice show an increase in the DP compartment [284] and elevated thymic cellularity [285]. PTEN heterozygous mice reveal autoimmune features and reduced Fas-dependent apoptosis [286], development of CD4+ T cell lymphomas, their T cells are characterized by enhanced proliferation and cytokine production and the thymocytes from these mice show decreased apoptosis [284].

From these results the picture that emerges is of PI3K and Akt as proteins important in preventing apoptosis in the thymus.

Also mTOR has been shown to play an important role in thymus development. Studies performed with Rapa demonstrated that pharmacological inhibition of mTOR interferes with the maturation of DP thymocytes. This results in an increase in TCR negative immature thymocytes [287]. Damoiseaux et al. [287] used two different rat models for their studies, the Lewis rats and the Brown Norway rats. They observed that in these rats treatment with Rapa led to thymic atrophy. Also Tian et al. [288] showed that Rapa treatment led to atrophy in the thymus of rats [288], by reducing the number of DP thymocytes accelerating their death. Luo et al. [289] showed that Rapa treatment led to strong thymic atrophy in mice. From a histological point of view Rapa caused the thinning of the thymic cortex. The authors did not show any effect on apoptosis, suggesting that thymic atrophy was caused by the decreased proliferation of thymocytes following Rapa treatment [289]. In a more recent report Kelly et al. [290] showed that Rapa treatment of DN thymocytes co-cultured with OP9-DL1 monolayers inhibited Notch-dependent proliferation of these cells *in vitro*. These DN cells, however, could still differentiate to DP cells, suggesting the importance of mTOR signalling for thymocytes proliferation rather

than differentiation [290].

Together existing evidences thus support a role for mTOR in T cell activation and differentiation. To further investigate this point, we took advantage of the availability in the Institute of TSC1 fl/fl mice and generated mice with T-lineage restricted TSC1 inactivation. Based on available data we hypothesized that TSC1 deletion might result in the inactivation of the TSC1/TSC2 complex. This in turn would perturb mTOR regulation, allowing the study of the contribution of this patway to mTORC1 and mTORC2 signalling and thymocyte development.

3.2 Material and methods

3.2.1 Mice.

TSC1 floxed mice [291] were crossed with Lck- and CD4-CRE deleter mice. Genotyping was performed using a PCR based approach with primers specific for TSC1: F4536 (5'-AGGAGGCCTCTTCTGCTACC-3'), R4830 (5'-CAGCTCCGACCATGAAGTG-3') and R6548 (5'-TGGGTCCTGACCTATCTCCTA-3') and for the CRE recombinase: FORWARD (5'-TATATCTTCAGGCGCGCGGT-3') and REVERSE (5'-GCAATCCCCAGAAATGCCAG-3'). For the TSC1 PCR the products are the band of the wt gene of 300 bp, the band of the floxed gene of 500 bp and the band of the deleted gene of 400 bp. The product of the CRE PCR is a band of 300 bp. For the TSC1 gene the conditions of PCR are: 94 °C for 2 min; 35 loops of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec; 72 °C for 7 min. For the CRE gene the PCR conditions are: 94 °C for 2 min; 35 loops of 94 °C for 30 sec, 62 °C for 30 sec and 72 °C for 30 sec; 72 °C for 7 min. Mice with T-lineage restricted conditional TSC1 deletion (TSC1ΔT) used for functional experiments have been backcrossed onto the C57BL/6J background for at least five generations and analyzed at 8 wk of age unless otherwise indicated. In all cases, age-matched littermates were used as controls. All mice were housed in a pathogen-free environment in accordance with the regulations of the San Raffaele Institute Institutional Animal Care and Use Committee.

3.2.2 Primary T cell cultures.

CD4+ T cells were purified by negative selection using anti-CD8 (clone KT1.9) and anti-I-Ab-d/I-E (clone B21-22) rat Abs and sheep anti-rat-coated magnetic beads (Dynal Biotech LTD., UK) to a purity of >95%. Cells were then stimulated on immobilized anti-CD3 and anti-CD28 mAb (0.5 μg/ml and 5 μg/ml) in the absence or in the presence of RAPA (100 nM) or of Recombinant Mouse TRAIL R2/TNFRSF10B Fc Chimera (50 ng/ml, R&D Systems).

Cell were always kept in RPMI-1640 medium (Invitrogen) with 5% FBS until lysis. This prevented inactivation of S6 phosphorylation.

3.2.3 Western blot analysis.

CD4+ purified cells were stimulated on immobilized anti-CD3 and anti-CD28 mAb (0.5 μg/ml and 5 μg/ml). When indicated cells were treated with Rapa (1 or 100 nM, Calbiochem), SL0101 (3 or 30 uM, Toronto Research Chemicals Inc.) or UO126 (10 uM, Promega). T cells and thymocytes extracts were washed twice with ice-cold PBS and lysed in lysis buffer containing SDS 2% and Tris-HCl 62.5 mM pH 6.8.

Proteins were quantified by BCA assay and appropriate amounts (30 or 50 μg) were separated on standard 10-15% SDS-PAGE. For Western blotting, gels transferred to nitrocellulose filters were first blocked for 1 h with 5% nonfat dry milk in PBA, and then incubated o.n. with the primary antibody diluted in PBS with 5% bovine serum albumin (BSA), washed in PBST (3-fold for 10 min), incubated for 1 h with the peroxidase-conjugated secondary antibody, washed again in PBST as described above and once in PBS. Photographic development was by chemiluminescence (ECL, Amersham Bioscience or Immobilon substrate, Millipore). Western blot bands were quantified by the ImageJ program (rsb.info.nih.gov/ij).

Anti-Akt, anti-P(S473)-Akt, anti-P(T308)-Akt, anti-4E-BP1, anti-TSC2, anti-TSC1, anti-GSK3β, anti-P(S9)-GSK3β, anti- P(Ser235/236)-S6 and anti-S6, anti-FoxO1, anti-P(T24)-FoxO1/P(T32)-FoxO3a, anti-P(T389)-P70S6K: Cell Signalling Technology (Milan, Italy); and actin, which was obtained from Santa Cruz Biotechnology Inc. Horseradish peroxidase-conjugated goat antimouse and anti-rabbit pAbs: Bio-Rad. The BCA Protein Assay Kit from Pierce.

Antibody anti-	Company	Cat. N°
FoxO1	Cell Signaling	2880
Bcl2A1	abcam	ab45413
H3	Upstate	06-755
BclXL	BD Transduction	556361
P53	Cell Signaling	2524
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Cell Signaling	9101
Phospho-Akt (Thr308)	Cell Signaling	2965
Akt	Cell Signaling	4685
Phospho-Akt (Ser473)	Cell Signaling	9271
4E-BP1	Cell Signaling	9452
TSC2	Cell Signaling	3990
TSC1	Cell Signaling	4906
S6	Cell Signaling	2317
Phospho-S6 (Ser235/236)	Cell Signaling	2211
Phospho-p70S6K (Thr389)	Cell Signaling	9205
Actin	Santa Cruz Biotechnology	Sc-1616
Phospho-FoxO1 (Thr24)/FoxO3a (Thr32)	Cell Signaling	9464

3.2.4 miRNA analysis.

RNA was extracted with mirVana miRNA Isolation Kit (Applied Biosystems) following manufacturer's instructions and its concentration was determined by spectrophotometry.

10 ng of RNA were used to generate cDNA templates for Real Time PCR using TaqMan MicroRNA Reverse Transcription (RT) Kit (Applied Biosystems). q-PCR was performed using Real Time Taqman universal master mix (Applied Biosystems) and primers of the TaqMan MicroRNA Assays. The primers used were mir-145 and snoRNA-202. Values were normalized to the concentration of snoRNA-202 RNA. q-PCR was performed using an ABI PRISM 7700 Sequence Detection System.

Specific mir-145 miRNA expression was normalized by housekeeping snoRNA-202 expression according to the ΔΔCT method.

3.2.5 CFSE proliferation assay.

As reported in 2.2.5

3.2.6 Quantitative real-time RT-PCR (qPCR).

As reported in 2.2.6

3.2.7 Statistical analysis.

As reported in 2.2.8

3.3 Results

3.3.1 TSC1 deletion perturbs mTOR signalling in thymocytes and in peripheral T cells.

To identify the role of the TSC1/TSC2 complex in thymic development, T-lineage restricted inactivation of TSC1 was adopted. Mice that carry floxed TSC1 alleles [291] were crossed with mice that express the CRE recombinase under the Lck promoter. This strategy allows the deletion of the TSC1 gene early on during thymic development, at the DN2 stage. Mice with T cell restricted silenced TSC1 (TSC1ΔT) were generated at the expected mendelian ratio. These mice did not phenotypically differ from their age-matched wild type littermates (TSC1WT). In Fig 3.1 A the presence of CRE recombinase and the different TSC1 alleles were evaluated by PCR in cDNA from thymi, spleens, lymphonodes and purified T cells from LCK-CRE/TSC1ΔT and LCK-CRE/TSC1WT mice. The CRE recombinase was present in the cDNA from all the LCK-CRE/TSC1ΔT samples while was absent in the LCK-CRE/TSC1WT samples. Most thymocytes and lymphocytes from LCK-CRE/TSC1ΔT mice showed the deleted form of the TSC1 allele. In the spleen from the LCK-CRE/TSC1ΔT animals just a percentage of cells showed the deleted form of the allele. Purification of CD4+ cells from the spleen of the LCK-CRE/TSC1ΔT mice confirmed that the TSC1 locus was deleted in this cell subset. In the cDNA from the LCK-CRE/TSC1WT mice all the TSC1 alleles were in the floxed form.

TSC1 and TSC2 expression was assessed also by western blot analysis in thymocytes and purified CD4+ T cells (Fig. 3.1 B, C). In samples from LCK-CRE/TSC1ΔT mice both TSC1 and TSC2 were not detectable. This could be explained by the fact that TSC1 has been shown to be important for TSC2 stabilization as it prevents TSC2 ubiquitin-mediated degradation [8, 9]. Also the mTORC1 and mTORC2-dependent pathways were analysed to assess whether inactivation of TSC1 could affect these signalling cascades. Extracts from unfractionated thymocytes and mature CD4-purified T

cells from LCK-CRE/TSC1ΔT but not LCK-CRE/TSC1WT mice revealed constitutive phosphorylation of p70S6K (Thr389) and of S6 (Ser235/236) in resting conditions. Following stimulation with CD3/CD28 for 30 min p70S6K and S6 phopshorylation increased in LCK-CRE/TSC1WT cells, while remained unchanged or was only modestly increased in LCK-CRE/TSC1ΔT cells. Rapa administration at the time of stimulation completely abrogated p70S6K and S6 in either cases, supporting the role of mTORC1. Phospho-S6 phosphorylation among thymocyte subsets was further investigated by intracellular staining (Fig. 3.1 D) . Result depict that constitutive phosphorylation of S6 starts to be detected in a fraction of DP cells from LCK-CRE/TSC1ΔT mice, and is most evident at the SP stage. The constitutive activation of mTORC1 liklely also accounted for an increase in cell size (Fig. 3.1 E) and protein content (Fig. 3.1 F). Indeed CD4SP, CD8SP thymocytes, mature CD4 and CD8 cells, and DP thymocytes, although to a lower extent, but not DN thymocytes or mature B cells from LCK-CRE/TSC1ΔT mice revealed a larger FSC when compared to those derived from LCK-CRE/TSC1WT littermates (Fig. 3.1 E). Likewise protein content of LCK-CRE/TSC1ΔT thymocytes is higher than that of LCK-CRE/TSC1WT cells (Fig. 3.1 F). We measured mTORC2 activity by looking at the phosphorylation of Akt on ser 473. After stimulation of LCK-CRE/TSC1WT thymocytes and of CD4+ T cells we could observe an increase in Akt phosphorylation. This was strongly reduced in LCK-CRE/TSC1ΔT thymocytes and absent in LCK-CRE/TSC1ΔT mature T cells (Fig. 3.1 B, C).

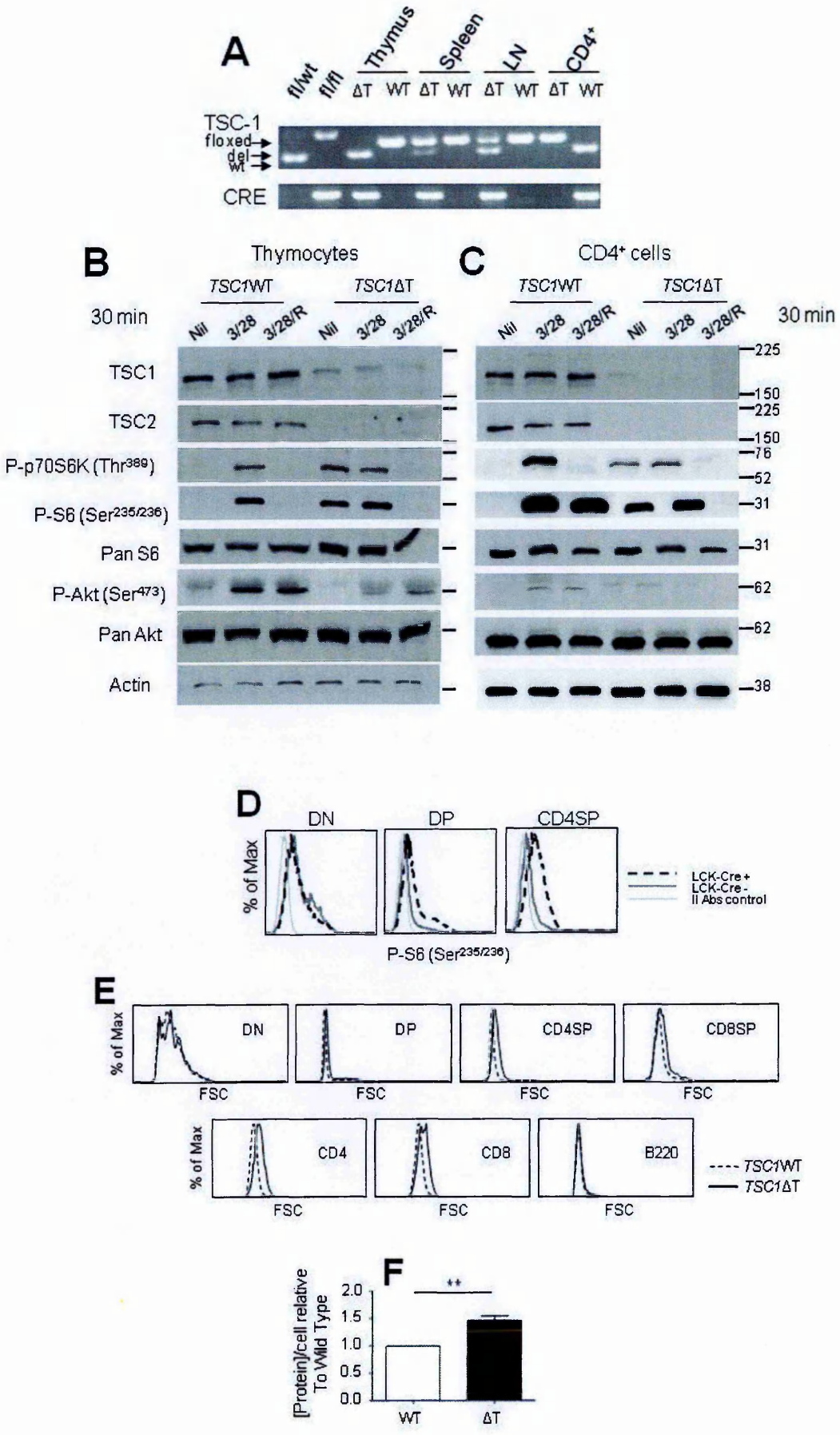

Fig 3.1 Deletion of TSC1 at the DN stage perturbs mTOR-signalling in developing thymocytes and mature T cells. TSC1WT and TSC1ΔT LCK-CRE mice were sacrificed

at 8 weeks of age. A) Genomic DNA was obtained from unfractionated thymocytes, splenocytes and lymphonodes cells, or from CD4+ T cells purified from spleens and analyzed by PCR with primers able to amplify the wt, floxed and deleted TSC1 allele and the CRE recombinase. B, C) Unfractionated thymocytes (B) or purified peripheral CD4+ T cells (C) from TSC1f/f, LCK-CRE- mice (TSC1WT) and from TSC1f/f, LCK-CRE+ (TSC1ΔT) mice were left untreated (Nil) or stimulated with anti-CD3 (0.5 ug/ml) and anti-CD28 (5 ug/ml) mAb for 30 min in the absence (3/28) or in the presence of Rapa (100 nM) (3/28/R). TSC1 and TSC2 levels, as well as relative phosphorylation of the mTORC1 target p70S6K (Thr389) and of its substrate S6 (Ser 235/236) and of the mTORC2 target Akt (Ser473) were assessed by western blot analysis. Actin is depicted as loading control, and relative migration of molecular weight markers is depicted. SDS-PAGE analyses were performed as detailed in Materials and Methods 3.2.3 with the indicated Ab. Molecular weight markers are indicated in KDa. D) Flow cytometry analysis of thymocytes subsets identified by electronic gate after staining with anti-CD4, anti-CD8 and anti-phosphoS6 (Ser235/236) Ab (dotted line TSC1ΔT cells; dark solid line TSC1WT cells, light solid line II Abs control). E) Flow cytometry analysis of thymocytes and lymphocytes subsets identified by electronic gate after staining with anti-CD4, anti-CD8 and anti-B220 mAb (dotted line TSC1WT cells; solid line TSC1ΔT cells). F) Protein concentration per cell, as determined by BCA assay, in TSC1WT and TSC1ΔT total thymocytes. Data are mean (±SE) of protein extracts made from 4 separate experiments. In E) statistical significance was evaluated with two-tailed t-Test.

This might be due to the failure of TSC1/TSC2 to activate mTORC2 [38], or to the presence of an mTORC1-dependent negative feedback loop. This was previously described in non-lymphoid cells to occur via an S6 Kinase-dependent downregulation of upstream activators of PI3K, such as the PDGF receptor and IRS-1 [125-127]. Our results support the existence of this loop also in TSC1 deficient T cells, as TSC-mediated signals failed to induce phosphorylation of Akt in Thr308 (PI3K-dependent) and also of MAPK in LCK-CRE/TSC1ΔT cells (Fig. 3.2 A, B).

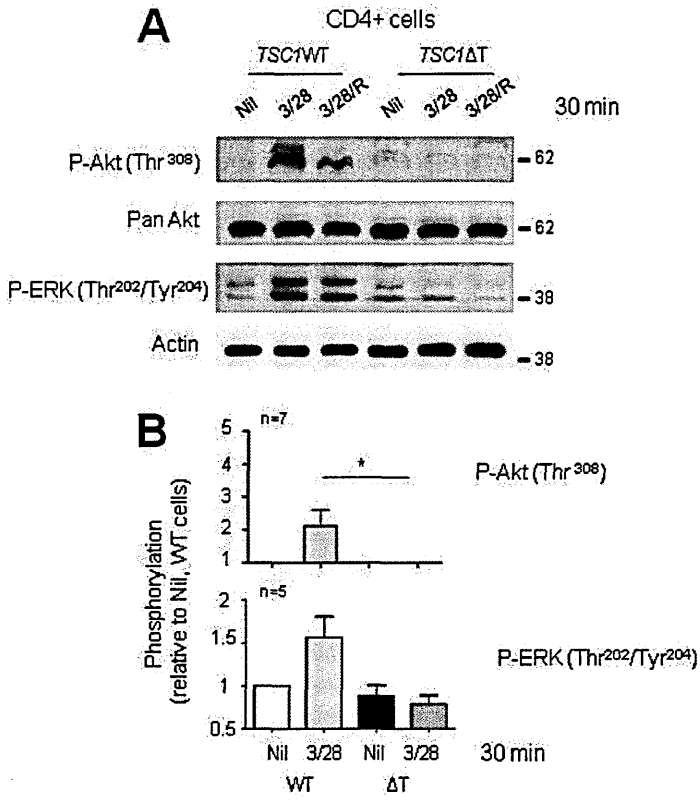

Fig 3.2 Constitutive mTORC1 hinders TCR-induced PI3K and MAPK activation in LCK-CRE TSC1ΔT mice. TSC1WT and TSC1ΔT LCK-CRE mice were sacrificed at 8 weeks of age. Purified peripheral CD4+ T cells were left untreated (Nil) or stimulated with anti-CD3 (0.5 ug/ml) and anti-CD28 (5 ug/ml) mAb for 30 min in the absence (3/28) or in the presence of Rapa (100 nM) (3/28/R). A) Phospho-Akt (Thr 308), Pan-Akt and Phospho-Erk (Thr 202, Tyr 204) levels were assessed by western blot analysis. Actin is depicted as loading control, and relative migration of molecular weight markers is depicted. SDS-PAGE analyses were performed as detailed in Materials and Methods 3.2.3 with the indicated Ab. Molecular weight markers are indicated in KDa. B) Independent experiments were compared by densitometric analyses. Expression levels are normalized on actin and shown as relative to TSC1-sufficient cells (±SE). Statistical significance was analysed by one-tail ANOVA test with Dunns post test.

Fig. 3.3 A, B shows the quantification of different western blot experiments pooled together in which the mTORC1 and mTORC2 targets were assessed.

These findings suggest that loss of TSC1 leads to constitutive mTORC1 signalling and reduced TCR-driven mTORC2 signalling, both at the central and peripheral level.

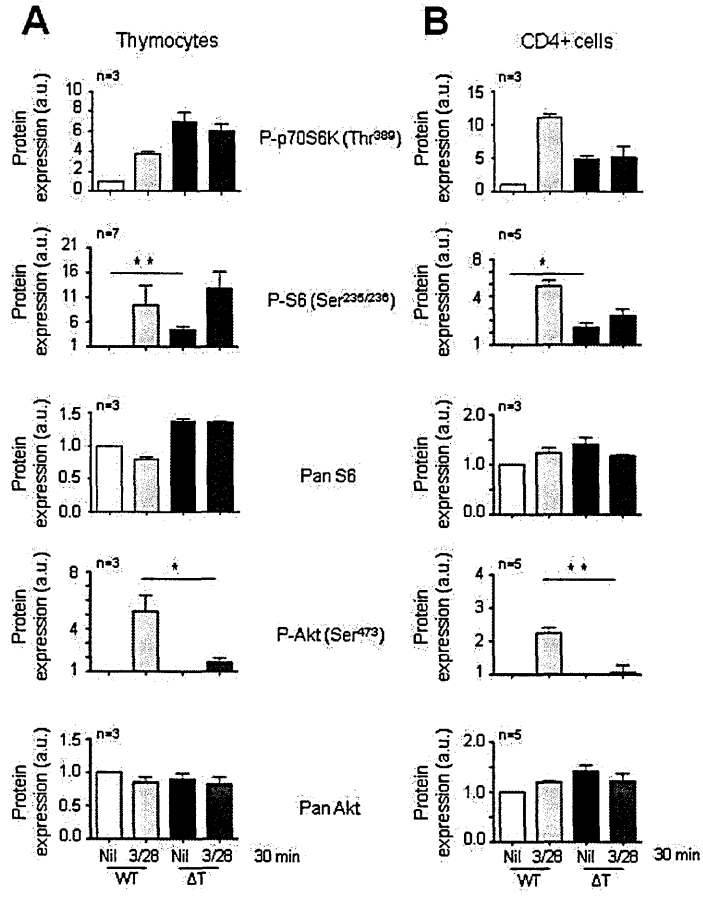

Fig 3.3 mTOR signalling in TSC1-sufficient and TSC1 deficient cells from LCK-CRE TSC1f/f mice. TSC1WT and TSC1ΔT LCK-CRE mice were sacrificed at 8 weeks of age. Unfractionated thymocytes (A) or purified peripheral CD4+ T cells (B) were left untreated (Nil) or stimulated with anti-CD3 (0.5 ug/ml) and anti-CD28 (5 ug/ml) mAb for 30 min in the absence (3/28) or in the presence of Rapa (100 nM) (3/28/R). Relative phosphorylation of the mTORC1 target p70S6K (Thr389) and of its substrate S6 (Ser235/236) and of the mTORC2 target Akt (Ser 473) and expression of S6 and Akt were assessed by western blot analysis. Independent experiments were compared by densitometric analyses. Expression levels are normalized on actin and shown as relative to TSC1-sufficient cells (±SE). Statistical significance was analysed by one-tail ANOVA test with Dunns post test.

3.3.2 TSC1 loss hinders T cell development.

Loss of TSC1 starting at the DN stage hindered proper T cell development and peripheral representation, as evidenced by the reduced cellularity of the thymus and the spleen (Fig. 3.4).

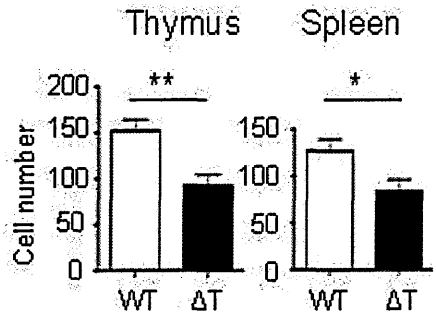

Fig 3.4 Defective T cell development in the absence of TSC1 in LCK-CRE TSC1f/f mice. TSC1WT and TSC1ΔT LCK-CRE mice were sacrificed at 8 weeks of age. A) Thymi and spleens recovered from TSC1WT and TSC1ΔT individual mice (n=14) were reduced to single cell suspension. Total cell count (±SE) is depicted.

By flow cytometry analyses we found that DN subsets were equally represented both in frequencies and total numbers (Fig. 3.5).

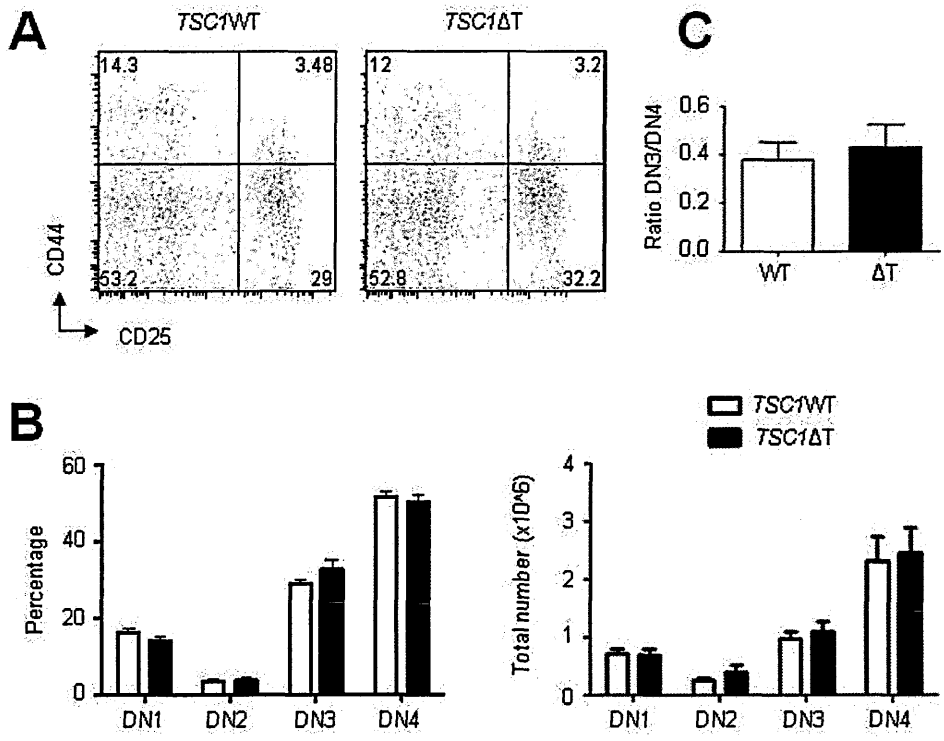

Fig 3.5 TSC1 deletion at the DN level in TSC1f/f LCK-CRE mice does not influence DN1-4 subsets. TSC1WT and TSC1ΔT LCK-CRE mice (n=9) were sacrificed at 8 weeks

of age. Thymi were recovered, reduced to single cell suspension and individually analyzed by flow cytometry. A) Representative dot plots depict DN1-4 cells after gating on viable DN (CD4- and CD8-) thymocytes. B) The frequency and number (± SE) of DN thymocyte subsets, identified by electronic gating, is depicted. Data were analysed comparing WT and KO subsets by the student t-test. No statistical difference was found. C) Ratio between DN3 and DN4 thymocytes.

In contrast, while the percentage of DP, and CD4SP/CD8SP was comparable among WT and LCK-CRE/TSC1ΔT mice, the total number of DP, CD4SP and CD8SP cells was severly reduced in the LCK-CRE/TSC1ΔT thymus when compared to WT ones (Fig. 3.6).

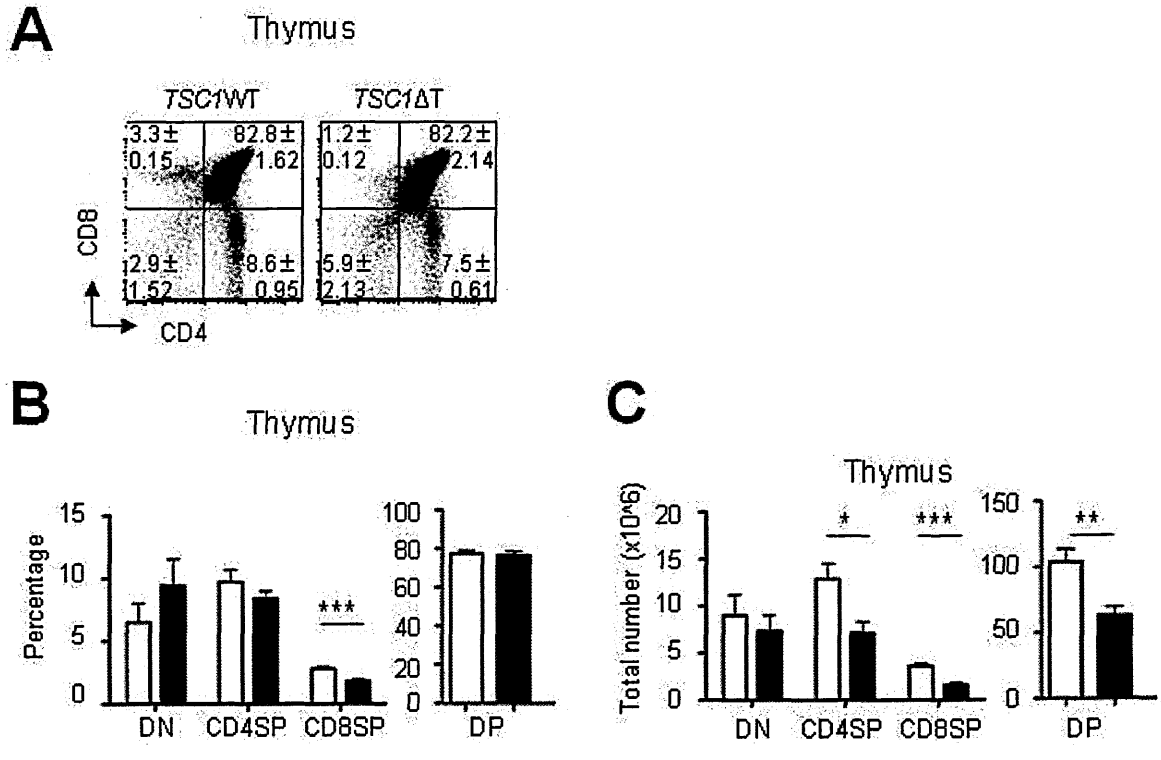

Fig 3.6 Defective T cell development in the absence of TSC1 in LCK-CRE TSC1f/f mice. TSC1WT and TSC1ΔT LCK-CRE mice were sacrificed at 8 weeks of age. Thymi were recovered and analyzed by flow cytometry after staining with anti-CD4 and anti-CD8 Ab. A) Representative dot plots depict CD4 and CD8 cells after gating on viable thymocytes. C) The frequency (±SE) of thymocyte subsets, identified by electronic gating, is depicted. FACS-generated percentages were multiplied by viable counts to define absolute thymocyte cell numbers. Statistical significance was evaluated with one-tail ANOVA test with Dunns post test.

In peripheral lymphoid organs, both the frequency and the total number of CD4+ and CD8+ mature T cells was lower in LCK-CRE/TSC1ΔT mice when compared to wt ones (fig. 3.7).

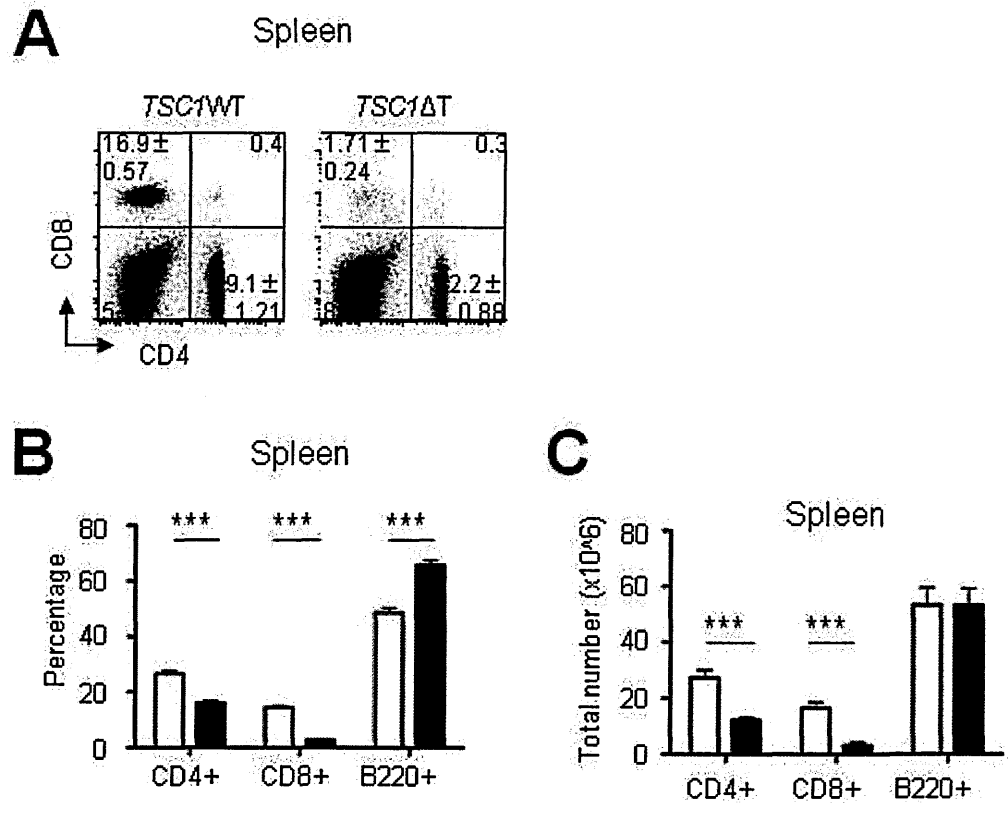

Fig 3.7 Defective T cell development in the absence of TSC1 in LCK-CRE TSC1f/f mice. TSC1WT and TSC1ΔT LCK-CRE mice were sacrificed at 8 weeks of age. Spleens were recovered and analyzed by flow cytometry after staining with anti-CD4, anti-CD8 and anti-B220 Ab. A) Representative dot plots depict CD4 and CD8 cells after gating on viable splenocytes. B, C) The frequency (±SE) of splenocyte subsets, identified by electronic gating, is depicted. FACS-generated percentages were multiplied by viable counts to define absolute lymphocyte cell numbers. Statistical significance was evaluated with one-tail ANOVA test with Dunns post test.

As deregulated AKT/PKB has been shown to alter thymocyte and T cells migration [223], we analyzed the possibility that reduced number of mature T cells might derive from accumulation of mature thymocytes in the thymus. To this purpose we enumerated CD24high and dim SP cells, which respectively identify immature and mature SP

thymocutes. Results depicted in Fig. 3.8 A and B indicate that thymi of LCK-CRE/TSC1ΔT mice and LCK-CRE/TSC1WT controls contain comparable frequencies of CD24 low cells. This suggests that SP LCK-CRE/TSC1ΔT mature SP thymocytes do not accumulate in the thymus. In contrast to young mice, mature SP cells were found to accumulate in great numbers in the thymi of aged (older than 18 months) LCK-CRE/TSC1ΔT mice (Fig. 3.8 C). Although this data might support a partial defect in homing properties, further investigation is still needed.

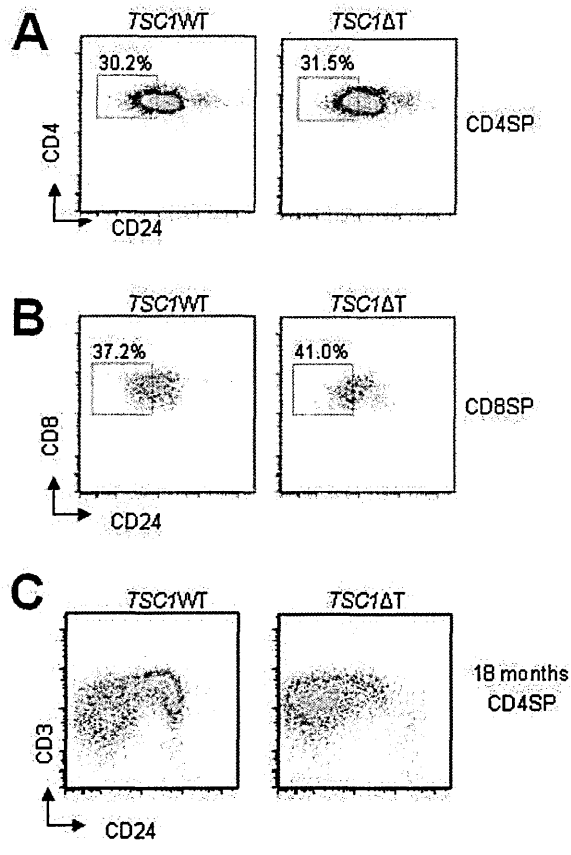

Fig 3.8 CD24 negative mature CD4SP and CD8SP cells are not enriched in TSC1ΔT LCK-CRE mice. A,B) TSC1WT and TSC1ΔT LCK-CRE mice (n=3) were sacrificed at 8 weeks of age. Thymi were recovered, reduced to single cell suspension and individually analyzed by flow cytometry after staining with CD4, CD8, CD24 and TCRβ Ab. A, B) Representative dot plots of CD4SP (A) and CD8SP (B), $TCR\beta^{high}$ cells. CD24 expression is shown on $TCR\beta^{high}$/CD4 or $TCR\beta^{high}$/CD8 SP thymocytes. Data is representative of 3 experiments. C) TSC1WT and TSC1ΔT LCK-CRE mice (n=3) were sacrificed at 18 months of age. Thymi were recovered, reduced to single cell suspension and individually analyzed by flow cytometry after staining with CD4, CD8, CD3, CD24 and TCRβ Ab. C) Representative dot plots of CD4SP $TCR\beta^{high}$ cells. CD24 expression is shown on $TCR\beta^{high}$/CD3 thymocytes.

Of note, the CD4:CD8 ratio was higher in the LCK-CRE/TSC1ΔT compared to the LCK-CRE/TSC1WT mice both in the thymus and in peripheral lymphoid organs (Fig. 3.9), suggesting that TSC1 might contribute to different extents to CD4 and CD8 subsets development.

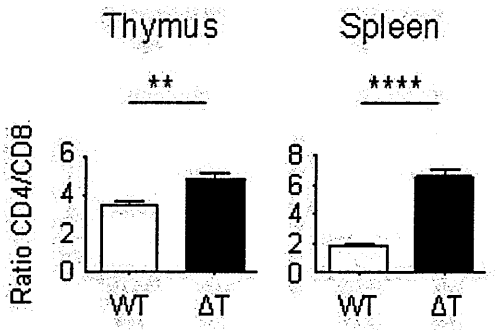

Fig 3.9 Defective T cell development in the absence of TSC1 in LCK-CRE TSC1f/f mice. TSC1WT and TSC1ΔT LCK-CRE mice were sacrificed at 8 weeks of age. The ratio between CD4 and CD8 positive cells is shown (±SE). Statistical significance was evaluated with two-tailed t-Test.

We also bred TSC1 floxed mice with mice expressing CRE under the CD4+ promoter. These mice started to express the CRE recombinase during the DP phase of differentiation [292]. In these mice no difference was detected in the percentages and number of DN, DP, CD4SP or CD8SP cells in thymi of CD4-CRE/TSC1ΔT or CD4-CRE/TSC1WT animals (Fig. 3.10 A, C), while a decrease in the percentage of CD4+ and CD8+ T cells in the peripheral organs of the CD4-CRE/TSC1ΔT mice compared to the CD4-CRE/TSC1WT ones was still detectable (Fig. 3.10 B, D). These results suggest that the timing of silencing of the TSC1 pathway is quite important for consequences on the thymus development and can explain the discrepancy between our data on the LCK-CRE TSC1ΔT mice and the Frap KO mice [103].

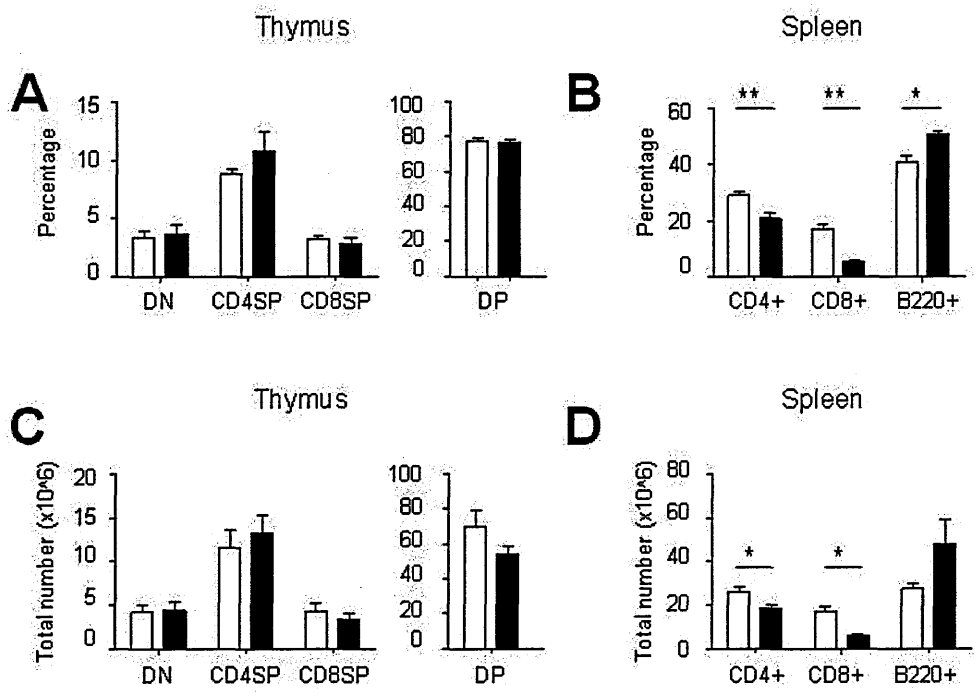

Fig 3.10 TSC1 deletion at the DP stage leads to peripheral lymphopenia in CD4-CRE TSC1f/f mice. TSC1WT and TSC1ΔT CD4-CRE mice were sacrificed at 8 weeks of age. Thymi (A, C) and spleen (B, D) from individual mice (n=7) were recovered, reduced to single cell suspension and analyzed by flow cytometry after staining with anti-CD4, anti-CD8 and anti-B220 Ab. The frequency (±SE) of thymocyte (A) and splenocyte (B) subsets, identified by electronic gating, is depicted. FACS-generated percentages were multiplied by viable counts to define absolute thymocyte and lymphocyte cell numbers (±SE), respectively depicted in C and D. Statistical significance was evaluated with one-tail ANOVA test with Dunns post test.

By further analysis of the peripheral T cells, the representation of CD4+, CD25+, Foxp3+ Treg cells was evaluated (Fig. 3.11 A) in LCK-CRE/TSC1 mice. No difference was observed between LCK-CRE/TSC1ΔT and LCK-CRE/TSC1WT mice. Instead CD44 high cells were increased in LCK-CRE/TSC1ΔT mice compared to their WT counterpart, probably this increase is a response to the peripheral lymphopenia that was detected (Fig. 3.11 B). From these results we can conclude that TSC1 is important for appropriate T cell development, and in particular for the passage from DN to DP cells.

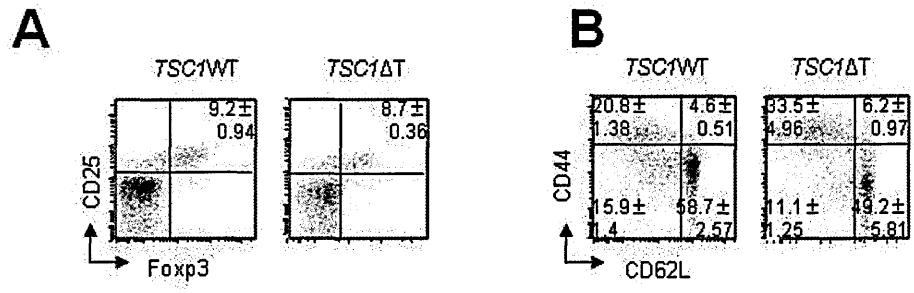

Fig 3.11 TSC1 deletion in LCK-CRE mice leads to an unbalance in memory vs effector cell populations. TSC1WT and TSC1ΔT LCK-CRE mice were sacrificed at 8 weeks of age. Spleen were recovered and analyzed by flow cytometry after staining with anti-CD4, anti-CD8, anti-CD44 and anti-CD62L or anti-CD25 (A, B) and then permeabilized and further stained with anti-Foxp3 Ab (A). Representative dot plots depict viable, CD4+ events.

This transition is strictly regulated by rearrangement and signalling of the TCRβ chain that together with the preTα chain forms the pre-TCR. For this reason TCR levels on developing thymocytes were evaluated by FACS analysis, both at the extracellular and the intracellular level. The majority of DN and the DP, CD4SP and CD8SP cells showed comparable expression of the TCR in LCK-CRE/TSC1ΔT and LCK-CRE/TSC1WT cells. In CD8SP cells the LCK-CRE/TSC1ΔT mice showed an increased population of TCR- cells (Fig. 3.12 A). We wondered whether this could be due to a faster progression of DN4 cells to iSP cells. Thus iSP cells were identified as CD8+ CD24- CD5+ cells. These cells are increased in percentage in LCK-CRE/TSC1ΔT thymi (Fig. 3.12 B). These cells do not express detectable TCR surface levels, but they express comparable intracellular TCR levels in LCK-CRE/TSC1ΔT and LCK-CRE/TSC1WT animals. Then the CD8SP population was re-analyzed excluding this iSP cells and in this new analysis LCK-CRE/TSC1ΔT and LCK-CRE/TSC1WT mice showed a similar surface TCR staining pattern (Fig. 3.12 C). These results indicate that the partial block in T cell development that was observed is probably due to events independent on the TCR rearrangement and signalling.

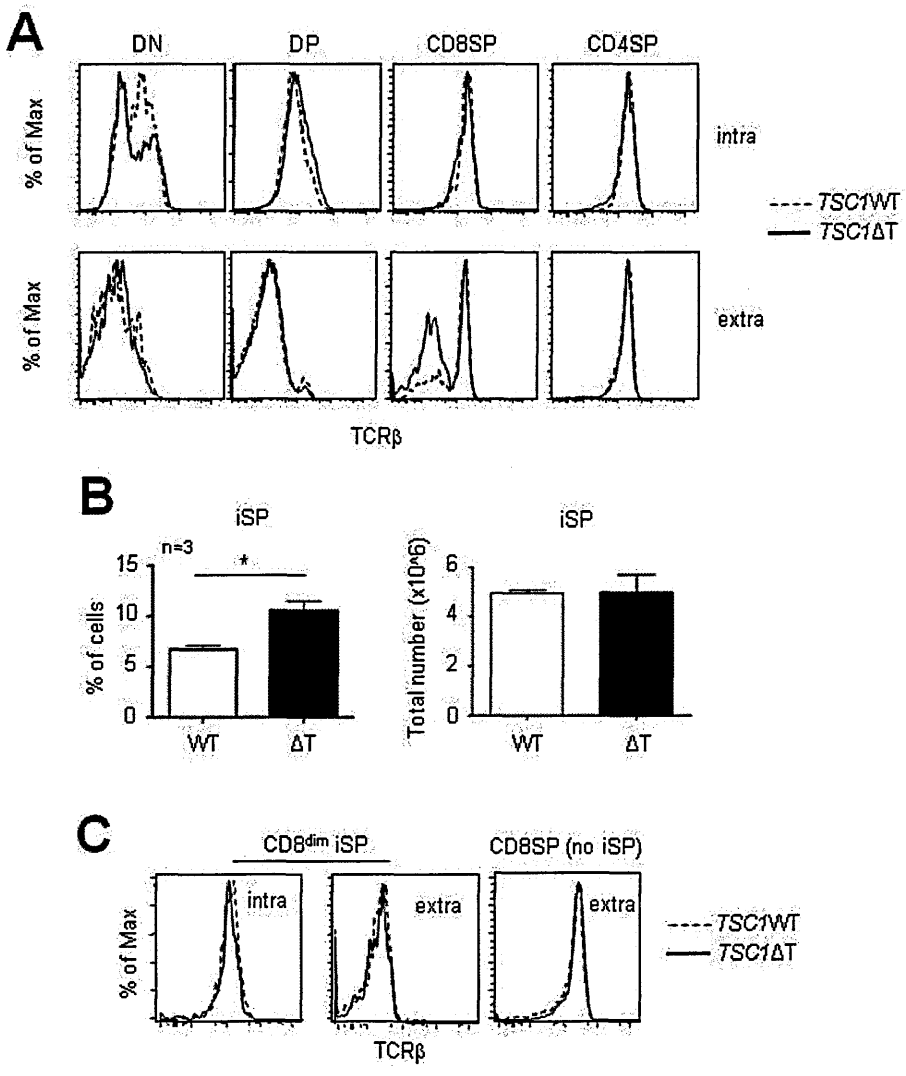

Fig 3.12 Proper TCR expression and faster DN to iSP transition in TSC1ΔT LCK-CRE mice. TSC1WT and TSC1ΔT LCK-CRE mice were sacrificed at 8 weeks of age. Thymocytes were analyzed for surface or intracellular TCRβ chain expression after staining with anti-CD4 and anti-CD8 Ab. A) Relative TCRβ levels are depicted on gated subpopulations. B-C) Cells were stained with anti-CD4, anti-CD8, anti-CD5 and anti-CD24/HSA Ab to identify iSP cells. The frequency and number (±SE) of iSP thymocytes within viable thymocytes is shown (B). In C, relative TCRβ levels of $CD8^{dim}$ iSP or CD8SP without iSP cells are depicted. In B statistical significance was evaluated with two-tailed t-Test.

3.3.3 TSC1ΔT thymocytes show a proliferative advantage.

Signalling from the pre-TCR induces several rounds of proliferation at the DN level and allows for the differentiation of these cells. We wondered whether the decreased

number of thymocytes in the LCK-CRE/TSC1ΔT mice could be due to a defect in the proliferative phase of development. Thus thymocyte proliferation was measured with two different assays. BrdU incorporation was analysed in the DN and DP cells. Cells were labelled with BrdU by injecting the mice 40 min prior to the sacrifice and analysed *ex vivo*. While there was no difference in BrdU incorporation in DN cells from LCK-CRE/TSC1ΔT and LCK-CRE/TSC1WT mice, there was an increase in BrdU incorporating cells in DP thymocytes from LCK-CRE/TSC1ΔT mice (Fig. 3.13 A). This was confirmed by Ki67 staining. Again there was no difference in Ki67 intracellular staining in DN cells, but iSP, DP, CD4SP and CD8SP cells from LCK-CRE/TSC1ΔT animals showed an increase in the percentage of positive cells compared to the WT littermates (Fig. 3.13 B). These results could possibly be explained by the fact that LCK-CRE/TSC1ΔT cells show hyperactivation of mTORC1 that could lead to increased cell size and an increase in cell proliferation.

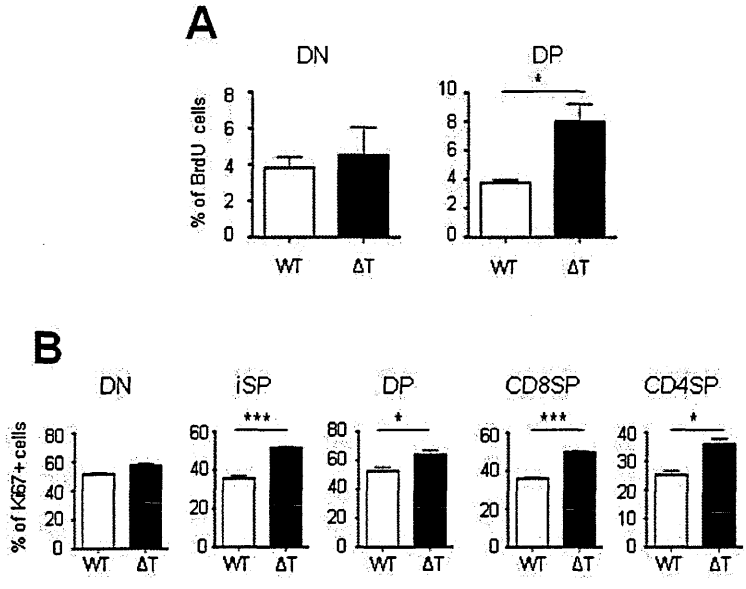

Fig 3.13 TSC1 deficient thymocytes reveal a proliferative advantage in LCK-CRE TSC1ΔT mice. A, B) TSC1WT and TSC1ΔT LCK-CRE mice were sacrified at 8 weeks of age. Thymocytes from individual mice (n=3) were surface stained with anti-CD4, anti-CD8, anti-CD5 and anti-CD24/HAS Ab to identify iSP cells, and then permeabilized to measure BrdU and Ki67. The relative frequency of BrdU (A) and Ki67+ (B) positive cells

within the gated thymocyte subsets is depicted (±SE). Statistical significance was evaluated with two-tailed t-Test.

3.3.4 TSC1ΔT thymocytes reveal an increase in cell death.

Another important mechanism that regulates thymic development and maturation is cell death. So we investigated whether the decreased number of cells we detected could be due to an increase in apoptosis of the LCK-CRE/TSC1ΔT cells. Cell death in the thymus was analysed by Annexin V and Topro-3 staining. A clear increase in the percentage of Annexin V/Topro-3+ cells *ex vivo* was evident in all the populations of LCK-CRE/TSC1ΔT thymocyte with the exception of DN cells (Fig. 3.14 A, B). Thymocyte were also kept in culture for 14 and 24 h. Beside DN cells the LCK-CRE/TSC1ΔT DP, CD4SP and CD8SP populations had a higher tendency to undergo apoptosis also when kept in culture (Fig. 3.14 C).

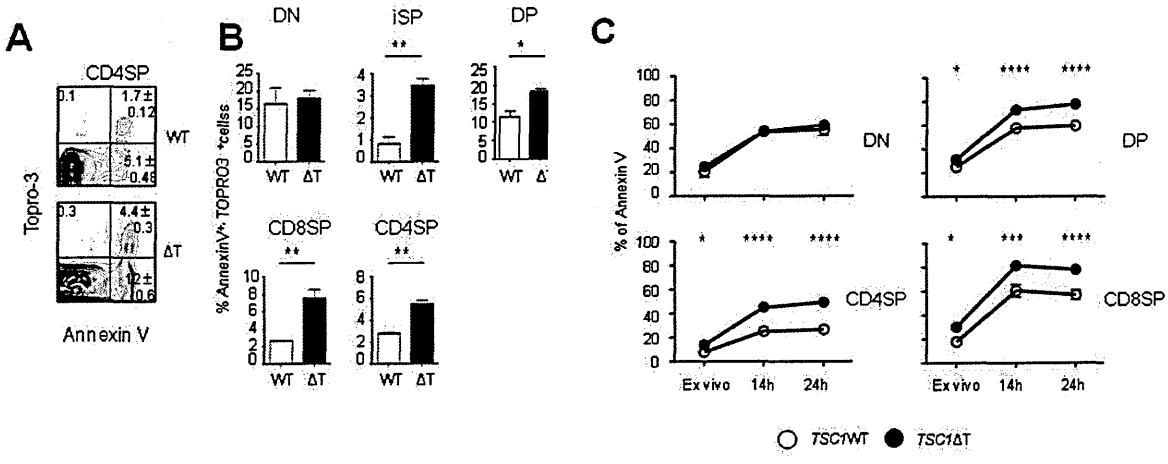

Fig 3.14 TSC1 deficient thymocytes reveal a higher apoptotic rate *ex vivo* and *in vitro* in LCK-CRE TSC1ΔT mice. A, B) Thymocyte subsets were identified by flow cytometry after staining with anti-CD4, anti-CD8, anti-CD5 and anti-CD24/HAS Ab. The presence of dying cells was determined by TOPRO-3 and Annexin V staining. Dot plots from representative CD4SP cells (A). Panel B depicts quantification of cumulative frequencies (±SE) of TOPRO-3+ and AnnexinV+ cells in 11 independently analyzed mice. C) Unfractionated thymocytes were analyzed *ex vivo* and after 14 and 24 hours of culture in complete medium. Thymocyte subsets were identified by flow cytometry after staining

with anti-CD4 and anti-CD8 and the frequency of dying cells was determined by Annexin V staining. One representative experiment of 11 is depicted. In B statistical significance was evaluated with two-tailed t-Test, in C with two-way ANOVA test with Bonferroni post test.

We further investigated which could be the molecular mechanism underlying this increased cell death. Akt was shown before to be less phosphorylated in the LCK-CRE/TSC1ΔT cells. Akt is known to play a key role in cellular processes such as acceleration of cell cycling and suppression of apoptosis (reviewed by Manning B. D. and Cantley L. C. [293]). Some of the Akt targets involved in cell proliferation and cell death were analysed. By western blot analysis the levels of p53, BclXL or Bcl2A1 and by RT PCR the Bax/BclXL ratio were evaluated. These levels were similar in LCK-CRE/TSC1ΔT and LCK-CRE/TSC1WT cells (Fig. 3.15 A, B). mRNA expression of other three genes involved in cell survival was analyzed. Thymocytes from LCK-CRE/TSC1ΔT mice showed an increase in the mRNA levels of the pro-apoptotic gene Trail and decreased levels of the pro-survival gene Cyclin D1 compared to cells from LCK-CRE/TSC1WT mice (Fig. 3.15 C).

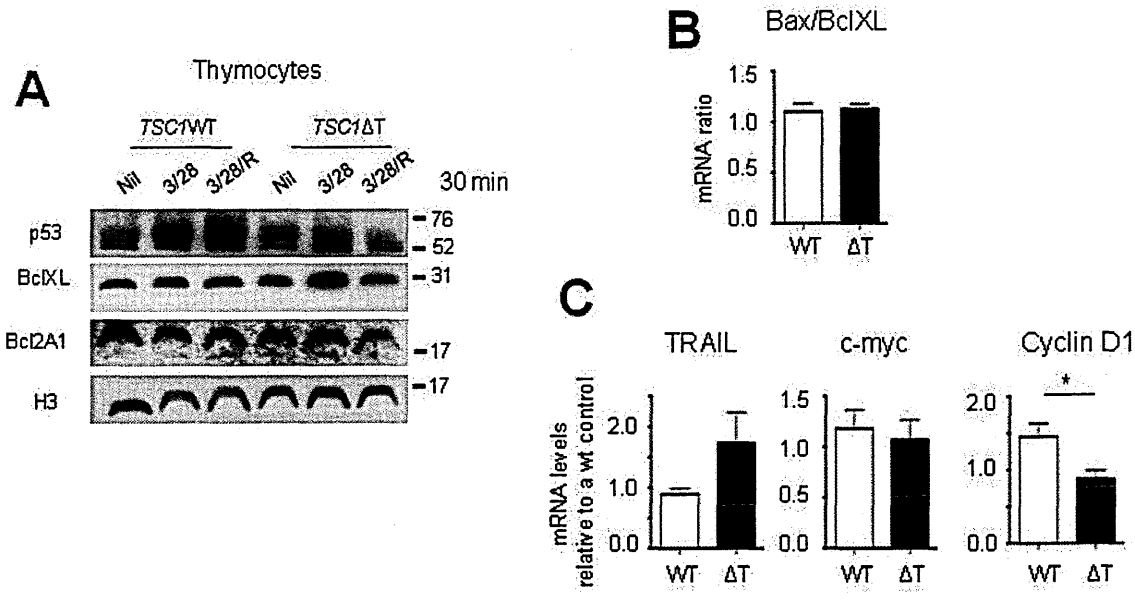

Fig 3.15 Analysis of intrinsic and extrinsic death pathways in TSC1WT and TSC1ΔT cells from LCK-CRE mice. A, Unfractionated thymocytes were left untreated (Nil) or stimulated with anti-CD3 and anti-CD28 Ab for 30 min in the absence or in the presence of Rapa (100 nM). P53, BclXL and Bcl2A1 levels were assessed by western blot analysis of

total cell extracts. B, C) RNA was derived from TSC1WT and TSC1ΔT thymocytes from individual mice (n=5) and analyzed by PCR (B) or quantitative PCR (C). The Bax/BclXL ratio (±SE) is shown (B). In C mRNAs were first normalized to the GAPDH housekeeping gene, and then expressed relatively to TSC1WT cells (±SE). Statistical significance was evaluated with two-tailed t-Test.

3.3.5 TSC1ΔT mature peripheral T cells show a proliferative advantage.

To investigate whether also mature T cells showed an increase in cell proliferation similar to what was observed in the thymus, splenocytes were labelled with CFSE and kept in culture with IL7 and CD3/CD28. In both the conditions of culture LCK-CRE/TSC1ΔT CD4+ and CD8+ cells proliferated more than T cells from LCK-CRE/TSC1WT mice (Fig. 3.16 A, C, D). The proliferative advantage of LCK-CRE/TSC1ΔT cells compared to LCK-CRE/TSC1WT ones observed when cells were kept in IL-7 did not seem to be due to altered expression of IL-7 receptor as CD127 surface levels were comparable between LCK-CRE/TSC1ΔT and LCK-CRE/TSC1WT CD4+ and CD8+ cells (Fig. 3.16 B). In order to address if this proliferative advantage could be due to increased mTORC1 activity we treated the cells with Rapa and we could observe that the proliferative advantage of the LCK-CRE/TSC1ΔT cells was abrogated by treatment with the drug (Fig. 3.16 C, D). This confirms that mTORC1 may be involved in the proliferative advantage of the LCK-CRE/TSC1ΔT cells. Treatment of LCK-CRE/TSC1ΔT cultures with Rapa showed that mTORC1 is important not only for the increased proliferation of LCK-CRE/TSC1ΔT cells, but also for their increased cell size. Treatment with Rapa, in fact, decreased the size both of CD4+ and CD8+ T lymphocytes (Fig. 3.16 E).

In spite of the increased proliferation we observed in the CD4+ and CD8+ compartment, LCK-CRE/TSC1ΔT mice were lymphopenic up to 30 weeks of age. In fact a lower frequency of CD4+ and CD8+ T cells, but not B220+ B cells, was detected in these mice for the whole period (Fig. 3.16 F). These data could suggest that lack of TSC1 may perturb the homeostatic control of mature T cells or may be the result of lower production of T cells from the thymus.

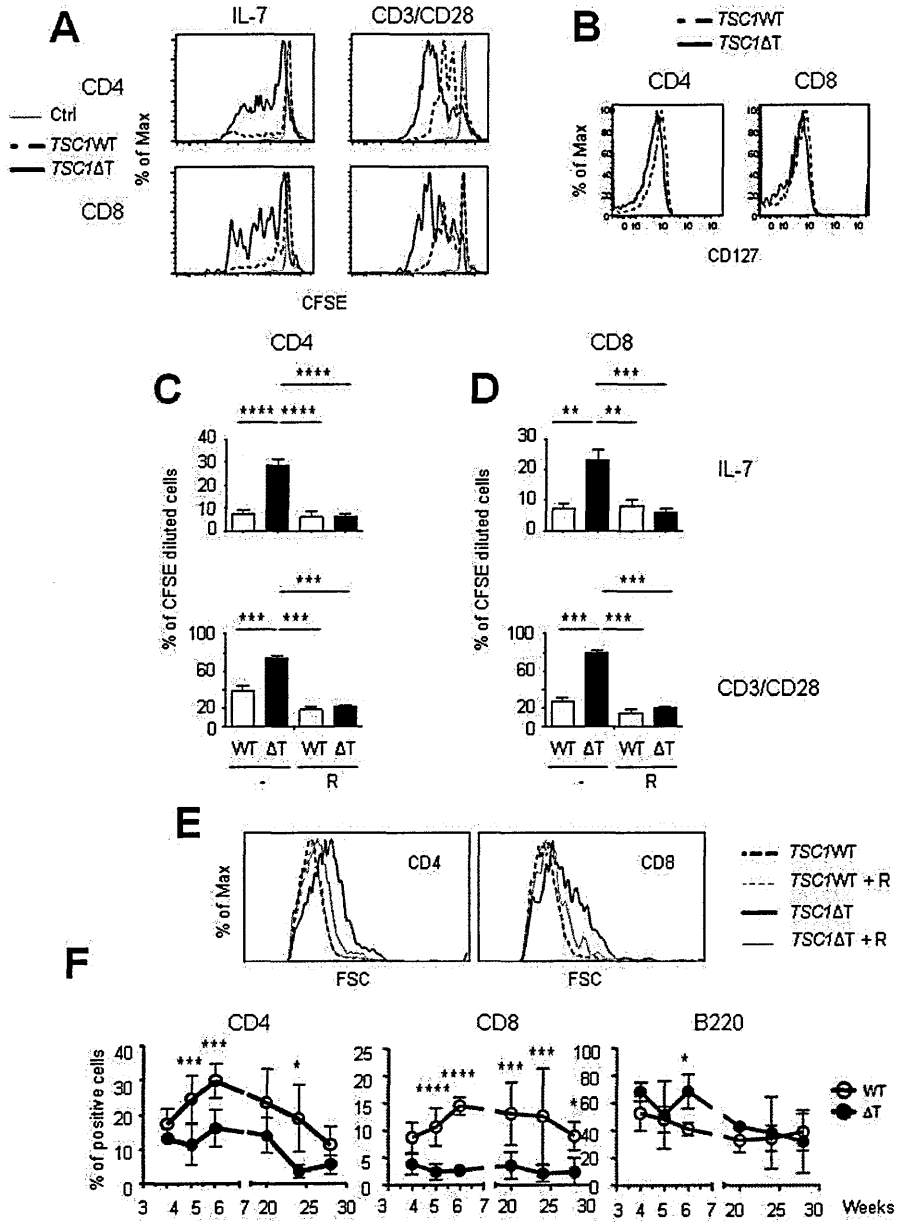

Fig 3.16 TSC1 deficient T cells reveal a proliferative advantage in TSC1ΔT LCK-CRE mice. A, C, D) TSC1WT and TSC1ΔT splenocytes were labelled with CFSE and cultured in complete medium in the absence (Ctrl) or in the presence of recombinant mouse IL-7 (0.5 ng/ml) or immobilized anti-CD3/CD28 mAb (0.01 ug/ml and 5 ug/ml) for 3 days. Relative CFSE content on gated viable CD4+ or CD8+ events is depicted in A. Rapamycin (100 nM) was provided to parallel cultures. Quantification of the frequency (±SE) of T cells with a CFSE-diluted profile is depicted in C for CD4+ T cells and in D for CD8+ T cells. B) Flow cytometry analysis of expression levels of CD127 on CD4+ and CD8+ lymphocytes subsets identified by electronic gate after staining with anti-CD4, anti-CD8 and anti-CD127 Ab (dotted line TSC1WT cells; solid line TSC1ΔT cells).
E) Flow cytometry analysis of lymphocytes subsets identified by electronic gate after staining with anti-CD4 and anti-CD8 Ab (dark dotted line TSC1WT cells –R; light dotted line TSC1WT cells +R; dark solid line TSC1ΔT cells –R; light solid line TSC1ΔT +R). Cells were kept in culture with IL-7 (0.5 ng/ml) for 2 days with or without Rapa (100 nM).
F) TSC1WT and TSC1ΔT mice were bled at the indicated times (4, 5, 6, 20, 24 and 28 weeks). The relative frequency (±SE) of CD4+, CD8+ and B220+ cells from TSC1WT

(n=7) and TSC1ΔT (n=7) mice is shown. In B and C statistical significance was evaluated with one-tail ANOVA test with Dunns post test, in E with two-way ANOVA test with Bonferroni post test.

3.3.6 TSC1ΔT mature T cells show an increase in apoptosis levels and deregulated FoxO signalling.

When mature T cells were cultured in IL-7 or CD3/CD28, LCK-CRE/TSC1ΔT cultures contained a higher proportion of dying cells when compared to the LCK-CRE/TSC1WT cultures. This increased cell death was basically insensitive to Rapa treatment (Fig. 3.17 A-E).

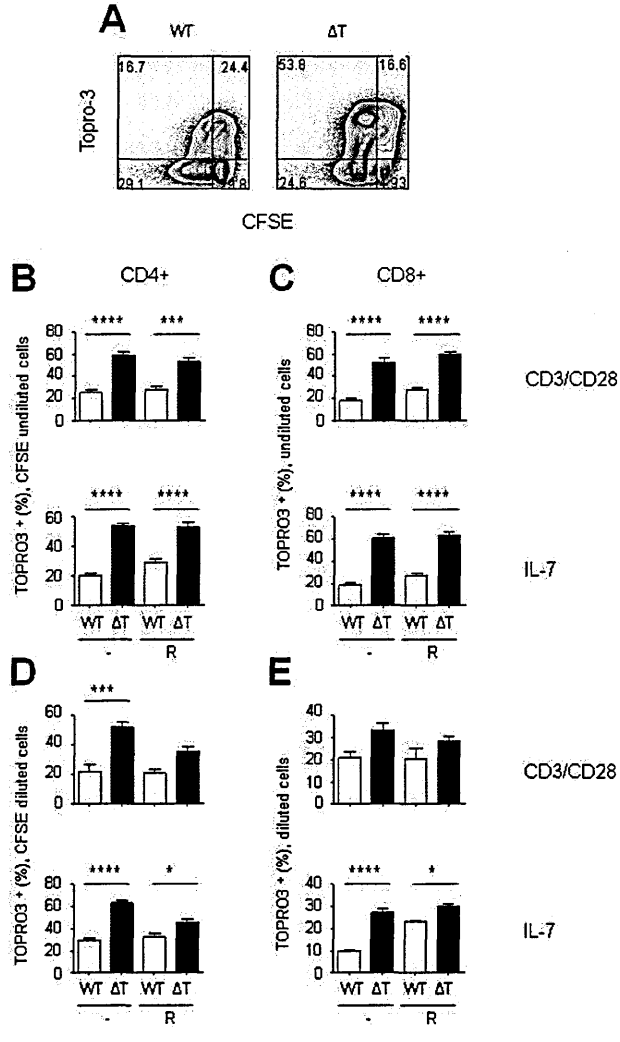

Fig 3.17 Increased cell death in TSC1ΔT LCK-CRE mature T cells is not Rapa-dependent. TSC1WT and TSC1ΔT splenocytes were labelled with CFSE and cultured in complete medium in the absence (Ctrl) or in the presence of recombinant mouse IL-7 (0.5 ng/ml) (B-E) or immobilized anti-CD3/CD28 mAb (0.01 ug/ml and 5 ug/ml) (A-E) for 3 days. Lymphocytes subsets were identified by flow cytometry after staining with anti-CD4 and anti-CD8 Ab. The presence of dying cells was determined by TOPRO-3 staining. Dot

plots from representative CD4+ cells stimulated with immobilized anti-CD3/CD28 mAb (0.01 ug/ml and 5 ug/ml) for 3 days (A). Relative CFSE content on gated viable CD4+ or CD8+ events is depicted in B-E. Rapamycin (100 nM) was provided to parallel cultures. Quantification of the frequency (±SE) of CD4+ T cells with a CFSE-diluted profile is depicted in B and D for CD4+ T cells and in C and E for CD8+ T cells. Panels B-E depict quantification of cumulative frequencies (±SE) of TOPRO-3+ cells in 7 independently analyzed mice. In B and C quantification of CFSE undiluted cells, in D and E quantification of CFSE diluted cells. Statistical significance was evaluated with one-tail ANOVA test with Dunns post test.

To further investigate a possible molecular mechanism responsible for this increased cell death we focused on the Akt pathway. Akt controls an important family of transcription factors, the FoxOs. When FoxOs are phosphorylated they are retained in the cytoplasm and thus inhibited [294], when they are in the unphosphorylated form, they can enter the nucleus and direct the transcription of specific genes that control proliferation as well as cell survival. When LCK-CRE/TSC1WT CD4+ T cells were stimulated with CD3/CD28 an increase in the phosphorylation of FoxO1 and FoxO3a was detected (Fig. 3.18 A). This increase in phosphorylation was lost in LCK-CRE/TSC1ΔT cells. Also typical FoxO target genes were differentially expressed in LCK-CRE/TSC1ΔT and LCK-CRE/TSC1WT cells. In accordance with reduced phosphorylation of FoxO in LCK-CRE/TSC1ΔT cells, TRAIL was expressed at higher levels in LCK-CRE/TSC1ΔT T cells compared to LCK-CRE/TSC1WT cells both at steady state and following TCR/CD28 stimulation (Fig. 3.18 B), while there was no difference in the mRNA levels of other FoxO target genes such as FasL and Bim and of the TRAIL-receptor (Fig. 3.18 B, C).

FoxO family members recently have been shown to function also as tumour suppressors *in vivo* [295] and the members of this family have been demonstrated to be able to inhibit expression of c-Myc. Another indication that FoxO may be constitutively active in LCK-CRE/TSC1ΔT cells was that both Cyclin D1 and c-Myc levels were lower in LCK-CRE/TSC1ΔT cells (Fig. 3.18 D). The lower levels of c-Myc in LCK-CRE/TSC1ΔT cells could be explained by increased levels of mir-145 (Fig. 3.18 E), a miRNA that silence c-Myc and that has been shown to be controlled by FoxOs proteins [296]. We thus think that FoxO regulation downstream of Akt could render the LCK-CRE/TSC1ΔT cells more prone to cell death.

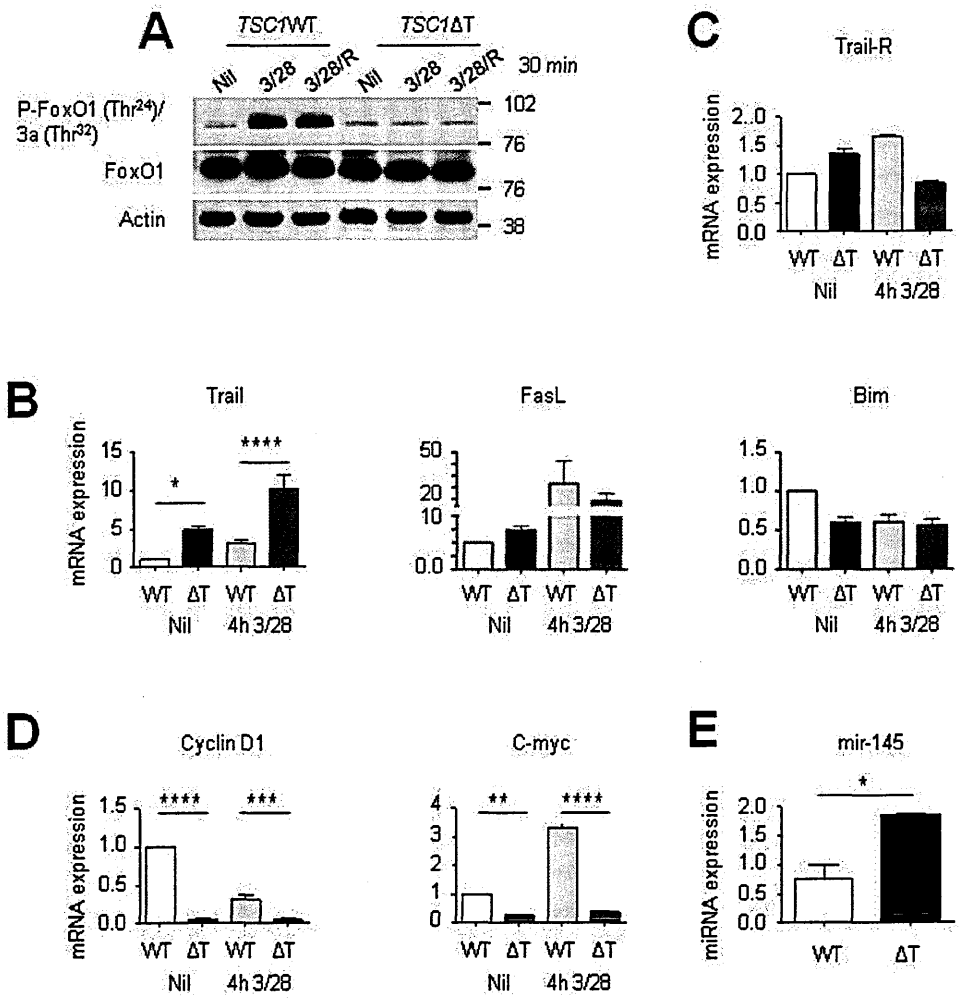

Fig 3.18 TSC1 deficiency favours FoxO-dependent signalling in TSC1ΔT LCK-CRE mice. A) CD4+ cells were purified from the lymphonodes of TSC1WT and TSC1ΔT mice (n=3) and left untreated (Nil) or stimulated with anti-CD3 and anti-CD28 mAb for 30 min

in the absence or in the presence of Rapa (100 nM). Phosphorylation of FoxO1 (Thr24)/3a (Thr32) was assessed by western blot analysis. Actin is depicted as loading control, and relative migration of molecular weight markers is depicted. B-D) CD4+ cells were purified from the lymphonodes of TSC1WT and TSC1ΔT mice (n=3) and left untreated (Nil) or stimulated with anti-CD3 and anti-CD28 mAb for 4 hours. mRNA was derived from naïve cells and cells stimulated with CD3/CD28 for 4 hours. mRNA levels for the indicated genes was quantified by quantitative PCR. Results depict the mean ± SE of 3-7 independent experiments. E) CD4+ cells were purified from the lymphonodes of TSC1WT and TSC1ΔT mice (n=3). Analysis of mir-145 expression by real-time PCR. The levels (±SE) of mir-145 were normalized on the snoRNA-202. In C and D statistical significance was evaluated with one-tail ANOVA test with Dunns post test, in E with two-tailed t-Test.

3.3.7 TRAIL induced cell death of TSC1ΔT T cells.

Having found that TRAIL is expressed to higher levels in LCK-CRE/TSC1ΔT T cells, we investigated whether mature T cells would show an increased propensity to undergo cell death, and whether this could be inhibited by the TRAILR2/Fc Fusion protein [297]. Splenocytes from LCK-CRE/TSC1WT and LCK-CRE/TSC1ΔT mice were cultured overnight in the absence or in the presence of TRAILR2/Fc Fusion protein, and cell death was measured by Annexin V and TOPRO-3 staining. Results depicted in Fig. 3.19 A and B indicate that LCK-CRE/TSC1ΔT T cell cultures contained a higher frequency of AnnexinV+, TOPRO-3+ cells both *ex vivo* and after an overnight culture. The addition of the TRAILR2/Fc chimeric protein completely abrogated differences among LCK-CRE/TSC1WT and LCK-CRE/TSC1ΔT cultures. Similar results were obtained by looking at CD3/CD28 and IL-7 driven cultures of CFSE-labelled LCK-CRE/TSC1WT and LCK-CRE/TSC1ΔT splenocytes. In the case of both undividing and dividing (Fig. 3.19 C, D) CD4+ and CD8+ T cells, LCK-CRE/TSC1ΔT T cells revealed a higher propensity to die when compared to LCK-CRE/TSC1WT cells, but differences were abrogated in the presence of TRAILR2/Fc chimeric protein (Fig 3.19 C, D).

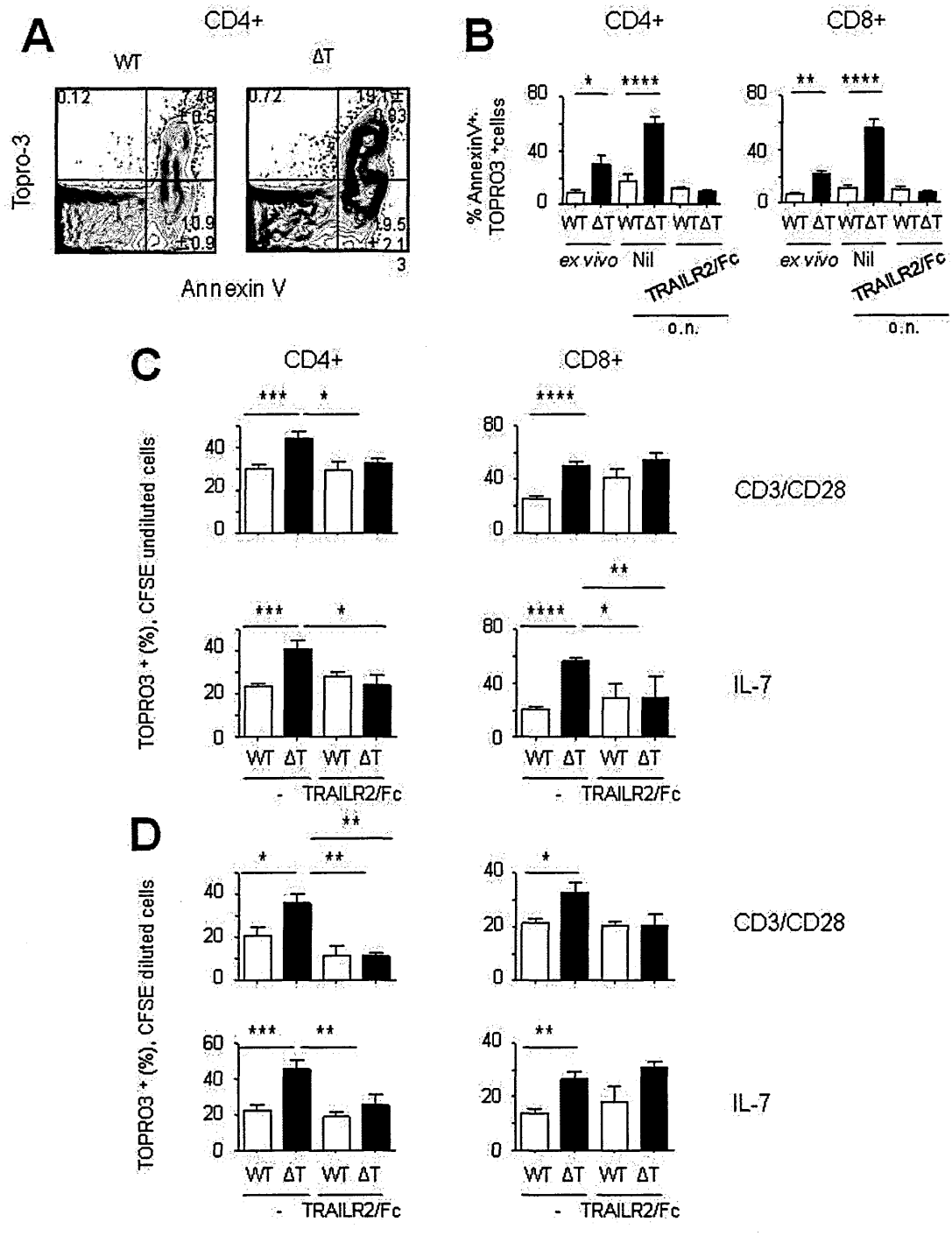

Fig 3.19 Increased cell death in TSC1ΔT LCK-CRE mature T cells is dependent on TRAIL. A, B) Lymphocytes subsets were identified by flow cytometry after staining with anti-CD4 and anti-CD8 Ab. The presence of dying cells was determined by TOPRO-3 and Annexin V staining. Dot plots from representative CD4+ cells analysed *ex vivo* (A). Panel B depict quantification of cumulative frequencies (±SE) of TOPRO-3+ and AnnexinV+ cells in 5 independently analyzed mice gated on CD4+ and CD8+ cells. Cells were analysed either *ex vivo* or were kept in medium o.n. with or without TRAILR2/Fc.
C, D) TSC1WT and TSC1ΔT splenocytes were labelled with CFSE and cultured in complete medium in the absence (Ctrl) or in the presence of recombinant mouse IL-7 (0.5 ng/ml) or immobilized anti-CD3/CD28 mAb (0.01 ug/ml and 5 ug/ml) for 3 days. Lymphocytes subsets were identified by flow cytometry after staining with anti-CD4 and

anti-CD8 Ab. The presence of dying cells was determined by TOPRO-3 staining. Relative CFSE content on gated viable CD4+ or CD8+ events is depicted. TRAILR2/Fc (50 ng/ml) was provided to parallel cultures. Quantification of the frequency (±SE) of CD4+ T cells with a CFSE-diluted profile is depicted for CD4 and for CD8+ T cells. Panels C and D depict quantification of cumulative frequencies (±SE) of TOPRO-3+ cells in 5 independently analyzed mice. In C quantification of CFSE undiluted cells, in D quantification of CFSE diluted cells. Statistical significance was evaluated with one-tail ANOVA test with Dunns post test.

Of note the TRAILR2/Fc chimeric protein did not have any effect on the proliferative advantage of LCK-CRE/TSC1ΔT T cells in response to IL-7 and CD3/CD28 (Fig. 3.20), consistent with the notion that this is mostly dependent on mTORC1 hyperactivation. Thus, unchecked FoxOs activity reduces C-Myc and Cyclin D1 expression, favours TRAIL expression and TRAIL-dependent cell death, accounting for decreased survival of LCK-CRE/TSC1ΔT T cells.

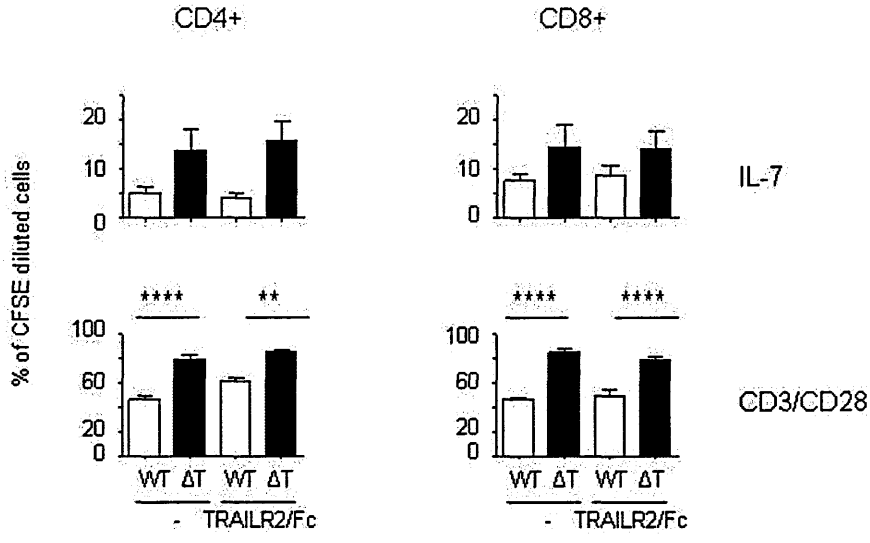

Fig 3.20 Increased proliferation in TSC1ΔT LCK-CRE T cells is insensitive to TRAIL inhibition. TSC1WT and TSC1ΔT splenocytes were labelled with CFSE and cultured in complete medium in the absence (Ctrl) or in the presence of recombinant mouse IL-7 (0.5 ng/ml) or immobilized anti-CD3/CD28 mAb (0.01 ug/ml and 5 ug/ml) for 3 days. Lymphocytes subsets were identified by flow cytometry after staining with anti-CD4 and anti-CD8 Ab. Relative CFSE content on gated viable CD4+ or CD8+ events is depicted. TRAILR2/Fc (50 ng/ml) was provided to parallel cultures. Quantification of the frequency (±SE) of T cells with a CFSE-diluted profile is depicted for CD4+ and for CD8+ T cells. Statistical significance was evaluated with one-tail ANOVA test with Dunns post test.

3.4 Discussion

In this part of the thesis are reported the data on the role of TSC1 in thymocyte development and peripheral homeostasis. To address the role of TSC1 in T cell development and function, we adopted a TSC fl/fl strategy and crossed mice with Lck- and CD4+-CRE deleters, thus silencing TSC1 during thymic development. We adopted a conditional KO strategy due to the lethal phenotype of complete KO animals. Inactivation of both TSC1 or TSC2 genes, in fact, was previously shown to lead to death of embryos in midembryogenesis because of cardiac malformation and liver hypoplasia [298, 299]. Mice heterozygous for the TSC1 or TSC2 genes develop renal and liver tumours.

Silencing of the TSC1 gene early on during thymocyte development (DN2/DN3), using the Lck-CRE deleters, hindered the generation of normal numbers of DP, CD4SP and CD8SP thymocytes and of peripheral CD4+ and CD8+ T cells, and the homeostasis of mature T cells. We found that TSC1 expression was almost completely lost in thymocytes, and absent in peripheral mature T cells. Loss of TSC1 was also paralleled by loss of TSC2, which is consistent with the idea that TSC1 is needed for TSC2 stability and for TSC1/TSC2 complex formation.

Given the fact that TSC is controlled by the PI3K-PDK-Akt pathway, and the notion that T cell conditional KO for proteins belonging to this pathway show problem in thymocyte development, the finding that TSC deletion might impact on thymocyte development was not unexpected. For example, deletion of a single allele of PDK1, the protein known to control phosphorylation, and thus activation, of Akt on thr308, prevents the proliferative expansion of α-β T cells in the thymus. The decreased number of thymocytes in these mice is due to the increased rate of apoptosis of PDK1 haplo-insufficient thymocytes [300]. Kelly et al. also demonstrated the fundamental role of PDK1 in mediating Notch induced T cell development [290].

The first differences between LCK-CRE/TSC1ΔT and LCK-CRE/TSC1WT animals can be detected at the DP stage. This does not appear to be due to defective TCRβ

chain rearrangement or suboptimal DN proliferation. TCRβ level, Ki67 positivity and BrdU incorporation, in fact, were comparable between LCK-CRE/TSC1ΔT and LCK-CRE/TSC1WT DN thymocytes.

Loss of TSC1 in LCK-CRE/TSC1ΔT mice prompted hyperactivation of mTORC1, an increase of cell size and a higher propensity to proliferate starting at the DP stage, but mostly evident at the SP stage of development. The increase in cell size was quite evident in CD4SP and CD8SP thymocytes and in peripheral CD4+ and CD8+ T cells (Fig. 3.1 E). In the DP population of thymocytes the increase in cell size was less evident. This was in spite deletion of TSC1 floxed gene was complete in DP cells, possibly due to limited S6 phopshorylation at this stage as determined by intracellular pS6 staining (Fig. 3.1 D). S6 phosphorylation is a dynamic process that varies in different thymocytes subpopulations [301]. Hinton et al. [301] showed that DN3 cells that express TCRβ contained phosphorylated S6. Also the great majority of DN4 cells were positive for phospho-S6 staining and expressed a preTCR complex that regulates the proliferation and differentiation of these cells [302]. Following the DN stage of development, thymocytes become DP cells that can go through cell death by neglect, when signalling via TCR is insufficient, or through negative selection when this signalling is too strong. A small percentage of cells that respond to TCR signalling in the right range go through the process of positive selection. This process allows the survival, proliferation and lineage commitment to mature CD4SP and CD8SP T cells. Hinton et al. [301] showed that SP cells and the majority of DP cells expressed low level of phospho-S6. Among DP cells the cells that were positive for S6 phosphorylation also expressed CD69, an activation marker that identifies TCR activated thymocytes [301]. This correlation between CD69 and S6 phosphorylation suggests that activation of the TCR can in turn activate PDK1/mTOR signalling during the process of thymocytes selection. We found that in the absence of in vitro activation, a fraction (about 20%) of DP cells and the majority of SP cells have constitutive phosphorylation of S6 phosphorylation in LCK-CRE/TSC1ΔT mice by

intracellular staining. Thus, in LCK-CRE/TSC1ΔT mice a fraction of DP thymocytes and most of SP cells appear to have constitutive mTORC1 activity in the absence of TCR ligation. This might also account for the increased cell size and augmented proliferation observed in these cells and in mature T cells (both CD4+ and CD8+).

Loss of TSC1/2 also hindered proper Akt phosphorylation. This might be due to defective mTORC2 activity or to a negative feedback loop from mTORC1. The latter was previously described in non-lymphoid cells to occur via a p70S6 Kinase-dependent down-regulation of upstream activators of PI3K, such as the PDGF receptor and IRS-1 [125-127]. The presence of this loop in LCK-CRE/TSC1ΔT T cells is suggested by the finding that TCR mediated signals do not induce proper phosphorylation of Akt in Thr308 and also of MAPK, at least at the time examined during the experimental work performed so far. It is possible that MAPK activity might be suboptimal or recovered at later times, which might explain preserved CD69 upregulation (not shown) and cell proliferation. Another important pathway for thymocyte proliferation, the Rho GTPase pathway, has not been investigated. In T cell progenitors proliferation and differentiation rely on both PDK1 and RhoA-dependent signalling pathways [303-305]. $PI(3,4,5)P_3$ signaling, in fact, has been linked also to signalling pathways mediated by the Rac/Rho family of GTPases, important regulators of thymocyte proliferation. Galandrini et al. [306] demonstrated that mice in which Rho GTPase activity was shut down early on during thymic development showed defects in fetal and adult thymopoiesis. By expressing *C. botulinum* C3 transferase (an inhibitor of Rho biological function) under the control of the p56lck proximal promoter the authors demonstrated that the mice were deficient in $CD44^+CD25^+$ pro-T cells and also in $CD44^-CD25^+$ early pre-T cells. Rho was essential for survival of these cells. By expressing C3 transferase under the control of the CD2 gene, Cleverley et al. [303] demonstrated that thymocytes numbers were reduced by 50- to 100- fold, similarly to what was found by using Lck proximal promoter. The main difference was that while in Lck-C3 mice the reduction in the number of thymocytes was mainly due to defects in cell survival of

thymocyte progenitors, the CD2-C3 transgenic mice showed a pre-T cell differentiation block [303]. It is difficult to speculate what could happen to Rho activation following silencing of TSC1. Rho GTPases can be regulated in a very complex way (reviewed by Cantrell D. A. [307]). It is possible to conceive that cells lacking TSC1 can regulate proliferation through the modulation of Rho activity possibly through feedback loops on proteins of the Rho pathway.

As a result of defective Akt phopshorylation, phosphorylation of FoxO1/3 was deregulated in LCK-CRE/TSC1ΔT mature T cells. FoxOs proteins are differentially regulated during T cell maturation. For instance FoxO3 and FoxO4 are down-regulated during maturation while FoxO1 is up-regulated during CD4+ and CD8+ T cell maturation and in CD4+Foxp3+Treg cells [308]. FoxO proteins are central for inhibiting cell cycling and for increasing apoptosis [294], in fact they act as tumour suppressors *in vivo* [295]. When a T cell is stimulated, the PI3K pathway is activated as well and this leads to Akt phosphorylation and thus inhibition of FoxO1 that is in turn phosphorylated and sequestered to the cytoplasm. In this way cell cycle progression can start [309]. It is quite peculiar that LCK-CRE/TSC1ΔT T cells are able to proliferate even if they cannot exclude FoxO from the nucleus. This could be due to constitutive activity of mTORC1 or other yet to be defined pathways able to overcome the inhibitory effect of nuclear FoxO. This latter possibility alo might account for proper maturation and exit of SP cells in the thymus. Indeed, while Akt defective T cells should fail to properly regulate homing receptors (reviewed by Finlay D. and Cantrell D. [223]), LCK-CRE/TSC1ΔT thymocytes completed their maturation and homed to secondary lymphoid organs. Preliminary experiments show that KLF2, which expression is controlled by FoxO, and some of its targets (CCR7, CD62L and S1P1) are not upregulated in LCK-CRE/TSC1ΔT cells. This is possibly again due to constitutive activation of mTORC1 and accounts for proper thymic egress of SP mature T cells.

Among the FoxO target genes, TRAIL expression was found to be increased in LCK-CRE/TSC1ΔT T lymphocytes when compared to LCK-CRE/TSC1WT controls. This nicely correlated with the higher propensity of the cells to undergo cell death. Accordingly, inhibition of TRAIL with a recombinant mouse TRAIL R2/Fc chimeric protein reduced cell death level in the LCK-CRE/TSC1ΔT cells to levels comparable to those of LCK-CRE/TSC1WT animals.

The expression of other two targets, c-Myc and Cyclin D1, instead, was decreased in LCK-CRE/TSC1ΔT compared to LCK-CRE/TSC1WT cells. FoxOs have been shown to regulate Myc through mir-145 in renal cell carcinoma (RCC) samples [296]. We found that the expression of mir-145 was increased in mature T cells from LCK-CRE/TSC1ΔT mice compared to LCK-CRE/TSC1WT mice. c-Myc, together with Cyclin D1, is a well known target of β-catenin, an important factor implied also in thymic development by different groups. Xu et al. [310] showed that T cell-specific deletion of β-catenin impaired T cell development at the β-selection checkpoint. Mulroy et al. [311] showed that, conversely, overexpression of β-catenin enhances generation of mature thymocytes. We didn't performed experiments aimed at evaluating the expression and activity of β-catenin in the LCK-CRE/TSC1ΔT mice. However by looking at c-Myc and Cyclin D1 levels it is possible to hypothesis that β-catenin in these KO cells is less active than in the WT ones. This could be explained by the effect of TSC1 loss on phosphorylation of Akt. When Akt is less phosphorylated it is less active and thus it does not phosphorylate and inhibit GSK3β activity. Active GSK3β can thus lead to β-catenin degradation. This could be another explanation for the decreased levels of c-Myc reported in the LCK-CRE/TSC1ΔT cells.

From the results reported in this part of the thesis it is clear that the TSC1/TSC2 complex is fundamental in the control of proliferation and cell death in developing

thymocytes and mature T lymphocytes. The LCK-CRE/TSC1ΔT mice however are characterized by a durable lymphopenia. This suggests that the increased cell death dominates over the increased proliferation. This could be due to the fact that in the KO cells we observed active FoxO. As Gan et al. [296] demonstrated, FoxO activity can act as a kind of brake to unrestrained progression of renal tumours by leading to cell cycle arrest and apoptosis of RCC cells. It could be possible that also in our model double inactivation of TSC1 and FoxO could lead to proliferation of leukemic cells and progression of lymphoma in LCK-CRE/TSC1ΔT mice in which hyperactive mTORC1 could lead to unrestrained proliferation not checked by FoxO pro-apoptotic activity.

Further understanding of the crosstalk between TSC/mTOR and FoxO pathways will be relevant in order to design proper therapeutic strategies that address immune-related diseases.

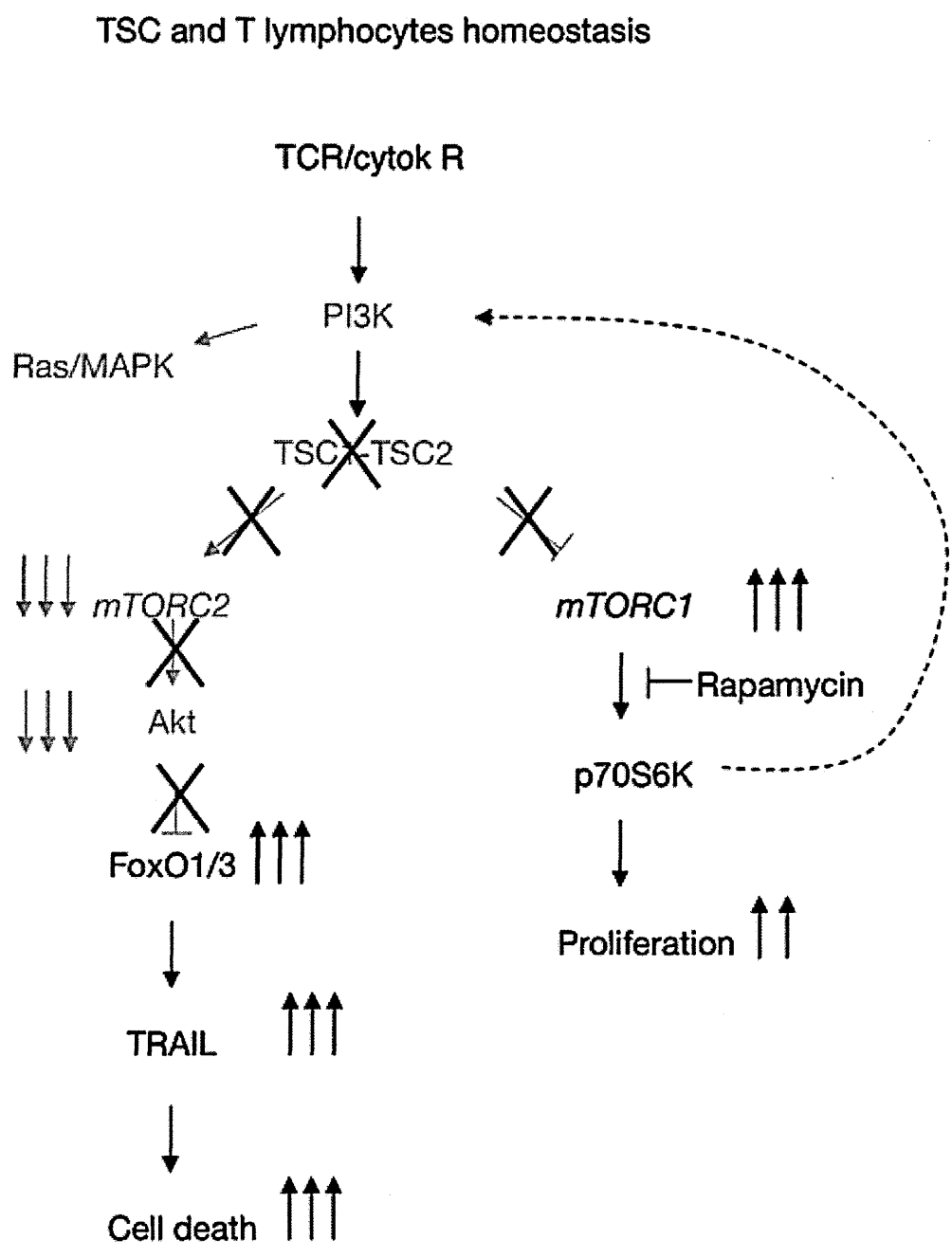

Fig VI. Effects of TSC silencing in T cells signalling. Loss of TSC1 leads to destabilization of TSC2 and lack of the TSC1/TSC2 complex activity. This lead to hyperactivation of mTORC1, increased S6K activity and thus higher cell proliferation. S6K can inhibit Akt activity through the feed-back loop. Akt activity could be further deregulated by loss of mTORC2 activity that could be a result of absent TSC1/TSC2 signalling. Akt down-regulation favours FoxO1/3a activity, increased transcription of its targets and this leads to a higher rate of apoptosis.

4. TSC2 controls proliferation and neurosecretory functions in nerve cells.

4.1 Introduction.

Proliferation and differentiation are interconnected events controlling homeostasis and functions of a numbers of cells besides those belonging to the immune system. Based on results obtained with T cells and developing thymocytes we became interested in evaluating the possible contribution of TSC and mTOR signalling to neural cells proliferation and differentiation. Thereafter will follow an introduction of existing evidences supporting the role of TSC and mTOR in neural functions.

4.1.1 Proliferation and differentiation in the central nervous system.

The adult central nervous system is characterized by the presence of mature non mitotic cells, the neurons. However, Altman and Das [312] described newly born cells also in the mature CNS suggesting that the process of neurogenesis might persist during adult life and not be restricted to the phases of embryonic development. Neural Stem Cells (NSC) are mainly responsible for neurogenesis as capable of proliferation and differentiation.

NSC are particular cells present mainly in two principal areas in the CNS: the subventricular zone (SVZ) (reviewed by Gage F. H. [313]) and the subgranular zone. To be defined as NSC they must possess particular features such as an undifferentiated phenotype, the ability to go through different rounds of proliferation, the competence for self maintenance and self-renewal and the possibility of a broad developmental fate (reviewed by Potten C. S. and Loeffler M. [314]). It is possible to recapitulate *in vitro* the processes of proliferation and differentiation that occur during neurogenesis *in vivo*. In the presence of mitogens such as Epidermal Growth Factor (EGF) and Fibroblast Growth Factor 2 (FGF2), rapidly dividing precursors from the SVZ can be induced to proliferate *in vitro*. These precursors posses most of the features of stem cells reported above [315, 316]. If these mitogens are removed from the culture these progenitors can be induced to

differentiate in astrocytes, oligodendrocytes and neurons, the main cell types of the CNS [317-319]. These processes of proliferation and differentiation can be thus finely tuned by activating different intracellular pathways.

4.1.2 Mouse models of Tuberous Sclerosis Complex

The TSC pathway has been shown to be critical within the nervous system. Patients with mutation in either TSC1 or TSC2 show many neurological abnormalities such as epilepsy [320], neurocognitive dysfunction [321] and disorders such as autism [322]. Conventional KO mice for either TSC1 or TSC2 die before brain development is complete. This renders impossible the study of TSC1 or TSC2 loss in formation of the brain and in cellular processes involving different kinds of neuronal cells. Because of this, several models of conditional KO mice have been generated that specifically silence TSC1 or TSC2 in different brain areas and neuronal populations.

Way et al. [323] used TSC2 floxed mice crossed with hGFAP-CRE transgenic mice in order to silence TSC2 in radial glial progenitor cells in the developing cerebral cortex and hippocampus. The TSC2ΔT mice showed post-natal megalencephaly and possible migration defects and the mice died between 3 and 4 weeks of age. The conditional KO mice presented brain pathology characterized by cortical and hippocampal lamination defects, hippocampal heteropias, enlarged dysplastic neurons and glia, abnormal myelination and astrocytosis. Loss of TSC2 in radial glia caused also myelin formation defects and increased number of oligodendrocytes [323].

Wang et al. [324] used a conditional TSC1 allele consisting of loxP elements upstream of exon 17 and downstream of exon 18, in this way both exons can be deleted by CRE-mediated recombination. They crossed these mice with Synapsin1-CRE transgenic mice so that the TSC1 gene was inactivated in neurons. Brain tissue sections from these mice did not show evident anatomic defects, loss of lamination or tuber formation. Instead extracellular recordings that were able to trace synaptic activity from neocortical slices

showed differences in the excitability between TSC1ΔT and TSC1WT mice. Whole-cell patch-clamp recordings revealed excitatory synaptic currents characterized by long duration and epileptiform discharge pattern in TSC1ΔT mice suggesting that synaptic excitation was not normal and seemed to lead to seizure generation in TSC1ΔT brain tissues. This appeared to be independent from the presence of cortical tubers, absent in the TSC1ΔT mice used for this study [324].

Ehninger et al. [325] crossed TSC1 floxed mice with mice carrying CRE under the αCaMKII promoter to specifically silence TSC1 in neurons of the postnatal forebrain. The conditional KO mice died within the first postnatal weeks. The few surviving TSC1ΔT mice developed severe macroencephaly and the brains of these KO animals weighted 2.5 times more than those of the control littermates. Brain enlargement was the result of neuronal hypertrophy and was accompanied by astrogliosis [325].

Ess et al. [326] used TSC1 floxed mice crossed with GFAP-CRE transgenic mice to generate astrocyte-restricted inactivation of the TSC1 gene. They demonstrated that brains from TSC1ΔT mice expressed markers of glia precursors such as vimentin and brain lipid binding protein (BLBP) at higher levels. This may suggest that astroglial cells from KO animals could be developmentally immature. Also several markers of neuroglial cell differentiation were aberrantly expressed in TSC1ΔT astrocytes supporting evidence for a developmental defect of neuroglial cell differentiation in the pathology of TSC [326].

Uhlmann et al. [327] silenced TSC1 in astrocytes by crossing TSC1 floxed mice with GFAP-CRE deleters. The authors showed that astrocyte-specific inactivation of TSC1 led to seizures and early death in the mice. The conditional KO mice presented astrogliosis and abnormal neuronal organization. Astrocyte-specific inactivation of TSC1 resulted also in increased cell proliferation [327]. The same group demonstrated that TSC1 is important not only for astrocytes cell proliferation but also for astrocytes cell size [328]. By using the same model they demonstrated that TSC1ΔT astrocytes presented an increase in cell size

compared to WT animals and they demonstrated that this was due to increased activation of the S6-kinase pathway [328].The same group used the same strategy for silencing TSC1 in the paper by Wong et al. [329]. In this report the authors demonstrated that astrocyte expression of glutamate transporters was downregulated in TSC1ΔT mice and that also physiological glutamate transporter currents from astrocytes in hippocampal slices were decreased in these mice [329].

Also Michael Wong's group made use of a mouse model of glia-restricted TSC1 inactivation by crossing TSC1 floxed mice with GFAP-CRE deleters [330-332]. In the paper by Jansen et al. [330] the authors showed that inward rectifier potassium (Kir) channels expression and current was reduced in TSC1ΔT astrocytes. Impairment of potassium uptake mediated by astrocyte Kir could take part in the epileptogenesis in TSC1ΔT mice. Hippocampal slices from TSC1ΔT mice, in fact, were characterized by increased sensitivity to potassium-induced epileptiform activity [330]. Zeng et al. [331] demonstrated that in the hippocampus of these conditional KO mice extracellular glutamate was elevated, in particular the elevated levels of synaptic glutamate led to impairment in hippocampal LTP and this was reflected in deficits in hippocampal dependent conditioning/learning tasks [331]. In the same mouse model Xu et al. [332] demonstrated that conditional KO mice had decreased expression of the astrocytic gap junction protein, Connexin 43. This was reflected by the fact that also astrocyte gap junction coupling was impaired in these mice leading to defects in potassium buffering. This could contribute to epilepsy characteristic of TSC1ΔT mice [332].

4.1.3 TSC/mTOR in the nervous system.

The severe neurological phenotype observed in TSC patients can be easily understood by thinking at the importance of the TSC/mTOR pathway in neurons, in particular in key processes such as neuronal development and synaptic transmission. In recent years many different labs started to study the role of mTOR in neuronal

development and synaptic plasticity. mTOR has been shown to be important for axon guidance, dendrite development, dendritic spine morphogenesis and different forms of long-term synaptic plasticity.

The navigation of axons to their targets is an extremely regulated process that occurs because of different signals. Campbell and Holt [333] demonstrated the key importance for mTOR in this process. By using Rapa they showed that inhibition of mTOR prevents repulsive turning and collapse of axon growth cones in response to Semaphorin 3A (a repellent molecule for the growth of axons). Inhibition of mTOR prevented also incorporation of ^{3}H-leucine into newly translated proteins [333]. So probably mTOR can affect growth cone guidance by its activity on protein synthesis. Also the complex TSC1/TSC2 plays a key role in axon guidance and formation. Ephrin-A can induce growth cone collapse. Loss of TSC2 accompanied by hyperactivation of Rheb can prevent this effect of Ephrin-A [334]. The TSC complex is important not only for axon guidance, but also for axon formation. Choi et al. [335] demonstrated that overexpression of TSC inhibits axon growth in neurons in culture while TSC knockdown induces multiple axons. They demonstrated that the TSC complex can affect axon formation by regulating the mTOR pathway [335].

mTOR has an effect also on dendritic arborisation as demonstrated by Jaworski et al. [336]. The authors showed that a constitutively active form of the PI3K subunit p110 and of Akt could increase dendrite branching in hippocampal neurons, in a similar way to PTEN silencing by RNAi. The effects of PI3K and Akt could be blocked by administration of Rapa and by silencing mTOR with RNAi strategies. They thus demonstrated that mTOR plays a key role in dendrite morphogenesis and by knocking down p70S6K they showed that mTOR could exert this function through activation of this kinase [336].

mTOR is not only involved in dendrite morphogenesis, it can also control dendritic spines morphology. Kumar et al. [337] showed that activation of the PI3K/Akt pathway resulted in increased soma size, increased dendritic size and complexity and alteration of

dendritic spine morphology. Chronic inhibition of mTOR by Rapa led to a reduction in dendrite and soma size, reduced dendritic complexity and spine and filopodia density [337]. These findings correlated with results in PTEN null mice in which the authors demonstrated higher spine density in neurons of dentate gyrus [338]. Results from Sabatini's lab uncovered the role of the TSC complex in neuronal morphology [339]. The authors demonstrated that the TSC complex could regulate both density and size of dendritic spines, in particular TSC1 lacking neurons showed increased size and decreased density of dendritic spines and increased AMPAR-mediated synaptic currents [339].

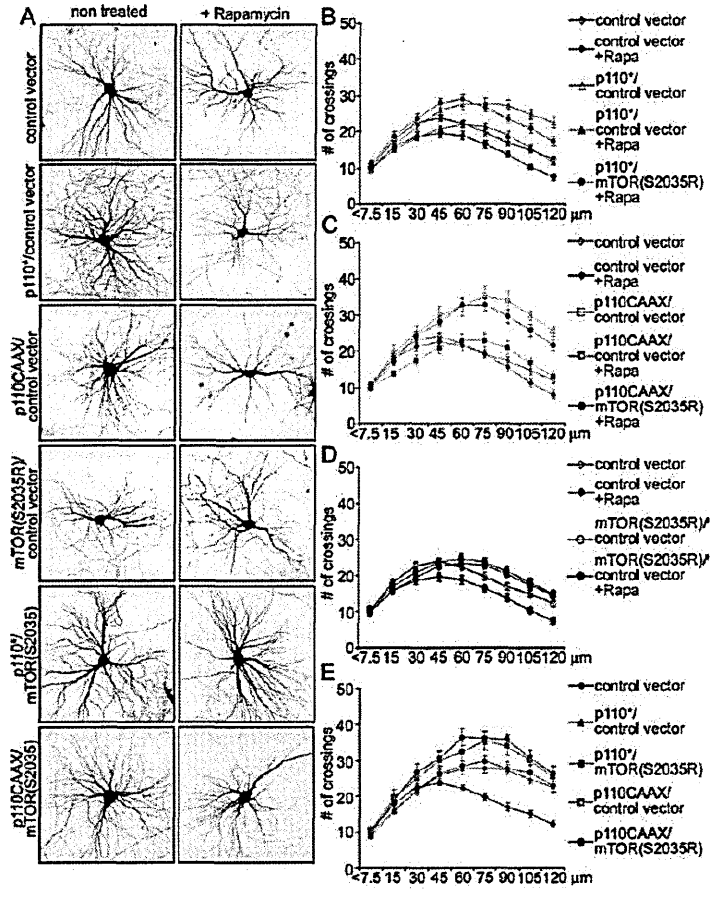

Fig VII. mTOR is central to dendrite arborization. mTOR is sufficient to mediate the effects of PI3K on dendrite branching. A) Micrographs of hippocampal neurons transfected at 7DIV. B-E) Sholl analysis of neurons in A. mTOR-S2035R is a mutant of mTOR resistant to Rapa. P110* and P11CAAX are constitutively active forms of PI3K. mTOR-S2035R blocks the reduction in dendrite number and complexity caused by Rapa (A, D). Overexpression of constitutive active PI3K leads to increase in dendrite branching that is sensitive to Rapa, mTOR-S2035R prevents Rapa effect (A, B, C). mTOR-S2035R does not increase the elaboration of dendrites due to p110* and p110CAAX. Jaworski J., The Journal of Neuroscience, 2005 [336]

The TSC/mTOR pathway plays a crucial role also in another fundamental neurological process that is synaptic plasticity. Synaptic plasticity is at the basis of mental processes such as memory and learning. Mice deficient for S6K1 and S6K2 showed context-specific associative fear memory deficits, problems in taste learning and a slight deficit in spatial learning [340]. Synaptic plasticity is compromised also in different models of Tuberous Sclerosis. In rats heterozygous for TSC2, von der Breile et al. [341] observed alteration in short-term synaptic plasticity and reduced LTP and Long Term Depression (LTD), another kind of synaptic plasticity [341]. Goorden et al. [342], by using TSC1 heterozygous mice, showed that these mice did not present evident cerebral pathology, but displayed signs of impaired cognitive function. Interfering with TSC1 led to spatial learning deficiencies, defective context conditioning and diminished social functioning [342].

It is easy to figure out that having and TSC and mTOR such fundamental roles in the life of a neuronal cell, alteration of one of these proteins or of their signalling pathways can lead to severe neurological diseases. Beside the tuberous sclerosis complex described above, another syndrome caused by altered TSC/mTOR signalling is neurofibromatosis type 1 (NF1). Johannessen et al. [343] demonstrated that in NF1 KO MEFs the PI3K pathway was hyperactived, and this resulted in inactivation of TSC2. Also NF1 KO tumour cells showed increased mTOR activity and treatment with Rapa could decrease their cell growth [343]. mTOR and TSC play a role also in neurodegenerative disorders such as Parkinson's disease (PD) and Huntington's disease (HD).

Engrailed 1 is a transcription factor of vital importance for dopaminergic neurons. Mice that do not express this factor, in fact, show motor problems similar to PD patients [344, 345]. Di Nardo et al. [346] demonstrated that Engrailed 1 translation is dependent on mTOR, thus suggesting a link between mTOR and PD.

Ravikumar et al. [347], instead, demonstrated mTOR involvement in HD. They showed that mTOR was sequestered into huntingtin aggregates in cell models, transgenic

mice and human brain. They also demonstrated that polyglutamine expansion impaired mTOR kinase activity, measured by S6K1 and 4E-BP1 phosphorylation, and mTOR-dependent translation [347]. After increase of mTOR activity they observed increased polyglutamine toxicity both in cells and flies. What's more, by inhibiting mTOR activity they observed improvements in behaviour and motor performance in a mouse model of Huntington disease [347].

4.1.4 The wtPC12/PC12-27 cell model.

wtPC12 cells resemble chromaffin cells and catecholaminergic neurons. In 1976 Greene et al. [348] established this clonal line from pheochromocytoma cells. wtPC12 cells are endowed with neurosecretory functions [348-350] and the ability to differentiate into neuron like cells upon NGF stimulation [348-350]. PC12-27 cells are a spontaneous mutant clone defective for neurosecretion [350-352]. The isolation of this clone was the fortuitous result of a study for obtaining the permanent overexpression of a constitutively activated form of α_s, a trimeric G protein subunit [351]. This clone, defective for neurosecretion, presents differences in morphology and in the proliferation ability compared to wtPC12 cells. Even if their rate of proliferation is quite different, proliferation of both clones is insensitive to Rapa administration (our results and [353]).

While wtPC12 cells are characterized by the low expression of the RE-1-specific Silencing Transcription factor (REST, or NRSF), PC12-27 cells express high levels of this transcriptional repressor [350, 352]. Although REST has been previously described to work as an oncogene and as a tumour-suppressor gene [354-358], its main activity appears to be linked to the process of neuronal differentiation. REST is known to repress transcription of hundreds of genes, many of which specific for neurons and neurosecretory cells. During maturation of nerve cell precursors the high REST, typical of stem cells, rapidly declines to very low levels, and this allows nerve cells to acquire their specific phenotypes [359, 360]. In contrast, non-nerve cells keep REST at high levels. The latter

property, which prevents the expression of REST target genes, has also been found to control cell proliferation, working as a tumour suppressor [354]. Rapidly proliferating and aggressive forms of cancer of non-nerve origin (lung, colorectum, breast, prostate) were shown in fact to lose REST expression and concomitantly acquire a faster proliferation rate and the expression of typical nerve-cell markers [355-358]. These events may be linked to a deregulation of Akt2 [361], induced by low REST. Kreisler et al. [361] demonstrated regulation of Akt2 by REST in a Small-Cell Lung Cancer (SCLC) cell line. In this particular model REST would act as a tumour suppressor. Its loss, in fact, would lead to deregulation of Akt ser473 phosphorylation and thus affect cell proliferation and survival.

The role of REST in controlling proliferation of nerve cells appears to be different. Low REST expression correlates to the low or absent proliferation of these cells. Medulloblastomas tumours and derived cell lines express high levels of REST, are poorly differentiated and capable of intense proliferation [355, 356, 362], which can be blocked by the forced expression of a dominant negative construct of REST [362].

Based on these premises we thus investigated the possibility that proliferation and neurosecretory functions of wtPC12 and PC12-27 cells might be under the control of TSC/mTOR.

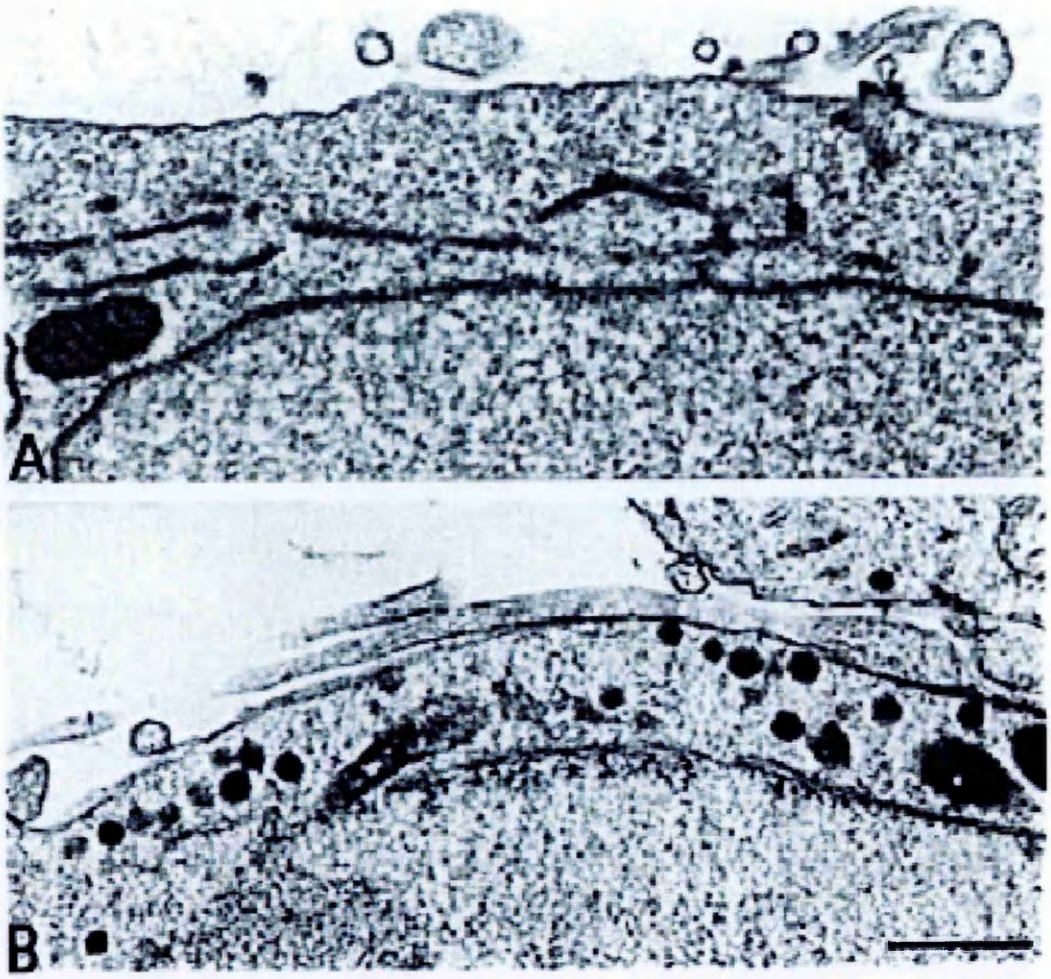

Fig VIII. Ultrastructural analysis of PC12-27 and wtPC12 cells. Electron microscopy of PC12-27 (A) and wtPC12 (B) cells. Cells in A lack vesicles in the cytoplasm near the plasmalemma. In B the same area is rich in typical dense granules. Malosio M.L., Journal of Physiology, 1999 [352]

4.2 Material and methods

4.2.1 PC12 cell cultures.

The wt PC12 and PC12-27 clones and subclones were grown at 37 °C. Media were supplemented with 2 mM l-glutamine, 100 U/ml penicillin, and streptomycin (Cambrex Bio Science Verviers S.p.r.l., Verviers, Belgium). PC12 wt and PC12-27 were grown in DMEM with 10% horse serum (Euroclone, Wetherby, United Kingdom) and 5% fetal clone III serum (Hyclone Laboratories, Logan, UT). Cells were always kept in medium until lysis. This prevented inactivation of S6 phosphorylation.

4.2.2 Transient and stable transfections.

Lipofectamine 2000TM (Invitrogen, Carlsbad CA, USA), was used for both transient and stable transfections. PC12-27 cells transfected with the Myc-tagged dominant negative construct DBD/REST together with the pcDNA3.1/hygro(+) vector carrying hygromycin resistance, were selected with the antibiotic. Stable subclones were grown in complete medium supplemented with 200 or 500 ug/mL of hygromycin B (Invitrogen), respectively. Upon transfection, the cells were grown in complete medium supplemented with puromycin 10 ug/mL.

The pcDNA3/Flag/TSC2 cDNA (gift of B. Manning) or the corresponding empty vector (for controls) was transiently transfected into PC12–27 cells that were processed 48h thereafter. To generate cells with stable TSC2 knockdown we transfected wtPC12 cells with TRC1 lentiviral plasmid vector pLKO.1-puro (Sigma-Aldrich) expressing control or TSC2 shRNAs. Forty-eight hours after transfection we added 2.5 μg/ml puromycin and selected clones. Knock-down of TSC2 was confirmed by qPCR and immunoblotting. Clones were then cultured in the presence of 2.5 μg/ml puromycin.

4.2.3 Western blot analysis.

wtPC12 and PC12-27 cells were treated with Rapa (100 nM, Calbiochem), endo-IWR1 (10 uM, Tocris); 1a, 25-dihydroxyvitamin D3 (100 nM, Sigma-Aldrich); quercetin (100 uM, Sigma-Aldrich). Total cell extracts of wtPC12 and PC12-27 cells obtained by suspending cells in lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, and protease inhibitors were rocked (15 min) and then centrifuged (13 000 g, 4°C, 15 min). Nuclear and cytoplasmic fractions were separated by gradient centrifugation. Proteins were quantified by BCA assay and appropriate amounts (30 or 50 μg) were separated on standard 10-15% SDS-PAGE. For Western blotting, gels transferred to nitrocellulose filters were first blocked for 1 h with 5% nonfat dry milk in PBA, and then incubated o.n. with the primary antibody diluted in PBS with 5% bovine serum albumin (BSA), washed in PBST (3-fold for 10 min), incubated for 1 h with the peroxidase-conjugated secondary antibody, washed again in PBST as described above and once in PBS. Photographic development was by chemiluminescence (ECL, Amersham Bioscience or Immobilon substrate, Millipore). Western blot bands were quantified by the ImageJ program (rsb.info.nih.gov/ij).

The IgG2a, rat-specific anti-chromograninB (ChgB) monoclonal antibody (mAb), was generated in Prof. Meldolesi laboratory. Other Abs were from commercial sources: anti-REST and anti-H2B polyclonal Abs (pAbs): Upstate; antisynaptotagmin1 and anti-β-tubulin mAbs: Synaptic Systems; anti-SNAP25 mAb: Sternberger-Monoclonals; anti-GFP mAb: Roche; anti-β-catenin and anti-paxillin mAbs: BD Transduction; anti-Akt, anti-P(S473)-Akt, anti-P(T308)-Akt, anti-4E-BP1, anti-TSC2, anti-TSC1, anti-GSK3β, anti-P(S9)-GSK3β, anti- P(Ser235/236)-S6 and anti-S6, and actin, which were obtained from Santa Cruz Biotechnology Inc. Horseradish peroxidase-conjugated goat antimouse and anti-rabbit pAbs: Bio-Rad. The BCA Protein Assay Kit from Pierce.

Antibody anti-	Company	Cat. N°
REST	Upstate	09-019
H2b	Upstate	05-1352
Synaptotagmin1	Synaptic Systems	105 311
β-tubulin	Synaptic Systems	302 302
SNAP25	Sternberger-Monoclonals	MMS-614R
GFP	Roche	11 814 460 001
β–catenin	BD Transduction	610153
Paxillin	BD Transduction	610055
Akt	Cell Signaling	4685
Phospho-Akt (Ser473)	Cell Signaling	9271
4E-BP1	Cell Signaling	9452
TSC2	Cell Signaling	3990
TSC1	Cell Signaling	4906
Phospho-GSK3β (Ser9)	Cell Signaling	9323
S6	Cell Signaling	2317
Phospho-S6 (Ser235/236)	Cell Signaling	2211
Phospho-p70S6K (Thr389)	Cell Signaling	9205
Actin	Santa Cruz Biotechnology	Sc-1616

4.2.4 Cell proliferation assay.

For the direct counting assays, wtPC12, PC12-27, PC12-27/DBD5 and wtPC12 cells transfected with ShRNAs and Δ90βCat constructs were plated at 1 x 10^4/well in a 24 well dish, incubated in culture medium and counted after 3, 4 and 5 days. Medium was replaced every 48 h. Rapamycin, 0.1 μM, 1a,25-dihydroxyvitamin D3 , 0.1 μM, quercetin, 100 μM, and endo-IWR1, 10 μM, were administered for 24 h during the 5th day of culture.

4.2.5 Luciferase assay.

β-Catenin transcription assay was performed using the Dual-luciferase reporter assay kit (Promega).

The assay was performed in 24-well dishes. Each well of a 24-well dish was cotransfected with lipofectamine 2000TM using standard conditions with the following constructs: 1 ug of the 16X TOPFLASH reporter plasmid (Oligos containing 4 TCF sites and an Asc1 overhang were

multimerized and cloned into Asc I/Mlu I sites of pTA (Clontech)) and 100 ng of SV40-Renilla-l.uc. Luciferase activity was measured 24 hr later, using a luminometer (GloMax Multi Detection System of Promega). All assays were normalized to Renilla levels.

4.2.6 Immunofluorescence.

Cell monolayers on coverslips were fixed with 4% formaldehyde for 10 min at room temperature and quenched in 0.1 M glycine, then permeabilized for 20 min in PBS containing 0.2% Triton X-100 and 1% BSA, and immunolabeled for 1 hr with either anti-paxillin mAb or phalloidin/FITC conjugate diluted in PBS with 1% BSA. Nuclei were stained with DAPI. Samples were studied in a Perkin-Elmer Ultraview ERS confocal microscope. Image deconvolution was performed in a wide field microscope of the Delta Vision system.

4.2.7 CFSE proliferation assay.

As reported in 2.2.5

4.2.8 Quantitative real-time RT-PCR (qPCR).

As reported in 2.2.6

4.2.9 Statistical analysis.

As reported in 2.2.8

4.3 Results

4.3.1 PC12-27 cells show a proliferative advantage and an altered morphology compared to wtPC12 cells.

PC12-27 cells are pheocromocytoma cells expressing high levels of the REST protein. These cells lack the neurosecretory phenotype typical of wtPC12 cells that are characterized by low levels of REST. Another important difference we observed was in their proliferative rate. First of all a viable count of these two clones was performed (Fig. 4.1 A). After 4 days it was clear that PC12-27 cells proliferated better than wtPC12, and this was even more evident after 5 days in culture. Cell proliferation was also evaluated by CFSE labelling. After 5 days of culture the CFSE content of PC12-27 cells was lower than that found in wtPC12 cells, indicative of faster proliferation (Fig. 4.1 B, C).

WT and defective cells did not differ only in their proliferative capacity but also in their morphology. PC12-27 cells were bigger than their wt counterpart (Fig. 4.1 D, E) as shown by the larger cell size evaluated by flow cytometry. PC12-27 cells contain also higher protein contents than wtPC12 cells (Fig. 4.1 F). Besides this PC12-27 cells also showed a particular morphology. While wtPC12 cells were round in shape, PC12-27 cells presented a flatter shape. Actin was distributed at the cortex of wtPC12 cytoplasm, while it was organized in thick fibers that crossed the whole cell in PC12-27. Also paxillin was differently distributed in these cells, in wtPC12 it was found in small, adjacent puncta all over the plasma membrane, in PC12-27 instead it formed larger structures at the tips of the actin fibers (Fig. 4.1 G). This phenotype was reminiscent of that acquired by HeLa cells following Rictor deletion [79].

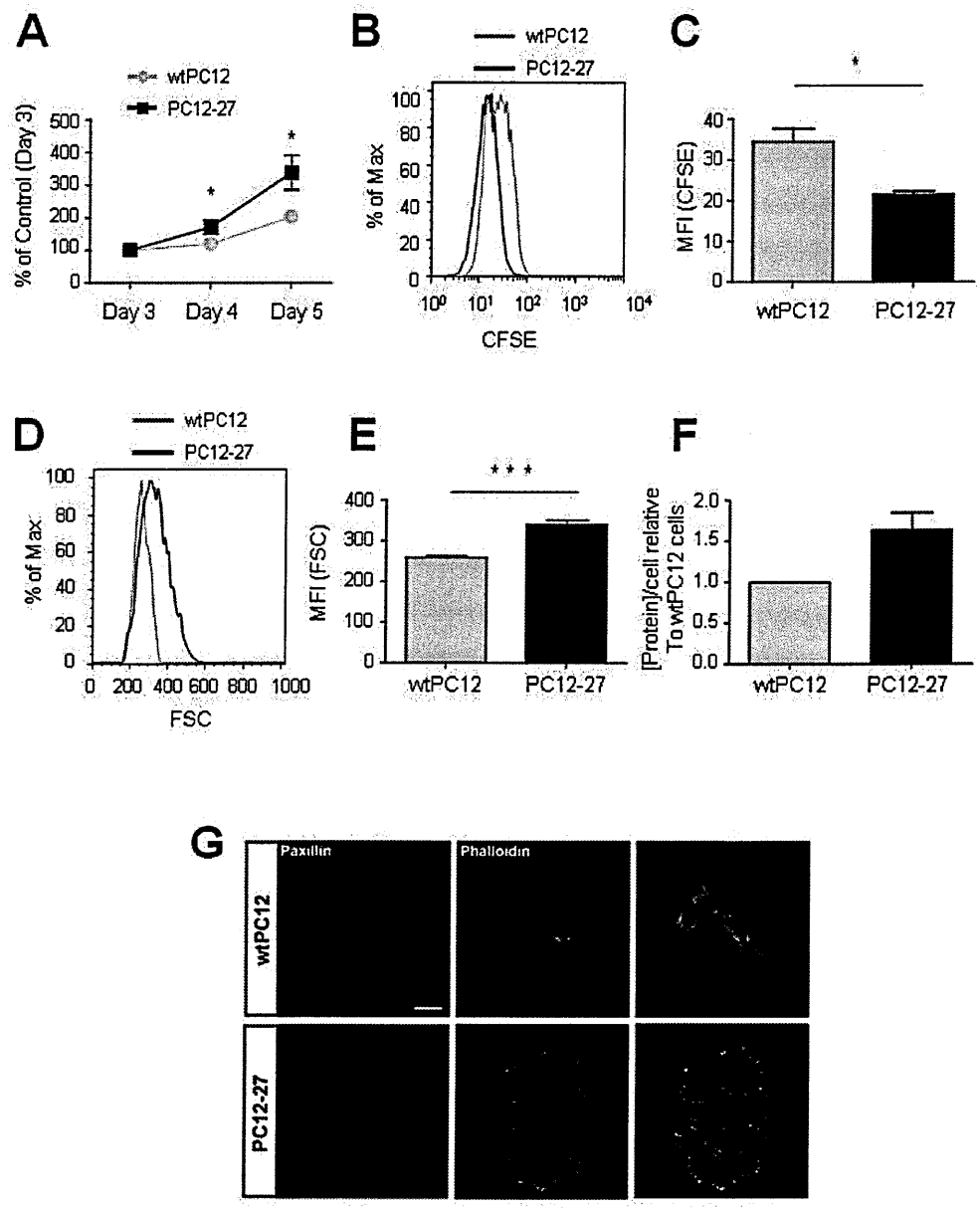

Fig 4.1 PC12-27 cells show a proliferative advantage and an altered morphology compared to wtPC12 cells. A) The same number of wtPC12 and PC12-27 cells was plated and then the viable cells were counted after 3, 4 and 5 days in culture. The results were normalized by the 3rd day of culture. B, C) Cells were labelled with CFSE the first day of culture and then CFSE dilution was evaluated after 5 days of culture. In B) the histogram of a representative experiment. In C) the quantification of 3 independent experiments (±SE). D) Histogram showing FSC profile of wtPC12 and PC12-27 cells, in E) the quantification of 3 experiments (±SE). F) Protein concentration per cell, as determined by BCA assay, in wtPC12 and PC12-27 cells. Data are mean (±SE) of protein extracts made from 3 separate experiments. Data are normalized on wtPC12 cells. G) Immunofluorescence staining of wtPC12 and PC12-27 cells labelled with Paxillin (left), phalloidin (middle) and the merge of the two together with DAPI staining (right). In A statistical significance was evaluated with two-way ANOVA test with Bonferroni post test, in C, E and F with two-tailed t-Test.

4.3.2 mTORC1 and mTORC2 signalling are perturbed in PC12-27 cells.

We thus decided to investigate whether differences between wtPC12 and PC12-27 cells could be attributed to mTOR signalling. We analyzed mTORC1 and mTORC2 activity by looking at the phosphorylation of some targets of these two complexes. For mTORC1 the phosphorylation of p70S6K (Thr389), 4E-BP1 and S6 (Ser235/236) and for mTORC2 of Akt in ser473 and its target GSK3β (Ser9) were assessed. An increase in S6 (Ser235/236) and p70S6K (Thr389) phosphorylation and in the mobility shift of 4EBP1 in PC12-27 cells compared to their wt counterpart was detected. This suggested constitutive mTORC1 activity in PC12-27 cells (Fig. 4.2). By looking at the phosphorylation of Akt (Ser473) and GSK3β (Ser9) it was found that these phosphorylation were decreased in the clone defective for neurosecretion compared to the wt one (Fig. 4.2). This was consistent with defective mTORC2 signalling. Fig. 4.2 A show representative blots, while Fig. 4.2 B depicts a pool of different experiments.

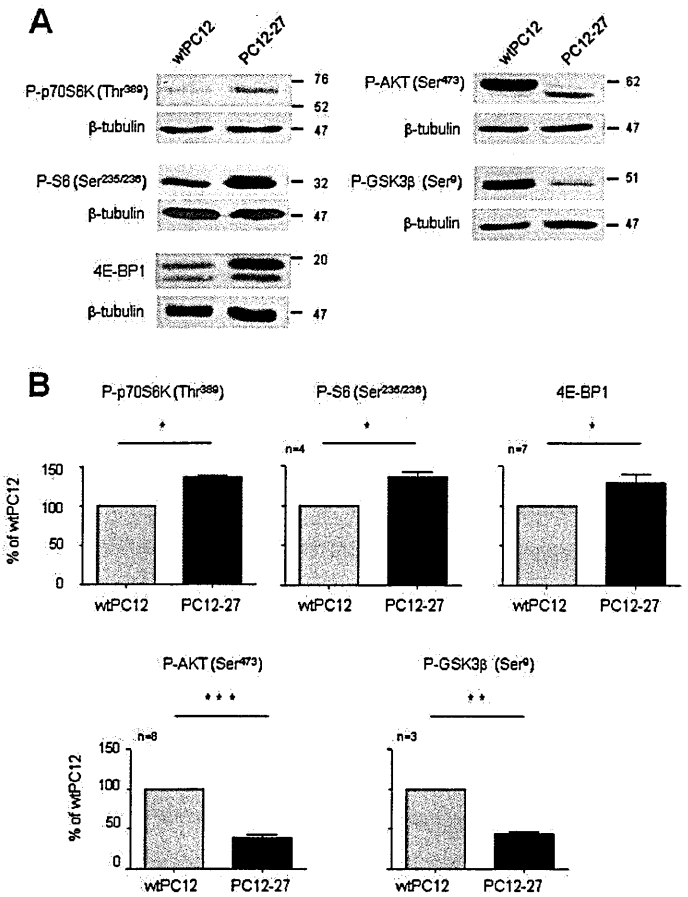

Fig 4.2 mTORC1 is hyperactivated and mTORC2 down-regulated in PC12-27 cells. SDS-PAGE analyses were performed as detailed in Materials and Methods 4.2.3.

Phosphorylation of the mTORC1 targets 4EBP1 and p70S6K (Thr 389), of p70S6K substrate S6 (Ser 235/236), of the mTORC2 target Akt (Ser 473) and of Gsk3β (Ser9) were assessed by western blot analysis (A and B). β-tubulin is depicted as loading control, and relative migration of molecular weight markers is depicted. One of 3-8 independent experiments is depicted. Molecular weight markers are indicated in KDa.
B) Independent experiments were compared by densitometric analyses. Expression levels are shown as relative to wtPC12 cells (±SE). In B statistical significance was evaluated with two-tailed t-Test.

4.3.3 Reduced TSC2 levels account for β–catenin upregulation and increased nerve cell proliferation.

This unbalance between mTORC1 and mTORC2 could be the consequence of a defect in TSC1/TSC2 signalling. As shown by Huang et al. [38], the TSC complex can regulate in an opposite way mTORC1 and mTORC2. The levels of TSC1 and TSC2 in wtPC12 and PC12-27 cells were thus investigated. While TSC1 was expressed to comparable extents in wt and PC12-27 cells, TSC2 levels were significantly lower in PC12-27 cells (Fig. 4.3 A, B).

PC12-27 cells overexpress the transcriptional repressor REST. We thus asked whether REST could be responsible for decreased TSC2 levels. The levels of TSC2 were analysed in a clone of PC12-27 cells stably transfected with a dominant negative construct able to inhibit the effect of REST, the PC12-27/DBD5 clone (Fig. 4.3 B). An increase in the levels of TSC2 was observed in the PC12-27/DBD5 clone compared to the PC12-27 cells, so a partial recovery in the levels of TSC2 appears to be due to a decrease in REST activity. The levels of TSC2 mRNA were then evaluated to check whether REST could have an effect in the transcription of the gene itself. Real time PCR experiments showed no difference in the transcription of TSC2 in wtPC12 and PC12-27 cells, suggesting a post-transcriptional control of TSC2 (Fig. 4.3 C).

After we established that TSC2 levels were decreased in the defective PC12-27 clone, we tried to understand which was the molecular mechanism responsible for the increased cell proliferation rate observed in these cells. We decided to assess if the hyperactivation of

mTORC1 could be responsible for this effect. While Rapa reduced S6 phosphorylation levels and the mobility of 4E-BP1 (Fig. 4.3 D), it did not have a significant effect on the growth of wtPC12 or PC12-27 cells (Fig. 4.3 E). These results suggested that proliferation in these cells did not appear to be regulated by mTORC1, or at least not only by mTORC1, in accordance with results from Powers et al. [353].

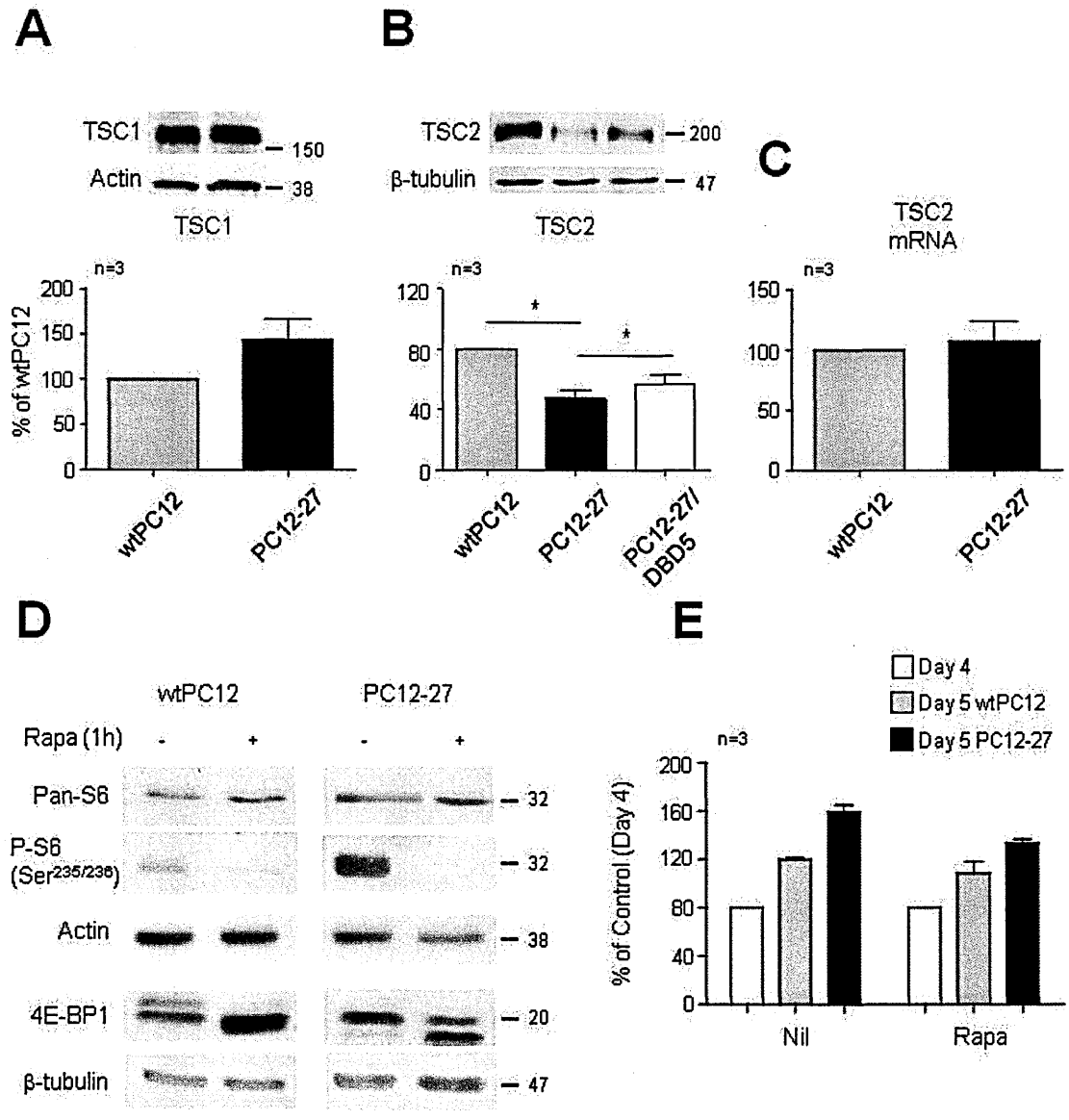

Fig 4.3 PC12-27 cells show reduced TSC2 levels and an increased Rapa-independent growth compared to wtPC12 cells. A, B, D) SDS-PAGE analyses were performed as detailed in Materials and Methods 4.2.3. Levels of TSC1 and TSC2 (A and B) and phosphorylation of the mTORC1 target 4EBP1 and of p70S6K substrate S6 (Ser 235/236) (D), were assessed by western blot analysis. Cell were treated with Rapa (100 nM) for 1 hour. β-tubulin and actin are depicted as loading controls, and relative migration of molecular weight markers is depicted. One of 3 independent experiments is depicted. Molecular weight markers are indicated in KDa. A, B) Independent experiments were compared by densitometric analyses. Expression levels are shown as relative to wtPC12 cells (±SE). C) TSC2 mRNA was first normalized to the GAPDH housekeeping gene, and

then expressed relatively to wtPC12 cells using the ΔΔCT method (±SE). E) The same number of wtPC12 and PC12-27 cells was plated and then the viable cells were counted after 4 and 5 days in culture. The results (±SE) were normalized by the 4th day of culture. 24h before counting the viable cells Rapa (100 nM) was administered. In B statistical significance was evaluated with one-tail ANOVA test with Dunns post test.

The TSC complex controls not only mTORC1 but also other important pathways for cell proliferation. One of these, the β-catenin pathway, is of key importance in the life of nerve cells in general. First the levels of β-catenin in the wt and defective clones were analysed. The nuclear levels of β-catenin were increased in the defective clone compared to the wt one (Fig. 4.4 A) as well as its transcriptional activity as assessed by a luciferase assay that evaluates the ability of transcription of β-catenin (Fig. 4.4 B). β-catenin activity was evaluated also by looking at the mRNA levels of some of its target genes by real time PCR. The mRNA levels of c-Myc, Cyclin D1 and REST were evaluated. REST, in fact, is another target of β-catenin. Again a significant increase in the levels of all of these genes was detected in PC12-27 compared to wtPC12 cells (Fig. 4.4 C). The increase in c-Myc mRNA levels in PC12-27 cells is strikingly higher compared to the increase in Cyclin D1 mRNA levels. The Cts (the fractional PCR cycle number at which the reporter fluorescence is greater than the threshold) of c-Myc and Cyclin D1 in wtPC12 are 28.75 and 21.75 respectively. These Cts indicate the presence of the mRNAs for these genes in the cells. The strong increase in PC12-27 cells in c-Myc mRNA levels compared to Cyclin D1 can be explained by the difference in the Ct between wtPC12 and PC12-27 cells. 7.61 in c-Myc compared to 4.08 in Cyclin D1 (Fig. 4.4 D-F).

These results indicate that together with increased levels of REST and decreased levels of TSC2, the defective clone is characterized by increased levels of β-catenin. We tried to evaluate whether this increased β-catenin activity could be the reason for the higher rate of proliferation we observed in PC12-27 cells. A viability count was thus performed by plating the cells with different β-catenin inhibitors. The drugs used for this assay were: 1α, 25-dihydrovitamin D3, known to induce the export of nuclear β-catenin to the

cytoplasm [363]; quercetin, a blocker of the β-catenin/TCF-LEF transcription [364]; and endo-IWR1, which favours β-catenin degradation [365]. After 4 days of culture the inhibitors were added to the cells and the cells were counted at day 5. The administration of 1α, 25-dihydrovitamin D3 provoked an almost complete block in cell proliferation in both wtPC12 and PC12-27 cells, suggesting that β-catenin plays a really important role in the proliferation of these cells. Quercetin and endo-IWR1, instead, caused a slight decrease in the proliferation of wtPC12 cells, but abrogated the proliferative advantage of the defective clone. The rate of proliferation of wtPC12 and PC12-27, in fact, is comparable (Fig. 4.4 G).

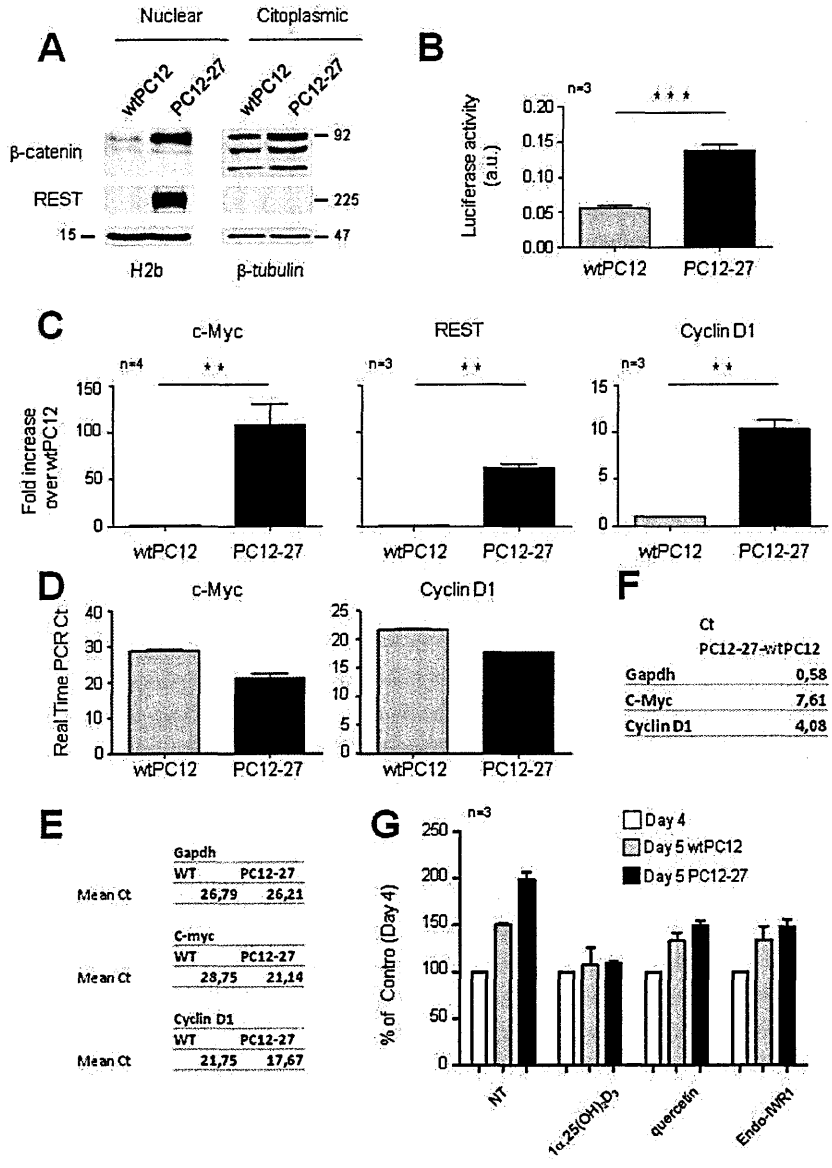

Fig 4.4 PC12-27 cells show increased β-catenin levels and an increased β-catenin dependent growth compared to wtPC12 cells. A) SDS-PAGE analyses were performed as detailed in Materials and Methods 4.2.3. β-catenin and REST protein levels were evaluated by western blot analysis in nuclear and cytoplasmic extracts of wtPC12 and

PC12-27 cells. β-tubulin and H2b are depicted as loading controls, and relative migration of molecular weight markers is depicted. One of 3 independent experiments is depicted. Molecular weight markers are indicated in KDa. B) A luciferase assay was performed on wtPC12 and PC12-27 cells. The cells were co-transfected with the reporter and the luciferase plasmids and luciferase activity (±SE) was measured 24h later. C) c-Myc, REST and Cyclin D1 mRNAs were normalized to the GAPDH housekeeping gene, and then expressed relatively to wtPC12 cells using the ΔΔCT method (±SE). D, E) Ct (the point at which the fluorescence crosses the threshold of real time PCR) for c-Myc and Cyclin D1. F) Difference in Ct between PC12-27 and wtPC12 cells for Gapdh, c-Myc and Cylin D1 genes. G) The same number of wtPC12 and PC12-27 cells was plated and then the viable cells were counted after 4 and 5 days in culture. The results (±SE) were normalized by the 4th day of culture. 24h before counting the viable cells the different inhibitors were administered. In B and C statistical significance was evaluated with two-tailed t-Test.

It has been proven that TSC1/TSC2 promotes the turnover of β-catenin [39-41, 366]. We thus wanted to demonstrate a direct link between the two proteins in PC12 cells as well. A full length construct of TSC2 was overexpressed by transiently transfecting PC12-27 cells (Fig. 4.5 A). Restoring TSC2 levels led to a decrease in β-catenin protein levels, a decrease in the mRNA levels of c-Myc and a reduction in the levels of the REST protein (Fig. 4.5 B). These results seem to further point to a direct link between TSC2 and β-catenin.

To further prove this link we moved to the wtPC12 cells. This clone was stably transfected with different shRNAs silencing the TSC2 gene. Different subclones were generated and the expression of β-catenin and REST were analysed. By western blot analysis an increase in the levels of REST and nuclear β-catenin was observed in the clones transfected with the shRNAs for TSC2 compared to the control clone transfected with a control shRNA that does not silence any human or mouse gene. In Fig. 4.5 C a representative blot, while in Fig. 4.5 D the quantification of different experiments. The decrease in TSC2 and following increase in β-catenin and REST levels correlated with an increase in their activity. β-catenin transcriptional activity was assessed by the luciferase assay and by looking at the transcription of c-Myc and Cyclin D1 (Fig. 4.5 E, F). The TSC2 shRNA clones analysed showed an increase in the luciferase activity compared to the control clone and higher mRNA levels of both Cyclin D1 and c-Myc by real time PCR

(Fig. 4.5 E, F). In order to evaluate whether the increase in REST levels was mirrored by an increase in its activity as well, the levels of two important REST targets, Synaptotagmin 1 and SNAP 25, were evaluated. The levels of both of these proteins were decreased in the TSC2-shRNA clones compared to the control one (Fig. 4.5 G). These results further prove the direct link between TSC2 and β-catenin. At this point we wanted to link TSC2 to proliferation as well. First of all a viability count was performed and an increase in the proliferation rate of the shRNA clones compared to the control one was observed (Fig. 4.5 H). To verify that this increase in proliferation was due to the effect of TSC2 silencing on β-catenin, the viability count was performed in the presence of quercetin and endo-IWR1, inhibitors of β-catenin. The administration of the inhibitors did not exert a strong inhibition on the control cells, but completely abrogated the proliferative advantage of the TSC2 KO clone (Fig. 4.5 I), thus indicating that the increased proliferation observed in the shRNA clones is probably due to the increase in β-catenin that was observed in these cells.

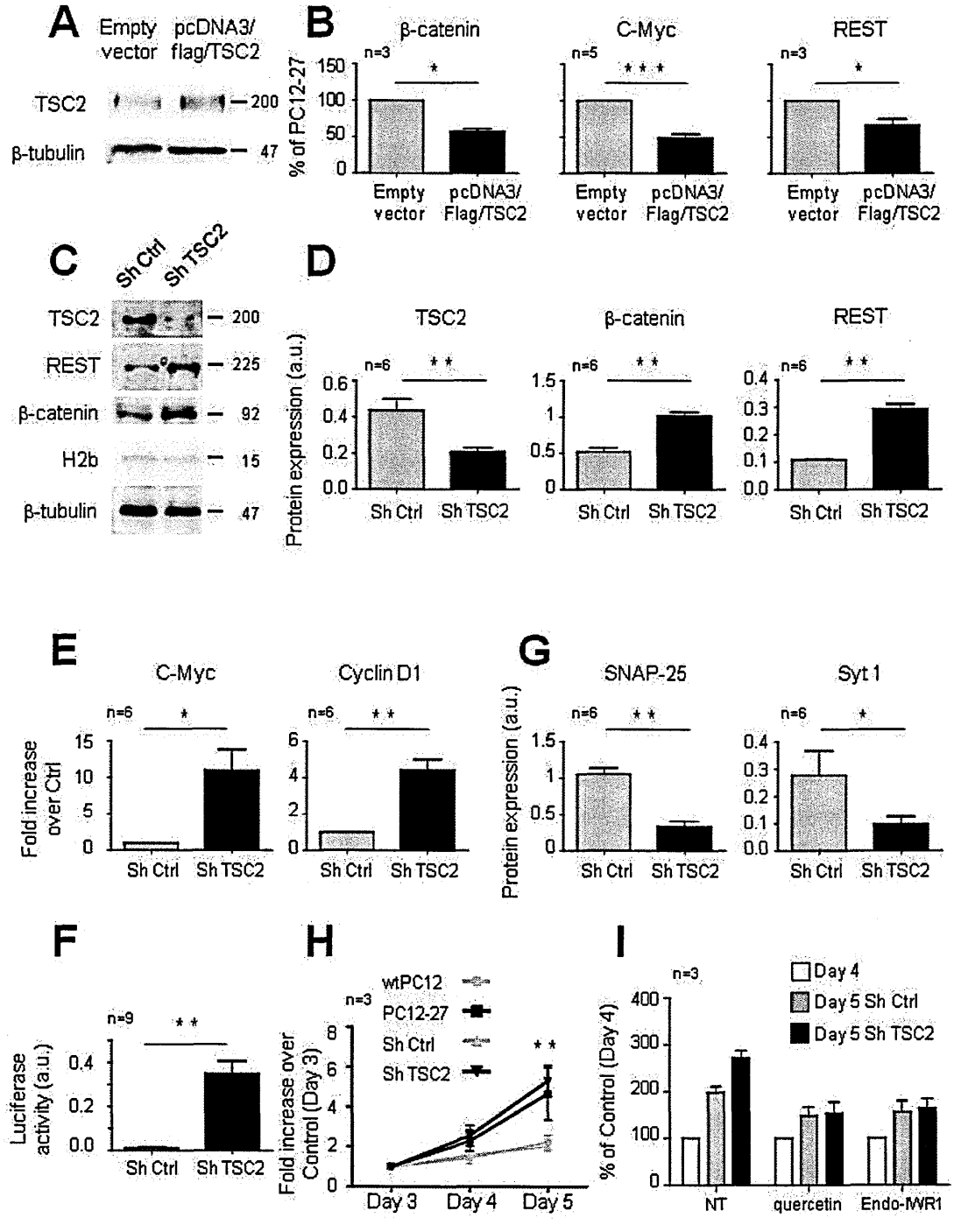

Fig. 4.5 TSC2 regulates β–catenin levels and proliferation of PC12 cells. A and B) PC12-27 cells were transfected with an empty vector and with a construct overexpressing TSC2: pcDNA3/flag/TSC2 A) SDS-PAGE analyses were performed as detailed in Materials and Methods 4.2.3. Levels of TSC2 were assessed by western blot analysis. β-tubulin is depicted as loading control, and relative migration of molecular weight markers is depicted. One of 3 independent experiments is depicted. Molecular weight markers are indicated in KDa. B) β-catenin and REST protein and c-Myc mRNA levels (±SE) were expressed relatively to PC12-27 cells transfected with the empty vector. C-I) wtPC12 cells were transfected with a control ShRNA (Sh Ctrl) and with a ShRNA silencing TSC2 (Sh TSC2). C and D) SDS-PAGE analyses were performed as detailed in Materials and Methods 4.2.3. Levels of TSC2, REST and β-catenin were assessed by western blot analysis. β-tubulin and H2b are depicted as loading controls, and relative migration of molecular weight markers is depicted. One of 3 independent experiments is depicted.

Molecular weight markers are indicated in KDa. D, G) Independent western blot experiments were compared by densitometric analyses. Expression levels (±SE) are shown as relative to wtPC12 cells transfected with a Ctrl ShRNA. F) A luciferase assay was performed on wtPC12 cells transfected with the ShRNA construct. The cells were co-transfected with the reporter and the luciferase plasmids and luciferase activity was measured 24h later. E) c-Myc and Cyclin D1 mRNAs (±SE) were expressed relatively to wtPC12 cells transfected with the Ctrl ShRNA. H, I) The same number of wtPC12 Ctrl and ShRNA transfected cells were plated and then the viable cells were counted after 3, 4 and 5 days in culture. The results (±SE) were normalized by the 3rd (H) or 4th (I) day of culture. 24h before counting the viable cells the different inhibitors were administered (I). In B, D-G statistical significance was evaluated with two-tailed t-Test, in H with two-way ANOVA test with Bonferroni post test.

As discussed above PC12-27 cells show morphological features typical of Rictor KO cells. The morphology of wtPC12 cells that were transfected with TSC2 shRNA was analysed by Immunofluorescence. While the clones transfected with a control shRNA do not show morphological differences compared to wtPC12 cells (Fig. 4.6), the TSC2 KO clones show morphological features typical of Rictor KO cells and are quite reminiscent of PC12-27 cells. Fig. 4.6 shows that these cells are flatter and more attached to the culture surface, phalloidin staining evidences the presence of actin fibers running through the whole cytoplasm. Also paxillin is redistributed with some accumulation in the peripheral cytoplasmic protusions.

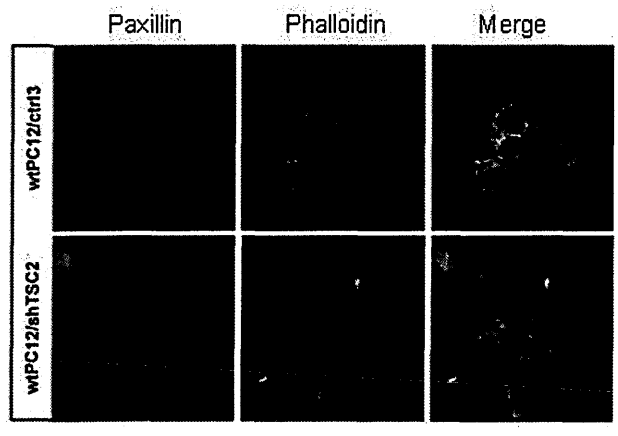

Fig 4.6 TSC2 silencing affects PC12 cells morphology. Immunofluorescence staining of PC12 cells labelled with Paxillin (left), phalloidin (middle) and the merge of the two together with DAPI staining (right). wtPC12 cells transfected with a Ctrl ShRNA (up) or a ShRNA silencing TSC2 (bottom).

4.4 Discussion

By the analysis of wtPC12 and PC12-27 nerve cells, we unveiled a new pathway linking TSC2 to proliferation and neurosecretory functions. Comparison of wtPC12 cells and PC12-27 cells, the first characterized by low levels of REST and proper neurosecretory functions, and the latter expressing high levels of REST and lacking neurosecretory functions [350], led to the identification in the latter of the loss of TSC2, and a gain in proliferative capacity. Loss of TSC2 in PC12-27 cells was paralleled by constitutive mTORC1 and defective mTORC2 signalling, and increase in cell size and proliferative potential and augmented β-catenin activity. Similar results and biological responses were achieved by knocking down TSC2 in wtPC12 cells by RNAi. Also in this case cells were enlarged in size and proliferated to faster extents when compared to control wtPC12 cells. The proliferative advantage of PC12-27 cells was insensitive to Rapa, suggesting that mTORC1 might not be involved in nerve cells proliferation, as also reported previously [353]. This proliferative advantage was rather dependent upon β-catenin increased activity. Indeed, proliferation of PC12-27 cells was inhibited by β-catenin inhibitors.

Thus loss of TSC2, either spontaneous (PC12-27) or mediated by ShRNA, allows β-catenin accumulation in the nucleus and confers a proliferative advantage. Decreased TSC2 also had a critical impact on cell morphology and neurosecretory functions. Indeed wtPC12 cells that were transfected with a TSC2 shRNA were flatter and more attached to the culture surface, phalloidin staining evidenced the presence of actin fibers running through the whole cytoplasm and paxillin was redistributed with some accumulation in the peripheral cytoplasmic protusions, thus showing morphological features typical of Rictor KO cells and were quite reminiscent of PC12-27 cells. Furthermore lowering TSC2 levels also had an impact on expression of neurosecretory proteins such as Synaptotagmin 1 and SNAP-25. Thus TSC2 controls proliferation, morphology and neurosecretory functions of nerve cells.

The ability of the TSC1/TSC2 complex to regulate β-catenin turnover was previously reported [39-41, 366]. The authors showed that β-catenin levels and activity is regulated by TSC1 and TSC2 and that β-catenin down-regulation could be due to the fact that TSC1 and TSC2 interact with components of the β-catenin degradation complex [40].

Also the link between β-catenin and REST was previously suggested. β-catenin has been shown to modulate transcription of the *REST* gene. Nishiara et al. [367] demonstrated that over-expression of β-catenin in chick spinal cord induces REST and Cyclin D1 [367].

The link between REST and TSC2, instead has never been reported in literature. We hypothesize that a possible mechanism through which REST could regulate TSC2 could be through REST regulation of a miRNA that targets TSC2 mRNA. REST has been found to repress the expression of non-coding RNAs such as miRNAs and maRNAs, acting at various posttranscriptional levels including RNA processing, editing and trafficking [368-371]. The possible effect of REST on non-coding RNAs that control TSC2 mRNA levels and/or stability could explain the fact that in PC12-27 cells a reduction in the protein levels of TSC2 was observed while TSC2 mRNA levels were not altered between wtPC12 and PC12-27 cells.

In collaboration with Prof. Meldolesi's group we have further studied the signalling loop composed by TSC2/β–catenin/REST. We demonstrated that favouring β–catenin activity mediates an increase in REST levels, a decrease in TSC2 and a faster proliferation rate. Likewise forcing REST expression in wtPC12 cells resulted in decreased levels of the TSC2 protein, β-catenin accumulation and again a proliferative advantage (Tomasoni et al., submitted).

By comparing wtPC12 and PC12-27 cells and also by genetically controlling expression level or activity of the different proteins involved, we unveiled a new loop

linking TSC2/β-catenin/REST. Of note this appears a feed forward loop as interfering with any of the players has direct consequences in the other two.

The consequences of the interconnection of the proteins of this loop should be further analysed as they could reveal of critical importance for the life of any cell. Further experiments performed in collaboration with Prof. Meldolesi's group demonstrated the existence of the loop also in another model of neural cells, the human NT2/D1 cells. This suggests that the pathway described can be present in other cellular models beside the PC12 cells. The existence of the TSC2/β-catenin/REST loop should be investigated in other cellular models such as primary cells, e.g. neurons or astrocytes. The existence of this pathway could be, in fact, a characteristic of transformed cells such as the PC12 and NT2/D1. Preliminary experiments performed in non-transformed TSC1ΔT and TSC1WT Neural Stem Cells (NSCs) by Laura Magri, a PhD student in Dr. Galli's group, suggest that there are no differences in β-catenin and REST levels in TSC1ΔT and TSC1WT NSCs.

The modification of the levels or activity of one of the three key players discussed above (such as nerve cell hyper-stimulation for REST [372]; activation of growth factor signalling for TSC2 [373]; activation of the canonical Wnt signalling for β-catenin [374]) would results in changes in the levels and activity of the others as well. So in analyzing the effect of the mutation of one factor researcher should take into consideration the possibility that the consequent responses could be on a bigger level.

Our results show how proliferation and neurosecretion can be regulated in opposite ways by a single signalling loop and it reflects what happens to rapidly proliferating nerve cells that lack almost completely neurosecretion.

The discovery of this new signalling loop could be of relevance also for therapeutic reasons. It suggests the use of combination of drugs that can address the different signalling pathways involved. The pathways we have studied are involved in a variety of

conditions such as cell growth, differentiation, function and metabolism [375, 376]. So the clinical approaches that could derive from inhibiting these factors could be of relevance in controlling proliferation and differentiation of nerve cells and in the prevention of neurodegeneration [377, 378].

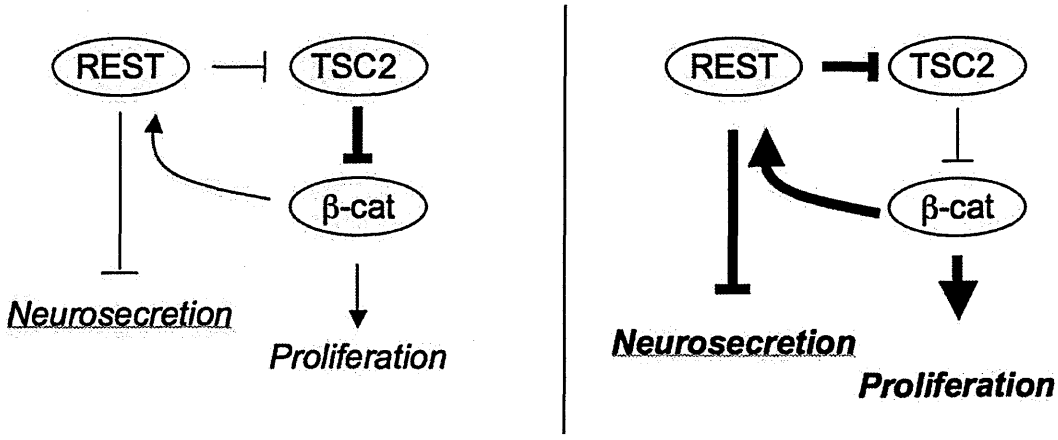

Fig IX. TSC2/β-catenin/REST signalling loop. PC12 cells (left) are characterized by high levels of TSC2 that lead to inhibition of β-catenin and consequently of REST. PC12-27 cells (right) are characterized by lower levels of TSC2 that thus cannot decrease β-catenin levels. β-catenin increased levels are accompanied by higher REST levels as well. The increased β-catenin and REST levels in the PC12-27 cells result in increased proliferation, positively controlled by β–catenin, and decreased neurosecretion, negatively controlled by REST.

5. General discussion

The TSC1/TSC2 and mTOR complexes are positioned at the crossroads of critical signalling cascades induced by growth promoting agents and energy and stress signals. The findings generated in this thesis underline some common mechanisms of regulation of proliferation and define new pathways by which TSC and mTOR might be interconnected to the acquisition or maintenance of functions typical of differentiated cells.

Under growth promoting conditions Akt becomes phosphorylated and by that inhibits the TSC1/TSC2 complex. This allows both mTORC1 and mTORC2 signalling, which controls aspects of cell growth, proliferation, differentiation and survival. mTORC1-dependent activation of p70S6K, in turn, prevents uncontrolled Akt-dependent cell proliferation by a negative feedback loop, involving IRS/PI3K and also other yet to be defined signalling modules, and leading to the inhibition of Akt phosphorylation and thus reactivation of the TSC complex. The negative feedback loop was originally described in MEFs WT or KO for TSC2 [127], but our data support the notion that it is also active in primary TCR/CD28-activated primary T cells. In contrast, the negative feed-back loop cannot be operational in TSC deficient cells, in which mTORC1 is constitutively active leading to increased proliferation.

Loss of TSC1 or TSC2 also hinders mTORC2 activity and by that Akt phosphorylation in Ser473, critical for full potential of the kinase. Indeed, reduced Akt signalling limits the uncontrolled growth of tumours lacking TSC2 [379, 380] and favours apoptosis because of the de-regulation of death-promoting factors such as the FoxO proteins.

Our data are in line with the model described above. We have shown that mTOR and TSC1/TSC2 are important for the proliferation of both primary T cells and established nerve cells. We have demonstrated that in cells in which mTOR is chemically inhibited by Rapa, proliferation is delayed and differentiation is strongly affected. Thymocytes and

mature lymphocytes in which TSC1 has been genetically silenced show an increase in proliferation. Moving to the PC12 cells model of nerve cells we observed an increase in the proliferation of PC12-27 cells that show reduced TSC2 levels compared to the wt clone and we proved TSC2 relevance for PC12 proliferation also by ShRNA mediated silencing of TSC2.

The way TSC1/TSC2 complex and mTOR can control proliferation or survival may differ according to the cell type. While in mature T cells the increased proliferation seems to be dependent on hyperactivation of mTORC1 as demonstrated by the fact that administration of Rapa could revert the increased proliferation of LCK-CRE/TSC1ΔT lymphocytes, in the PC12 cells we have demonstrated that the increased cell proliferation was due to increased β-catenin signalling. Furthermore, in mature CD4+ T cells TSC1/TSC2 balances proliferation and cell death by regulating mTORC1 and mTORC2, respectively. Whether loss of TSC1 has a direct impact on mTORC2 activity or whether it is indirect via the feedback loop from mTORC1 remains to be clarified. Regardless, loss of TSC1 favoured mTORC1 hyperactivation and loss of mTORC2-dependent Akt phosphorylation of FoxOs, and by that increased TRAIL expression and TRAIL-dependent cell death.

In PC12 cells loss of TSC2 leads to stabilization of β-catenin promoting cell proliferation, and caused an upregulation of REST (Tomasoni et al., submitted) leading to the shut off of neurosecretory functions. The effect of TSC2 on β-catenin in PC12 cells seems to differ from the possible activity of the TSC1/TSC2 complex in thymocytes from LCK-CRE/TSC1ΔT mice. While β-catenin targets c-Myc and Cyclin D1 were lower in cells from LCK-CRE/TSC1ΔT mice, these targets were expressed at higher level in PC12-27 cells characterized by low level of TSC2. This could be explained by the fact that the TSC1/TSC2 complex controls β-catenin stability either by direct regulation or by its indirect activity on Gsk3β. From the results reported above it is reasonable to hypothesise

that in LCK-CRE/TSC1ΔT mice low levels of TSC1/TSC2 complex lead to decreased phosphorylation and activation of Akt that in turn cannot phosphorylate and thus inhibit Gsk3β. This would lead to activation of Gsk3β that then would de-stabilize β-catenin. In PC12-27 cells, instead, Gsk3β control of β-catenin does not seem to play a crucial role. Even if these cells show decreased phosphorylation of Gsk3β and thus the kinase should be more active on its target β-catenin, the PC12-27 cells show increased levels and activity of β-catenin. Experiments performed on wtPC12 and PC12-27 cells using LiCl, an inhibitor of Gsk3β, showed that while LiCl could increase β-catenin activity, evaluated by luciferase assay, in wtPC12 cells, LiCl treatment had little effect in PC12-27 cells, again hinting at the fact that in these cells probably it's the direct control of TSC2 on β-catenin levels that plays a critical role in the regulation of this protein.

The picture that appears from the results obtained with thymocytes, mature primary T cells and nerve cells clones depicts a situation in which the interaction among different signalling cascades can control in a coordinated way many different cellular processes. By touching one signalling pathway also the others are perturbed and thus the many diverse processes controlled by all of them. The different complexes thus orchestrate these most diverse processes in a finely regulated way. In case of alteration of this delicate balance the consequences for the cell can be severe. The unbalance towards excessive proliferation can lead to uncontrolled growth typical of many aggressive tumours. The loss of differentiation can have important effects during development and results in dysfunctions of many systems.

The processes described above are at the basis of most severe pathologies such as tumours growth, neurological and immune diseases. By understanding the molecular mechanisms underlying these conditions and how different signalling molecules are involved in the generation of these illnesses could help in finding new therapeutic

protocols that act by addressing the different pathways involved in the generation of the diseases.

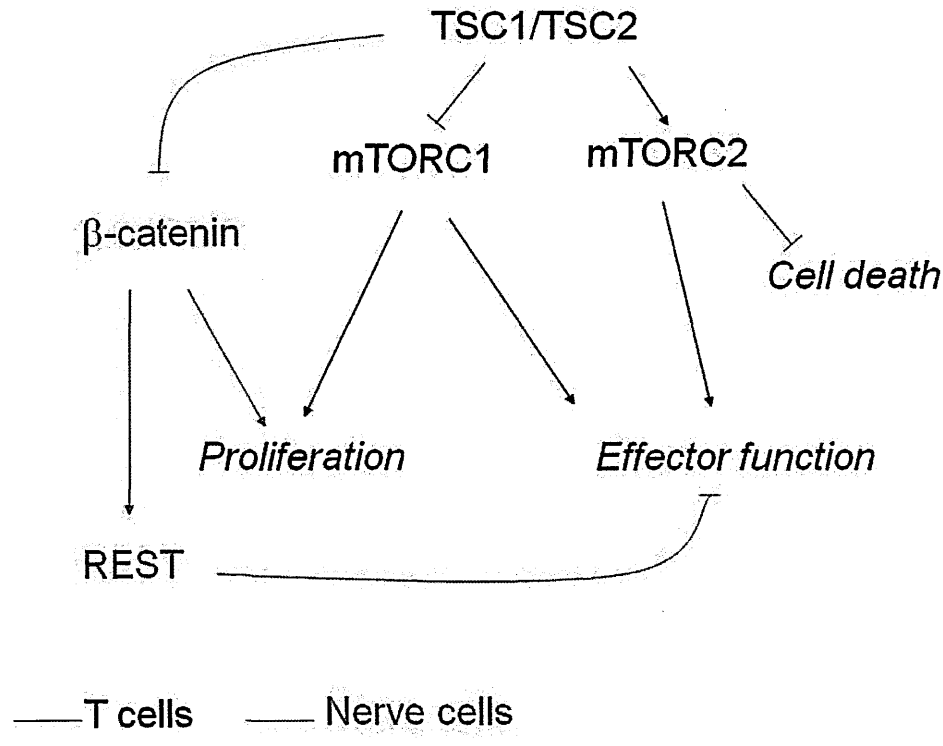

Fig X. TSC/mTOR pathway in T and nerve cells proliferation and differentiation. The TSC1/TSC2 complex can control both mTORC1 and mTORC2 and β-catenin. By inhibiting mTORC1 and β-catenin and activating mTORC2 it can affect different cellular processes such as proliferation and differentiation of both T cells and nerve cells. In our model the effect of the TSC complex on the mTOR complexes plays a central role in T cells biology, while the effect of the TSC complex on β-catenin and REST is important in nerve cells functions.

6. Abbreviations

3-phosphoinositide-dependent protein kinase 1	PDK1
AMP-activated protein kinase	AMPK,
Antigen	Ag
Antigen-Presenting Cells	APCs
Bovine Serum Albumin	BSA
Brain Lipid Binding Protein	BLBP
ChromograninB	ChgB
Cyclin-Dependent Kinase 1	CDK1
Cyclosporine A	CSA
DEP-domain-containing mTOR-interacting protein	Deptor
DNA methyltransferase	Dnmt
Double Negative	DN
Double Positive	DP
Early-phase Long Term Potentiation	E-LTP
Epidermal Growth Factor	EGF
eukaryotic Elongation Factor 2 Kinase	eEF-2K
eukaryotic Initiation Factor 4E	eIF4E
eIF4E-binding protein 1	4E-BP1
Fibroblast Growth Factor 2	FGF2
FKBP12–Rapamycin Binding domain	FRB
Glycogen Synthase Kinase 3β	GSK3β
Huntington's disease	HD
Immunoreceptor Tyrosine-based Activation Motifs	ITAMs
Insulin Receptor Substrate 1	IRS1
Intermediate Single Positive	iSP

Linker for Activation of T cell	LAT
Long Term Depression	LTD
Lymphangioleiomyomatosis	LAM
Lymphocytic choriomeningitis virus	LCMV
Major Histocompatibility Complex	MHC
mammalian Lethal with Sec13 protein 8	mLST8
mammalian stress-activated protein kinase interacting protein	mSIN1
mammalian Target of Rapamycin	mTOR
medullary Thymic Epithelial Cells	mTECs
messenger RNA	mRNA
Methylated DNA Immunoprecipitation	MeDIP
mTOR Complex 1	mTORC1
mTOR Complex 2	mTORC2
Negative Regulatory Domain	NRD
Neural Stem Cell	NSC
Neurofibromatosis type 1	NF1
p70S6 Kinase	S6K
Parkinson's disease	PD
Peroxisome Proliferator-Activated Receptor-γ	PPARγ
Phospho-Inositide 3-Kinase	PI3K
PI3K-related Kinase	PIKK
Pleckstrin Homology	PH
PMA and Ionomycin	P/I
Polymerase I	Pol I
Programmed Cell Death 4	PDCD4
Proline-Rich Akt Substrate 40 kDa	PRAS40
Promyelocytic leukemia	PML

Protein Kinase Cα	PKCα
Rapamycin	Rapa
Ras homolog enriched in brain	Rheb
RE-1-specific Silencing Transcription factor	REST
regulatory-associated protein of mTOR	raptor
Retinoblastoma	Rb
Ribosomal Protein genes	RP genes
RAPTOR-independent companion of mTOR	Rictor
S6K1 aly/REF-like target	SKAR
Serum- and Glucocorticoid-induced protein Kinase 1	SGK1
Signal Transducer and Activator of Transcription	STAT
Single Positive	SP
Small-Cell Lung Cancer	SCLC
Stem Cell Factor	SCF
Sterol Regulatory Element Binding Protein 1	SREBP1
Subventricular zone	SVZ
T Cell Factor	TCF
T Cell Receptor	TCR
T Cell Receptor Interacting Molecule	
TRIM	
Tract of oligopyrimidine	TOP
Transcription Initiation Factor IA	TIF-IA
Tuberous Sclerosis complex	TSC
Unfolded Protein Response	UPR
Untranslated regions	UTRs
Very Late Antigen 4	VLA4
ζ-chain associated protein of 70 kDa	ZAP-70

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