Study of the molecular mechanisms responsible for E2F1-induced mTORC1 activation

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Study of the molecular mechanisms responsible for E2F1-induced mTORC1 activation

A thesis submitted by Nathalie Meo Evoli for the fulfillment of the degree of *Doctor of Philosophy*

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Introduction

Cancer progression is a multistep process of acquisition of several biological capabilities which allows normal cells to evolve to a neoplastic state. The malignant state can be defined by the ability of cancer cells to survive, proliferate and disseminate. The acquisition of these properties can occur at various times during tumorigenesis and via different mechanisms in distinct tumor types, but it is made possible by two enabling characteristics: firstly, the development of genomic instability in cancer cells, which is necessary to create random mutations that can enable hallmark capabilities; secondly, an enabling characteristic implicates the inflammatory state of the neoplastic mass which serves to promote tumor progression. At least six different hallmark capabilities of cancer cells which are acquired during tumorigenesis have been summarized:

sustaining proliferative signaling, resisting cell death, evading growth suppressors, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis (Hanahan and Weinberg, 2011). Although it is true that all these characteristics are necessary for cancer progression, each different tumor can be more dependent on one or more particular capabilities. As the deregulation of the biological functions is due to a specific setting of mutations in each tumor, the understanding of the interconnections between the mutations, the molecular pathways, and the biological capabilities can help us to address the research study toward the development of new cancer drugs against therapeutic targets in a more tumor-specific manner.

Probably, the fundamental feature of neoplastic cells is the ability to sustain chronic proliferation. In normal tissues, the homeostasis of cell number and size is maintained by controlling the production of growth-promoting signals that drive the entry into the cell growth-and-division cycle. Cancer cells are able to continuously regulate progression through the cell cycle as well as cell growth (that is, increase in cell size) through the deregulation of these signals. Thus, many tumors contain mutations resulting in constitutive activation of signaling pathways usually initiated by activated growth factor receptors. At the same time, cancer cells

must acquire the ability to evade the cellular programs which normally act to counteract the proliferative signalings. Many of these programs are controlled by the actions of the tumor suppressor genes which serve as stress sensors that can trigger apoptosis or senescence. The Rb-E2F and MDM2-p53 pathways are among the most frequently mutated in human tumors. They operate in two complementary networks by integrating signals from various extracellular and intracellular sources and, in response, they control either the cell progression through the growth-and-division cycle or the activation of senescence or apoptotic programs (Burkhart and Sage, 2008).

The "pocket protein" RB has been described more than 20 years ago as a crucial negative regulator of the cell cycle through the inhibition of the E2F transcription factor. The role of RB in the progression from the G1 to the S cell cycle phase is due to its capacity to bind to the E2F family proteins and hence inhibit E2F-dependent transcription of genes that promote DNA synthesis and cell cycle advancement (Dick and Rubin, 2013).

In addition to the well-described role of the E2F family in the regulation of the cell cycle, during the last few years many other functions associated with this family of transcription factors have been emerging. In particular,

recent works showed that the increased E2F1 expression is predominantly associated with high-grade tumors or metastases and unfavorable patient survival prognosis, suggesting that E2F1's oncogenic properties extend beyond the simple ability to stimulate aberrant growth of neoplastic cells (Alla et al., 2010; Lee et al., 2010).

In our research, we focused on the study of the oncogenic properties of E2F1 not related to the cell cycle progression, and we aimed at investigating whether the activity of this oncogene could regulate other biological processes that can contribute to tumor promotion and progression. Specifically, one of the new targets that we found to be regulated by E2F1 is the mTORC1 signaling, and we are interested in investigating the molecular mechanism underlying this finding. The mTORC1 pathway functions as one of the major sensors of the nutritional and energetic conditions of the cells and carefully controls several biosynthetic and catabolic processes. Given the pivotal importance of this pathway and its implication in cancer, it could be possible that the oncogenic properties of E2F1 could be associated with its ability to regulate mTORC1.

E2F1 signature is emerging as being associated with the invasive phenotype and metastasis, as mentioned above, but the molecular mechanisms underlying this process are not known to date. The capability of acti-

vating invasion and metastasis to promote tumor progression is one of the hallmarks of cancer. This malignant property is acquired during a multistep process named the invasion-metastasis cascade. The cascade involves a succession of cell-biologic events which starts with cancer cells local invasion of the stroma, then penetration into the blood or lymphatic vessels, and circulation to distant sites. The dissemination is followed by the escape of cancer cells from vessels to form small nodules (micrometastases) that can finally grow into macroscopic tumors, this last step being termed "colonization" (Talmadge and Fidler, 2010).

Several biological processes are involved in the invasion-metastasis cascade. Firstly, during local invasion cancer cells often present alterations in their shape as well as in their attachment to adjacent cells and to the extracellular matrix (ECM) (Berx and van Roy, 2009). In addition, a developmental regulatory program, known as the "epithelial - mesenchymal transition" (EMT), has been shown to be implicated in orchestrating most events of the invasion-metastasis cascade except the final step of colonization (Micalizzi et al., 2010). Another process involved in the facilitation of invasion is the inflammation of the tumors' boundaries. The assembly of macrophages at the tumor periphery can support local invasion by producing the extracellular matrix-degrading enzymes such as

metalloproteinases and cysteine cathepsin proteases (Joyce and Pollard, 2009). Finally, the acidification of the extracellular milieu of tumors is another crucial mechanism related to invasion and metastasis. The low extracellular pH (pH_e) is required for the activation of several types of proteases including cathepsins, metalloproteases and gelatinases. Accordingly, solid tumors show a characteristic extracellular acidosis, with pH values as low as 6.5 or 6. pH homeostasis in any cell type is a complicated process that involves many proteins and buffer systems. In tumor cells, these mechanisms are even more complex as they have evolved to maintain the intracellular compartment slightly more alkaline (pH_i 7.4 or more) and the extracellular environment more acidic than in normal cells. Since a change in the pH_i/pH_e ratio as low as 0.1 can affect several biological processes such as enzyme function, proliferation, migration, invasion and metastasis of cancer cells, a tight regulation of the tumor-specific pH homeostasis has evolved (Neri and Supuran, 2011). A better understanding of the sophisticated molecular mechanisms responsible for maintaining the alkaline pH_i and the acidic pH_e in tumor cells could help us to find new therapeutic targets to restore both pH towards normal values and inhibit tumor growth.

1.1 The E2F family of transcription factors

The E2Fs are a large family of transcription factors which consists in the effectors of the "pocket protein" RB. This family plays a crucial role in cell cycle progression, but, moreover, is involved in many other biological processes such as apoptosis, differentiation, development, and growth. In mammals, the family is composed of 8 different members that belong to activator and repressor subclasses. *E2F1* was the first member of the family to be cloned. In this work, we aim at studying the oncogenic functions of E2F1 beyond the ones already recognized.

1.1.1 The E2Fs proteins

The E2Fs are a family of transcription factors containing one or more conserved DNA binding domains (DBDs) that allow the binding to various target promoters. Based on results from several in vitro studies, the E2F family has been traditionally divided into activator (E2F1-E2F3) and repressor (E2F4-E2F8) subclasses, as it is shown in Fig.1.1.

Most E2F family members (E2F1-E2F6) bind to DNA as heterodimers with one of three dimerization partner (DP) proteins, TFDP1, TFDP2 and TFDP3, whereas heterodimerization is mediated by the leucine zip-

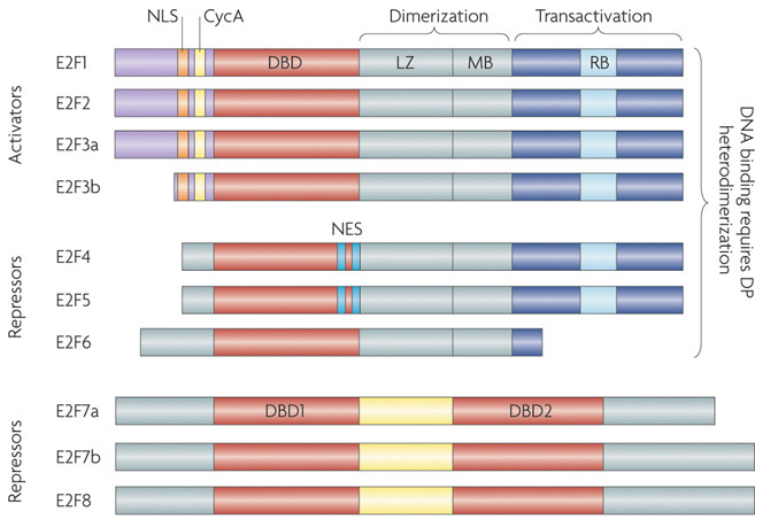

Figure 1.1: The mammalian E2F family of transcription factors. The 8 members of the E2F family transcription factors are expressed from eight chromosomal loci that encode nine different gene products (Chen et al., 2009).

per (LZ) and marked box (MB) domains. E2F1-E2F3 bind exclusively to RB within the transactivation domain (RB) and show an amino-terminal nuclear localization signal (NLS) sequence. Unlike the activators, E2F4 associates with all three pocket protein family members and E2F5 binds mainly to p130, while both have nuclear export signals (NES) that mediate their export to the cytoplasm. Repressors E2F6-E2F8 do not possess the transactivation domain, and so they repress the E2F-responsive genes without binding to the pocket proteins (Chen et al., 2009). E2F7 and E2F8 can also specifically mediate the repression of E2F activators such

as E2F1. In particular, they can directly bind as heterodimers or homodimers to the E2F1 promoter and repress its expression during S and G2 phases to restrain E2F1's function (Di Stefano et al., 2003).

1.1.2 The cell cycle regulation by the E2F family

The first function that was described for the E2F family is the regulation of the cell cycle (De Gregori et al., 1995). The timely movement of cells through the four cell cycle phases is regulated by the cyclin-CDK complexes that are responsible for the post-translational modifications of RB and related pocket proteins. These modifications coordinate the subsequent waves of E2F-dependent transcription activation and repression during the distinct phases (Murray, 2004) (Fig. 1.2).

In particular, in quiescent (G0) cells, the repression of the E2F-responsive genes is achieved by the ubiquitously expressed E2F4 and E2F5 bound to pocket proteins and other co-repressors. Upon mitogenic stimulation, the release of E2F repressors is due to the sequential phosphorylation of RB by activated cyclin-dependent kinases. These post-translational modifications lead to the loss of RB function and thus result also in the accumulation in G1 phase of newly synthesized free E2F1, E2F2 and E2F3. The released E2F activators are therefore able to initiate a tran-

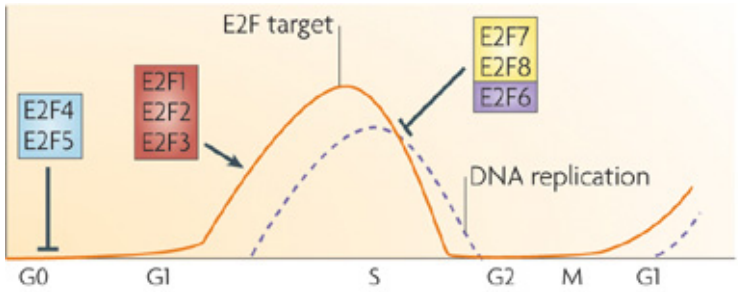

Figure 1.2: The activity of E2F family members during the cell cycle. The phosphorylation of the pocket proteins causes a shift in the activity of the E2F repressors and activators which drives the progression through the different phases of the cell cycle. Adapted from Chen et al., 2009.

scriptional program driving cells into S phase. The transcriptional activity of E2F activators is then attenuated a the end of S phase and in G2 by the action of the repressors E2F6, E2F7 and E2F8 independent of pocket protein binding. At the conclusion of G2 phase, RB is dephosphorylated and can sequester again the E2F activators (Chen et al., 2009).

It has been shown that in quiescent or differentiated cells, the association of the pocket protein-bound E2F4 and E2F5 with several co-repressors, such as histone deacetylases and the DNA methyltransferase DNMT1, leads to chromatin compaction and transcription inhibition. On the contrary, when RB is hyperphosphorylated, the open chromatin configuration and the transcription initiation are sustained by the recruitment to specific gene promoters of the transcription factor TFIID and other co-

activators, such as histone acetyltransferases, p300 and CBP, together with E2F activators (Brehm et al., 1998; Taubert et al., 2004).

1.1.3 The role of E2F in apoptosis

In addition to the extensive knowledge of E2F role in cell proliferation, it is important to note that a number of E2F family members are known to be responsive to DNA damage and thus, can act as tumor suppressors. More than 15 years ago, it was shown that E2F1 is induced in response to various DNA-damaging agents, including ionizing radiation, UV radiation, and a number of chemotherapeutic drugs (Blattner et al., 1999). This response primarily involves an increase in E2F1 protein stability and, at least in some cases, is associated with the induction of apoptosis. Indeed, it is well known that the ectopic expression of E2F1 alone can trigger apoptosis in cultured mammalian cells (Johnson and DeGregori, 2006).

Although the ability to induce apoptosis was traditionally attributed to E2F1, recent works showed that E2F2 and E2F3 can also mediate apoptosis induction (Opavsky et al., 2007; Martinez et al., 2010). Even though the three E2F activators are induced and stabilized after DNA damage, it seems that at least the ability of E2F3 to promote apoptosis

is mediated through the transcriptional upregulation of E2F1. E2F7 and E2F8 are also upregulated in response to DNA-damaging drugs, but, in this case, E2F7 and E2F8 suppress apoptosis by repressing the *E2F1* gene promoter (Martinez et al., 2010; Zalmas et al., 2008).

The mechanism by which E2F1 can induce apoptosis has been well studied and it is known that the tumor suppressor behavior of E2F1 is associated with its capacity to activate the p53 or p73 pathways of intrinsic cell death (Biswas and Johnson, 2012). A number of post-translational modifications on E2F1 allow it to respond to DNA damage and induce cell death. Specifically, E2F1 is phosphorylated by ATM (ataxia telangiectasia mutated)/ATR (ataxia-telangiectasia and Rad3-related) and checkpoint kinase 2 (CHK2), three kinases that are activated by double strand DNA breaks. E2F1 is also acetylated by p300/CBP associated factor (PCAF; also known as KAT2B), an acetyltransferase that has been implicated in transcriptional control. Furthermore, several basic residues of E2F1 are also demethylated in response to DNA damage signaling. These damage-inducible post-translational modifications play key roles in regulating E2F1 stabilization and its interactions with protein partners, as well as with specific sites in the genome, such as the p73 gene promoter (Biswas and Johnson, 2012).

In addition to p73 induction, E2F1 can also regulate directly the expression of other apoptotic genes, such as caspases, Apaf1 and Bcl-2 homology region 3 (BH3)-only proteins (Hershko and Ginsberg, 2004; Moroni et al., 2001). Moreover, the E2F1-induced apoptotic cell death can be also mediated by p53 activation. This effect is mostly due to the capacity of E2F1 to activate the transcription of the tumor suppressor $p14^{ARF}$. This protein drives the accumulation of p53 via the direct association and inhibition of the p53-ubiquitin ligase, MDM2. Hence, E2F1 can trigger p53 accumulation by inducing the expression of $p14^{ARF}$ which blocks the MDMD2-dependent degradation of p53 (Parisi et al., 2002).

Finally, results from our group have shown that the intrinsic pathway, but not the extrinsic one, is involved in E2F1-induced apoptosis. Specifically, E2F1 activation promotes apoptosis through the accumulation of ROS, the consequent Bax oligomerization and translocation to the mitochondria, as well as by upregulating BimL expression levels (Espada et al., 2012).

1.1.4 Functions of E2F in differentiation and development

Several laboratories were able to identify many E2F-responsive genes beyond those involved in proliferation, DNA repair and apoptosis, including genes that participate in biological processes as diverse as cell differentiation, metabolism, and animal development.

Various studies on KO animal models highlighted key roles of different E2Fs in differentiation and development. For example, E2F1-3 are involved in cell survival, proliferation and development of myeloid cells (Trikha et al., 2011). E2F3 specifically mediates neuronal migration and differentiation *in vivo* (Andrusiak et al., 2011; McClellan and Slack, 2007). E2F4 promotes adipocyte differentiation and mediates the development of the ventral telencephalon through a genetic interaction with the Sonic Hedgehog morphogenetic pathway (Landsberg et al., 2003; McClellan and Slack, 2007). E2F6 participates in the recruitment of Polycomb proteins to specific target promoters driving homeotic transformations during the development of the axial skeleton (Storre et al., 2002).

1.1.5 E2F1 functions in autophagy

The role of E2F1 in autophagy regulation is controversial. Some reports pointed out that E2F1 promotes autophagy by upregulating the expression of autophagy genes such as *LC3*, *ATG1*, *ATG5* and *DRAM* (Polager et al., 2008). According to this theory, others claimed that the transactivation domain of E2F1 is not essential for the induction of autophagy driven by the oncogene (Garcia-Garcia et al., 2012), and identified *BNIP3* as a direct E2F1 target gene required for hypoxia-induced autophagy (Tracy et al., 2007). On the other hand, others showed that downregulation of E2F1 results in high levels of autophagy, suggesting that regulation of Bcl-2 expression by E2F1 is involved in this process (Jiang et al., 2010).

As autophagy can have conflicting effects on tumor progression, either acting as a barrier to tumorigenesis or promoting autophagic cell death depending on the conditions (White and DiPaola, 2009), it would be useful to understand the role of autophagy in tumor cells where the Rb-E2F pathway is deregulated.

1.1.6 Role of E2F1 in metabolism

Recently, it has been shown that E2F1 is able to repress key genes that regulate energy homeostasis and mitochondrial functions, adding a new function to the oncogene in metabolism regulation.

Specifically, E2F1 knockout mice exhibit a switch from glycolytic to oxidative metabolism (Blanchet et al., 2011). The hypothesis of E2F1 being a promotor of the glycolytic flux is also supported by other data demonstrating that E2F1 regulates the transcription of the glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. This enzyme controls the levels of fructose-2,6-bisphosphate that allosterically activates the first rate-limiting glycolytic enzyme, phosphofructokinase-1 (Fernández de Mattos et al., 2002). Moreover, E2F1 contributes to lipid synthesis, glucose production and insulin secretion, thus having a strong impact on overall metabolism regulation (Escoté and Fajas, 2014).

1.1.7 E2F1 in human cancer

The amplification of the *E2F1* or *E2F3* gene locus has been reported as a frequent genetic event in a high number of malignancies, such as hepatocellular carcinoma, bladder cancer, retinoblastoma, liposarcoma,

and melanoma. Overexpression of *E2F1* or *E2F3* has also been detected in glioblastoma and lung, ovarian, breast, gastric and colon cancer (Chen et al., 2009). The kind and frequency of mutations of E2F1 alone in several cancer types are shown in Fig. 1.3.

Interestingly, the increased E2F1 expression is predominantly associated with high-grade tumors or metastases and poor patient prognosis, suggesting that E2F1 oncogenic properties extend beyond the simple ability to stimulate aberrant proliferation of neoplastic cells. Regarding this

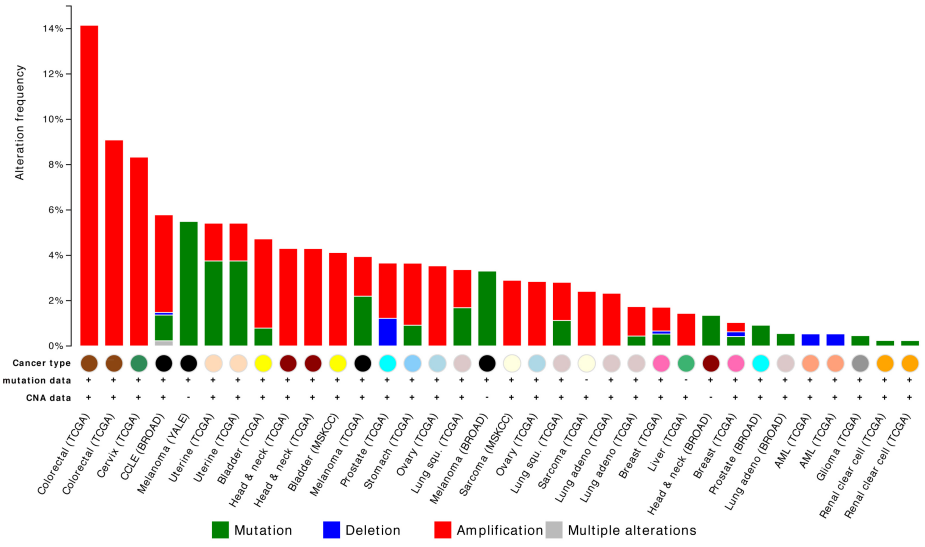

Figure 1.3: Cross-cancer alterations summary for E2F1. The most frequent alterations in human cancer for *E2F1* locus are amplifications. Adapted from cBioPortal for Cancer Genomics.

aspect, it has been shown that E2F1 overexpression drives melanoma progression and promotes the invasiveness of the metastatic cell line (Alla et al., 2010). Alla and coworkers reported that E2F1 depletion is associated with an increased expression of E-cadherin and with a reduction of cell invasion and motility, without having any effect on growth rates. While *in vivo*, E2F1-knockout cells showed a drastic reduction in metastatic growth in xenografts. Other works reported that high expression of E2F1 and its associated target genes are strong indicators for the invasive progression of breast and bladder tumors (Lee et al., 2010; Zhang et al., 2000). Presently, it is not known how E2F1 accomplishes tumor progression and how it can promote invasiveness and metastasis.

1.1.8 The role of E2F1 in cell growth

Results from our research group demonstrated that human E2F1 induces cellular growth by modulating mTORC1 activity. We showed that the activation of cell growth and mTORC1 by E2F1 is dependent on both E2F1's ability to bind to DNA and to regulate gene transcription, and that moreover, the effect is independent of the canonical PI3K/Akt/TSC1-TSC2 pathway (Real et al., 2011).

Specifically, we used the 4-hydroxitamoxifen inducible system described

in section 3.1.1, and we demonstrated by flow cytometry that E2F1 activation promotes cell size increase in G1 cells. The change in size observed is similar to the one detected with serum addition, while rapamycin treatment completely blocks the growth effect of E2F1, confirming that mTORC1 is a mediator of this effect (Fig. 1.4).

E2F1 overexpression causes the activation of the mTORC1 downstream targets, S6K1 and S6, but not the phosphorylation of TSC2 and AKT, at odds with insulin treatment (Fig. 1.5). Moreover, either the overexpression of TSC2 or the knockdown of Akt do not interfere with E2F1-induced mTORC1 activation (Fig. 1.6A-B), thus corroborating that the Akt/TSC1-TSC2 pathway is not involved in the activation of mTORC1 triggered by E2F1.

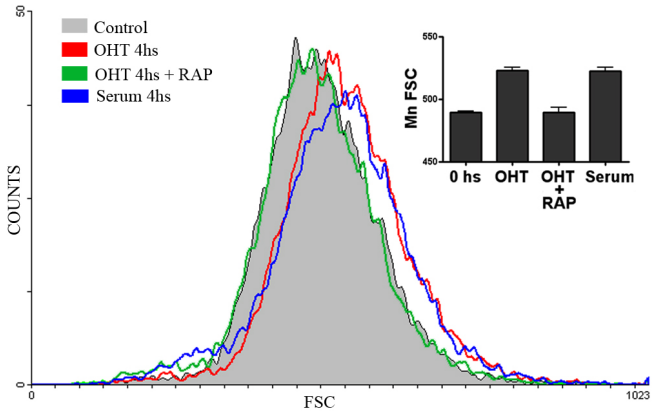

Figure 1.4: E2F1 controls cellular size. Adapted from Real et al., 2011.

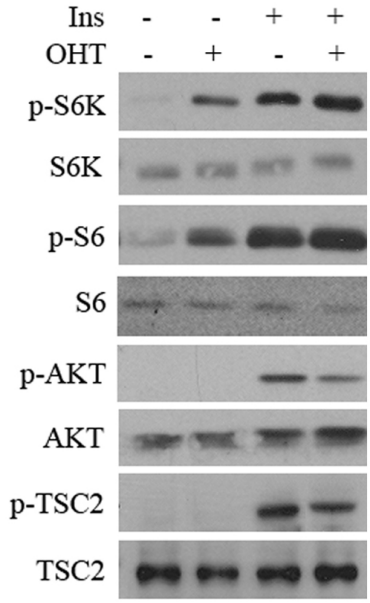

Figure 1.5: E2F1 activates mTORC1 downstream targets, but not insulin pathway effectors. Adapted from Real et al., 2011.

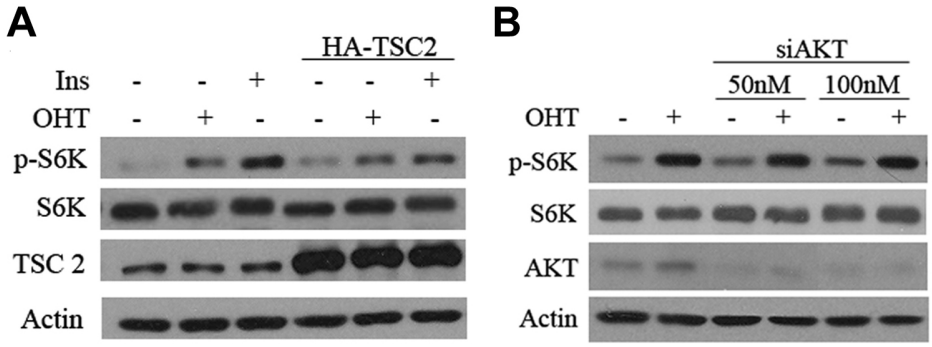

Figure 1.6: Both TSC2 overexpression and Akt interference do not interfere with E2F1-induced mTORC1 activation. Adapted from Real et al., 2011.

1.2 The mTOR signaling

TOR (*target of rapamycin*) is a serine/threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family. It is a highly conserved protein from yeast to human which responds to several environmental cues, including growth factors, nutrients, energy status as well as stress. Under favorable conditions, TOR promotes cell growth by stimulating biosynthetic pathways, including protein synthesis, and by inhibiting cellular catabolism, such as through repression of the autophagy pathway.

Mammalian TOR (mTOR) interacts with several proteins to form two different complexes termed mTOR complex 1 (mTORC1) and 2 (mTORC2). The two complexes have different sensitivities to rapamycin as well as upstream regulators and downstream effectors. The mTORC1 complex is composed of: mTOR, regulatory-associated protein of mTOR (RAPTOR), mammalian lethal with SEC13 protein 8 (mLST8; also known as GβL), 40 kDa Pro-rich AKT substrate (PRAS40) and DEP domain-containing mTOR-interacting protein (DEPTOR), the former two being positive regulators and the latter two being negative regulators of mTORC1. The mTORC2 complex consists of mTOR, mLST8, DEPTOR, rapamycin-insensitive companion of mTOR (RICTOR), mam-

malian stress-activated map kinase-interacting protein 1 (mSin1) and protein observed with rictor 1 and 2 (protor 1/2) (Laplante and Sabatini, 2012). Rapamycin binds to the FKBP12 protein and forms a gain-of-function complex which interacts with and inhibits mTOR when it is part of mTORC1 but not mTORC2.

Compared to mTORC1, much less is known about the mTORC2 pathway. mTORC2 signaling has been traditionally described to be insensitive to nutrients, but responsive to growth factors like insulin. However, it has been recently shown that in certain starvation conditions mTORC2 can respond to amino acids stimulation, via PI3K/Akt signaling activation (Tato et al., 2011). A potential upstream mechanism of mTORC2 regulation involving ribosomes has been described, where insulin-stimulated PI3K signaling promotes the binding of mTORC2 to ribosomes and thus, its activation (Zinzalla et al., 2011). Among the downstream effectors of mTORC2 there are several members of the AGC subfamily of kinases including Akt, serum- and glucocorticoid-induced protein kinase 1 (SGK1), and protein kinase C-α (PKC-α). In particular, mTORC2 directly phosphorylates Akt in Ser473, a site required for its maximal activation (Sarbassov et al., 2005). Together with other effectors such as paxilin and Rho GTPases, the activation of PKC-α by mTORC2 reg-

ulates the cell shape by controlling the actin cytoskeleton assembly (Jacinto et al., 2004). Much more is known about the downstream effectors and the upstream regulators of mTORC1, which we will summarize in the next sections.

1.2.1 The downstream effectors of mTORC1

Among the various processes controlled by the mTORC1 complex we can enumerate protein synthesis, lipid synthesis, energy metabolism, autophagy, and lysosome biogenesis (Fig. 1.7).

The downstream targets directly phosphorylated by mTORC1 which promote protein synthesis are the translational regulators eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and S6 kinase 1 (S6K1). Translation initiation is a limiting step in protein synthesis, where the recruitment of the small ribosomal subunit to mRNA requires the assembly of the eukaryotic translation initiation factor 4F (eIF4F) complex on the 5' cap structure of mRNA (Laplante and Sabatini, 2012).

The mTORC1 phosphorylation of 4E-BP1 prevents its binding to the cap-binding protein eIF4E. The free eIF4E is therefore able to bind to

eIF4G and so to participate in the formation of the eIF4F complex which is required for the initiation of cap-dependent translation.

The activation of S6K1 by phosphorylation leads, through a variety of effectors, to an increase in mRNA biogenesis, as well as translational initiation and elongation. The main targets of S6K1 are the ribosomal protein S6 (RPS6), a component of the 40S ribosomal subunit, the eukaryotic translation elongation factor 2 kinase (eEF2K), the cap-binding protein 80 (CBP80), and the eukaryotic translation initiation factor 4B (eIF4B). S6K1 also phosphorylates PDCD4 (programmed cell death 4), a tumor suppressor that binds to eIF4A, and SKAR, a mediator of splicing and mRNA transport (Ma and Blenis, 2009). All these post-translational modifications regulated by S6K1 facilitate the assembly of the pre-initiation complex and the cap-dependent translation. Moreover, mTOR and S6K1 together activate the regulatory element tripartite motif-containing protein-24 (TIF-1A), promoting its interaction with RNA Polymerase I (Pol I) and the expression of ribosomal RNA (rRNA) (Mayer et al., 2004). Finally, mTORC1 directly phosphorylates and inhibits Maf1, a RNA polymerase (pol) III inhibitor, and so induces the synthesis of tRNA and 5S rRNA by Pol III (Kantidakis et al., 2010).

mTORC1 not only regulates the production of proteins, but also controls

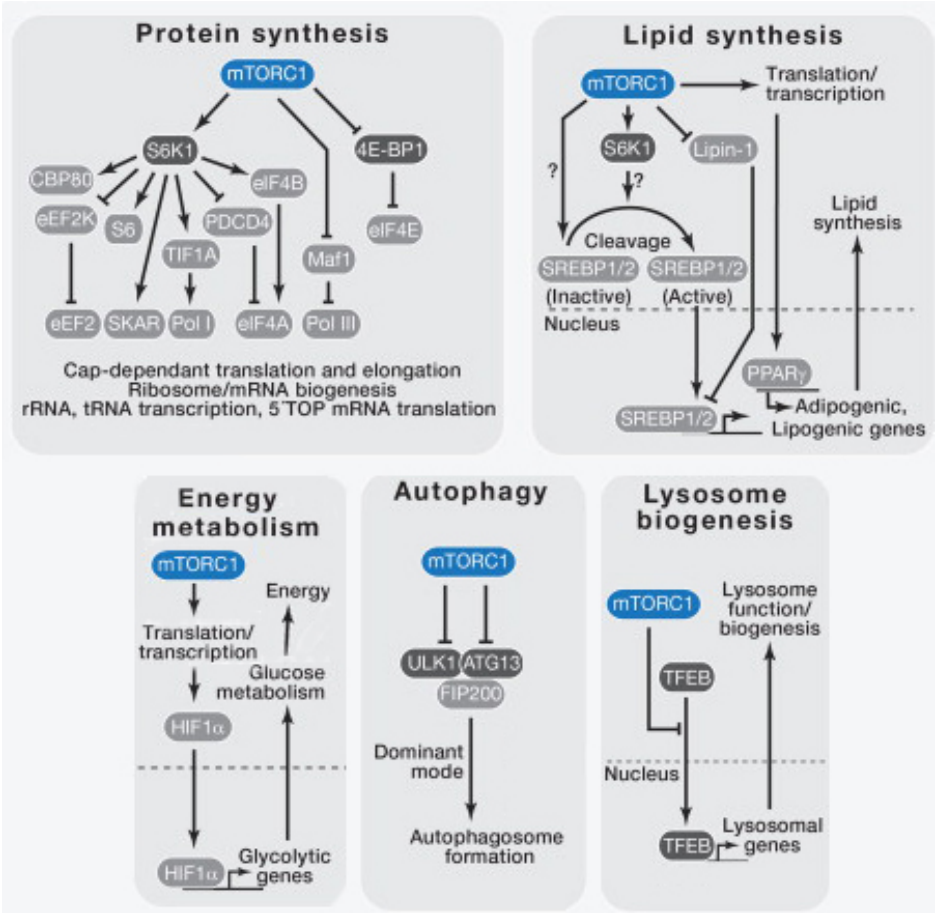

Figure 1.7: The mTORC1 downstream effectors. The main downstream effectors of mTORC1 in regulating protein synthesis, lipogenesis, energy metabolism and autophagy. Adapted from Laplante and Sabatini, 2012.

the biosynthesis of lipids which are required to generate membranes in proliferating cells. Together with Akt, mTORC1 is necessary to promote nuclear accumulation of the sterol regulatory element binding protein 1/2

(SREBP1/2) transcription factors that control the expression of several genes involved in fatty acid and cholesterol synthesis (Porstmann et al., 2008). In addition, mTORC1 phosphorylates Lipin-1, preventing it from entering the nucleus and suppressing SREBP1/2 function and levels (Peterson et al., 2011). Finally, mTORC1 also promotes the expression and activity of peroxisome proliferator-activated receptor γ (PPAR-γ), a major regulator of adipogenesis (Zhang et al., 2009).

mTORC1 is also able to positively regulate cellular metabolism and ATP production and to negatively regulate catabolic processes such as autophagy. In particular, mTORC1 stimulates the stabilization and accumulation of the hypoxia inducible factor 1α (HIF1α), thus increasing the glycolytic flux (Hudson et al., 2002). Upon nutrient rich conditions, mTORC1 inhibits the assembly of the ULK1/Atg13/FIP200 complex required for the autophagosome formation, by directly phosphorylating ULK1 (Jung et al., 2009). In addition to inhibiting autophagy initiation, mTORC1 also negatively regulates the biogenesis of lysosomes through the modulation of the transcription factor EB (TFEB), which controls many genes necessary for lysosomal maturation and function. Specifically, it has been shown that mTORC1 directly phosphorylates TFEB, thus preventing its nuclear entry and activity (Settembre et al., 2012).

All the downstream effectors mentioned in this section are summarized in Fig. 1.7.

1.2.2 The upstream regulators of mTORC1

Many upstream signals converge to the tumor suppressor complex tuberous sclerosis 1 (TSC1; also known as *hamartin*) and TSC2 (also known as *tuberin*). This heterodimer is a key negative regulator of mTORC1 that functions as a GTPase-activating protein (GAP) for the Ras homolog enriched in brain (Rheb) GTPase. Rheb is a crucial activator of mTORC1 which directly interacts with and stimulates the complex, when it is found in the GTP-bound state. As TSC1/2 has GAP activity toward Rheb, it negatively regulates mTORC1 by converting Rheb into its inactive GDP-bound state (Inoki et al., 2003).

Among the various stimuli which signal through the TSC complex, we can enumerate growth factors, stress, energy status and inflammation.

TSC1/2-dependent mTORC1 regulation

As shown in Fig. 1.8, growth factors, such as insulin and insulin-like growth factor 1 (IGF1), stimulate the PI3K and Ras pathways that

act by inhibiting TSC1/TSC2. The effector kinases of these pathways, protein kinase B (Akt/PKB), extracellular-signal-regulated kinase 1/2 (ERK1/2), and ribosomal S6 kinase (RSK1), directly phosphorylate the TSC1/TSC2 complex to inactivate it and thus activate mTORC1 (Manning et al., 2002; Ma et al., 2005). In particular, growth factors or related hormones stimulate receptor tyrosine kinases and G protein-coupled receptor which in turn activate PI3K. PI3K promotes the accumulation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) which induces the PDK1-mediated activation of Akt. It has been recently shown that Akt also activates mTORC1 in a TSC1/2-independent manner by phosphorylating and causing the dissociation from raptor of PRAS40, an mTORC1 inhibitor (Sancak et al., 2007).

In addition to growth factors, proinflammatory cytokines, such as tumor necrosis factor-α (TNFα), can activate mTORC1. In this case, the downstream effector which phosphorylates and inactivates TSC1 is IKB kinase β (IKKβ) (Lee et al., 2007).

The canonical Wnt pathway, a major regulator of cell growth and proliferation, also signals to mTORC1 through TSC1/2. Specifically, Wnt activates mTORC1 via inhibiting glycogen synthase kinase 3β (GSK3-β), which normally phosphorylates TSC2 and stimulates its GAP activity

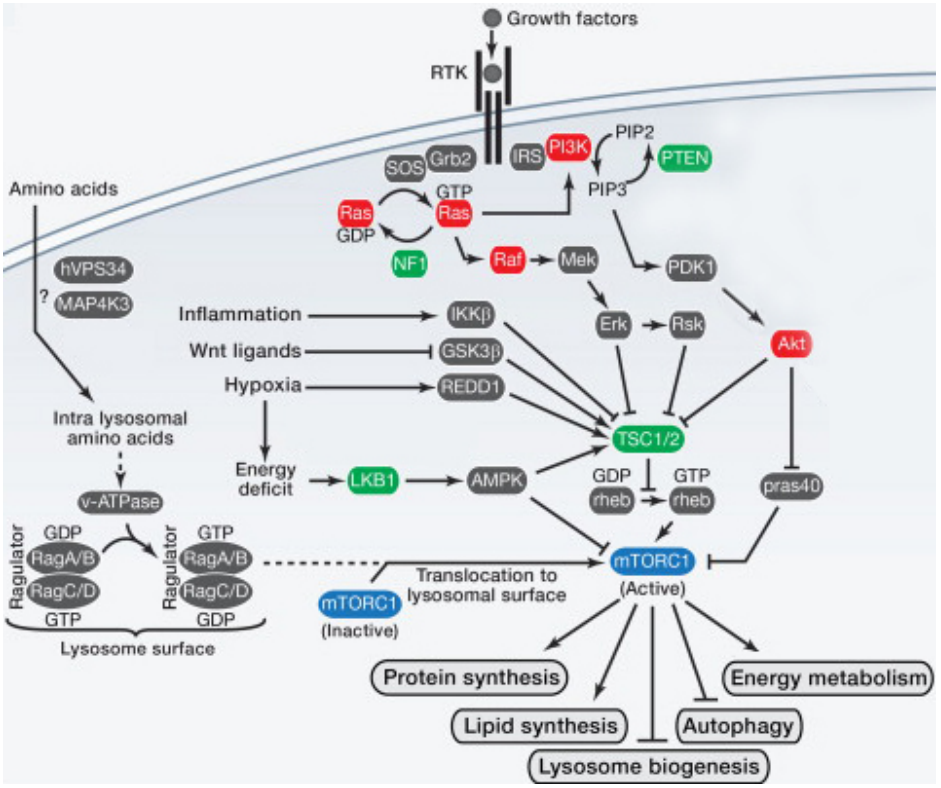

Figure 1.8: The mTORC1 signaling. Growth factors, stress, energy status, inflammation and nutrients are all upstream signals which converge to modulate mTORC1 activity in a TSC1/2 dependent or independent manner. Adapted from Laplante and Sabatini, 2012.

towards Rheb. In particular, it has been shown that GSK3β phosphorylates TSC2 on Ser1341 and Ser1337, and that these two phosphorylation events require priming by the AMPK-mediated phosphorylation of Ser1345 (Inoki et al., 2006).

Lastly, low energy and oxygen levels also contribute to a TSC1/2-mediated

mTORC1 inhibition. Under hypoxic conditions, the transcription factor HIF1α is stabilized and drives the expression of a set of genes, including *REDD1*. REDD1 competes with TSC2 for the inhibitory binding of 14-3-3. Thus, hypoxia-induced REDD1 prevents the inhibitory binding of 14-3-3 to TSC2 and eventually leads to the inhibition of mTORC1 signaling (DeYoung et al., 2008). AMP-activated protein kinase (AMPK; also known as PRKAB1) serves as another main "energy sensor" for mTORC1. AMPK can be activated under various conditions of cellular stress, particularly those that increase the level of AMP or the AMP to ATP ratio. Active AMPK phosphorylates TSC2 on Ser1345 and enhances its GAP activity, resulting in the inhibition of mTORC1 (Inoki et al., 2006). A recent study proposed a TSC2-independent mechanism by which AMPK can signal to mTORC1. In particular, like Akt, AMPK directly phosphorylates Raptor on two well-conserved serine residues. This phosphorylation induces 14-3-3 binding to raptor, leading to the inhibition of mTORC1 activity (Gwinn et al., 2008).

mTORC1 regulation by nutrients

The presence of nutrients is also a main cue for mTORC1 stimulation. Amino acids are the most crucial signals for mTORC1 activation, since

growth factors cannot efficiently activate mTOR when amino acids are limiting. Unlike the other stimuli, amino acids do not signal through the TSC complex and the molecular mechanism through which mTORC1 senses their intracellular amount remains elusive to date. However, several works have recently described that the mechanism implicates components residing at the lysosomal surface, where mTORC1 needs to be translocated for activation. This relocalization has been proposed to promote the interaction of mTORC1 with the activator Rheb, which is itself thought to be anchored to the lysosome (Jewell et al., 2013).

In 2008, two research groups independently discovered that the RAS superfamily Rag GTPases mediate mTORC1 activation in response to amino acid signals (Kim et al., 2008; Sancak et al., 2008). Mammals have four Rag proteins, RagA to RagD, which form heterodimers where the activation state is reflected by their guanine nucleotide state. In particular, the presence of amino acids promotes the formation of the active complex configuration, in which RagA and RagB are GTP-bound and RagC and RagD are GDP-bound. The active RagA/B·GTP-RagC/D·GDP complex can bind directly to Raptor and consequently induce the translocation of mTORC1 from a cytoplasmic localization to the lysosomal surface, where the Rag GTPases dock on a multisubunit

complex called Ragulator and where mTORC1 can bind to and be activated by Rheb (Sancak et al., 2010). This means that although TSC1/2-dependent positive signals can modulate the guanine nucleotide state of Rheb, only if amino acids are available mTORC1 can translocate to the lysosome and be activated (Fig. 1.8). The Ragulator complex acts as a scaffold for the active Rag complex at the lysosome and functions as a guanine exchange factor (GEF) for RagA/B, promoting the GTP loading of Rag A/B and the consequent activation of mTORC1 (Bar-Peled et al., 2012).

A recent work demonstrated that V-ATPase is involved in amino acid sensing to regulate mTORC1 activation. It has been shown that amino acids modulate the interaction of V-ATPase with Rag GTPases and Ragulator on the lysosomal membrane (Zoncu et al., 2011). In particular, the accumulation of amino acids within the lysosome is necessary to signal to V-ATPase and consequently enhance the GEF activity of Ragulator. This leads to the formation of the active Rag GTPase conformation (RagA/B·GTP-RagC/D·GDP), which can then recruit mTORC1 to the lysosome and induce its activation (Fig. 1.9). Although the precise amino acid sensor that stimulates V-ATPase is unknown, the disruption of V-ATPase activity inhibits mTORC1 lysosomal localization and acti-

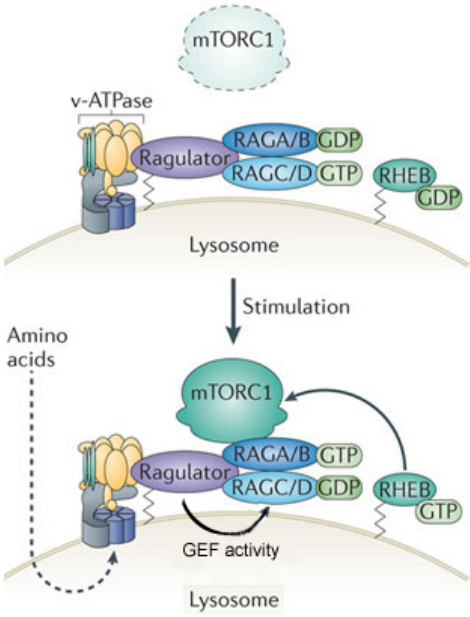

Figure 1.9: mTOR activation at the lysosome. Amino acids accumulate within the lysosomal lumen and signal V-ATPase through an "inside-out" mechanism, leading to the formation of the Rag GTPase active complex and to the recruitment of mTOR to the lysosome. Adapted from Jewell et al., 2013.

vation, highlighting a crucial role for V-ATPase in the formation of the active complex.

Not only the V-ATPase activity is required for mTORC1 activation, but also the cytoplasmic pH (pH_i) seems to be relevant in this regulation. About this mechanism, conflicting data have been reported by different groups. Some reports pointed out that the decrease in cytoplasmic pH correlates with the inhibition of mTORC1 activity. Specifically, the

protonophoric activity induces the cytoplasmic acidification and results in mTORC1 inhibition in a Akt, ERK and AMPK-independent manner (Fonseca et al., 2012). On the contrary, another work reported that nutrient addition provokes a decrease in pH_i which correlates with mTORC1 activation (Korolchuk et al., 2011). Interestingly, the results obtained by Korolchuk and coworkers demonstrated that the nutrient-dependent decrease in pH_i influences lysosomal positioning which in turn modulates mTORC1 activity. In particular, the lower pH_i correlates with an increase in the binding to lysosomes of proteins, such as KIF2A and ARL8, which in turn drive lysosomes' movement to the cell periphery and finally lead to mTORC1 activation.

At least other two proteins have been implicated in amino acid sensing by mTORC1: mitogen-activated protein kinase kinase kinase kinase 3 (MAP4k3) (Yan et al., 2010), and mammalian vacuolar protein sorting 34 homolog (hVps34) (Gulati et al., 2008). Yan and coworkers reported that amino acids withdrawal induces the dephosphorylation of MAP4k3 via the PP2A complex. The dephosphoryation of MAP4k3 causes its inactivation and, subsequently, the inhibition of mTORC1 signaling. Gulati and coworkers showed that amino acids induce a rise in intracellular Ca^{2+}, which triggers the binding of Ca^{2+}/calmodulin (CaM) to hVps34

that is required for lipid kinase activity and increased mTORC1 signaling. Finally, Leucyl-tRNA synthetase (LeuRS) has been shown to be involved in mTORC1 activation in response to intracellular leucine levels (Han et al., 2012).

1.2.3 The mTOR pathway in cancer

Taking into account the great number of cellular processes regulated by mTORC1, like growth, survival and energy metabolism, it is not surprising that the mTOR signaling is a pathway frequently deregulated in cancer cells. Many genes of the PI3K signaling are often mutated in human cancers, such as TSC1/2, serine threonine kinase 1 (LKB1), PTEN, Akt or PI3K (Laplante and Sabatini, 2012; Beauchamp and Platanias, 2013). p-4E-BP1 and pS6 overexpressions were also found to be associated with poor prognosis in several human tumors (Zhou et al., 2010; Armengol et al., 2007). All these mutations' informations lead us to speculate that the oncogenic hyperactivation of the mTOR signaling sustains several biological processes required for cancer cell growth, survival, and proliferation.

1.3 pH regulation in cancer cells

The dysregulation of pH is probably emerging as one of the new hallmarks of cancer. In normal cells, the activity and the amount of several membrane proton pumps and transporters are tightly regulated to maintain the intracellular pH within a narrow range (7.1-7.2) which is lower than the extracellular pH, normally around ~ 7.4. On the contrary, cancer cells have a higher pH_i of $\geqslant 7.4$ and a lower pH_e of $\sim 6.7-7.1$. This "reversed" pH gradient enables cancer progression by promoting several processes, including cell proliferation, apoptosis evasion, metabolic adaptation, migration and invasion (Webb et al., 2011).

The frequent hypoxic conditions and the upregulation of some oncogenes (e.g., *RAS, MYC, AKT*) experienced by tumors lead to a shift in the energetic metabolism from the mitochondrial oxidative phosphorylation towards glycolysis. This means that both oncoproteins' hyperactivation and hypoxia can independently increase the levels of HIF1α and HIF2α transcriptions factors, which in turn upregulate glycolysis (Jones and Thompson, 2009). Furthermore, the glycolytic phenotype can become hardwired even after reoxygenation because the obtained metabolic intermediates (that is, lactate and pyruvate) can be used for the biosynthesis of amino acids, nucleotides and lipids, thus providing a

selective advantage to proliferating tumor tissues (Warburg effect). This oncogenic metabolism generates an excess of glycolytic products, lactate and H^+ which should produce an intracellular acidosis. It is well known that intracellular acidification is cytotoxic by inducing apoptosis. However, cancer cells usually have neutral to alkaline pH_i in an acidic extracellular environment, implying that they have evolved various mechanisms to extrude intracellular protons in order to maintain physiological pH_i. The acidification of the extracellular milieu confers a selective advantage to cancer cells over normal cells, which undergo apoptosis in response to such an acidic extracellular environment. Moreover, the low pH_e promotes cancer progression, invasion and metastasis by inducing the degradation of the extra-cellular matrix due to increased activity of acid-activated proteases. Finally, increased pH_i also affects cancer cell functions. It promotes cell proliferation, the cytoskeletal remodeling for directed cell migration, and limits apoptosis (Neri and Supuran, 2011; Damaghi et al., 2013).

Actin cytoskeleton is a major pH-sensitive system in the cells. The assembly of globular (G-actin) to filamentous (F-actin) plays several roles in cancer cell processes such as vesicle trafficking, contraction, migration and invasion. Actin assembly requires $pH_i > 7.2$ and variations of 0.3-0.4

units induce severe alterations in actin filament assemblies and architectures, suggesting that the disruption of the alkaline pH_i could negatively regulate the mobility of cancer cells and eventually affect metastasis (Webb et al., 2011).

In particular, the ADF/cofilin family consists in ADF (actin depolymerizing factor), cofilin 1 and cofilin 2, which are pH-sensitive proteins that sever and nucleate actin filaments. It has been recently shown that the local increase in pH at the cytoplasmic side of the plasma membrane decreases the affinity of cofilin for binding PtdIns(4,5)P_2. The dissociation of cofilin from membrane PtdIns(4,5)P_2 increases its activity and leads to the formation of local protrusions and consequently, to cell motility (Bravo-Cordero et al., 2013).

Moreover, the dynamic remodeling of actin filaments at focal adhesion sites is also a pH_i-sensitive mechanism. Focal adhesion (FA) remodeling is a rate-limiting process during migration of adherent cells. Actin filaments do not directly bind to the cytoplasmic domain of integrins but to integrin-associated FA proteins, such as talin and vinculin. It has been shown that talin is a pH sensor whose binding to actin filaments decreases at pH > 7.2, permitting faster focal adhesion turnover and increased migration (Srivastava et al., 2008).

The dysregulation of pH in cancer cells is due to changes in the expression and activity of various plasma membrane proteins such as pumps and transporters that facilitate H^+ efflux, import of weak bases and export of weak acids, in order to maintain the alkaline pH_i and the acidic pH_e. The main players involved in the regulation of tumor pH are shown in Fig.1.10 and include: carbonic anhydrases such as CA2, CA9 and CA12; the vacuolar ATPase (V-ATPase); Na^+/HCO_3^- co-transporters; the Na^+-driven Cl^-/HCO_3^- exchanger NDCBE; the monocarboxylate transporters such as MTC1-4; Na^+/H^+ exchanger 1, NHE1; and the anion exchangers AE1-3 (Damaghi et al., 2013).

The carbonic anhydrase family consists of metalloenzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate and protons ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$). CA9 and CA12 are transmembrane CAs which hydrate the CO_2 generated as the final product of oxidative phosphorylation to produce HCO_3^- and an extracellular proton. The HCO_3^- can diffuse or re-enter into the cell via bicarbonate transporters or anion exchangers. Intracellular CAs (e.g., CA2) dehydrate the HCO_3^- into aqueous CO_2 in a reaction consuming a proton. By mass action, the CO_2 is exported across the bilayer or through aquaporins. The two carbonic anhydrases CA9 and CA12 are overexpressed in many tumors, and

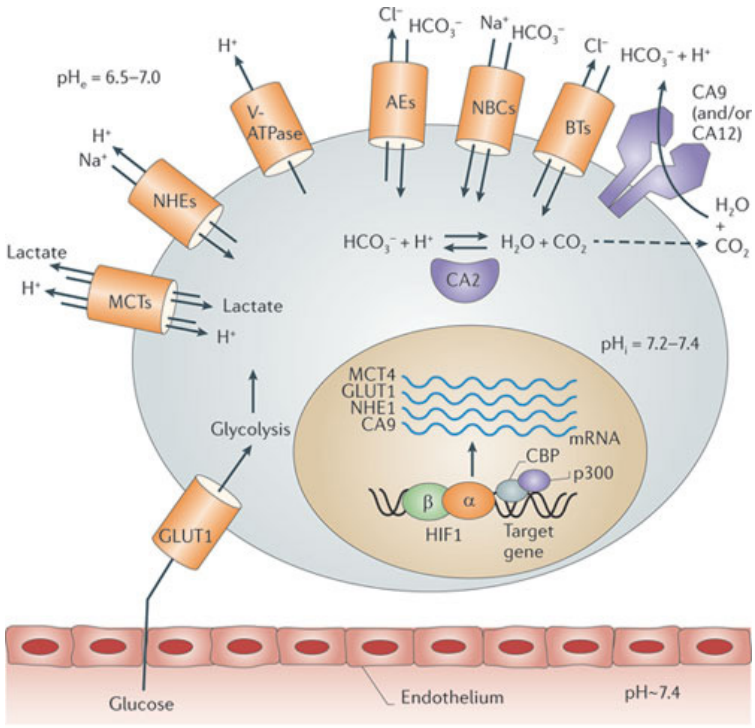

Figure 1.10: pH regulation in cancer cells. The most important proteins involved in regulating pH within tumor cells are: carbonic anhydrases (CA2, CA9 and CA12); the vacuolar ATPase (V-ATPase); Na^+/HCO_3^- co-transporters (NBCs); the Na^+-driven Cl^-/HCO_3^- exchangers (BTs); the monocarboxylate transporters (MTCs); Na^+/H^+ exchangers (NHEs); and anion exchangers (AEs). GLUT1 transports glucose into tumor cells (Neri and Supuran, 2011).

are associated with cancer progression and response to therapy (Chiche et al., 2009).

The HCO_3^- transporters facilitate the movement of the membrane-impermeant HCO_3^- ion across biological membranes. They are phylogenetically

clustered into three classes: electroneutral Cl^-/HCO_3^- exchangers, the NBC family of Na^+/HCO_3^- co-transporters and the anion transporters of the SLC26 family. The physical and functional interactions between these HCO_3^- transporters and the various carbonic anhydrase isoforms suggest that they could form a complex. Carbonic anhydrases produce the HCO_3^- substrate for transport, whereas the HCO_3^- transporters translocate the membrane-impermeant HCO_3^- ion either inside or outside the cell to alkalinize or acidify the pH_i.

NHE1 is the most common isoform of the Na^+/H^+ exchanger family. The NHE transporters are ubiquitously expressed in all mammalian cells and are crucial proteins involved in pH regulation. They use the powerful sodium electrochemical gradient to extrude H^+ in response to an acidification of the cytosolic pH. In cancer cells, NHE1 hyperactively extrudes protons into the extracellular space thereby contributing to its acidification. In particular, it has been shown that upregulation of NHE1 activity is a main factor that promotes invasion and metastasis in breast and cervical cancer (Amith and Fliegel, 2013; Chiang et al., 2008).

Monocarboxylate transporters (MCTs) facilitate the transport of monocarboxylic acids (such as lactate, pyruvate and ketone bodies) across plasma and mitochondrial membranes. As mentioned above, in tumors,

hypoxia and oncogenic mutations sustain high glycolytic rates which promote the conversion of pyruvate to lactate and result in the production of high amounts of lactic acid. Indeed, MCTs are often overexpressed in many tumors to regulate the efflux of lactate and byproducts of glycolysis in order to maintain physiological pH_i. As a result, overexpression of these transporters contributes to extracellular acidification and has been associated with poor prognosis and cancer progression in many tumors such as, for example, breast carcinoma and neuroblastoma (Pinheiro et al., 2010; Fang et al., 2006).

Finally, an emerging crucial player in the maintenance of malignant tumor environment is the vacuolar H^+-ATPase (V-ATPase). Indeed, it has become recently evident that overexpressions of different V-ATPase subunits are associated with invasive phenotypes and cancer metastasis (Lu and Qin, 2011). This enzyme is known to be involved in many physiological processes which we shall describe in the following section.

1.3.1 The vacuolar H^+-ATPase

V-ATPase is an evolutionarily highly conserved enzyme which pumps protons from the cytoplasm outside the cell and into intracellular compartments, in an ATP-dependent manner. The protein participates in several cellular processes, such as pH regulation, endocytosis, intracellular trafficking, maturation of endosomes and degradation of recycling vesicles. Recent studies showed that hyperactivation of V-ATPase is associated with malignant transformation, invasion and metastasis.

The V-ATPase structure

The mammalian V-ATPase is composed of two domains. The first one is the cytosolic catalytic V1 domain, which consists of eight subunits (A-H) and is responsible for ATP hydrolysis. The second one is the transmembrane V0 domain, which is composed of five subunits (a, c, c", d, and e) and is responsible for proton translocation. Many of these subunits have isoforms which are either ubiquitous or selectively expressed in specific cell types.

As shown in Fig. 1.11, the ATP catalytic V1 domain is made of a hetero-hexamer of alternating A- and B-subunits. The rotation of the central

rotor is due to a conformational change in the AB heterohexamer driven by the ATP hydrolysis on the A-subunit. The evolutionary conserved D-subunit works as the main axle of the rotor and is associated with other regulatory subunits, such as the F-subunit and the d-subunit. This last couples the axle to the ion pump components of the rotor. The transmembrane V0 domain consists of a rotating ring of b- and c-subunits, which together with the a-subunit create the channel where protons flow

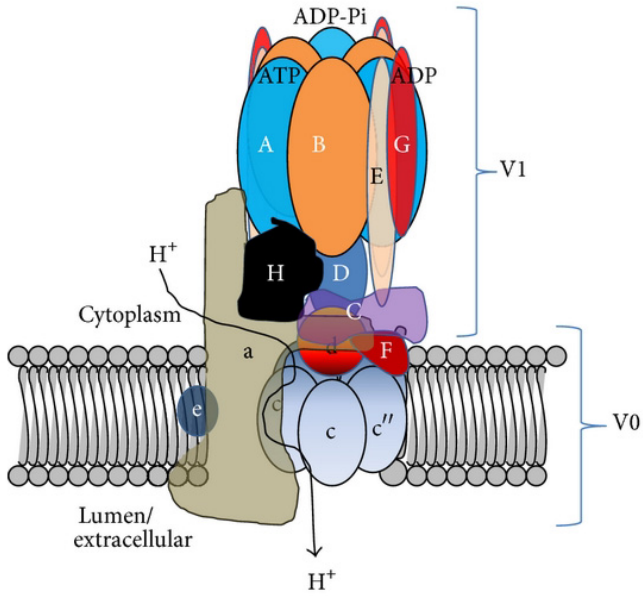

Figure 1.11: Structure and subunit composition of the V-ATPase complex. ATP hydrolysis occurs on the A-subunit and the conformational change in the AB heterohexamer promotes the turning of the central rotor and the efflux of protons through the membranes (Holliday, 2014).

due to the rotation of the b/c-subunit ring. Finally, a collar domain containing C- and H-subunits serves to link the transmembrane a-subunit to the V1 domain (Lu and Qin, 2011; Holliday, 2014).

The V-ATPase regulation

Considering the complexity of the V-ATPase multisubunit structure, it is not surprising that cells have evolved a coordinate regulation of the expression levels of the various subunits to allow the assembly of the active enzyme. Different studies revealed that both transcriptional and post-transcriptional mechanisms control the production of the correct amount of the ubiquitous and cell-type specific isoforms, in a certain cell at a particular time (Holliday, 2014).

To date, the mechanisms regulating V-ATPase activity are not well understood yet in mammalian systems. Many studies in yeast models pointed out that the main mechanism of regulation is the reversible assembly of the V1 and V0 components. It has been shown that the reversible disassembly of V-ATPase is triggered by glucose deprivation, and some glycolytic enzymes, such as aldolase, can modulate this process. In particular, both in mammals and in yeast the E-subunit has been identified as the binding site of aldolase, which is sensitive to the pres-

ence of glucose in the medium (Lu et al., 2007). Moreover, in human, the a-subunit has been reported to bind to another glycolytic enzyme, phosphofructokinase-1, but it is not yet known whether this interaction is relevant to V-ATPase regulation (Su et al., 2003).

The C-subunit has been suggested to play a role in the regulation of V-ATPase reversible disassembly. It has been proposed that some unknown environmental stimulus leads to a conformational change in the C-subunit which breaks its interaction with the EG stator, in order to release the V1 complex (Toei et al., 2010; Forgac, 2007).

Another mechanism that regulates proton flux via the V-ATPase is the change in pump density at the plasma membrane through reversible fusion of V-ATPase-containing vesicles. For example, in the renal epithelial cells, glucose induces the fusion of membrane vesicles containing high density of V-ATPases with the apical membrane. At the same time it stimulates the assembly of the V1 and V0 subunits, through a mechanism in part regulated by PI-3 kinase (Sautin et al., 2005). Finally, it has been shown that the actin cytoskeleton is also involved in modulating the density of V-ATPases at the plasma membrane (Beaulieu et al., 2005).

V-ATPase functions

Given the relevance of V-ATPase in regulating intracellular and extracellular pH, the activity of such enzyme may in turn modulate several biological process. Besides its role in the activation of mTORC1 complex, which we already described in section 1.2.2, intracellular V-ATPase is known to be involved in the regulation of endosome maturation, autophagy, endocytosis, and intracellular trafficking (Hurtado-Lorenzo et al., 2006; Saftig and Klumperman, 2009; Settembre et al., 2013). Moreover, the plasma membrane V-ATPase has pivotal importance in many different processes, such as renal acidification, bone resorption, sperm maturation and homeostasis of cytoplasmic pH (Toyomura et al., 2003; Sautin et al., 2005; Forgac, 2007).

V-ATPase is the primary enzyme responsible for the gradual acidification occurring during endosomal maturation. The pump lowers the pH from values around 6.2 in early endosomes, to a final pH around 4.5-5 in mature lysosomes (Forgac, 2007). The endolysosomal system is a dynamic network of vesicles connecting the plasma membrane to the lysosomes. It unfolds along the gradual maturation of endosomes, from the budding of early endosomes at the plasma membrane to their complete maturation into lysosomes. During this process, lysosomes receive several essential

proteins, including lysosomal hydrolases, and V-ATPase activity mediates the proton transport into the organelles. The extracellular material that is intended for degradation reaches the lysosomes through the endocytic pathway, while intracellular components are transported to the lysosomes by autophagy. The autophagic program enables cells to break down cellular organelles, such as ribosomes and mitochondria, allowing the resulting catabolites to be recycled and used for biosynthesis and energy metabolism. Specifically, intracellular vesicles named autophagosomes envelope intracellular organelles and then fuse with lysosomes wherein degradation occurs. In this manner, in the stressed and nutrient-limited conditions, cells can generate metabolites that support survival. (Levine and Kroemer, 2008). Hence, the role of autophagy in degradation and recycling is strictly dependent on lysosomal function.

As lysosomes are the main catabolic compartment of cells and V-ATPase is a major factor in regulating their maturation, clearly such protein has in turn a crucial role in the autophagic process. Indeed, it is well known that V-ATPase inhibition blocks lysosomal acidification, thereby impairing lysosomal digestive functions and degradation of vesicles (Mijaljica et al., 2011).

Besides its relevance in endosomal maturation and autophagy, V-ATPase

activity is also essential for endocytosis and intracellular trafficking. It has been shown that V-ATPase inhibition impairs the budding and formation of endosome-derived carrier vesicles, leading to a blockage of endocytic trafficking between early and late endosomes (Clague et al., 1994; Hurtado-Lorenzo et al., 2006).

The activity of V-ATPase has furthermore been implicated in the efficiency of Notch signaling response. The intracellular vesicles represent an important sorting station as they contain proteins (for example, endocytosed receptors) and lipids destined to be targeted back either to the cell membrane or to the lysosomal degradation. Therefore, the faster the receptor can be recycled and targeted back to the plasma membrane, the more efficiently cells can respond to a certain signal molecule. Hence, in cells depleted for V-ATPase, Notch accumulates in an expanded lysosome-like compartment leading to a reduction in the signaling response (Vaccari et al., 2010). We can speculate that, in cancer cells, hyperactivation of V-ATPase probably influences many signaling pathways and increases the sensitivity to growth factors.

Finally, the B- and C- subunits of V-ATPase have been shown to bind to actin. C-subunit interaction with microfilaments occurs after V-ATPase disassembly and could be involved in organizing the cross-linking of mi-

crofilaments into higher order structures (Vitavska et al., 2005). The existence of interactions between V-ATPase and cytoskeleton suggests an additional function for the enzyme in the regulation of cell mobility and membrane trafficking.

The V-ATPase in cancer

Several studies pointed out that V-ATPase contributes to invasion, metastasis and endosome-dependent drug resistance. The process of acidification of the extracellular environment in tumor cells is associated with the activation of many matrix-degrading proteases such as cathepsins, metalloproteases and gelatinases. Alike, the plasma membrane V-ATPase can promote the acidification of the extracellular space directly, while the intracellular V-ATPase may facilitate the same process by inducing the acidification of intracellular vesicles which are targeted to exocytosis. In this manner, V-ATPase hyperactivation in cancer cells contributes to the degradation of the extracellular matrix and to invasion (Damaghi et al., 2013; Neri and Supuran, 2011).

Accordingly, overexpression of both a1 and a3 subunit isoforms greatly increases the invasiveness of breast cancer cells as also increases the expression of V-ATPases at the plasma membrane (Capecci and Forgac,

2013). Others have reported that siRNA downregulation of the c subunit suppresses cancer growth and metastasis in an hepatocellular carcinoma mouse model (Lu et al., 2005). Moreover, V-ATPase inhibition reduces cell migration *in vitro* and metastatic dissemination in xenograft models (Wiedmann et al., 2012). Interestingly, the V-ATPase localization at the plasma membrane fosters the acquisition of a more metastatic phenotype in human breast cancer cells (Sennoune et al., 2004). Finally, V-ATPase hyperactivation has been shown to interfere with the absorption of chemotherapy drugs and consequently, to participate in multi-drug resistance in cancer (Pérez-Sayáns et al., 2010).

2

Objectives

The aim of this thesis work is to study the molecular pathways regulated by E2F1 that may be involved in its oncogenic properties. Previous results of our Research Group demonstrated that the overexpression of mammal E2F1 induces cellular growth by activating the mTORC1 pathway (Real et al., 2011). Consistent with this observation, tumors from transgenic mice in which E2F1 is overexpressed possess high mTORC1 activity, suggesting that the effects of E2F1 on tumorigenesis may be largely mediated through mTORC1 (Ladu et al., 2008). Taking into account the relevance of this finding and the well known role of the mTOR pathway in cancer, the general aim of the Thesis is to elucidate the molecular mechanism by which E2F1 regulates mTORC1. Specifically, since previous data from our group demonstrated that E2F1 drives the

activation of mTORC1 independently of the Akt-TSC1/2 pathway, we first decided to investigate whether E2F1 was inducing mTORC1 activity by modulating any nodes of the amino acids signaling. To this end, we focused on the following five objectives:

1 - Role of E2F1 in mTOR subcellular localization and amino acids' implication in this process.

2 - Function of E2F1 in intracellular trafficking.

3 - Effect of E2F1 activity in autophagy.

4 - Characterization of the mechanism by which E2F1 regulates mTORC1 activity and intracellular trafficking: Role of V-ATPase and KIF2A.

5 - Study of E2F1 implication in cell migration.

3 Results

3.1 Role of E2F1 in mTOR subcellular localization and amino acids' implication in this process

3.1.1 E2F1 induces the translocation of mTOR into the lysosomes

In order to elucidate the mechanism of mTORC1 activation by E2F1, we investigated the subcellular localization of mTOR after E2F1 induction. Previous reports demonstrated that mTORC1 stimulation by amino acids is mediated by its translocation into the lysosome, where the complex can be activated by interacting with Rheb (Zoncu et al., 2011). To this end, we performed immunofluoroscence analysis using antibodies against

endogenous mTOR and E2F1.

This study was performed using the human osteosarcoma cell line U2OS stably transfected with the ER-E2F1 plasmid. The stable cell line overexpresses the fusion protein ER-E2F1, which consists in the human E2F1 conjugated to the estrogen receptor domain. This system allows us to regulate E2F1 activity by modulating its subcellular localization. The fusion protein is stably expressed in the cytosol and the addition of 4-hydroxitamoxifen (OHT) induces its translocation to the nucleus where E2F1 regulates gene transcription (Fig. 3.1).

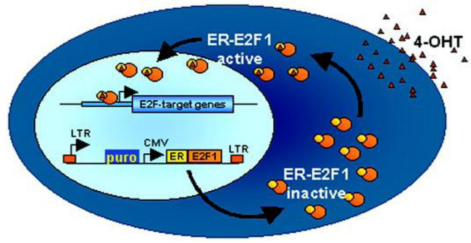

Figure 3.1: The 4-hydroxitamoxifen inducible system. The addition of 4-hydroxitamoxifen (OHT) to the culture medium promotes the translocation of the stably overexpressed fusion protein ER-E2F1 into the nucleus, thereby inducing the transcription of E2F1 target genes.

ER-E2F1 U2OS cells were serum-starved overnight and treated with OHT during 6h. As shown in Fig. 3.2, in E2F1 induced cells, mTOR was found to localize much more in some kind of vesicular structures compared to the control, where mTOR presented a more cytoplasmic staining. We

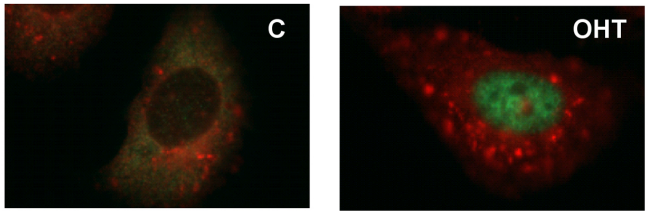

Figure 3.2: E2F1 promotes mTOR translocation to subcellular vesicle structures. Serum-deprived ER-E2F1 U2OS cells were cultured in the absence (C) or in the presence of 4-hydroxitamoxifen (OHT). At 6 h after treatment, immunofluorescence assay was performed as described in Materials and Methods using mTOR and E2F1 primary antibodies. The red signal represents mTOR staining, the green signal represents E2F1 staining.

could also observe that the ER-E2F1 inducible system was perfectly working, as E2F1 totally translocated into the nucleus after OHT treatment.

To next identify in which type of vesicles mTOR was localizing after E2F1 activation, we performed other immunostaining studies using three different subcellular markers: the early endosomal antigen 1 (EEA1), the cis-Golgi Matrix protein (GM130) and the lysosome-associated membrane glycoprotein 2 (LAMP2). As shown in Fig. 3.3, in OHT treated cells, mTOR was perfectly colocalizing with the LAMP2 marker, while no colocalization was observed with EEA1 or GM130 markers. These data strongly suggest that E2F1 activates the mTORC1 signaling by a mechanism that involves the translocation of mTORC1 to lysosomes.

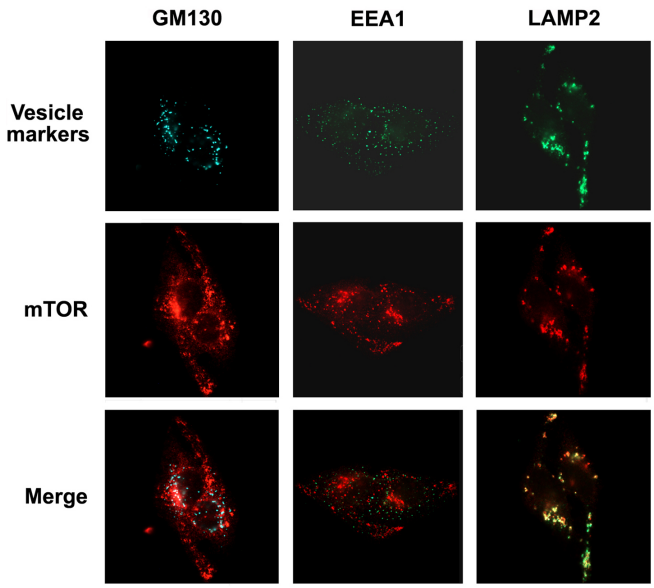

Figure 3.3: E2F1 induces mTOR translocation to lysosomes. Serum-deprived ER-E2F1 U2OS cells were cultured in the presence of 4-hydroxitamoxifen. At 6 h after treatment, immunofluorescence assay was performed as described in Materials and Methods using primary antibodies against showed proteins. Merge panels indicate the colocalization of antibody signals.

Further immunostaining experiments using mTOR and LAMP2 antibodies allowed us to quantify the change induced by E2F1 from the cytoplasmic to the lysosomal localization of mTOR. After 6h of OHT treatment, the colocalization of mTOR with the LAMP2 marker was increased by approximately 40% compared to the control (Fig. 3.4).

As the amino acids-induced recruitment of mTORC1 to lysosomes is an essential step for the activation of mTORC1 by mitogenic signals

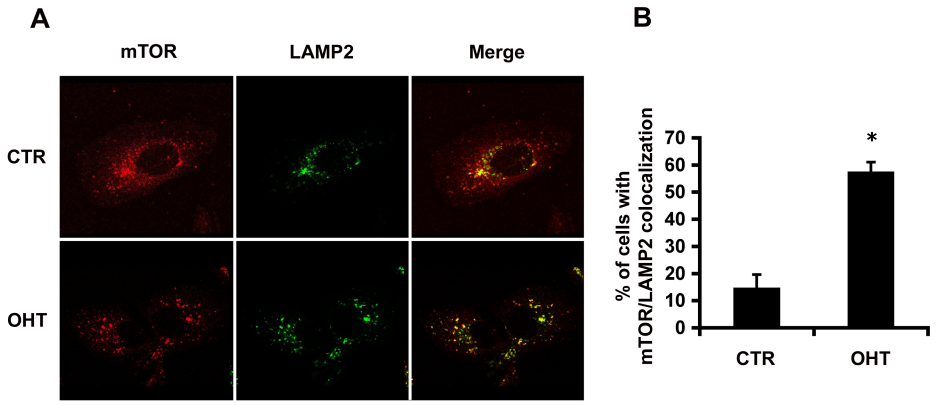

Figure 3.4: E2F1 promotes mTOR colocalization with the LAMP2 marker. (A) Serum-deprived ER-E2F1 U2OS cells were cultured in the absence (CTR) or in the presence of 4-hydroxitamoxifen (OHT). At 6 h after treatment, immunofluorescence assay was performed as described in Materials and Methods using primary antibodies against showed proteins. Merge panels indicate the colocalization of antibody signals. (B) Quantification of % of cells with mTOR/LAMP2 colocalization is shown.

(Sancak et al., 2010), we further investigated whether the E2F1-induced activation of mTORC1 requires the presence of amino acids. To answer this question, we starved the cells overnight for amino acids and serum, we treated them with OHT and/or with a previous addition of leucine or insulin, and we subsequently performed Western Blot and immunofluorescence analysis. As shown in Fig. 3.5A, in the absence of amino acids, overexpression of E2F1 was not able to induce the phosphorylation of S6K1 either alone or in the presence of insulin. However, high induction of S6K1 phosphorylation was observed when E2F1 was activated in the

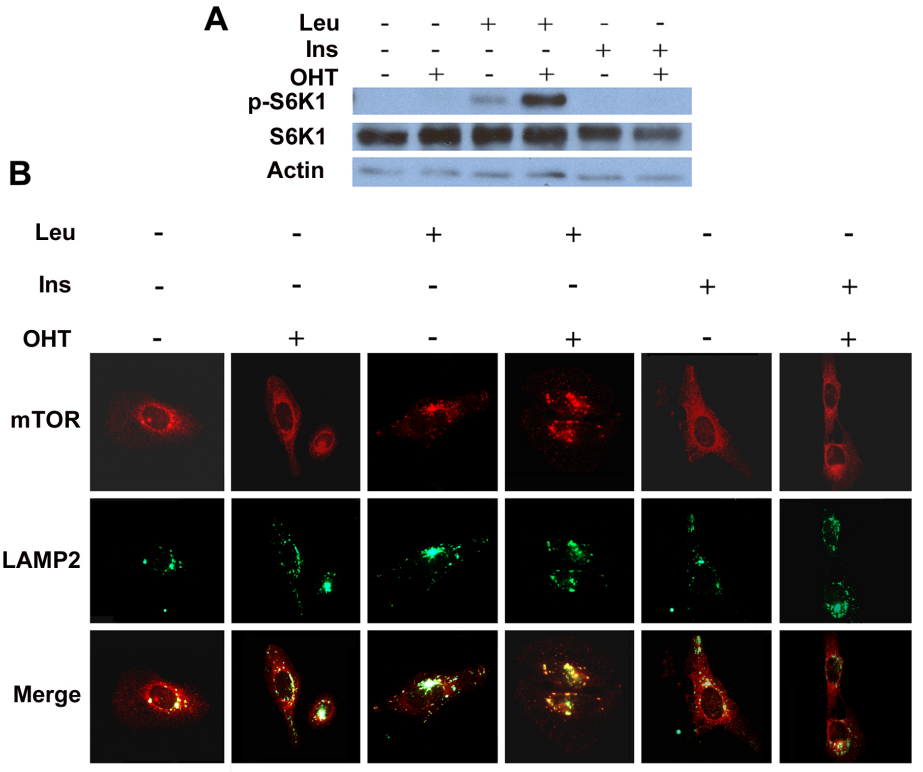

Figure 3.5: The E2F1-induced activation of mTORC1 requires the presence of amino acids. ER-E2F1 U2OS cells were serum and amino acids starved for 15 hours, treated with OHT for 6 hours and/or pre-treated with insulin (Ins) or leucine (Leu) for 30 min. (A) Expression of the indicated proteins was determined by Western Blot analysis. (B) Immunofluorescence assay was performed as described in Materials and Methods using primary antibodies against showed proteins. Merge panels indicate the colocalization of antibody signals.

presence of leucine, and the level of phosphorylation was even higher than in the leucine condition alone. In accordance with this result, the translocation of mTORC1 to lysosomes was only detected in the conditions where leucine was present (Fig. 3.5B). These data indicate that E2F1 alone is not able to replace amino acids in their function to promote mTORC1 translocation to lysosomes, however, it enhances such process if nutrients are present.

Since the lysosomal RagGTPase family of proteins mediates the interaction of mTORC1 with lysosomes in the presence of amino acids, we hypothesized that E2F1 could promote the translocation of mTORC1 to lysosomes through a similar mechanism. Hence, we used U2OS ER-E2F1 cells stably transfected with FLAG-tagged RagB to perform pull down assays against RagB protein. We serum-starved the cells overnight before adding OHT, treated them with the cross-linking reagent dithiobis (succinimidylpropionate) prior to lysis, and we extracted the proteins to measure the extent of Rag interaction with mTORC1. The results from FLAG immunoprecipitation experiments showed that the binding of mTOR and Raptor to RagB was increased after E2F1 activation, demonstrating the translocation of both proteins to the lysosomal compartment (Fig. 3.6). These data indicate that E2F1 activation promotes

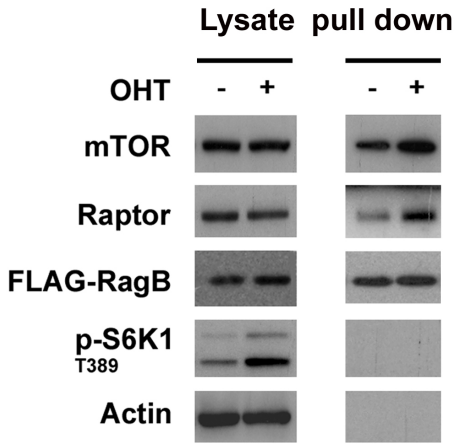

Figure 3.6: E2F1 activation increases mTORC1 binding to RagB. Stably transfected ER-E2F1/ FLAG-RagB U2OS cells were serum deprived and cultured in the absence (-) or in the presence of 4-hydroxitamoxifen (+) for 6 h. Proteins were cross-linked as described in Materials and Methods and immunoprecipitated using FLAG antibody. Expressions of the indicated proteins were determined by Western Blot analysis.

the translocation of mTORC1 into the lysosome and increases the physical interaction of mTORC1 with the lysosomal protein RagB.

3.1.2 Activation of mTORC1 by E2F1 does not depend on amino acids' uptake

As the amino acids drive the translocation of mTORC1 to the lysosomal surface and also the movement of endosomes toward the cell periphery (Sancak et al., 2010; Korolchuk et al., 2011), we hypothesized that

	CTR 4h	OHT 4h	CTR 8h	OHT 8h
ASP	119,04	88,95	111,49	82,82
THR	25,77	34,77	29,92	54,42
SER	12,12	14,11	14,44	17,26
ASN	6,55	5,65	10,06	11,40
GLU	143,12	152,01	149,78	199,22
GLN	6,04	13,76	18,29	34,76
GLY	48,38	50,11	47,09	61,63
ALA	29,23	28,52	27,22	33,20
VAL	9,23	12,56	10,34	15,08
MET	2,72	2,80	2,92	4,02
CYST	1,72	2,29	1,58	3,88
ILE	5,54	8,35	7,48	11,52
LEU	9,75	12,21	10,38	13,42
TYR	6,11	6,83	5,87	7,78
PHE	5,22	6,99	6,59	8,09
ORN	2,95	2,95	2,39	2,89
LYS	10,72	12,15	11,36	12,47
HIS	3,41	4,52	4,51	4,65
ARG	6,37	5,70	6,27	6,88

Table 3.1: Effect of E2F1 on intracellular amino acids concentration. Serum-deprived ER-E2F1 U2OS cells were cultured in the absence (CTR) or in the presence of 4-hydroxitamoxifen (OHT). At indicated times, amino acids concentration was measured as described in Material and Methods. Concentration is given as μmols/mg total protein.

E2F1 activation could drive both processes by inducing an uptake of amino acids within the cell. To test this possibility, we analyzed the intracellular amount of free amino acids in E2F1 induced or not conditions. The activation of E2F1 led to a small increase in the intracellular level of essential amino acids, particularly branched-chain amino acids, suggesting a potential role in mediating the E2F1-induced mTORC1 activation (Fig. 3.7A, Table 3.1).

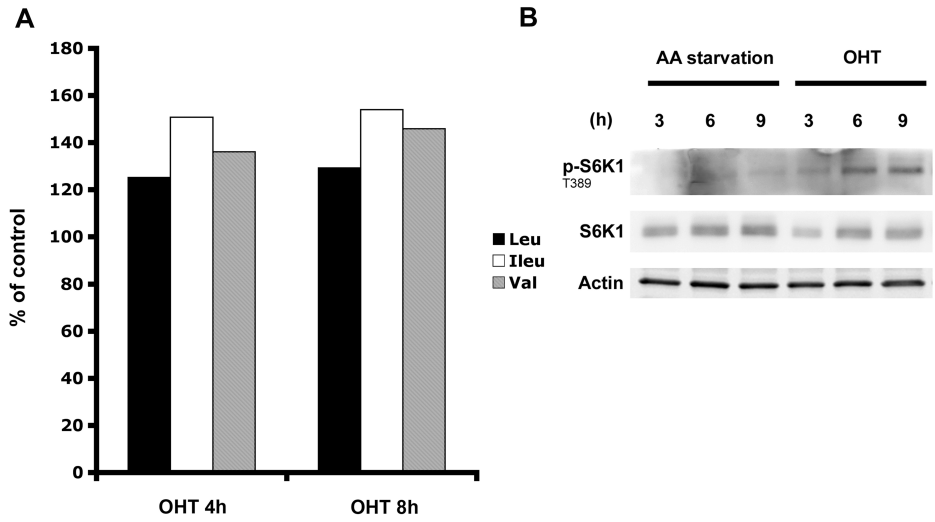

Figure 3.7: AA uptake is not required for the E2F1-induced mTORC1 activation. (A) Serum-deprived ER-E2F1 U2OS cells were cultured in the absence or in the presence of 4-hydroxitamoxifen (OHT). At the indicated times, free amino acids levels were measured from the supernatant as described in Materials and Methods. Results are shown as a % respect to 4-hydroxitamoxifen untreated conditions. (B) ER-E2F1 U2OS cells were serum/amino acids starved for 2 h and then cultured in the absence or in the presence of 4-hydroxitamoxifen (OHT). At the showed times, expression of the indicated proteins was determined by Western Blot analysis.

However, the small increase detected in the concentration of leucine, isoleucine and valine does not prove alone to be sufficient for the activation of mTORC1 modulated by E2F1. In order to verify the implication of amino acids in this response, cells were first deprived of amino acids during 2 hours, then E2F1 was induced, and S6K1 T389 phosphorylation was monitored as a marker of mTORC1 activation. The Western Blot

analysis showed that although 2 hours of amino acids deprivation reduced their intracellular levels to lower amount (data not shown), E2F1 induction was still able to promote mTORC1 activation (Fig. 3.7B). Thus, we could conclude that the uptake of essential amino acids is not involved in the acute activation of mTORC1 by E2F1.

It is important to note that this finding and the data reported in Fig. 3.5 are not contradictory, as they reflect different conditions. In the case of Fig. 3.7B, the only two hours of amino acid deprivation are sufficient to create a condition where no further amino acids can be up-taken by the cells, but where the low amount of intra-cellular amino acids left is still sufficient to promote the activation of mTOR triggered by E2F1. Differently, in the case of Fig. 3.5, the overnight starvation of amino acids totally abrogates the E2F1-induced mTORC1 activation.

3.2 Function of E2F1 in intracellular trafficking

3.2.1 E2F1 induces lysosomal movement to the cell periphery

Since E2F1 modulates the subcellular localization of mTOR into the lysosomes, and as it is known that lysosomal positioning plays a role in

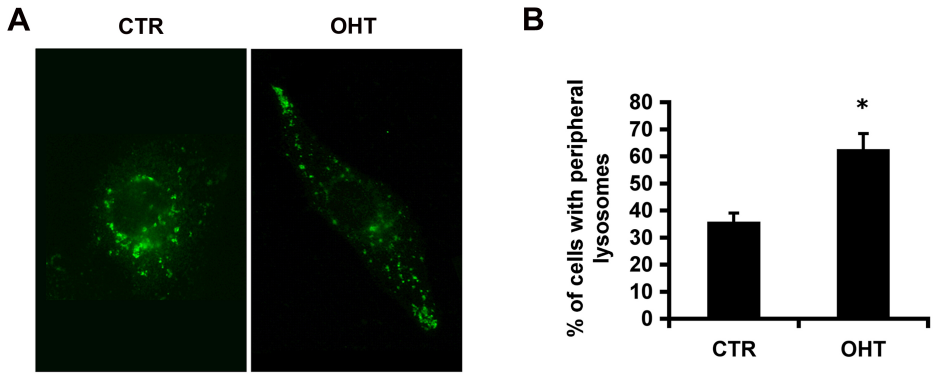

Figure 3.8: E2F1 induces lysosomal trafficking. (A) Serum-deprived ER-E2F1 U2OS cells were cultured in the absence (CTR) or in the presence of 4-hydroxitamoxifen (OHT) for 6 h. Immunofluorescence assay was performed as described in Materials and Methods using primary antibody against LAMP2. (B) Quantification of peripheral lysosomes localization is shown.

regulating mTORC1 signaling and autophagy (Korolchuk et al., 2011), we decided to investigate whether E2F1 activity was affecting lysosomal trafficking and distribution. To this end, we performed analysis by immunofluorescence microscopy of endogenous LAMP2 protein. As shown in Fig. 3.8, the activation of E2F1 produced a significative change in the intracellular distribution of LAMP2-positive vesicles. Whereas serum-deprived cells showed a predominant perinuclear distribution of LAMP2-positive particles, activation of E2F1 induced the relocalization of LAMP2-positive particles toward the cell periphery. The quantification analysis showed that the percent of peripheral lysosomes shifts from

around 35% in the control to 65% in the E2F1-induced condition.

In order to verify this finding *in vivo*, we tracked the lysosomes' movement in live cells. We transiently transfected U2OS ER-E2F1 cells with a LAMP1-GFP expressing plasmid and, after serum starvation, we added OHT at the time point zero. We then tracked lysosomal mobility by live imaging employing time-lapse microscopy during 16h. The image analysis confirms the time-dependent repositioning of lysosomes toward the cell periphery in the OHT treated condition (Movie 1).

To corroborate the function of E2F1 in regulating endosomal traffick-

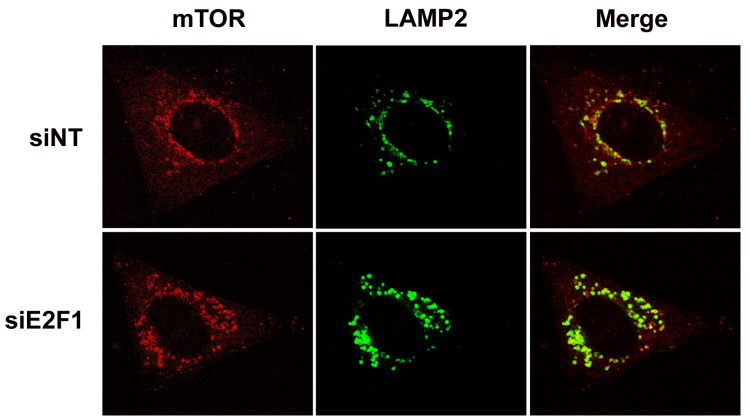

Figure 3.9: E2F1 is required for lysosomal trafficking. U2OS cells were transfected with non target siRNA (siNT) or E2F1 siRNA. Cells were serum-deprived and immunofluorescence was performed as described in Materials and Methods using primary antibodies against specified proteins at 48 h after transfection.

ing, we performed a RNA interference experiment to knockdown the endogenous E2F1 in U2OS wt cells. Consistent with the overexpression experiment, siRNA depletion of endogenous E2F1 led to an accumulation of enlarged LAMP2 particles, which localized in the perinuclear region (Fig. 3.9). These results demonstrate that E2F1 activation plays an important role in redirecting lysosomes to the cell periphery.

3.2.2 Raptor, but not mTOR activity, is required for the E2F1-regulated lysosomal movement

As E2F1 is able to trigger the lysosomal repositioning toward the cell periphery, we next asked whether the E2F1-induced mTORC1 activity was implicated in this process. To answer this question, we performed the same kind of immunofluorescence staining of endogenous LAMP2 and mTOR proteins, as described in section 3.2.1, but in the presence of rapamycin, an mTORC1 allosteric inhibitor. The immunofluorescence analysis showed that treatment with rapamycin did not alter the E2F1-induced LAMP2 or mTORC1 relocalization to the cell periphery, despite abrogating mTORC1 basal activity, as measured by S6K1 T389 and 4E-BP1 T37/T46 phosphorylation (Fig. 3.10). These data were also consistent with the live cell imaging of ectopically expressed LAMP1-

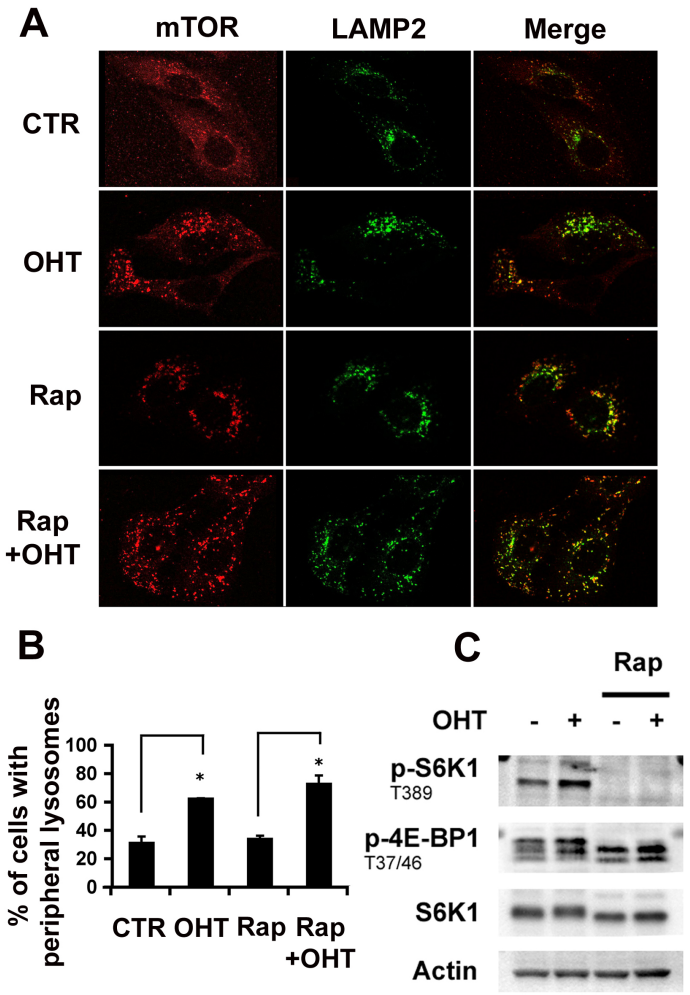

Figure 3.10: Effect of mTORC1 activity in lysosomal trafficking. (A-C) Serum-deprived ER-E2F1 U2OS cells were cultured in the absence (CTR) or in the presence of 4-hydroxitamoxifen (OHT) and pre-treated or not with rapamycin (Rap) for 6 h. (A) Immunofluorescence assay was performed as described in Materials and Methods using primary antibodies against showed proteins. Merge panels indicate the co-localization of antibodies signals. (B) Quantification of peripheral lysosomes localization is shown. (C) Expression of the indicated proteins was determined by Western Blot analysis at 6 h after treatment.

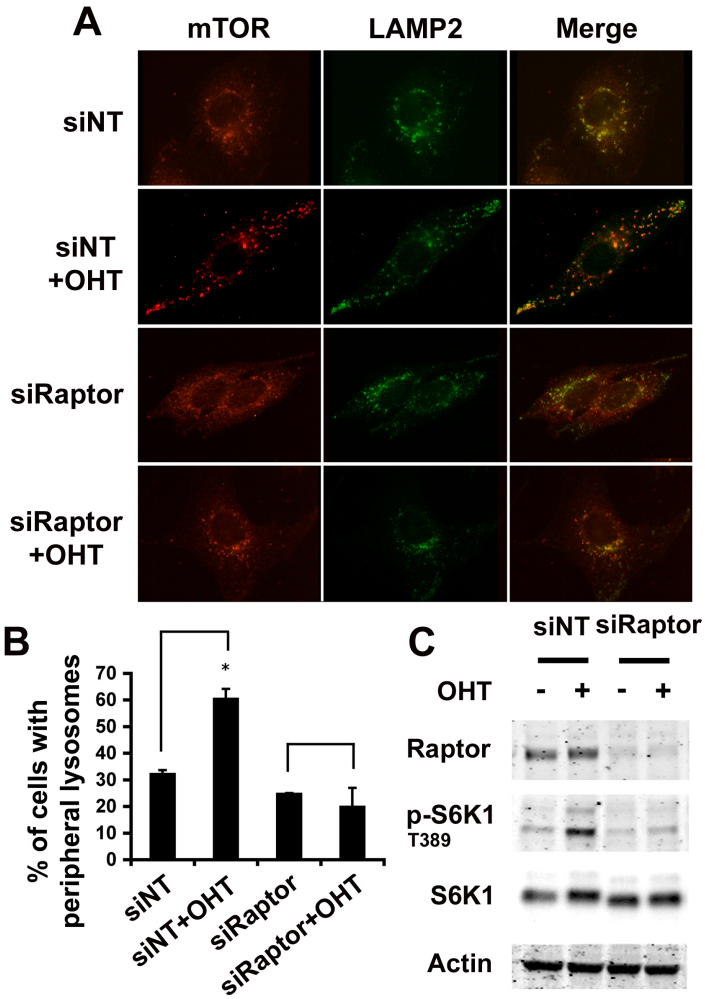

Figure 3.11: Effect of Raptor depletion in lysosomal trafficking. (A-C) ER-E2F1 U2OS cells were transfected with non target siRNA (siNT) or Raptor siRNA for 48 h. Cells were serum-starved and treated or not (CTR) with 4-hydroxitamoxifen (OHT). (A) Immunofluorescence assay was performed as described in Materials and Methods using primary antibodies against showed proteins. Merge panels indicate the co-localization of antibodies signals. (B) Quantification of peripheral lysosomes localization is shown. (C) Expression of the indicated proteins was determined by Western Blot analysis at 6 h after treatment.

GFP. The image analysis confirms that rapamycin treatment did not abrogate the time-dependent repositioning of lysosomes toward the cell periphery regulated by E2F1 (Movie 2).

To further investigate the implication of mTORC1 activity in the E2F1-regulated lysosomal movement, we analyzed the effect of the depletion of Raptor, an essential component of the mTORC1 complex. The knock-down of Raptor impaired both mTORC1 activation, as measured by S6K1 T389 phosphorylation, and the movement of LAMP2 containing lysosomes to cell periphery (Fig. 3.11). This finding indicates that Raptor is needed to promote the peripheral lysosomal localization induced by E2F1. Taken together, the results suggest that mTORC1 activity is not necessary for lysosomal trafficking induced by E2F1, but that the presence of Raptor is required for this response.

3.3 Effect of E2F1 activity in autophagy

3.3.1 E2F1 represses autophagy

The mTORC1 complex negatively regulates the first phase of autophagy, by inhibiting the phagophore nucleation, while the lysosome positioning can regulate the last phase of autophagy by modulating the rate of

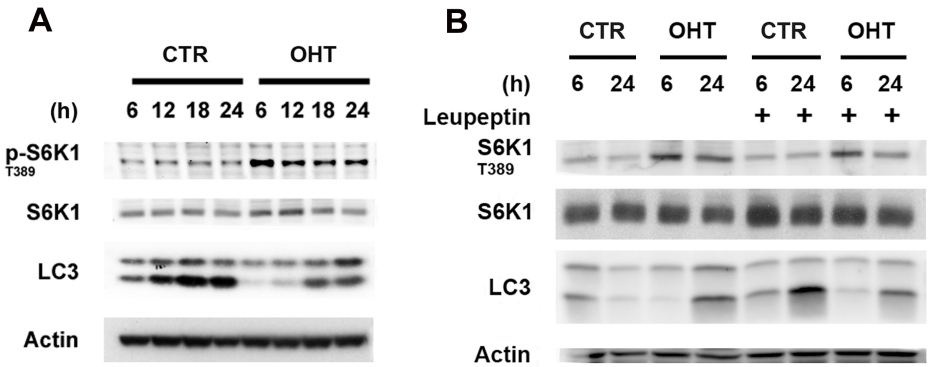

Figure 3.12: E2F1 represses autophagy. Serum-deprived ER-E2F1 U2OS cells were treated or not treated (CTR) with 4-hydroxitamoxifen (OHT), in the presence (+) or in the absence of leupeptin. At showed times, expression of the indicated proteins was determined by Western Blot analysis.

autophagosome-lysosome fusion (Jung et al., 2009; Korolchuk et al., 2011). As E2F1 regulates lysosomal trafficking and mTORC1 activity, we speculated that it could also play a role in controlling autophagy. To investigate the function of E2F1 on autophagy, cells were deprived overnight of serum and the effect of E2F1 activity on the conversion of LC3-I to LC3-II was monitored by Western Blot analysis. In serum deprived cells, we observed a time-dependent accumulation of LC3-II, but this response was markedly suppressed by overexpression of E2F1 (Fig. 3.12A). The blocking of the autophagy flux, by the addition of the protease inhibitor leupeptin, confirmed E2F1's role as a repressor of autophagy, since in this setting as well we observed smaller accumulation

of LC3-II in the OHT treated conditions respect to the controls (Fig. 3.12B).

Consistent with these findings, immunofluorescence analysis showed that LC3 and LAMP2 formed prominent punctae and localized throughout the perinuclear region upon serum starvation, while in E2F1-induced cells, LC3 staining was more diffuse, less abundant, and LAMP2 vesicles were localized at the cell periphery (Fig. 3.13).

The lower amount of LC3-II and the disseminated localization of LC3 found in E2F1 activated conditions indicate that autophagosomes formation is inhibited by E2F1, very likely through the activation of mTORC1

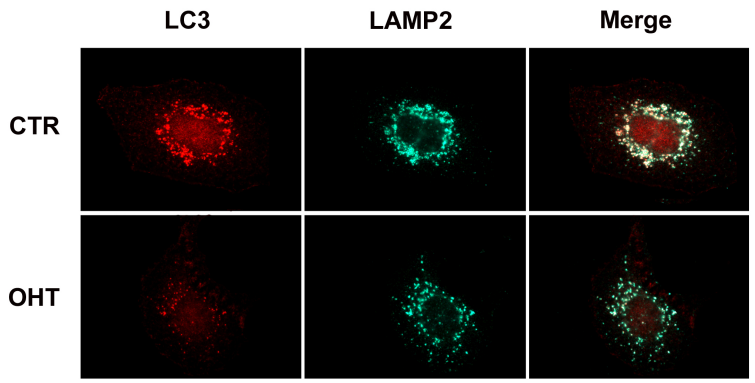

Figure 3.13: Effect of E2F1 activity on LC3 localization. Serum-deprived ER-E2F1 U2OS cells were cultured in the absence (CTR) or in the presence of 4-hydroxitamoxifen (OHT). Immunofluorescence assay was performed as described in Materials and Methods using primary antibodies against showed proteins at 6 h after treatment.

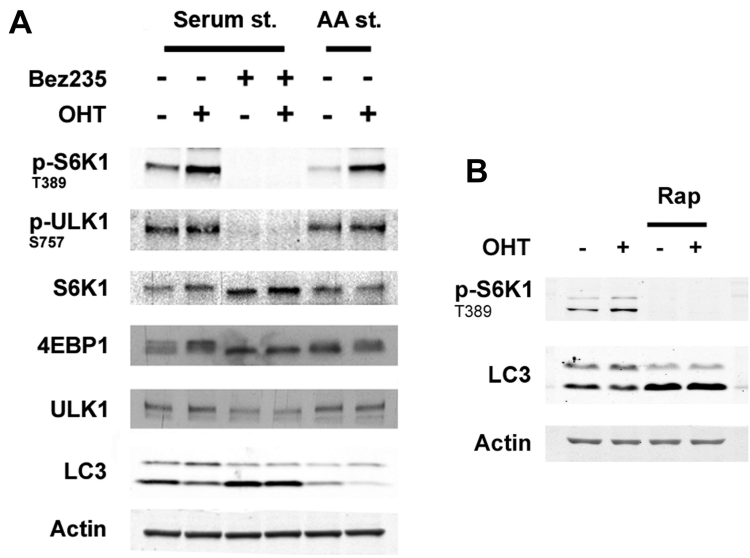

Figure 3.14: Effect of mTOR inhibitors on the E2F1-regulated autophagy. Serum-deprived or amino acids/serum deprived ER-E2F1 U2OS cells were treated (+) or not treated (-) with 4-hydroxitamoxifen (OHT) in the presence (+) or in the absence (-) of Bez235 or rapamycin (Rap). Expression of the indicated proteins was determined by Western Blot analysis at 6 h after treatment.

(Levine and Kroemer, 2008). Accordingly, incubation with BEZ235, a dual mTOR/PI3K inhibitor, totally abrogated the negative effect of E2F1 on autophagy, as measured by the increase in LC3-II levels in both conditions with or without E2F1 induction (Fig. 3.14A). As expected, BEZ235 treatment inhibited the phosphorylation of the mTOR targets S6K1, ULK1 and 4E-BP1. Similar results were obtained in the presence of rapamycin (Fig. 3.14B). Thus, these results demonstrate that E2F1

is a negative regulator of autophagy and suggest that the migration of lysosomes to the cell periphery as well as the activation of mTORC1 could both contribute to this response.

3.4 Characterization of the mechanism by which E2F1 regulates mTORC1 activity and intracellular trafficking: Role of V-ATPase and KIF2A

3.4.1 The microtubule-dependent trafficking is necessary for E2F1-induced mTORC1 activation

Considering the role of lysosomal positioning in mTORC1 regulation (Korolchuk et al., 2011), we decided to examine whether the E2F1-regulated lysosomal trafficking was involved in mTORC1 activation. Given that microtubules are required for the transport of lysosomes within the cell, we investigated whether the alteration of microtubules depolymerization by nocodazole treatment could affect the action of E2F1 on lysosomal distribution and mTORC1 signaling. Immunofluorescence analysis showed that LAMP2 vesicles failed to migrate to the cell periphery in the presence of nocodazole (Fig. 3.15A). Likewise, the treatment with 30 μM nocodazole strongly inhibited the E2F1-induced mTORC1 activation, as

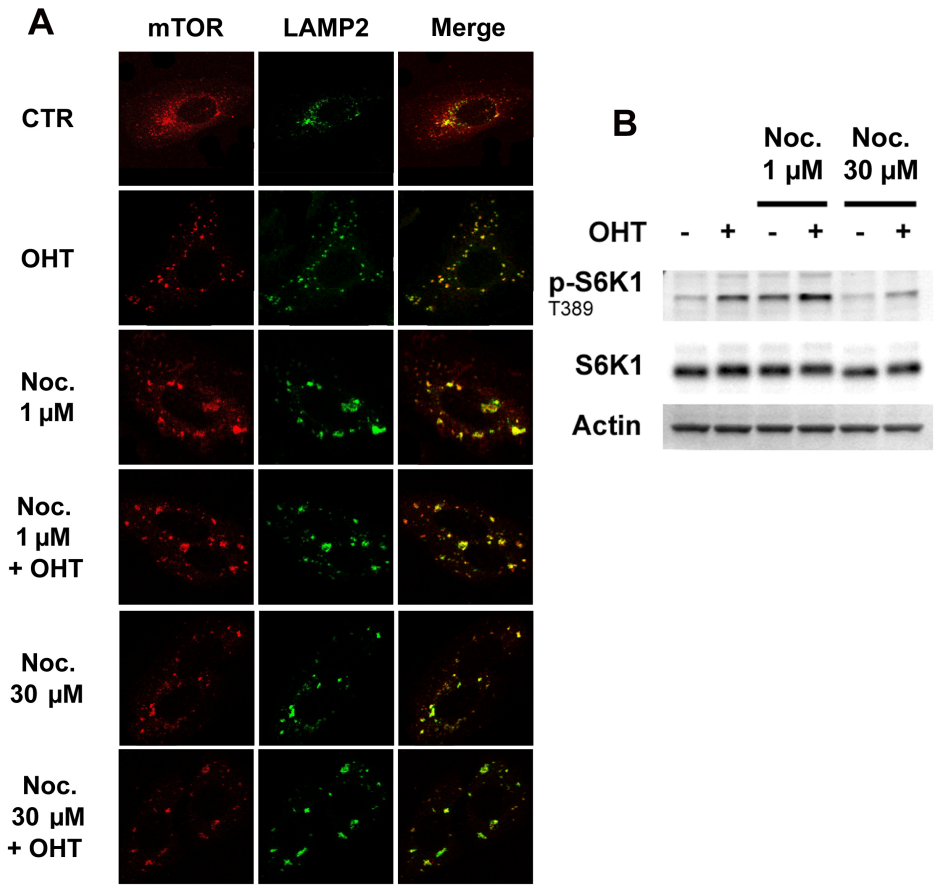

Figure 3.15: Effect of nocodazole on the E2F1-regulated endosomal trafficking and mTORC1 activity. Serum-deprived ER-E2F1 U2OS cells were cultured in the absence (CTR) or in the presence of 4-hydroxitamoxifen (OHT) and pre-treated (Noc) or not with nocodazole for 6 h at the indicated concentration. (A) Immunofluorescence assay was performed as described in Materials and Methods using primary antibodies against showed proteins. (B) Expression of the indicated proteins was determined by Western Blot analysis in the absence (-) or in the presence (+) of 4-hydroxitamoxifen (OHT).

measured by S6K1 T389 phosphorylation (Fig. 3.15B). Unexpectedly, at lower concentration of Nocodazole (1 μM), mTORC1 activity was higher than in untreated cells, either in the presence or in the absence of E2F1 induction. In these conditions, cells showed an accumulation of larger LAMP2 vesicles similar to those found in E2F1-depleted cells and to those previously reported as a temporal accumulation of lysosomes intermediates (Le Blanc et al., 2005). These data support a role for microtubules in the E2F1-induced mTORC1 activation and lysosomal trafficking.

3.4.2 E2F1 regulates KIF2A transcription, a kinesin required for mTORC1 activation

We next hypothesized that E2F1 could modulate lysosomal trafficking and mTORC1 activity by regulating microtubules' functionality. The mechanism of intracellular transport involves molecular motor proteins that carry cargo directionally along a cytoskeletal track (myosins along actin and kinesins and dyneins along microtubules). The members of the kinesin and dynein families of motor proteins generate directional movement along microtubules using ATP and they are also responsible for organizing cytoskeletal filaments. The anterograde (microtubule plus-end-

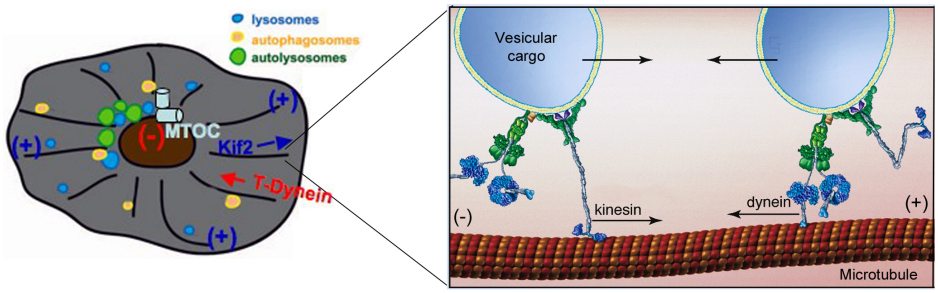

Figure 3.16: The intracellular transport. The kinesin and dynein families carry out respectively the anterograde (microtubule plus-end-directed) and the retrograde (minus-end-directed) movements along microtubules filaments, allowing the transports of vesicular cargoes within the cell. Adapted from Vale, 2003.

directed) and the retrograde (minus-end-directed) movements are carried out by the kinesin and dynein families respectively (Fig. 3.16). Kinesins have been reported to transport several vesicular cargoes such as mitochondria, lysosomes, endoplasmic reticulum, and various anterograde-moving vesicles in axons (Vale, 2003).

To investigate whether E2F1 modulates mTORC1 activity by regulating microtubules function, we tested the role of kinesin KIF2A and ARL8B in this process, as both have been reported to mediate mTORC1 stimulation by nutrients (Korolchuk et al., 2011). KIF2A is a member of the plus end-directed microtubule-dependent motor family, and ARL8B (ADP-ribosylation factor-like 8B) is a Arf-like GTPase protein which binds to lysosomes and contributes to their anterograde movement toward the

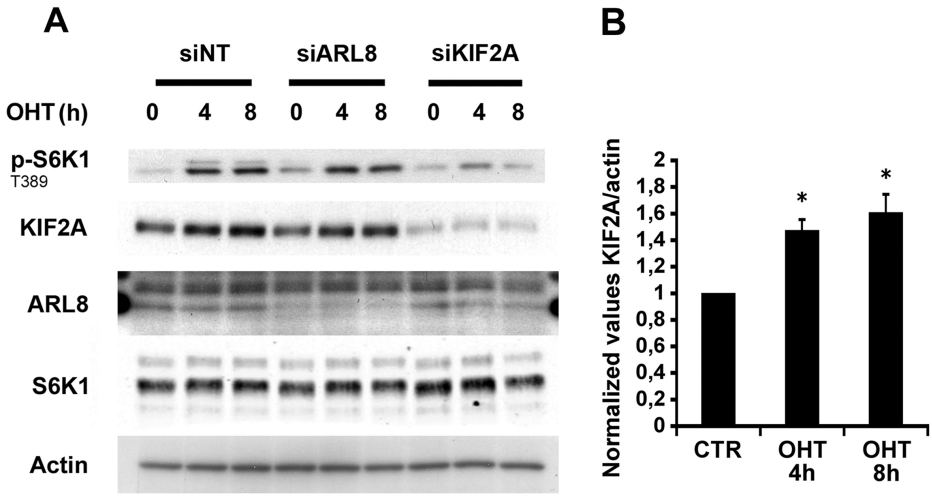

Figure 3.17: KIF2A, a new target of E2F1, is essential for mTORC1 activation. ER-E2F1 U2OS cells were transfected with non target siRNA (siNT) or ARL8B or KIF2A siRNAs for 48h. Cells were serum-deprived and treated (OHT) or not (CTR) with 4-hydroxitamoxifen at the indicated times. (A) Expression of the indicated proteins was determined by Western Blot analysis (B) Quantification of KIF2A protein levels.

cell periphery (Hofmann and Munro, 2006). To this end, we performed siRNA interference experiments against these two proteins. As shown in Fig. 3.17A, the depletion of KIF2A levels by siRNA treatment inhibited the mTORC1 activation induced by E2F1 measured as S6K1 T389 phosphorylation, while ARL8B knockdown had no measurable effect. Interestingly, KIF2A protein levels increased after E2F1 overexpression both in control and in ARL8B depleted cells, as shown in the densitometric analysis of the Western Blot experiments (Fig. 3.17A-B).

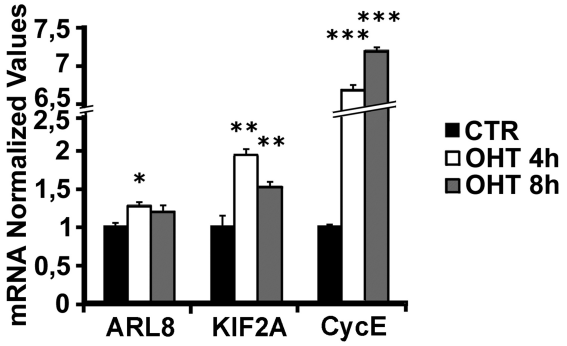

Figure 3.18: E2F1 upregulates KIF2A mRNA levels. ER-E2F1 U2OS cells were serum-deprived and treated (OHT) or not (CTR) with 4-hydroxitamoxifen at the indicated times. Indicated mRNA levels were measured by real-time PCR as described in Materials and Methods.

Taking this finding into account, we decided to measure by quantitative Real-time PCR the levels of the KIF2A transcript in these conditions. In accordance with the change at the protein level, the KIF2A mRNA amount was also upregulated in a time-dependent manner in E2F1-induced cells. The increase in cyclin E mRNA levels, a well-known E2F1 target, was used as a positive control for E2F1 transcriptional activity (Fig. 3.18).

The role of E2F1 in the regulation of KIF2A expression was confirmed by siRNA depletion of endogenous E2F1, which abolished KIF2A expression and suppressed mTORC1 activation, as measured by a decrease in S6K1 T389 phosphorylation and by an increase in LC3-II (Fig. 3.19A-B).

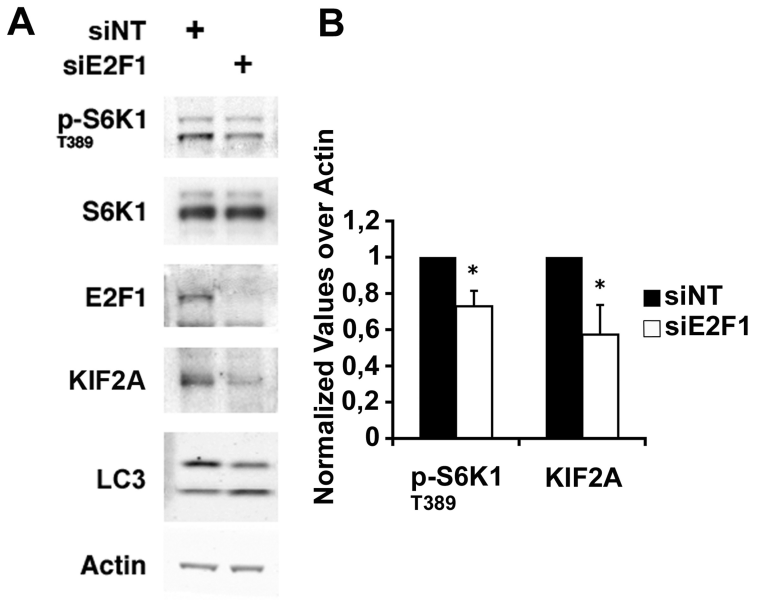

Figure 3.19: KIF2A is required for the E2F1-driven mTORC1 activation. U2OS cells were transfected (+) or not with non target siRNA (siNT) or E2F1 siRNA for 48 h and serum-starved for 15 h. (A) Expression of the indicated proteins was determined by Western Blot analysis. (B) Quantification of indicated protein levels.

We next explored the mechanism of KIF2A upregulation triggered by E2F1. Considering that E2F1 is a transcription factor, we investigated whether KIF2A control occurred at the transcriptional level. As the human KIF2A promoter has not been previously characterized, we firstly identified a ∼1300 bp genomic fragment of the human gene KIF2A 5' UTR-flanking region that was predicted to have promoter activity. We subcloned the genomic region indicated in a luciferase reporter vector,

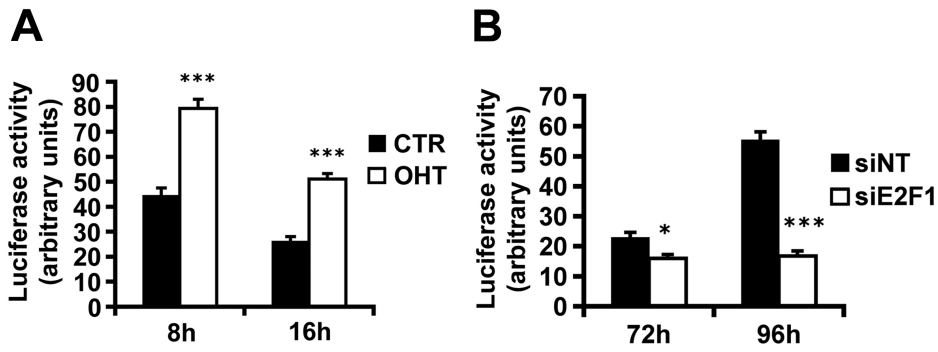

Figure 3.20: E2F1 upregulates KIF2A at the transcriptional level. (A) ER-E2F1 U2OS cells were co-transfected with the KIF2A-luciferase reporter plasmid together with the CMV-renilla vector, serum-deprived and treated (OHT) or not (CTR) with 4-hydroxitamoxifen. The luciferase activity was measured at the indicated times and the values were normalized over renilla activity and over μg of proteins. (B) U2OS cells were transfected with non target siRNA (siNT) or E2F1 siRNA. After 24 h cells were co-transfected with the KIF2A-luciferase reporter plasmid together with the CMV-renilla vector and serum-starved. The luciferase activity was measured at the indicated times and the values were normalized over renilla activity and over μg of proteins.

and we transfected the cells with the obtained reporter (KIF2A-luciferase) to perform the luciferase assay. In this setting, E2F1 overexpression by OHT addition resulted in a two-fold increase in the luciferase activity, whereas siRNA depletion of E2F1 caused a significant decrease in the reporter activity (Fig. 3.20A-B).

Next, we asked whether increased KIF2A expression could be sufficient to induce mTORC1 activation, as reported by others (Korolchuk et al., 2011). Unexpectedly, as shown in Fig. 3.21, the ectopic expression of a

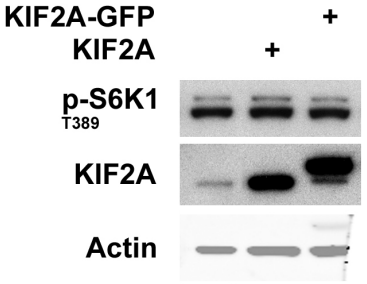

Figure 3.21: KIF2A overexpression does not enhance mTORC1 activity. U2OS cells were transfected with GFP-KIF2A and KIF2A-pLX304 expression plasmids (+) or not (-) as indicated. After 48 h, expression of the indicated proteins was determined by Western Blot analysis.

KIF2A cDNA or a GFP-tagged KIF2A cDNA failed to induce mTORC1 activation. Taken together, the results described in this section demonstrate that E2F1 enhances KIF2A expression at the transcriptional level and that, although KIF2A basal levels are required for E2F1-induced mTORC1 activation, the increase in the kinesin levels alone does not appear sufficient for this induction.

3.4.3 E2F1 enhances V-ATPase activity, which modulates lysosomal movements and mTORC1 activity

As V-ATPase has been functionally implicated in vesicular trafficking and mTORC1 signaling, we investigated whether E2F1 regulated V-ATPase activity (Zoncu et al., 2011; Hurtado-Lorenzo et al., 2006; Clague et al.,

1994). To examine this hypothesis, we measured the V-ATPase activity by monitoring the pH of individual lysosomes after E2F1 induction. To this end, we loaded endosomes with dextran that was coupled to both fluorescein isothiocyanate (FITC) and to rhodamine B, and we followed the changes in fluorescence intensity by live-cell imaging system. The FITC fluorescence decreases with acid pH, whereas the rhodamine signal acts as a pH-independent control (Majumdar et al., 2007). We analyzed the fluorescence intensities of both signals and we converted them to pH values through a calibration method. Thus, we obtained the changes

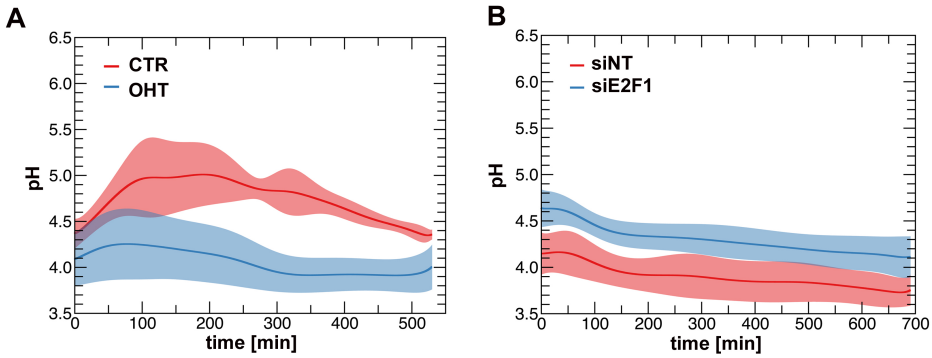

Figure 3.22: E2F1 enhances V-ATPase activity. (A) ER-E2F1 U2OS cells were loaded with dextran labeled with FITC and with Rhodamine B for 24 h. After 15 h of serum starvation, cells were treated (OHT) or not (CTR) with 4-hydroxitamoxifen and intra-lysosomal pH was measured as described in Materials and Methods. (B) U2OS cells were transfected either with non target siRNA (siNT) or E2F1 siRNA and then loaded with dextran labeled with FITC and with Rhodamine B for 24 h. Intra-lysosomal pH was measured at 48 h after transfection as described in Materials and Methods.

in endosomal pH values in time and we found that the mean pH of lysosomes shifts from 4.3-4.8, in serum deprived cells, to 3.8-4.2 in E2F1-induced cells, suggesting that V-ATPase activity is enhanced by E2F1 (Fig. 3.22A).

The role of E2F1 in the regulation of V-ATPase was further supported by the siRNA-depletion experiment of endogenous E2F1, which led to an increase in the lysosomal pH compared to the control (Fig. 3.22B). The rise of the intra-lysosomal pH value should imply a decrease in lysosomal protease activity. Accordingly, the increase in lysosomal pH induced by E2F1 knockdown was associated with the accumulation of LC3-II at the protein level, as discussed above (Fig. 3.19A).

The finding that E2F1 overexpression leads to an increase of V-ATPase activity implicates that the extrusion of intracellular protons is enhanced in this condition. Consistent with this observation, activation of E2F1 also correlated with an increase in intracellular pH, as measured by using a pH-sensitive fluorescence dye 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester (BCECF). To measure pH_i, we incubated the ER-E2F1 U2OS cells with the fluorescent dye after treatment, and we performed immunofluorescence studies to analyze the pH_i-dependent green fluorescence intensity of the images. As shown in Fig.

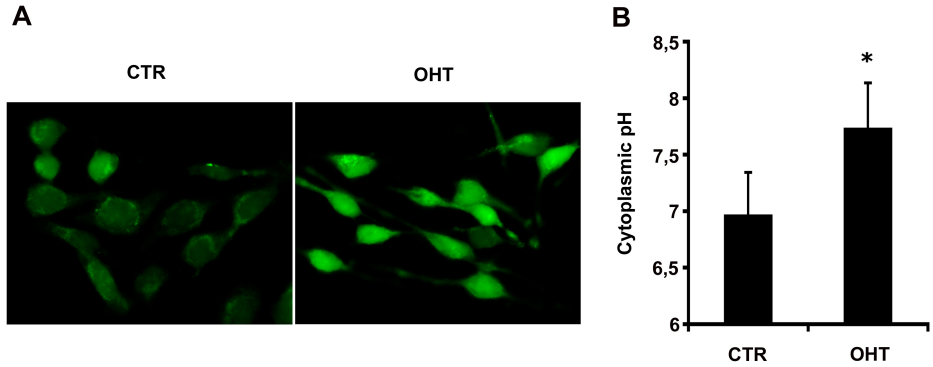

Figure 3.23: E2F1 activity increases the intracellular pH. (A-B) ER-E2F1 U2OS cells were serum-starved, cultured in the absence (CTR) or in the presence of 4-hydroxitamoxifen (OHT) and incubated with the BCECF dye for 30 minutes. At 20 h after treatment, intracellular pH was measured as described in Material and Methods. (A) A representative immunofluorescence image is shown.

3.23, serum deprived cells presented a pH_i of approximately 7.0, whereas E2F1 overexpression shifted the pH_i values to a more basic range (approximately 7.7).

The above findings raise the possibility that E2F1 mediates lysosome redistribution and increases mTORC1 signaling by inducing V-ATPase activation. To corroborate such hypothesis, we initially tested the effects of concanamycin, a specific V-ATPase inhibitor on both responses. The results showed that such treatment impaired E2F1-induced redistribution of lysosomes to the cell periphery and induced the formation of large LAMP2-positive vesicles (Fig. 3.24A). These vesicles could have origi-

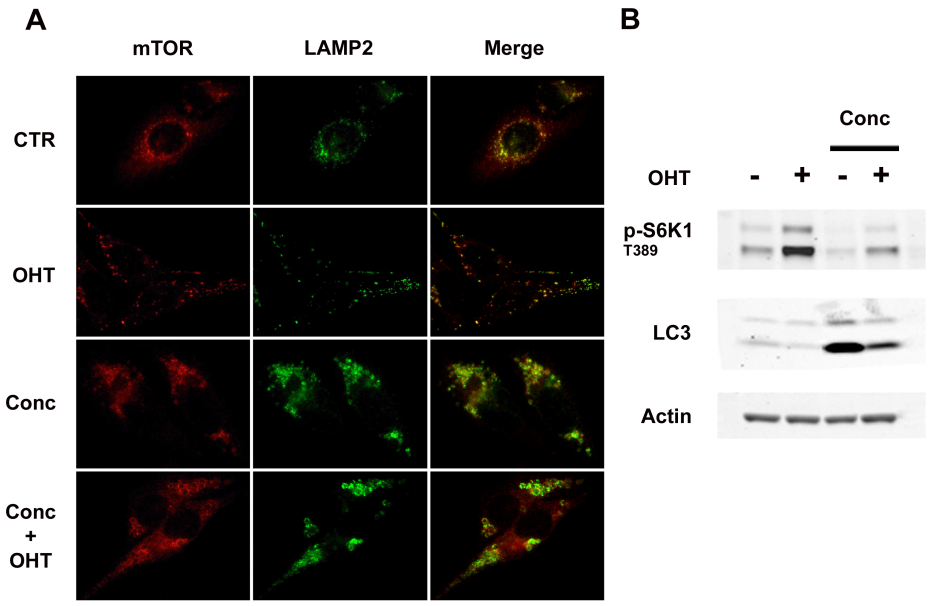

Figure 3.24: Effect of concanamycin on mTORC1 activity and lysosomal trafficking. Serum-deprived ER-E2F1 U2OS cells were cultured in the absence (CTR) or in the presence of 4-hydroxitamoxifen (OHT) with or without concanamycin (Conc). (A) At 6 h after treatment, immunofluorescence assay was performed as described in Material and Methods using primary antibodies against showed proteins. Merge panels indicate the co-localization of antibody signals. (B) Expression of the indicated proteins was determined by Western Blot analysis.

nated because of the blocking of early endosome to lysosome maturation due to the inhibition of endosomal acidification, as it has been previously reported by others (Hurtado-Lorenzo et al., 2006; Vaccari et al., 2010). In parallel, the Western Blot analysis showed that concanamycin treatment inhibited the E2F1-induced phosphorylation of S6K1 T389 (Fig. 3.24B). In the same experiment we could also notice that the treatment

with concanamycin was indeed blocking the lysosomal protease activity, as LC3-II was accumulating in these conditions, and, furthermore, that E2F1 activity was inhibiting the autophagy flux, as reported in section 3.3.1.

To support the above findings, which indicate the implication of V-ATPase in E2F1's function, we performed additional interference exper-

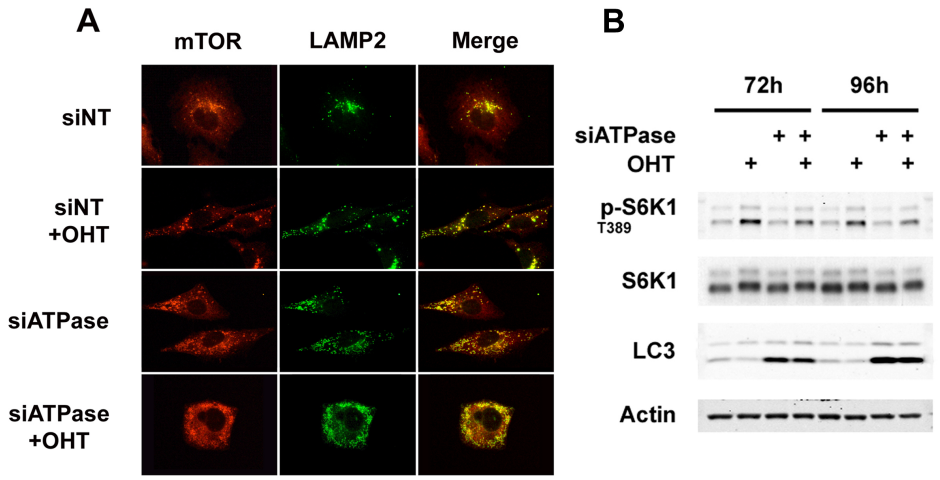

Figure 3.25: V-ATPase in required for the E2F1-induced mTORC1 activation and lysosomal trafficking. ER-E2F1 U2OS cells were transfected with non target siRNA (siNT) or ATP6V0C siRNAs (siATPase), serum deprived and cultured in the absence (CTR) or in the presence of 4-hydroxitamoxifen (OHT). (A) At 6 h after treatment, immunofluorescence assay was performed as described in Material and Methods using primary antibodies against showed proteins. Merge panels indicate the co-localization of antibody signals. (B) Expression of the indicated proteins was determined by Western Blot analysis.

iments to down-modulate the amount of the V0 subunit C (ATP6V0C) of the V-ATPase. The siRNA interference provoked both the formation of large LAMP2-positive vesicles and the inhibition of S6K1 T389 phosphorylation induced by E2F1, similarly to the effects obtained following concanamycin treatment (Fig. 3.25A-B).

To rule out the possibility that the change detected in the V-ATPase activity after E2F1 induction could be mediated by mTORC1, we monitored the effect of rapamycin treatment on intra-lysosomal pH by using the live-cell imaging system described above. Even in the presence of ra-

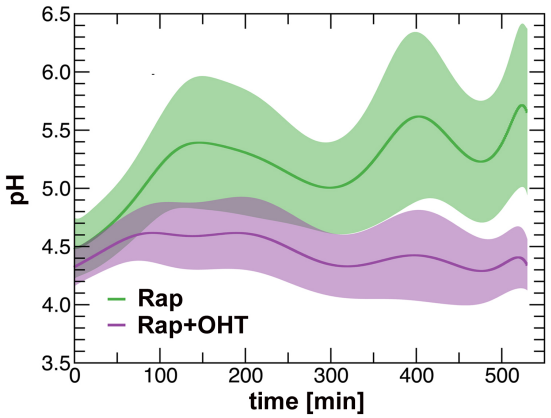

Figure 3.26: Effect of rapamycin on intra-lysosomal pH. ER-E2F1 U2OS cells were loaded with dextran labeled with FITC and with Rhodamine B for 24 h. After 15 h of serum starvation, cells were treated (OHT) or not with 4-hydroxitamoxifen in the absence or in the presence (Rap) of rapamycin, and intra-lysosomal pH was measured as described in Material and Methods.

pamycin, the intra-lysosomal pH values measured in E2F1-induced cells were lower than in non-induced cells, corroborating that mTORC1 activity was not required for the increased protons' flux into lysosomes regulated by E2F1 (Fig. 3.26).

Next, we investigated the mechanism by which E2F1 upregulates V-ATPase activity. It is known that V-ATPase activity requires the reversible association of the V1 domain with the membrane inserted V0 domain of the V-ATPase complex (Kane and Smardon, 2003). Since we had detected an increase in the physical interaction between mTORC1 and RagB after E2F1 overexpression (Fig. 3.6), we tested whether E2F1 also induced the association of the V1 domain with the RagGTPase complex (Zoncu et al., 2011). To this end, we evaluated the extent of RagB interaction with the C1 subunit of the V1 complex (ATP6V1C1) in U2OS ER-E2F1 cells stably transfected with a FLAG-tagged RagB as mentioned in section 3.1.1. The results from FLAG immunoprecipitation experiments showed that E2F1 induced the binding of ATP6V1C1 to RagB, suggesting that, in addition to mTORC1 complex, E2F1 is also capable of recruiting the V1 subunit to lysosomes and of activating V-ATPase activity (Fig. 3.27). Aldolase B, a glycolytic enzyme that has been also reported to be associated with V-ATPase, was similarly de-

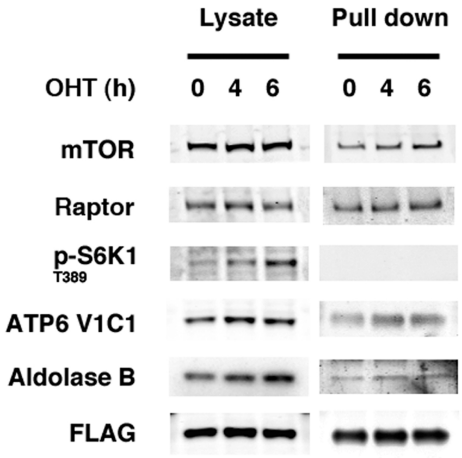

Figure 3.27: E2F1 activity promotes ATP6V1C1 binding to the RagGTPase complex. Stably transfected ER-E2F1/ FLAG-RagB U2OS cells were serum starved and treated with 4-hydroxitamoxifen (OHT) at the indicated times. Proteins were cross-linked as described in Material and Methods and immunoprecipitated using FLAG antibody. Expressions of the indicated proteins were determined by Western Blot analysis.

tected in the FLAG-tagged RagB immunoprecipitated (Lu et al., 2007). Although the amount of aldolase B was upregulated by E2F1 activation, the association of the glycolytic enzyme with RagB did not change.

Taken together, the results presented here demonstrate that E2F1 activity induces lysosomal acidification, implying that E2F1 enhances V-ATPase activity. Moreover, E2F1 promotes the recruitment of the V-ATPase subunit ATP6V1C1 to the RagB/mTORC1 complex, suggesting that the association of V1 subunit with lysosomes could be the mecha-

nism of V-ATPase activation. Finally, the increase in V-ATPase activity is essential for the E2F1-induced mTORC1 activation and lysosomal trafficking.

3.4.4 V-ATPase is involved in E2F1-dependent regulation of KIF2A

As both V-ATPase and KIF2A are required for E2F1-induced mTORC1 activation, we investigated their mutual dependence in this response. Thus, we performed a double siRNA knockdown experiment against ATP6V0C subunit and KIF2A. As shown in Fig. 3.28, the repression of E2F1-induced S6K1 T389 phosphorylation was stronger when both ATP6V0C and KIF2A were depleted together than when either was depleted alone. Moreover, the response to KIF2A depletion alone was weaker compared to those obtained for V-ATPase, even if full KIF2A downregulation was obtained. This observation led us to speculate that the only $\sim$ 40% reduction in KIF2A levels observed after E2F1 depletion was unlikely to be the sole cause for mTORC1 downregulation in these conditions (Fig. 3.19A). For example, the activity of V-ATPase, that we observed to be inhibited in E2F1-depleted conditions measuring by an increase in lysosomal pH, could be essential for mTORC1 activa-

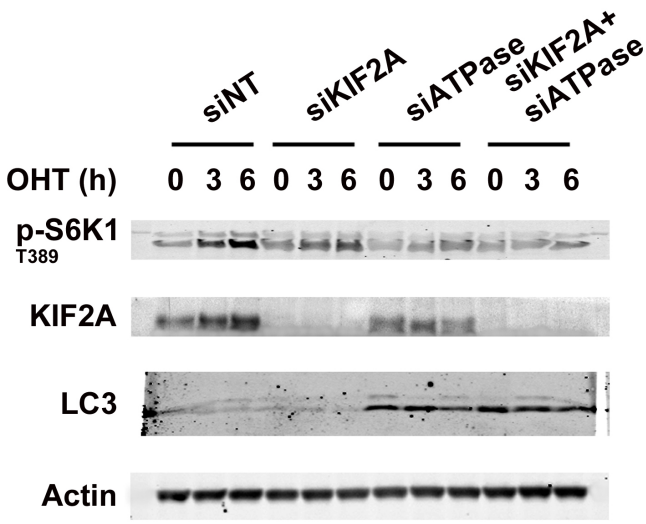

Figure 3.28: Effect of the combined depletion of V-ATPase and KIF2A on the E2F1-induced mTORC1 activation. ER-E2F1 U2OS cells were transfected with non target siRNA (siNT)/ KIF2A siRNA (siKIF2A)/ ATP6V0C (siATPase) as indicated. Cells were serum-deprived for 15 h and treated with 4-hydroxitamoxifen (OHT) at the indicated times. Expression of the indicated proteins was determined by Western Blot analysis.

tion independently of KIF2A levels (Fig. 3.22B). To answer this question, we performed another siRNA knockdown experiment of endogenous E2F1, where we overexpressed an exogenous KIF2A-GFP construct to recover the decrease in KIF2A levels after E2F1 depletion. In support of our hypothesis, KIF2A overexpression did not rescue the reduction of S6K1 T389 phosphorylation in response to E2F1 knockdown (Fig. 3.29).

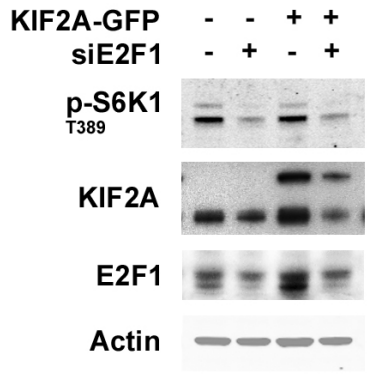

Figure 3.29: KIF2A overexpression does not rescue mTORC1 repression due to E2F1 depletion. U2OS cells were co-transfected with non-target (-) or E2F1 siRNAs and with empty (-) or GFP-KIF2A expression vector (+) and then serum starved. After 72 h, expression of the indicated proteins was determined by Western Blot analysis.

Interestingly, we noted in the double knockdown experiment that ATP6V0C depletion abolished the increase in KIF2A expression detected after OHT treatment, suggesting a V-ATPase-dependent regulation of KIF2A expression (Fig. 3.28). To test this possibility, we measured the effect of V-ATPase inhibition on the E2F1-regulated KIF2A transcription, by using the KIF2A-luciferase reporter assay described in section 3.4.2. The results indicate that the V-ATPase knockdown abolished the increase detected in the luciferase activity 10 hours after E2F1 activation (Fig. 3.30A), suggesting that V-ATPase activity is required for the E2F1-regulated transcription of KIF2A.

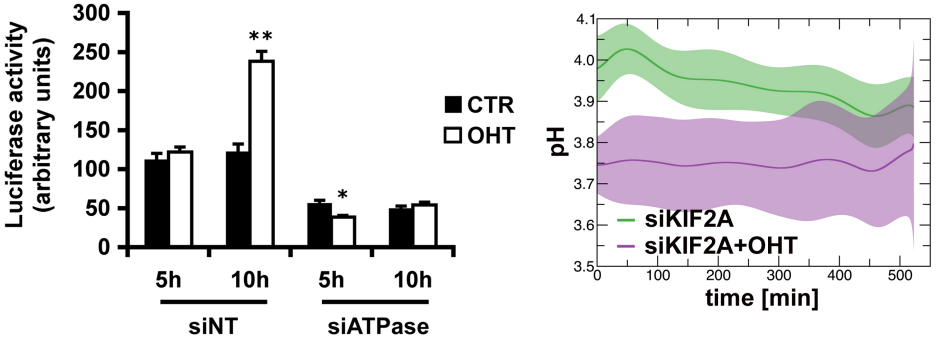

Figure 3.30: The E2F1-regulated KIF2A transcription is V-ATPase-dependent, whereas the V-ATPase activity is KIF2A-independent. (A) ER-E2F1 U2OS cells were transfected with non target siRNA (siNT) or ATP6V0C siRNA (siATPase). After 24 h, cells were co-transfected with the KIF2A-luciferase reporter plasmid together with the CMV-renilla vector, serum-deprived and treated (OHT) or not (CTR) with 4-hydroxitamoxifen. The luciferase activity was measured at the indicated times and the values were normalized over renilla activity and over μg of proteins. (B) ER-E2F1 U2OS cells were transfected with KIF2A siRNA for 24 h, then loaded with dextran labeled with FITC and with Rhodamine B for 24 h. After 15 h of serum starvation, cells were treated or not with 4-hydroxitamoxifen (OHT) and intra-lysosomal pH was measured as described in Material and Methods.

Conversely, we also explored the possibility that KIF2A expression levels could be involved in the regulation of V-ATPase activity. To this end, we measured the effect of KIF2A knockdown on the change in the intra-lysosomal pH modulated by E2F1, by using the live-cell imaging system described in section 3.4.3. As shown in Fig. 3.30B, depletion of KIF2A did not impair the E2F1-induced endosomal acidification, demonstrating that the regulation of V-ATPase activity by E2F1 is independent of KIF2A

expression. Taken together, these data demonstrate that E2F1 activates KIF2A expression through the modulation of V-ATPase activity and that, by contrast, the V-ATPase regulation is independent of KIF2A levels. Furthermore, the results show that KIF2A over-expression alone cannot rescue the repression of mTORC1 activity due to E2F1 depletion.

3.5 Study of E2F1 implication in cell migration

3.5.1 E2F1 regulates cytoskeleton assembly

Given the implication of cytoskeleton assembly and of focal adhesions turnover in cell migration, we firstly investigated whether E2F1 activity

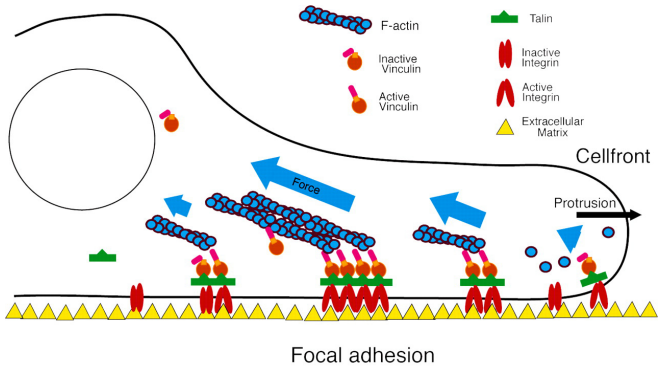

Figure 3.31: The role of cytoskeleton in cell motility. Vinculin and talin are the adaptor proteins which connect actin filaments to integrins and regulate focal adhesions turnover during cell motility. Adapted from Humphries et al., 2007

was affecting the cytoskeleton structure of the cells (Srivastava et al., 2008; Bravo-Cordero et al., 2013). To this end, we performed an immunofluorescence staining of actin filaments and of vinculin. Vinculin is a key adaptor protein which, together with talin, connects actin filaments to integrins and regulates focal adhesions turnover (Fig. 3.31). The force transmission across these proteins from the extracellular matrix to the cytoskeleton has been shown to be necessary for cell migration (Humphries et al., 2007; Grashoff et al., 2010).

Immunofluorescence analysis showed that E2F1 overexpression led to a

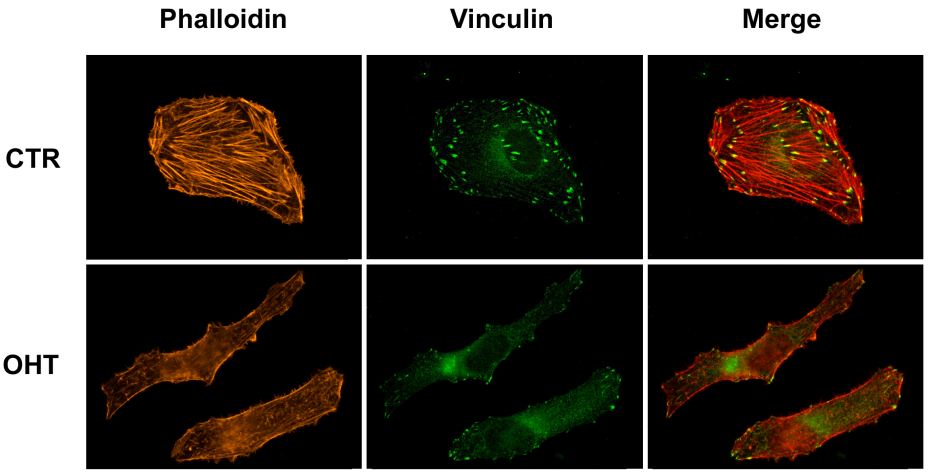

Figure 3.32: E2F1 induces a cytoskeletal re-arrangement. Serum-deprived ER-E2F1 U2OS cells were cultured in the absence (CTR) or in the presence of 4-hydroxitamoxifen (OHT) for 15 h and stained with phalloidin-red. Immunofluorescence assay was performed as described in Materials and Methods using primary antibody against vinculin.

general disassembly of the actin structure and to the loss of most vinculin staining (Fig. 3.32). Moreover, we could observe that the activation of E2F1 also correlated with a change in shape towards a spindle cell form. These results indicate that E2F1 activity induces a general re-arrangement of the cytoskeleton.

3.5.2 E2F1 activity is required for cell migration

The dynamical remodeling of actin filaments, their linkage to the extracellular matrix through integrins, together with the cycles of assembly and turnover of focal adhesions are all processes required for cell motility and migration. In particular, it has been shown that cells depleted of vinculin have reduced adhesion to the ECM and acquire increased migration rates (Saunders et al., 2006). Taking into account that E2F1 was able to drive a general re-arrangement of the cytoskeleton together with a decrease in vinculin levels, we investigated whether cell motility and migration were also affected by E2F1 activity. Hence, we performed a "wound healing" assay, where we depleted endogenous E2F1 and we followed the cell migration rate by monitoring the closure of the gap. As shown in Fig. 3.33, E2F1 knockdown drastically inhibited the "healing" of the wound, suggesting that E2F1 activity is necessary to promote cell

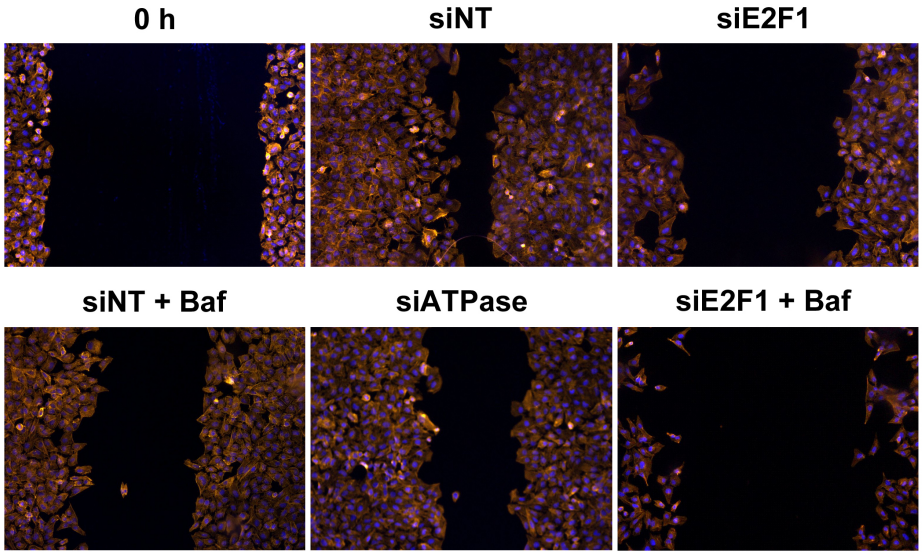

Figure 3.33: E2F1 is required for cell migration. U2OS cells were transfected with non target siRNA (siNT)/ E2F1 siRNA (siE2F1)/ ATP6V0C (siATPase) as indicated and treated (Baf) or not with Bafilomycin. The scratch was performed at time point 0 (0 h) for all the conditions and after 24 h cells were stained with phalloidin-red and DAPI. Immunofluorescence assay was performed as described in Materials and Methods.

migration.

In light of the well studied role of V-ATPase in cell migration and invasion (Sennoune et al., 2004; Wiedmann et al., 2012; Capecci and Forgac, 2013), we compared the effect of E2F1 depletion to V-ATPase inhibition in cell migration. To this end, we used in the same "wound healing" experiment a siRNA against the V0 subunit C (ATP6V0C) of the V-ATPase or the proton pump's inhibitor Bafilomycin.

Both ATP6V0C knockdown and Bafilomycin treatment repressed the closure of the gap at an intermediate level compared to E2F1-depleted condition, while E2F1 knockdown together with Bafilomycin treatment did not show any additional effect in blocking migration, compared to E2F1-depleted condition alone (Fig. 3.33). These data demonstrate that E2F1 is required for cell migration and suggest that V-ATPase activity could mediate the effect of E2F1 in migration. Additionally, depletion of E2F1 has a stronger effect in blocking migration compared to V-ATPase inhibition.

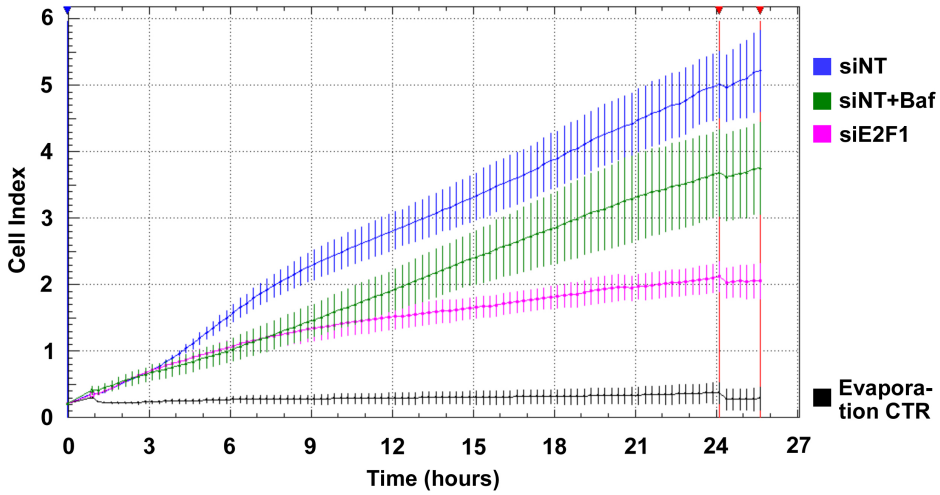

Figure 3.34: E2F1 activity is necessary for cell migration in collagen I. U2OS cells were transfected with non target siRNA (siNT) or E2F1 siRNA (siE2F1) and treated (Baf) or not with Bafilomycin as indicated. After 24h of transfection cells were seeded in Boyden chambers and the migration assay was performed as described in Materials and Methods.

To confirm the role of E2F1 in cell migration, we analyzed the effect of E2F1 depletion and V-ATPase inhibition in a cell migration assay (Real Time Cell Analysis system xCELLigence). In this assay, treated cells were first seeded in a Boyden chamber coated with collagen I and then, the number of cells invading the matrix was monitored *in vivo* in time. As shown in Fig. 3.34, the depletion of E2F1 deeply impaired the ability of cells to migrate, and additionally, this repression was stronger compared to the Bafilomycin treated condition. These data demonstrate that E2F1 is required for cell migration. Furthermore, depletion of E2F1 has a stronger effect in blocking migration compared to V-ATPase inhibition.

4 Discussion

Oncogenic events can affect multiple intracellular signaling networks that involve interconnections and crosstalks between the individual signaling pathways. The specific deregulation of these integrated networks orchestrates the acquisition of the hallmark capabilities that identify tumor cells (Hanahan and Weinberg, 2011). The understanding of the interconnections between the mutations, the molecular pathways, and the biological capabilities can help us to address the research study toward the development of new cancer drugs against therapeutic targets in a more tumor-specific manner.

The oncogenic properties of E2F1 have been traditionally associated with its ability to regulate the G1/S transition and S-phase entry (Stevens and Thangue, 2003). However, it has become increasingly evident that other

biological processes associated with malignant transformation are also regulated by E2F1, including cell growth, autophagy, invasiveness and metastasis (Real et al., 2011; Jiang et al., 2010; Polager et al., 2008; Alla et al., 2010; Zhang et al., 2000). In spite of the importance of these processes, the molecular mechanisms by which E2F1 modulates these responses are not extensively understood. The aim of this Thesis is to study the oncogenic properties of E2F1 beyond those already recognized, such as its function in cell cycle progression. Based on our previous data showing that E2F1 induces cellular growth by activating the mTORC1 pathway (Real et al., 2011), we focused on elucidating the underlying molecular mechanisms by which E2F1 mediates mTORC1 activation. Our work yielded novel observations concerning the ability of E2F1 in regulating V-ATPase activity, intracellular trafficking and autophagy repression. Specifically, we demonstrated that E2F1 enhances the activity of V-ATPase, the major regulator of lysosomal pH. By modulating this activity, E2F1 is capable of regulating lysosomal biology, thus leading to the activation of mTORC1, to the relocalization of lysosomes to the cell periphery and to the repression of the autophagy flux.

E2F1 promotes mTOR translocation into the lysosomes and amino acids uptake is not responsible for E2F1-induced mTORC1 activation

We showed that, similarly to the amino acids signaling, E2F1 activation promotes the translocation of mTOR to lysosomes and also induces an increase in the binding of mTORC1 to the lysosomal protein RagB. The presence of amino acids is an essential step for the recruitment of mTOR into the lysosomes, thereby rendering the complex sensitive to mitogenic signals (Sancak et al., 2008). Although E2F1 overexpression mimics the effect of nutrients, it requires at least a basal level of amino acids to promote mTORC1 activation. Upon an overnight amino acids starvation, E2F1 alone is not able to replace amino acids in translocating mTOR into lysosomes. However, it enhances this process in the presence of nutrients. Several studies reported that the uptake of nutrients is frequently enhanced in tumor cells through upregulation of various amino acid transporters (Ganapathy et al., 2009). Moreover, it has been shown that increased levels of LAT1 and LAT2 amino acid transporters can induce mTORC1 signaling in prostate cancer cells (Wang et al., 2011). However, we demonstrated that, upon two hours of amino acids starvation only, E2F1 is still able to activate mTORC1, probably because the

intracellular pool of amino acids left in such condition is still sufficient for the response, but no more can be up-taken by the cells. These data exclude the possibility that essential amino acid uptake is responsible for E2F1-induced activation of mTORC1, but still suggest that the presence of a basal level of amino acids is essential for this process.

E2F1 activation promotes the association of mTORC1 with RagB, thus indicating that the lysosomal protein is involved in the activation of mTOR regulated by E2F1. It is possible that the increased binding rate correlates with a change in the guanine nucleotide state of the RagGTPase complex, which is known to be responsible for the recruitment of mTOR into the lysosome (Sancak et al., 2010). However, we cannot discard other mechanisms of regulation independent of the RagGTPase complex activity, and further studies are required to elucidate whether E2F1 induces the formation of the active RagA/B·GTP-RagC/D·GDP complex.

E2F1 regulates lysosomal trafficking to the cell periphery

In addition to the ability of modulating the subcellular localization of mTOR, we demonstrated that E2F1 also regulates the peripheral localization of lysosomes. Localization of lysosomes to the cell periphery and increased lysosomal exocytosis sustain the establishment of an acidic

and active protease environment, which is required to promote invasive growth and angiogenesis (Kallunki et al., 2013).

The peripheral lysosomal localization has been recently related to mTORC1 activation (Korolchuk et al., 2011). It has been reported that the decrease in pH_i modulated by nutrients correlates with an increase in the recruitment of proteins such as KIF2A and ARL8 to lysosomes. These drive lysosomes' movement to the cell periphery and, thus, lead to mTORC1 activation. However, other reports pointed out that the decrease in cytoplasmic pH correlates with the inhibition of mTORC1 activity (Fonseca et al., 2012). We demonstrated that E2F1 overexpression induces mTORC1 translocation to lysosomes, where it is activated, and causes an alkalization of intracellular pH together with a peripheral localization of lysosomes. Thus, put in the context of published data, our results suggest that intracellular acidification is not responsible for the peripheral movement of lysosomes nor for mTORC1 activation induced by E2F1. On the contrary, our data support the theory proposed by Fonseca and coworkers which correlates the activation of mTORC1 with a more alkaline pH_i. Further experiments are necessary to clarify the role of pH_i in the regulation of mTORC1 activity and of lysosomal positioning.

mTORC1 activity is not involved in the E2F1-regulated lysosomal movement, but Raptor is required for this response

Our immunofluorescence analysis revealed that mTORC1 activity is not necessary for the peripheral movement of lysosomes regulated by E2F1. However, the depletion of Raptor totally abrogates this response. These results indicate that the role of E2F1 in regulating endosomal trafficking is not dependent on its ability to activate mTORC1, although it requires the presence of Raptor. It is well known that Raptor functions as an essential scaffold protein for the formation of the active mTORC1 complex at the lysosome and its depletion provokes a cytoplasmic dispersion of mTOR (Sancak et al., 2008), but there was no evidence for an implication of this protein in endosomal trafficking until now. We can speculate that, in addition to the function in the assembly of mTORC1 complex, Raptor could have a role as a scaffold in the formation of the endosomal cargo.

E2F1 acts as an autophagy repressor

We demonstrated that activation of E2F1 represses autophagy both in serum and two hours amino acid starvation conditions. The ability of E2F1 to act as an autophagy repressor is in agreement with its role in driving lysosomes to cell periphery and in activating mTORC1. Lyso-

somes are not only essential for trafficking of endosomal cargo, but also for autophagolysosome formation and degradation of recycling vesicles. The accumulation of lysosomes in the perinuclear region, under serum starvation conditions, facilitates their fusion rate with autophagosomes that mostly originate in that area. At the same time, the inhibition of mTORC1 promotes the formation of new autophagosomes, thus leading to the activation of the autophagy flux in starvation conditions (Jung et al., 2009; Korolchuk et al., 2011; Levine and Kroemer, 2008).

To date, the role of E2F1 in autophagy has been controversial. Some reports pointed out that E2F1 promotes autophagy by upregulating the expression of autophagy genes such as LC3, ATG1, ATG5 and DRAM (Polager et al., 2008). On the other hand, others showed that downregulation of E2F1 results in high levels of autophagy and they suggested that regulation of Bcl-2 expression by E2F1 is involved in this process (Jiang et al., 2010). Our finding that E2F1 represses autophagy supports the model where tumor suppressor genes involved in the upstream inhibition of mTORC1 signaling (e.g. PTEN, TSC1, TSC2) stimulate autophagy and where, conversely, mTORC1-activating oncogenes (e.g. class I PI3K and Akt) inhibit autophagy (Levine and Kroemer, 2008).

E2F1 regulates KIF2A transcription, a kinesin required for mTORC1 activation

Our data demonstrated that functional microtubule filaments are required for the effect of E2F1 on lysosomal trafficking and mTORC1 activation. We identified kinesin KIF2A as a novel transcriptional target of E2F1. As mentioned above, KIF2A has been shown to participate in lysosomes' maturation, lysosomal peripheral dispersion and mTORC1 activation (Korolchuk et al., 2011). Although KIF2A basal levels are required for E2F1-induced mTORC1 activation, the overexpression of the kinesin is incapable of rescuing mTORC1 activity in E2F1-depleted conditions. In addition, the overexpression of KIF2A alone does not increase mTORC1 activity in normal growth conditions. These data indicate that the increase in KIF2A levels triggered by E2F1 does not contribute to mTORC1 activation. However, we cannot totally exclude that the decrease in KIF2A levels due to E2F1 depletion has an effect on mTORC1 activity. In fact, in this condition, other E2F1 targets are negatively regulated. Such targets, in turn, could be essential for mTORC1 activation, independently of the recovery of KIF2A levels by overexpression. Furthermore, as KIF2A is involved in other cellular processes such as mitotic chromosome movement or the maintenance of spindle polarity, we can-

not discard the possibility of KIF2A implication in other E2F1's functions (Zhu et al., 2005). In this regard, it has been shown that over-expression of KIF2A promotes the progression and metastasis of squamous cell carcinoma of the tongue (Wang et al., 2010). Whether E2F1's effects on metastasis are mediated by its ability to induce KIF2A is unknown at present.

E2F1 enhances V-ATPase activity, thereby regulating lysosomal trafficking and mTORC1 activity

E2F1 activation induces acidification of lysosomes, while depletion of endogenous gene produces alkalization of these vesicles. Since V-ATPase is the main contributor for maintaining the intracellular acidic milieu of lysosomes, our results imply that V-ATPase activity is modulated by E2F1 (Saftig and Klumperman, 2009). V-ATPase activity is associated with a number of cellular processes related to lysosomal biology, including mTORC1 regulation and endosomal trafficking (Hurtado-Lorenzo et al., 2006; Zoncu et al., 2011). Consistently, the regulation of both processes by E2F1 was abolished by treatment with V-ATPase inhibitor concanamycin A, or by depletion of the essential subunit for V-ATPase function, APT6V0C, indicating an essential role for the proton pump on E2F1's functions. The fact that regulation of V-ATPase by E2F1

was also detected in KIF2A depleted cells implies that this process is independent of KIF2A regulation.

The V-ATPase holoenzyme consists of the membrane inserted V0 domain which is responsible for the proton pore and the peripheral V1 domain responsible for ATP hydrolysis. Reversible association of V1 and V0 domains has been reported as the main mechanism of V-ATPase regulation (Toei et al., 2010; Kane and Smardon, 2003). Accordingly, we demonstrated that the E2F1-induced acidification of lysosomes is accompanied by an increased association of the C1 subunit of the V1 domain, ATP6V1C1, with the V-ATPase/RagB lysosomal complex, suggesting that this binding could be the mechanism by which E2F1 enhances V-ATPase activity. In addition, it has been shown that the interaction between the V1 domain of V-ATPase and Rag GTPases is regulated by amino acids to promote the lysosomal recruitment of mTORC1 (Zoncu et al., 2011). Our data demonstrated that E2F1 induces also an increase in the binding of mTORC1 to RagB, suggesting that once V-ATPase is activated by E2F1, RagB is needed for the recruitment of mTORC1 to lysosomes and its activation.

Interestingly, a central role of ATP6V1C1 subunit has been described in yeast in holoenzyme disassembly/reassembly. In response to glucose,

this subunit binds to V-ATPase and enhances its activity by assembling the V0 and V1 domain (Forgac, 2007). Moreover, overexpression of the ATP6V1C1 subunit has been detected in oral squamous and hepatocellular carcinomas, and depletion of ATP6V1C1 subunit by siRNA resulted in a suppression of growth and metastasis in *in vitro* and *in vivo* models of hepatocellular carcinoma (Lu et al., 2005; Otero-Rey et al., 2008). The emerging roles of ATP6V1C1 in modulating V-ATPase activity and in promoting metastasis are aspects of interest for further studies in the implication of the subunit in E2F1' functions.

On the other hand, glycolytic enzymes such as aldolase and phosphofructokinase-1 (PFK-1) have been found to be physically associated with V-ATPase in yeast. Specifically, it has been shown that aldolase depletion disrupts V-ATPase activity through the disassembly of the complex. These findings suggest that the ATP-generating glycolytic pathway is coupled to the ATP-hydrolyzing proton pump (Lu et al., 2007; Su et al., 2003). Our results from FLAG-RagB co-immunoprecipitation experiments demonstrated that aldolase B is associated with mTOR/RagB complex. Although the amount of aldolase B bound to RagB does not change following E2F1 induction, conformational changes due to the increase of its activity might regulate V-ATPase activity.

In addition to the possibility of V-ATPase activation being regulated by E2F1 through the direct assembly of the complex, the modulation of V-ATPase activity could also be and indirect effect due to the metabolic functions of E2F1. Interestingly, it has been shown that E2F1 regulates oxidative metabolism *in vivo*. It has been demonstrated that E2F1 knockout mice exhibit a switch from glycolytic to oxidative metabolism (Blanchet et al., 2011). Moreover, E2F1 regulates the transcription of the glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. This enzyme controls the levels of fructose-2,6-bisphosphate that allosterically activates the first rate-limiting glycolytic enzyme, phosphofructokinase-1. (Fernández de Mattos et al., 2002). Our data demonstrated that E2F1 activity also increases the amount of aldolase B, supporting the hypothesis that E2F1 is a promotor of the glycolytic flux. We can speculate that activation of key regulatory glycolytic enzymes by E2F1 would provide energy to the proliferating cells and, in parallel with up-regulating V-ATPase activity, may extrude excessive cytosolic protons coming from glycolytic flux. On the other hand, it is important to note that also the intracellular pH can directly modulate the glycolytic rate. The activity of lactate dehydrogenase, which converts pyruvate to lactate and regenerates NAD^+ for glycolysis, is maximal at pH 7.5, and phosphofructokinase-1 is a pH sensor with an increased activity between

pH 7.0 and 7.5 (Webb et al., 2011). Thus, an alkaline pH_i (fitting with a high activity of V-ATPase an of other ions transporters) promotes glycolysis, while the association of glycolytic enzymes to V-ATPase regulates the activity of the proton pump. Taking into account the reliance of tumor cells on glycolysis and the implication of V-ATPase in invasiveness, it will be critical in future studies to elucidate at the molecular level the mechanism by which these two processes are linked in a contest where E2F1 is deregulated, leading to invasive metastatic growth.

E2F1 is required for cell migration

We demonstrated that E2F1 activation leads to a general re-arrangement of the cytoskeleton together with down-regulating the levels of vinculin. It is known that cytoskeleton assembly and remodeling of actin filaments at focal adhesion sites are all processes necessary for cell motility and migration (Saunders et al., 2006; Bravo-Cordero et al., 2013). Accordingly, our results also demonstrated that E2F1 is required for cell migration. In fact, E2F1 depletion deeply impairs both the closure of the "wound" and the spread of the cells across a collagen I matrix, in a xCELLigence migration assay.

Further studies are needed to assess how E2F1 activation can modify the cytoskeleton assembly and which are the effectors of this regula-

tion. Nevertheless, by analyzing the actin immunostaining experiment, we can speculate about a potential implication of E2F1 in the epithelial-mesenchymal transition (EMT). E2F1 overexpression leads to a spindle cell shape change, to the assembly of a different type of actin filaments and to a reduction in the number of cell-cell contacts. It is intriguing to consider that the transition from a cuboidal to a spindle cell shape, as well as the replacement of the actin cytoskeleton by stress fibers, are both peculiar markers of the epithelial-mesenchymal transition (EMT). This developmental regulatory program has been shown to be involved in orchestrating most events of the invasion-metastasis cascade. The transition to the mesenchymal state allows the cell to lose the cell-cell junctions, to increase the motility, invade the stroma and resist to apoptosis (Micalizzi et al., 2010). The stress fibers that form during the EMT exhibit contractile properties required to promote migration (Vallenius, 2013). Additional experiments should be performed to assess whether the actin filaments that form after E2F1 overexpression actually are stress fibers. Interestingly, preliminary experiments of our laboratory demonstrated that E2F1 activity controls E-cadherin levels, a well established marker of the EMT (Berx and van Roy, 2009). These intriguing data do indicate that a potential implication of E2F1 in the regulation of the EMT shall be further investigated.

Another mechanism of regulation that could be involved in the cytoskeleton re-arrangement is the increase in intracellular pH_i induced by E2F1. Specifically, the increase in pH_i modulates the activity of several proteins responsible of regulating the nucleation of actin filaments and the turnover of focal adhesions, thereby promoting the formation of local protrusions and cell migration (Webb et al., 2011; Bravo-Cordero et al., 2013; Srivastava et al., 2008). Further studies are necessary to investigate the potential implication of pH_i in this response.

The capacity of cancer cells to migrate and to invade the host stroma are all processes required to promote the metastatic dissemination (Talmadge and Fidler, 2010; Joyce and Pollard, 2009). Our data showing that E2F1 is required for cell migration are consistent with the theory of E2F1 being a promotor of invasiveness. E2F1 is found over-expressed in various human cancers, including breast, hepatocellular carcinomas, glioblastoma and ovarian cancer (Chen et al., 2009). Several studies indicated a high correlation between E2F1 overexpression and metastasis. For example, E2F1 overexpression drives melanoma progression and promotes the invasiveness of the metastatic cell line, without affecting proliferative activity (Alla et al., 2010). Moreover, both expression of E2F1 and the gene expression signature reflecting activation of E2F1 are

strong predictors of the invasive progression of breast and bladder tumors (Lee et al., 2010; Zhang et al., 2000).

Our novel observations concerning the ability of E2F1 to regulate the peripheral movement of lysosomes and to enhance V-ATPase activity help us to better understand the role of E2F1 in invasion and metastasis. Indeed, the peripheral localization of lysosomes together with V-ATPase hyperactivation induce the acidification of the extracellular environment that allows the activation of several matrix-degrading proteases, thus facilitating invasiveness (Capecci and Forgac, 2013; Wiedmann et al., 2012; Lu et al., 2005; Kallunki et al., 2013). Furthermore, the finding described above that E2F1 overexpression correlates with an alkalization of cytosolic pH is also in agreement with a malignant role of E2F1. The intracellular pH of tumor cells is frequently higher than normal cells. This alkalization of the intracellular compartment is optimal for promoting cell proliferation, cell survival, directed cell migration, and, in addition, it has been shown to be sufficient to induce tumorigenicity in cultured fibroblasts (Lu and Qin, 2011; Neri and Supuran, 2011; Webb et al., 2011). Although the alkalization of cytosolic pH is consistent with the data of E2F1-induced V-ATPase activation, we should take into account the complexity of pH_i regulation. Therefore, we cannot exclude that

the alkalization of pH_i triggered by E2F1 could also be mediated by other ions transporters. Taking together all these novel results, we can conclude this work by proposing to test pharmacological inhibition of V-ATPase as a means to break metastatic processes in E2F1 over-expressing tumors.

5 Conclusions

- **1** - E2F1 induces the translocation of mTORC1 into the lysosomes and promotes its association with the lysosomal protein RagB. The activation of mTORC1 modulated by E2F1 requires the presence of a basal level of amino acids but it is not due to an uptake of nutrients within the cells.

- **2** - E2F1 induces the trafficking of lysosomes to the cell periphery. The peripheral movement of lysosomes regulated by E2F1 does not depend on mTORC1 activity, but it requires the presence of Raptor.

- **3** - E2F1 represses the autophagy flux, both in serum starvation condition and in short amino acids deprivation condition. The in-

hibition of autophagy is probably mediated through the ability of E2F1 to activate mTORC1 and to regulate the peripheral movement of lysosomes.

- **4** - The microtubules' functionality is necessary for E2F1-induced lysosomal trafficking and mTORC1 activation. E2F1 regulates KIF2A transcription, a kinesin required for mTORC1 activation, however, the increase of KIF2A levels is not responsible for the activation of mTORC1 induced by E2F1.

- **5** - E2F1 enhances V-ATPase activity, and this modulation is required for E2F1-induced mTORC1 activation and lysosomal trafficking. The regulation of V-ATPase by E2F1 is independent of mTORC1 activity.

- **6** - The E2F1-regulated KIF2A transcription is V-ATPase activity-dependent, while the increase of V-ATPase activity modulated by E2F1 is independent of KIF2A levels.

- **7** - E2F1 overexpression correlates with an alkalization of cytosolic pH, consistently with the data showing the E2F1-induced acidification of lysosomal pH.

- **8** - E2F1 induces the association of RagB with the C1 subunit of

the V1 complex (ATP6V1C1). This binding could be the mechanism through which E2F1 activates V-ATPase and mTORC1.

- **9** - E2F1 regulates cytoskeleton assembly and down-modulates the levels of vinculin. Moreover, E2F1 is required for cell migration in collagen I matrix.

6 Materials and Methods

6.1 Cell culture

Human bone osteosarcoma cell line (U2OS) was purchased from American Type Culture Collection. The U2OS cell line expresses wild type p53 and Rb, but lacks p16. The cells have a doubling time of approximately 30 hours and exhibit epithelial adherent morphology.

U2OS cells were grown in 100 mm culture dishes with Dulbecco's modified Eagle medium (DMEM) high glucose (Gibco) with: 4 mM L-Glutamine, 4500 mg/L glucose and 1mM sodium pyruvate; and supplemented with 1X Penicillin-Streptomycin solution (Sigma-Aldrich) and 10% of heat-inactivated fetal bovine serum (Gibco). For all studies,

cells were incubated at 37°C, 5% CO_2 and 90-95% of relative humidity. Unless indicated otherwise, cells were serum-starved overnight before starting the experiments.

To develop the U2OS ER-E2F1 stable cell line, cells were transfected with the expression plasmid and integration was selected with toxic concentration (750 μg/mL) of Geneticin Selective Antibiotic (G418) (Sigma-Aldrich). The ER-E2F1 plasmid consists of the human E2F1 gene conjugated to the estrogen receptor domain. The inducible system allows to regulate E2F1 activity by modulating its subcellular localization. The addition of 400 nM (Z)-4-hydroxitamoxifen (OHT) (Calbiochem) to the culture medium allows the translocation of the fusion protein into the nucleus and the transcriptional induction of the E2F1 responsive genes.

For assays using inhibitors, cells were pre-incubated for 30 minutes in serum-starving media in the presence of Rapamycin (Sigma-Aldrich), BEZ235 (Novartis), Concanamycin A (Sigma-Aldrich), Nocodazole (Sigma-Aldrich) or Leupeptin (Sigma-Aldrich).

6.2 Transfection procedures

Plasmid transfections were performed following manufacturer's instructions in opti-MEM medium (Life Technologies) using Lipofectamine 2000 (Life Technologies). GFP-KIF2A expression vector was provided by Dr. Gohta Goshima (Nagoya University), KIF2A-pLX304 was purchased from DNASU (Arizona State University), LAMP1-GFP was purchased from Addgene and pCMVHAER-E2F1 was provided by Dr. Kristian Helin (University of Copenaghen). Plamid reporter vector KIF2A-luciferase was obtained as reported in section 6.7.

siRNA transfections were performed following manufacturer's instructions in opti-MEM medium (Life Technologies) using Lipofectamine RNAiMAX (Life Technologies) during 48h at a final concentration of 40-80 nM. The following siRNAs were used: non-silencing CTR (GCAUCAGUGUCACGUAAUA) was purchased from Sigma-Aldrich, E2F1 siRNA (sc-29297) was purchased from Santacruz Biotechnology (USA); ARL8B (J-020294-09-0005), KIF2 (L-004959-00-0005), Raptor (L-004107-00-0005) and ATP6V0C (L-017620-01-0005) siRNAs were purchased from Thermo Scientific.

6.3 Western Blot

Cells were washed once with ice-cold PBS and lysed with a cell scraper in Lysis Buffer [20 mM Tris-HCl pH 8.0, 10 mM EDTA, 150 mM NaCl, 1% Triton-X100 with a supplement of 1 mM DTT and 1/100 dilution of phosphatase and protease inhibitor cocktail (Sigma Aldrich)]. Lysates were harvested in eppendorf tubes, incubated for 20 min at 4°C in a rotatory shaker and finally centrifuged at maximum speed to recover the supernatant. Quantification of protein concentrations was performed with Bradford technique (BIO-RAD Protein assay, BIO-RAD). Equal amounts of protein lysate were subjected to 12, 10 or 8% sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis and electrophoretically transferred to PVDF membranes (Millipore). The membranes were then blocked with Fluorescent Blocker (Millipore) for 1 hour at room temperature and incubations with primary antibodies were performed in 5% non-fat dry milk or 5% BSA in TBS-T solution overnight (TBS with 0,1% Tween20). The primary antibodies used were the following: anti-human E2F1 (1:500), anti-S6K1 (1:3000) and anti-Aldolase B (1:1000) from Santacruz Biotechnology, anti-KIF2A (1:3000) from Abnova Corporation, anti-Arl8 (1:1000) from Proteintech Group, anti-LC3 (1:2000) from MBL International, anti-FLAG (1:5000) from Sigma Aldrich, anti-

ATP6V1C1 (1:1000) from Bionova, anti-raptor (1:1000) from Millipore, anti-pS6K1 (1:2000), anti-p4EBP1 (1:3000), anti-4EBP1 (1:3000), anti-pULK1 (1:500), anti-ULK1 (1:500), anti-mTOR (1:1000), and anti-actin (1:2000) from Cell Signaling Technology. Incubations with secondary antibodies IRDye 680LT Donkey anti-Mouse or anti-Goat, and 800CW Goat Anti-Rabbit were performed in 0,01% SDS in TBS-T (LI-COR Biosciences). Blots were scanned with an Odyssey detection system (LI-COR Biosciences).

6.4 Immunofluorescence analysis

Cells were grown and treated on glass coverslips inserted into a 6-well plate, fixed in 4% paraformaldehyde for 30 min at 4°C, permeabilized with 0,1%Triton-X100 in PBS Glycine 20 mM for 10 min, blocked in 1% bovine serum albumin in PBS Glycine 20 mM, and incubated with primary antibodies in blocking buffer for 1 h at 37°C. The following antibodies were used for immunofluorescence analysis: mTOR rabbit monoclonal (1:150) from Cell Signaling Technology; E2F1 mouse monoclonal (1:400) from Santa Cruz Biotechnology; LC3 rabbit polyclonal (1:300) from MBL International, LAMP2 mouse monoclonal (dilution

1:300), mouse GM130 (1:300), mouse EEA1 (1:300) and mouse vinculin (1:300) from BD Biosciences Pharmingen. For actin staining we used Rhodamine Phalloidin from Molecular Probes. As secondary antibodies, we used fluorophore-labeled Rhodamine Red goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes), incubated at 1:300 dilution in blocking solution for 45 minutes at 37°C. Coverslips were then washed and mounted in ProLong Gold Antifade Reagent with DAPI nuclear stain (Molecular Probes). Fluorescence was detected with the Leica spectral confocal microscope TCS SP5 using a 63X N.A 1.4 objective and LAS AF software. Fluorophores were excited with Argon laser 15% for 488 nm, DPSS 561 for 555 nm and Diode laser for 405 nm. Images were analyzed with ImageJ software.

6.5 Quantitative Real-time PCR

After treatment, total RNA was extracted using Trizol (Invitrogen) following manufacturer's instructions. 1μg of total RNA was subjected to reverse transcription and the resulting cDNA samples were used (diluted 1:100) in PCR amplification using LightCycler 480 SYBR Green I Master (Roche Applied Science). The sequences of the PCR primers used were

as followed: Cyc E, 5'-AGCAACACCCTCTTCTGCAG-3' (forward) and 5'-CTTGTGTCGCCATATACCGG-3' (reverse); KIF2A, 5'-GCCTTTGATGACTCAGCTCC-3' (forward) and 5'-CTTCCAGTCTGCCCATAAGC-3' (reverse); ARL8B, 5'-GAAGGAAGAGATGGAGCTGAC-3' (forward) and 5'-GAAGCCCACTGTGGGTATCAT-3' (reverse); Actin, 5'-AATGTGGCCGAGGACTTTGATTGC-3' (forward) and 5'-AGGATGGCAAGGGACTTCCTGTAA-3' (reverse). Calculation of relative mRNA was done using Light Cycler 480 software.

6.6 Cross-linking and FLAG pull down assay

After treatment, U2OS cells stably expressing FLAG-RagB and ER-E2F1 were washed twice with ice-cold PBS and incubated for 7 minutes at room temperature with 1 mM DSP cross-linker reagent (Thermo Scientific) in PBS supplemented with protease and phosphatase inhibitors cocktails (Sigma Aldrich). 1M Tris-HCl (pH 7.5) was added 1:10 to quench DSP and cells were washed twice prior to lysis in ice-cold RIPA buffer [150mM NaCl, 50 mM HEPES (pH 7.4), 1 mM EDTA, 1% NP-40, 1% deoxycholate, 0.1% SDS] with protease and phosphatase inhibitors

cocktails (Sigma Aldrich). The soluble fractions from cell lysates were isolated by centrifugation at 13,000 rpm for 5 minutes in a microfuge. For immunoprecipitations, 30 μl of a 33% slurry of anti-FLAG M2 beads (Sigma Aldrich) was added to each lysate and incubated with rotation overnight at 4°C. Immunoprecipitates were washed three times with RIPA buffer supplemented with 500 mM NaCl and once with normal RIPA buffer. Immunoprecipitated proteins were denatured by the addition of 40 μl of sample buffer 1X followed by boiling for 5 minutes, resolved by 4%-20% Criterion TGX Gel (BIO-RAD) electrophoresis, and then analyzed by immunoblotting.

6.7 Luciferase assay

KIF2A-Luciferase reporter vector was obtained as following. A 1246 bp genomic fragment of the human gene KIF2A 5'-UTR-flanking region (Position: chromosome 5: 61601054-61602299) was predicted to have promoter activity by using Genome browser software. The genomic fragment was obtained by PCR amplification using U2OS genomic DNA as the template and then subcloned into the KpnI and XhoI sites of pGL3-Basic luciferase reporter vector (Promega). The integrity of the construct,

KIF2A-Luciferase, was confirmed by sequencing. For the luciferase assay, cells were transfected using lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Specifically, cells were co-transfected with 3 μg of KIF2A-Luciferase reporter vector and 0,5 μg of CMV-Renilla control plasmid for 24 hours. After re-plating and treatment, cells were harvested and lysed with cell lysis buffer for luciferase assay (Promega). Luciferase and Renilla activities were measured using the Dual-Luciferase Reporter Assay System according to manufacturer's instructions (Promega). Luciferase activities values were then normalized over Renilla activities values and over μg of total proteins.

6.8 Measurement of lysosomal pH in live cells

ER-E2F1 U2OS cells were plated in 8 wells chamber slide (Ibidi) and loaded with a 70,000-Da dextran that was coupled to FITC and to Rhodamine (Fluorescein isothiocyanate-dextran, Rhodamine B isothiocyanate-dextran) (Sigma Aldrich) at 1 mg/ml during 20 hours in serum starved conditions. FITC fluorescence decreases with acid pH, whereas rhodamine acts as a pH-independent control (Majumdar et al., 2007). Af-

ter washing, cells were treated as indicated. Fluorescence was analyzed on the Leica TCS SP5 spectral Live confocal microscope using a 63X N.A 1.4 objective and LAS AF software. Time-lapse images were taken from six regions for each condition every minute during 20 hours using excitation wavelengths of 488 for FITC and 568 for Rhodamine. The fluorescence intensity of the images was analyzed using ImageJ software. As a result, the red and green signals as a function of time were obtained. These curves featured a noisy pattern, so that each of them was smoothened by a polynomial interpolation. The green-to-red ratio was computed from each of these smoothened curves and then from these an average curve with standard deviation was computed for all six positions. Calibration was performed by incubation of the cells with media adjusted between 5.0 to 8.0 pH values containing 10 μM of nigericin (Panreac Sciences) and 10 μM valinomycin (Sigma Aldrich).

6.9 Live-cell imaging analysis of lysosomal trafficking

ER-E2F1 U2OS cells were plated in 8 wells chamber slide (Ibidi) and transiently transfected for 24h to express LAMP1-GFP. After overnight

serum starvation, cells were treated as described. Fluorescence was analyzed on the Leica TCS SP5 spectral Live confocal microscope using a 63X N.A 1.4 objective and LAS AF software, within an incubation chamber XL LSM710 S1 (PeCon GmbH, Germany) with a heating insert P-LabTek S1. GFP fluorophore was excited with Argon laser 15%. Time-lapse images were taken from six regions for each sample every minute for 20 hours. Images were analyzed with ImageJ software and converted into avi format to be edited with Final Cut software.

6.10 Measurement of intracellular pH

ER-E2F1 U2OS cells were grown on glass coverslips, treated as described and loaded with 3 μM of 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester (BCECF, AM) (Molecular Probes) in PBS for 10 min at room temperature. The BCECF AM reagent is a membrane-permeant fluorescent dye which diffuses into the cytosol and then is intracellular retained by its negative charge. The fluorescence excitation profile of the dye is pH-dependent. Coverslips were then washed and fixed in 4% paraformaldehyde. Images were obtained using a Nikon epifluorescence microscope at an excitation wavelength of 488 nm and

an emission of 540 nm. Calibration was performed by incubation of cells with extracellular buffers adjusted between 6.0 to 8.0 pH values containing 10 μM of Nigericin (Panreac Sciences). The fluorescence intensity of the images was analyzed using ImageJ software.

6.11 Analysis of intracellular levels of amino acids

$10^{-}7$ Cells were washed 4 times with PBS, scraped and resuspended. Proteins were precipitated with perchloric acid and supernatants were neutralized with potassium carbonate. Amino acids concentrations were analysed by ion exchange chromatography with nynhydrin detection (Biochrom 30, Chromsystems, UK).

6.12 Scratch assay

Cells were plated into glass coverslips and treated as indicated. The scratch was performed in each condition at time point 0 and cells were re-placed in the incubator for different time points. Coverslips were fixed

at the indicated time points in 4% paraformaldehyde for 30 min. Cells were stained with Rhodamine Phalloidin (Molecular Probes) for 1 h at 37°C, washed and then mounted in ProLong Gold Antifade Reagent (Molecular Probes). Images were obtained using a Nikon epifluorescence microscope at an excitation wavelength of 540 nm and an emission of 565 nm.

6.13 Cell migration assay

The experiment was performed by using the The Real-time cell analyzer DP instrument (RTCA DP) from Roche. The RTCA DP Analyzer is located inside a tissue culture incubator and has integrated stations for CIM-Plates 16 (Roche). The principle of this technique is based on the electrical impedance caused by the migration of the cells from the upper chamber to the lower chamber of the CIM-Plates 16, in response to chemoattractant. The RTCA DP Analyzer automatically selects wells for measurement and continuously transfers measured impedance data to the computer, which are converted to Cell Index values. Thus, the Cell Index is a quantitative measure of the number of cells that migrate from the upper chamber through the membrane into the bottom chamber, and

is an indicator of the cell capacity to invade the matrix and migrate.

Briefly, the wells of both sides of the upper chamber were firstly coated with 0,1 μg/μL of collagen type I (Sigma Aldrich), then the lower chamber was filled with medium with serum as chemoattractant, whereas the upper chamber was filled with medium without serum. The pre-treated cells were resuspended and re-plated in the upper chamber wells without serum. The assembled chambers were loaded on the RTCA DP Analyzer instrument and, after 1 hour of instrument equilibration, the cell migration was monitored during 15 hours. The RTCA Software 1.2 was used to analyze the data.

6.14 Statistic analysis

Data were analyzed by Excel program and GraphPad Prism4 software. Results are presented as Mean $\pm$SEM, for n $= 3$ to n $= 6$. Experimental data sets were compared by a two-sampled, two-tailed and unequal SD Student's t-test. Values of $^{*}P < 0.05$, $^{**}P < 0.005$ and $^{***}P < 0.0005$ were considered statistically significant.

7

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Bibliography

Vijay Alla, David Engelmann, Annett Niemetz, Jens Pahnke, Anke Schmidt, Manfred Kunz, Stephan Emmrich, Marc Steder, Dirk Koczan, and Brigitte M. Pützer. E2F1 in Melanoma Progression and Metastasis. *Journal of the National Cancer Institute*, 102(2):127–133, 2010.

Schammim R. Amith and Larry Fliegel. Regulation of the Na^+/H^+ Exchanger (NHE1) in Breast Cancer Metastasis. *Cancer Research*, 73 (4):1259–1264, 2013.

Matthew G. Andrusiak, Kelly A. McClellan, Delphie Dugal-Tessier, Lisa M. Julian, Sonia P. Rodrigues, David S. Park, Timothy E. Kennedy, and Ruth S. Slack. Rb/E2F Regulates Expression of

Neogenin during Neuronal Migration. *Molecular and Cellular Biology*, 31(2):238–247, 2011.

Gemma Armengol, Federico Rojo, Josep Castellví, Carmela Iglesias, Miriam Cuatrecasas, Berta Pons, José Baselga, and Santiago Ramón y. Cajal. 4E-Binding Protein 1: A Key Molecular "Funnel Factor" in Human Cancer with Clinical Implications. *Cancer Research*, 67(16): 7551–7555, 2007.

Liron Bar-Peled, Lawrence D. Schweitzer, Roberto Zoncu, and David M. Sabatini. Ragulator Is a GEF for the Rag GTPases that Signal Amino Acid Levels to mTORC1. *Cell*, 150(6):1196 – 1208, 2012.

Elspeth M. Beauchamp and Leonidas C. Platanias. The evolution of the TOR pathway and its role in cancer. *Oncogene*, 32(34):3923–3932, 08 2013.

Valérie Beaulieu, Nicolas Da Silva, Nuria Pastor-Soler, Christopher R. Brown, Peter J. S. Smith, Dennis Brown, and Sylvie Breton. Modulation of the Actin Cytoskeleton via Gelsolin Regulates Vacuolar H^+-ATPase Recycling. *Journal of Biological Chemistry*, 280(9):8452–8463, 2005.

Geert Berx and Frans van Roy. Involvement of Members of the Cadherin

Superfamily in Cancer. *Cold Spring Harbor Perspectives in Biology*, 1 (6), 2009.

Anup K. Biswas and David G. Johnson. Transcriptional and Nontranscriptional Functions of E2F1 in Response to DNA Damage. *Cancer Research*, 72(1):13–17, 2012.

Emilie Blanchet, Jean-Sebastien Annicotte, Sylviane Lagarrigue, Victor Aguilar, Cyrielle Clape, Carine Chavey, Vanessa Fritz, Francois Casas, Florence Apparailly, Johan Auwerx, and Lluis Fajas. E2F transcription factor-1 regulates oxidative metabolism. *Nature Cell Biology*, 13(9): 1146–1152, 09 2011.

Christine Blattner, Alison Sparks, and David Lane. Transcription Factor E2F-1 Is Upregulated in Response to DNA Damage in a Manner Analogous to That of p53. *Molecular and Cellular biology*, 19(5): 3704–3713., May 1999.

Jose Javier Bravo-Cordero, Marco A. O. Magalhaes, Robert J. Eddy, Louis Hodgson, and John Condeelis. Functions of cofilin in cell locomotion and invasion. *Nature Reviews Molecular Cell Biology*, 14(7): 405–415, 07 2013.

Alexander Brehm, Eric A. Miska, Dennis J. McCance, Juliet L. Reid, An-

drew J. Bannister, and Tony Kouzarides. Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature*, 391(6667): 597–601, 02 1998.

Deborah L. Burkhart and Julien Sage. Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nature Reviews Cancer*, 8(9): 671–682, 09 2008.

Joseph Capecci and Michael Forgac. The Function of Vacuolar ATPase (V-ATPase) a Subunit Isoforms in Invasiveness of MCF10a and MCF10CA1a Human Breast Cancer Cells. *Journal of Biological Chemistry*, 288(45):32731–32741, 2013.

Hui-Zi Chen, Shih-Yin Tsai, and Gustavo Leone. Emerging roles of E2Fs in cancer: an exit from cell cycle control. *Nature Reviews Cancer*, 9 (11):785–797, 11 2009.

Yihan Chiang, Cheng-Yang Chou, Keng-Fu Hsu, Yu-Fang Huang, and Meng-Ru Shen. EGF upregulates Na^+/H^+ exchanger NHE1 by post-translational regulation that is important for cervical cancer cell invasiveness. *Journal of Cellular Physiology*, 214(3):810–819, 2008.

Johanna Chiche, Karine Ilc, Julie Laferrière, Eric Trottier, Frédéric Dayan, Nathalie M. Mazure, M. Christiane Brahimi-Horn, and Jacques

Pouysségur. Hypoxia-Inducible Carbonic Anhydrase IX and XII Promote Tumor Cell Growth by Counteracting Acidosis through the Regulation of the Intracellular pH. *Cancer Research*, 69(1):358–368, 2009.

Michael J. Clague, Sylvie Urbe, Fernando Aniento, and Jean Gruenberg. Vacuolar ATPase activity is required for endosomal carrier vesicle formation. *Journal of Biological Chemistry*, 269(1):21–24, 1994.

Mehdi Damaghi, Jonathan W. Wojtkowiak, and Robert J. Gillies. pH Sensing and Regulation in Cancer. *Frontiers in Physiology*, 4(370), 2013.

James De Gregori, Timothy F. Kowalik, and Joseph R. Nevins. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. *Molecular and Cellular Biology*, 15(8):4215–24, 1995.

Maurice P. DeYoung, Peter Horak, Avi Sofer, Dennis Sgroi, and Leif W. Ellisen. Hypoxia regulates TSC1/2–mTOR signaling and tumor suppression through REDD1-mediated 14–3–3 shuttling. *Genes & Development*, 22(2):239–251, 2008.

Luisa Di Stefano, Michael R. Jensen, and Kristian Helin. E2F7, a novel

E2F featuring DP-independent repression of a subset of E2F-regulated genes. *The EMBO Journal*, 22(23):6289–6298, 12 2003.

Frederick A. Dick and Seth M. Rubin. Molecular mechanisms underlying RB protein function. *Nature Reviews Molecular Cell Biology*, 14(5): 297–306, 05 2013.

Xavier Escoté and Lluís Fajas. Metabolic adaptation to cancer growth: From the cell to the organism. *Cancer Letters*, Article in Press, April 2014.

Lilia Espada, Nathalie Meo-Evoli, Patricia Sancho, Sebastian Real, Isabel Fabregat, Santiago Ambrosio, and Albert Tauler. ROS Production Is Essential for the Apoptotic Function of E2F1 in Pheochromocytoma and Neuroblastoma Cell Lines. *PLoS ONE*, 7(12):e51544, 12 2012.

Jun Fang, Quintin J. Quinones, Trevor L. Holman, Michael J. Morowitz, Qun Wang, Huaqing Zhao, Frank Sivo, John M. Maris, and Miriam L. Wahl. The H^+-Linked Monocarboxylate Transporter (MCT1/SLC16A1): A Potential Therapeutic Target for High-Risk Neuroblastoma. *Molecular Pharmacology*, 70(6):2108–2115, 2006.

Silvia Fernández de Mattos, Eric W-F Lam, and Albert Tauler. An E2F-binding site mediates the activation of the proliferative isoform of 6-

phosphofructo-2-kinase/fructose-2,6-bisphosphatase by phosphatidylinositol 3-kinase. *Biochem. J.*, 368(1):283–291, 2002.

Bruno D. Fonseca, Graham H. Diering, Michael A. Bidinosti, Kush Dalal, Tommy Alain, Aruna D. Balgi, Roberto Forestieri, Matt Nodwell, Charles V. Rajadurai, Cynthia Gunaratnam, Andrew R. Tee, Franck Duong, Raymond J. Andersen, John Orlowski, Masayuki Numata, Nahum Sonenberg, and Michel Roberge. Structure-Activity Analysis of Niclosamide Reveals Potential Role for Cytoplasmic pH in Control of Mammalian Target of Rapamycin Complex 1 (mTORC1) Signaling. *Journal of Biological Chemistry*, 287(21):17530–17545, 2012.

Michael Forgac. Vacuolar ATPases: rotary proton pumps in physiology and pathophysiology. *Nature Reviews Molecular Cell Biology*, 8(11): 917–929, 11 2007.

Vadivel Ganapathy, Muthusamy Thangaraju, and Puttur D. Prasad. Nutrient transporters in cancer: Relevance to warburg hypothesis and beyond. *Pharmacology & Therapeutics*, 121(1):29 – 40, 2009.

Aracely Garcia-Garcia, Humberto Rodriguez-Rocha, Michael T. Tseng, Roberto Montes de Oca-Luna, H. Sam Zhou, Kelly M. McMasters, and Jorge G. Gomez-Gutierrez. E2F-1 lacking the transcriptional activity

domain induces autophagy. *Cancer Biology & Therapy*, 13(11):1091–1101, 09 2012.

Carsten Grashoff, Brenton D. Hoffman, Michael D. Brenner, Ruobo Zhou, Maddy Parsons, Michael T. Yang, Mark A. McLean, Stephen G. Sligar, Christopher S. Chen, Taekjip Ha, and Martin A. Schwartz. Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics. *Nature*, 466(7303):263–266, 07 2010.

Pawan Gulati, Lawrence D. Gaspers, Stephen G. Dann, Manel Joaquin, Takahiro Nobukuni, Francois Natt, Sara C. Kozma, Andrew P. Thomas, and George Thomas. Amino Acids Activate mTOR Complex 1 via Ca^{2+}/CaM Signaling to hVps34. *Cell Metabolism*, 7(5):456 – 465, 2008.

Dana M. Gwinn, David B. Shackelford, Daniel F. Egan, Maria M. Mihaylova, Annabelle Mery, Debbie S. Vasquez, Benjamin E. Turk, and Reuben J. Shaw. AMPK Phosphorylation of Raptor Mediates a Metabolic Checkpoint. *Molecular Cell*, 30(2):214 – 226, 2008.

Jung Min Han, Seung Jae Jeong, Min Chul Park, Gyuyoup Kim, Nam Hoon Kwon, Hoi Kyoung Kim, Sang Hoon Ha, Sung Ho Ryu, and Sunghoon Kim. Leucyl-tRNA Synthetase Is an Intracellular Leucine

Sensor for the mTORC1-Signaling Pathway. *Cell*, 149(2):410 – 424, 2012.

Douglas Hanahan and Robert A. Weinberg. Hallmarks of Cancer: The Next Generation. *Cell*, 144(5):646–674, 03 2011.

Tzippi Hershko and Doron Ginsberg. Up-regulation of Bcl-2 Homology 3 (BH3)-only Proteins by E2F1 Mediates Apoptosis. *Journal of Biological Chemistry*, 279(10):8627–8634, 2004.

Irmgard Hofmann and Sean Munro. An N-terminally acetylated Arf-like GTPase is localised to lysosomes and affects their motility. *Journal of Cell Science*, 119(8):1494–1503, 2006.

Shannon L. Holliday. Vacuolar H^+-ATPase: An Essential Multitasking Enzyme in Physiology and Pathophysiology. *New Journal of Science*, 2014:21, 2014.

Christine C. Hudson, Mei Liu, Gary G. Chiang, Diane M. Otterness, Dawn C. Loomis, Fiona Kaper, Amato J. Giaccia, and Robert T. Abraham. Regulation of Hypoxia-Inducible Factor 1 alpha Expression and Function by the Mammalian Target of Rapamycin. *Molecular and Cellular Biology*, 22(20):7004–7014, 2002.

Jonathan D. Humphries, Pengbo Wang, Charles Streuli, Benny Geiger, Martin J. Humphries, and Christoph Ballestrem. Vinculin controls focal adhesion formation by direct interactions with talin and actin. *The Journal of Cell Biology*, 179(5):1043–1057, 2007.

Andres Hurtado-Lorenzo, Mhairi Skinner, Jaafar El Annan, Masamitsu Futai, Ge-Hong Sun-Wada, Sylvain Bourgoin, James Casanova, Alan Wildeman, Shaliha Bechoua, Dennis A. Ausiello, Dennis Brown, and Vladimir Marshansky. V-ATPase interacts with ARNO and Arf6 in early endosomes and regulates the protein degradative pathway. *Nature Cell Biology*, 8(2):124–136, 02 2006.

Ken Inoki, Yong Li, Tian Xu, and Kun-Liang Guan. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes & Development*, 17(15):1829–1834, 2003.

Ken Inoki, Hongjiao Ouyang, Tianqing Zhu, Charlotta Lindvall, Yian Wang, Xiaojie Zhang, Qian Yang, Christina Bennett, Yuko Harada, Kryn Stankunas, Cun yu Wang, Xi He, Ormond A. MacDougald, Ming You, Bart O. Williams, and Kun-Liang Guan. TSC2 Integrates Wnt and Energy Signals via a Coordinated Phosphorylation by AMPK and GSK3 to Regulate Cell Growth. *Cell*, 126(5):955 – 968, 2006.

Estela Jacinto, Robbie Loewith, Anja Schmidt, Shuo Lin, Markus A. Ruegg, Alan Hall, and Michael N. Hall. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nature Cell Biology*, 6(11):1122–1128, 11 2004.

Jenna L. Jewell, Ryan C. Russell, and Kun-Liang Guan. Amino acid signalling upstream of mTOR. *Nature Reviews Molecular Cell Biology*, 14(3):133–139, 03 2013.

Hong Jiang, Vanesa Martin, Candelaria Gomez-Manzano, David G. Johnson, Marta Alonso, Erin White, Jing Xu, Timothy J. McDonnell, Naoki Shinojima, and Juan Fueyo. The RB-E2F1 Pathway Regulates Autophagy. *Cancer Research*, 70(20):7882–7893, 2010.

David G. Johnson and James DeGregori. Putting the Oncogenic and Tumor Suppressive Activities of E2F into Context. *Current Molecular Medicine*, 6(7):731–738, 2006.

Russell G. Jones and Craig B. Thompson. Tumor suppressors and cell metabolism: a recipe for cancer growth. *Genes & Development*, 23 (5):537–548, 2009.

Johanna A. Joyce and Jeffrey W. Pollard. Microenvironmental regulation of metastasis. *Nature Reviews Cancer*, 9(4):239–252, 04 2009.

Chang Hwa Jung, Chang Bong Jun, Seung-Hyun Ro, Young-Mi Kim, Neil Michael Otto, Jing Cao, Mondira Kundu, and Do-Hyung Kim. ULK-Atg13-FIP200 Complexes Mediate mTOR Signaling to the Autophagy Machinery. *Molecular Biology of the Cell*, 20(7):1992–2003, 2009.

Tuula Kallunki, Ole D. Olsen, and Marja Jaattela. Cancer-associated lysosomal changes: friends or foes? *Oncogene*, 32(16):1995–2004, 04 2013.

Patricia N. Kane and Anne N. Smardon. Assembly and regulation of the yeast vacuolar H^+-ATPase. *Journal of bioenergetics and biomembranes*, 35(4):313–21, august 2003.

Theodoros Kantidakis, Ben A. Ramsbottom, Joanna L. Birch, Sarah N. Dowding, and Robert J. White. mTOR associates with TFIIIC, is found at tRNA and 5S rRNA genes, and targets their repressor Maf1. *Proceedings of the National Academy of Sciences of the United States of America*, 107(26):11823–11828, 2010.

Eunjung Kim, Pankuri Goraksha-Hicks, Li Li, Thomas P. Neufeld, and Kun-Liang Guan. Regulation of TORC1 by Rag GTPases in nutrient response. *Nature Cell Biology*, 10(8):935–945, 08 2008.

Viktor I. Korolchuk, Shinji Saiki, Maike Lichtenberg, Farah H. Siddiqi, Esteban A. Roberts, Sara Imarisio, Luca Jahreiss, Sovan Sarkar, Marie Futter, Fiona M. Menzies, Cahir J. O'Kane, Vojo Deretic, and David C. Rubinsztein. Lysosomal positioning coordinates cellular nutrient responses. *Nature Cell Biology*, 13(4):453–460, 04 2011.

Sara Ladu, Diego F. Calvisi, Elizabeth A. Conner, Miriam Farina, Valentina M. Factor, and Snorri S. Thorgeirsson. E2F1 Inhibits c-Myc-Driven Apoptosis via PIK3CA/Akt/mTOR and COX-2 in a Mouse Model of Human Liver Cancer. *Gastroenterology*, 135(4):1322–1332, 10 2008.

Rebecca L. Landsberg, Julia E. Sero, Paul S. Danielian, Tina L. Yuan, Eunice Y. Lee, and Jacqueline A. Lees. The role of E2F4 in adipogenesis is independent of its cell cycle regulatory activity. *Proceedings of the National Academy of Sciences of the United States of America*, 100(5):2456–2461, 2003.

Mathieu Laplante and David M. Sabatini. mTOR Signaling in Growth Control and Disease. *Cell*, 149(2):274–293, 04 2012.

Isabelle Le Blanc, Pierre-Philippe Luyet, Veronique Pons, Charles Ferguson, Neil Emans, Anne Petiot, Nathalie Mayran, Nicolas Demau-

rex, Julien Faure, Remy Sadoul, Robert G. Parton, and J. Gruenberg. Endosome-to-cytosol transport of viral nucleocapsids. *Nature Cell Biology*, 7(7):653–664, 07 2005.

Dung-Fang Lee, Hsu-Ping Kuo, Chun-Te Chen, Jung-Mao Hsu, Chao-Kai Chou, Yongkun Wei, Hui-Lung Sun, Long-Yuan Li, Bo Ping, Wei-Chien Huang, Xianghuo He, Jen-Yu Hung, Chien-Chen Lai, Qingqing Ding, Jen-Liang Su, Jer-Yen Yang, Aysegul A. Sahin, Gabriel N. Hortobagyi, Fuu-Jen Tsai, Chang-Hai Tsai, and Mien-Chie Hung. IKKβ Suppression of TSC1 Links Inflammation and Tumor Angiogenesis via the mTOR Pathway. *Cell*, 130(3):440 – 455, 2007.

Ju-Seog Lee, Sun-Hee Leem, Sang-Yeop Lee, Sang-Cheol Kim, Eun-Sung Park, Sang-Bae Kim, Seon-Kyu Kim, Yong-June Kim, Wun-Jae Kim, and In-Sun Chu. Expression Signature of E2F1 and Its Associated Genes Predict Superficial to Invasive Progression of Bladder Tumors. *Journal of Clinical Oncology*, 28(16):2660–2667, 2010.

Beth Levine and Guido Kroemer. Autophagy in the Pathogenesis of Disease. *Cell*, 132(1):27–42, 01 2008.

Ming Lu, David Ammar, Harlan Ives, Fred Albrecht, and Stephen L. Gluck. Physical Interaction between Aldolase and Vacuolar H^+-

ATPase Is Essential for the Assembly and Activity of the Proton Pump. *Journal of Biological Chemistry*, 282(34):24495–24503, 2007.

Xiaodong Lu and Wenxin Qin. Vacuolar H^+-ATPase in Cancer Cells: Structure and Function. *Atlas of Genetics and Cytogenetics in Oncology and Haematology*, 16(3):252–259, september 2011.

Xiaodong Lu, Wenxin Qin, Jinjun Li, Ning Tan, Dongning Pan, Haitao Zhang, Li Xie, Genfu Yao, Huiqun Shu, Ming Yao, Dafang Wan, Jianren Gu, and Shengli Yang. The Growth and Metastasis of Human Hepatocellular Carcinoma Xenografts Are Inhibited by Small Interfering RNA Targeting to the Subunit ATP6L of Proton Pump. *Cancer Research*, 65(15):6843–6849, 2005.

Li Ma, Zhenbang Chen, Hediye Erdjument-Bromage, Paul Tempst, and Pier Paolo Pandolfi. Phosphorylation and Functional Inactivation of TSC2 by Erk: Implications for Tuberous Sclerosisand Cancer Pathogenesis. *Cell*, 121(2):179 – 193, 2005.

Xiaoju Max Ma and John Blenis. Molecular mechanisms of mTOR-mediated translational control. *Nature Reviews Molecular Cell Biology*, 10(5):307–318, 05 2009.

Amitabha Majumdar, Dana Cruz, Nikiya Asamoah, Adina Buxbaum, Ist-

van Sohar, Peter Lobel, and Frederick R. Maxfield. Activation of Microglia Acidifies Lysosomes and Leads to Degradation of Alzheimer Amyloid Fibrils. *Molecular Biology of the Cell*, 18(4):1490–1496, 2007.

Brendan D. Manning, Andrew R. Tee, Nicole M. Logsdon, John Blenis, and Lewis C. Cantley. Identification of the Tuberous Sclerosis Complex-2 Tumor Suppressor Gene Product Tuberin as a Target of the Phosphoinositide 3-Kinase/Akt Pathway. *Molecular Cell*, 10(1): 151 – 162, 2002.

Luis A. Martinez, Elzbieta Goluszko, Hui-Zi Chen, Gustavo Leone, Sean Post, Guillermina Lozano, Zhenping Chen, and Anne Chauchereau. E2F3 Is a Mediator of DNA Damage-Induced Apoptosis. *Molecular and Cellular Biology*, 30(2):524–536, 2010.

Christine Mayer, Jian Zhao, Xuejun Yuan, and Ingrid Grummt. mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. *Genes & Development*, 18(4):423–434, 2004.

Kelly A. McClellan and Ruth S. Slack. Specific In Vivo Roles for E2Fs in Differentiation and Development. *Cell Cycle*, 6(23):2917–2927, 12 2007.

Douglas S. Micalizzi, Susan M. Farabaugh, and Heide L. Ford. Epithelial-Mesenchymal Transition in Cancer: Parallels Between Normal Development and Tumor Progression. *Journal of Mammary Gland Biology and Neoplasia*, 15(2):117–134, 2010.

Dalibor Mijaljica, Mark Prescott, and Rodney J. Devenish. V-ATPase engagement in autophagic processes. *Autophagy*, 7(6):666–668, 06 2011.

Cristina M. Moroni, Emma S. Hickman, Eros Lazzerini Denchi, Greta Caprara, Elena Colli, Francesco Cecconi, Heiko Muller, and Kristian Helin. Apaf-1 is a transcriptional target for E2F and p53. *Nature Cell Biology*, 3(6):552–558, 06 2001.

Andrew W. Murray. Recycling the Cell Cycle. *Cell*, 116(2):221–234, 2014/04/30 2004.

Dario Neri and Claudiu T. Supuran. Interfering with pH regulation in tumours as a therapeutic strategy. *Nature Reviews Drug Discovery*, 10(10):767–777, 10 2011.

Rene Opavsky, Shih-Yin Tsai, Martin Guimond, Anjulie Arora, Jana Opavska, Brian Becknell, Michael Kaufmann, Nathaniel A. Walton, Julie A. Stephens, Soledad A. Fernandez, Natarajan Muthusamy,

Dean W. Felsher, Pierluigi Porcu, Michael A. Caligiuri, and Gustavo Leone. Specific tumor suppressor function for E2F2 in Myc-induced T cell lymphomagenesis. *Proceedings of the National Academy of Sciences of the United States of America*, 104(39):15400–15405, 2007.

Eva M. Otero-Rey, Manuel Somoza-Martín, Francisco Barros-Angueira, and Abel García-García. Intracellular pH regulation in oral squamous cell carcinoma is mediated by increased V-ATPase activity via overexpression of the ATP6V1C1 gene. *Oral Oncology*, 44(2):193 – 199, 2008.

Tiziana Parisi, Alessandra Pollice, Antonio Di Cristofano, Viola Calabrò, and Girolama La Mantia. Transcriptional Regulation of the Human Tumor Suppressor p14ARF by E2F1, E2F2, E2F3, and Sp1-like Factors. *Biochemical and Biophysical Research Communications*, 291(5): 1138 – 1145, 2002.

Mario Pérez-Sayáns, José Manuel Somoza-Martín, Francisco Barros-Angueira, Pilar Gayoso Diz, José Manuel Gándara Rey, and Abel García-García. Multidrug resistance in oral squamous cell carcinoma: The role of vacuolar ATPases. *Cancer Letters*, 295(2):135–143, 9 2010.

Timothy R. Peterson, Shomit S. Sengupta, Thurl E. Harris, Anne E. Carmack, Seong A. Kang, Eric Balderas, David A. Guertin, Katherine L. Madden, Anne E. Carpenter, Brian N. Finck, and David M. Sabatini. mTOR Complex 1 Regulates Lipin 1 Localization to Control the SREBP Pathway. *Cell*, 146(3):408 – 420, 2011.

Céline Pinheiro, André Albergaria, Joana Paredes, Bárbara Sousa, Rozany Dufloth, Daniella Vieira, Fernando Schmitt, and Fátima Baltazar. Monocarboxylate transporter 1 is up-regulated in basal-like breast carcinoma. *Histopathology*, 56(7):860–867, 2010.

Shirley Polager, Matan Ofir, and Doron Ginsberg. E2F1 regulates autophagy and the transcription of autophagy genes. *Oncogene*, 27(35): 4860–4864, 2008.

Thomas Porstmann, Claudio R. Santos, Beatrice Griffiths, Megan Cully, Mary Wu, Sally Leevers, John R. Griffiths, Yuen-Li Chung, and Almut Schulze. SREBP Activity Is Regulated by mTORC1 and Contributes to Akt-Dependent Cell Growth. *Cell Metabolism*, 8(3):224–236, 9 2008.

Sebastian Real, Nathalie Meo-Evoli, Lilia Espada, and Albert Tauler. E2F1 Regulates Cellular Growth by mTORC1 Signaling. *PLoS ONE*, 6(1):e16163, 01 2011.

Paul Saftig and Judith Klumperman. Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function. *Nature Reviews Molecular Cell Biology*, 10(9):623–635, 09 2009.

Yasemin Sancak, Carson C. Thoreen, Timothy R. Peterson, Robert A. Lindquist, Seong A. Kang, Eric Spooner, Steven A. Carr, and David M. Sabatini. PRAS40 Is an Insulin-Regulated Inhibitor of the mTORC1 Protein Kinase. *Molecular Cell*, 25(6):903 – 915, 2007.

Yasemin Sancak, Timothy R. Peterson, Yoav D. Shaul, Robert A. Lindquist, Carson C. Thoreen, Liron Bar-Peled, and David M. Sabatini. The Rag GTPases Bind Raptor and Mediate Amino Acid Signaling to mTORC1. *Science*, 320(5882):1496–1501, 2008.

Yasemin Sancak, Liron Bar-Peled, Roberto Zoncu, Andrew L. Markhard, Shigeyuki Nada, and David M. Sabatini. Ragulator-Rag Complex Targets mTORC1 to the Lysosomal Surface and Is Necessary for Its Activation by Amino Acids. *Cell*, 141(2):290 – 303, 2010.

Dos D. Sarbassov, David A. Guertin, Siraj M. Ali, and David M. Sabatini. Phosphorylation and Regulation of Akt/PKB by the Rictor-mTOR Complex. *Science*, 307(5712):1098–1101, 2005.

Ruth M. Saunders, Mark R. Holt, Lisa Jennings, Deborah H. Sutton,

Igor L. Barsukov, Andrey Bobkov, Robert C. Liddington, Eileen A. Adamson, Graham A. Dunn, and David R. Critchley. Role of vinculin in regulating focal adhesion turnover. *European Journal of Cell Biology*, 85(6):487 – 500, 2006.

Yuri Y. Sautin, Ming Lu, Andrew Gaugler, Li Zhang, and Stephen L. Gluck. Phosphatidylinositol 3-Kinase-Mediated Effects of Glucose on Vacuolar H^+-ATPase Assembly, Translocation, and Acidification of Intracellular Compartments in Renal Epithelial Cells. *Molecular and Cellular Biology*, 25(2):575–589, 2005.

Souad R. Sennoune, Karina Bakunts, Gloria M. Martínez, Jenny L. Chua-Tuan, Yamina Kebir, Mohamed N. Attaya, and Raul Martínez-Zaguilán. Vacuolar H^+-ATPase in human breast cancer cells with distinct metastatic potential: distribution and functional activity. *American Journal of Physiology - Cell Physiology*, 286(6):C1443–C1452, 05 2004.

Carmine Settembre, Roberto Zoncu, Diego L. Medina, Francesco Vetrini, Serkan Erdin, SerpilUckac Erdin, Tuong Huynh, Mathieu Ferron, Gerard Karsenty, Michel C. Vellard, Valeria Facchinetti, David M. Sabatini, and Andrea Ballabio. A lysosome-to-nucleus signalling mechanism

senses and regulates the lysosome via mTOR and TFEB. *The EMBO Journal*, 31(5):1095–1108, 02 2012.

Carmine Settembre, Alessandro Fraldi, Diego L. Medina, and Andrea Ballabio. Signals from the lysosome: a control centre for cellular clearance and energy metabolism. *Nature Reviews Molecular Cell Biology*, 14 (5):283–296, 05 2013.

J. Srivastava, G. Barreiro, S. Groscurth, A. R. Gingras, B. T. Goult, D. R. Critchley, M. J. S. Kelly, M. P. Jacobson, and D. L. Barber. Structural model and functional significance of pH-dependent talin–actin binding for focal adhesion remodeling. *Proceedings of the National Academy of Sciences of the United States of America*, 105(38):14436–14441, 2008.

Craig Stevens and Nicholas B. La Thangue. E2F and cell cycle control: a double-edged sword. *Archives of Biochemistry and Biophysics*, 412 (2):157 – 169, 2003.

Jorg Storre, Hans-Peter Elsosser, Miriam Fuchs, Diana Ullmann, David M. Livingston, and Stefan Gaubatz. Homeotic transformations of the axial skeleton that accompany a targeted deletion of E2F6. *EMBO reports*, 3(7):695–700, 07 2002.

Ya Su, Aiwu Zhou, Rafia S. Al-Lamki, and Fiona E. Karet. The a-Subunit of the V-type H^+-ATPase Interacts with Phosphofructokinase-1 in Humans. *Journal of Biological Chemistry*, 278(22):20013–20018, 2003.

James E. Talmadge and Isaiah J. Fidler. AACR Centennial Series: The Biology of Cancer Metastasis: Historical Perspective. *Cancer Research*, 70(14):5649–5669, 2010.

Irantzu Tato, Ramon Bartrons, Francesc Ventura, and José Luis Rosa. Amino acids activate mammalian target of rapamycin complex 2 (mTORC2) via PI3K/Akt signaling. *Journal of Biological Chemistry*, 286(8):6128–6142, 2011.

Stefan Taubert, Chiara Gorrini, Scott R. Frank, Tiziana Parisi, Miriam Fuchs, Ho-Man Chan, David M. Livingston, and Bruno Amati. E2F-Dependent Histone Acetylation and Recruitment of the Tip60 Acetyltransferase Complex to Chromatin in Late G1. *Molecular and Cellular Biology*, 24(10):4546–4556, 2004.

Masashi Toei, Regina Saum, and Michael Forgac. Regulation and Isoform Function of the V-ATPases. *Biochemistry*, 49(23):4715–4723, 2010. PMID: 20450191.

Takao Toyomura, Yoshiko Murata, Akitsugu Yamamoto, Toshihiko Oka,

Ge-Hong Sun-Wada, Yoh Wada, and Masamitsu Futai. From lysosomes to the plasma membrane: Localization of vacuolar type H^+-ATPase with the a3 isoform during osteoclast differentiation. *Journal of Biological Chemistry*, 278(24):22023–22030, 2003.

Kristin Tracy, Benjamin C. Dibling, Benjamin T. Spike, James R. Knabb, Paul Schumacker, and Kay F. Macleod. BNIP3 Is an RB/E2F Target Gene Required for Hypoxia-Induced Autophagy. *Molecular and Cellular Biology*, 27(17):6229–6242, 2007.

Prashant Trikha, Nidhi Sharma, Rene Opavsky, Andres Reyes, Clarissa Pena, Michael C. Ostrowski, Martine F. Roussel, and Gustavo Leone. E2F1-3 Are Critical for Myeloid Development. *Journal of Biological Chemistry*, 286(6):4783–4795, 2011.

Thomas Vaccari, Serena Duchi, Katia Cortese, Carlo Tacchetti, and David Bilder. The vacuolar ATPase is required for physiological as well as pathological activation of the Notch receptor. *Development*, 137(11):1825–1832, 2010.

Ronald D. Vale. The Molecular Motor Toolbox for Intracellular Transport. *Cell*, 112(4):467–480, 02 2003.

Tea Vallenius. Actin stress fibre subtypes in mesenchymal-migrating cells. *Open Biology*, 3(6), 2013.

Olga Vitavska, Hans Merzendorfer, and Helmut Wieczorek. The V-ATPase Subunit C Binds to Polymeric F-actin as Well as to Monomeric G-actin and Induces Cross-linking of Actin Filaments. *Journal of Biological Chemistry*, 280(2):1070–1076, 2005.

Cheng-Qin Wang, Xun Qu, Xiao-Ying Zhang, Cheng-Jun Zhou, Gui-Xiang Liu, Zuo-Qing Dong, Feng-Cai Wei, and Shan-Zhen Sun. Overexpression of kif2a promotes the progression and metastasis of squamous cell carcinoma of the oral tongue. *Oral oncology*, 46(1):65–69, 01 2010.

Qian Wang, Charles G. Bailey, Cynthia Ng, Jessamy Tiffen, Annora Thoeng, Vineet Minhas, Melanie L. Lehman, Stephen C. Hendy, Grant Buchanan, Colleen C. Nelson, John E.J. Rasko, and Jeff Holst. Androgen receptor and nutrient signaling pathways coordinate the demand for increased amino acid transport during prostate cancer progression. *Cancer Research*, 71(24):7525–7536, 2011.

Bradley A. Webb, Michael Chimenti, Matthew P. Jacobson, and Diane L.

Barber. Dysregulated pH: a perfect storm for cancer progression. *Nature Reviews Cancer*, 11(9):671–677, 09 2011.

Eileen White and Robert S. DiPaola. The Double-Edged Sword of Autophagy Modulation in Cancer. *Clinical Cancer Research*, 15(17): 5308–5316, 2009.

Romina M. Wiedmann, Karin von Schwarzenberg, Andrea Palamidessi, Laura Schreiner, Rebekka Kubisch, Johanna Liebl, Christina Schempp, Dirk Trauner, Gyorgy Vereb, Stefan Zahler, Ernst Wagner, Rolf Müller, Giorgio Scita, and Angelika M. Vollmar. The V-ATPase-Inhibitor Archazolid Abrogates Tumor Metastasis via Inhibition of Endocytic Activation of the Rho-GTPase Rac1. *Cancer Research*, 72(22):5976–5987, 2012.

Lijun Yan, Virginie Mieulet, Darren Burgess, Greg M. Findlay, Katherine Sully, Julia Procter, Jozef Goris, Veerle Janssens, Nick A. Morrice, and Richard F. Lamb. PP2AT61$_\epsilon$ Is an Inhibitor of MAP4K3 in Nutrient Signaling to mTOR. *Molecular Cell*, 37(5):633 – 642, 2010.

Panagiotis L. Zalmas, Xiujie Zhao, Anne L. Graham, Rebecca Fisher, Carmel Reilly, Amanda S. Coutts, and Nicholas B. La Thangue. DNA-

damage response control of E2F7 and E2F8. *EMBO reports*, 9(3): 252–9, January 2008.

Hui H. Zhang, Jingxiang Huang, Katrin Duvel, Bernard Boback, Shulin Wu, Rachel M. Squillace, Chin-Lee Wu, and Brendan D. Manning. Insulin Stimulates Adipogenesis through the Akt-TSC2-mTORC1 Pathway. *PLoS ONE*, 4(7):e6189, 07 2009.

Shi Yu Zhang, Shao Chen Liu, Luma F. Al-Saleem, Dana Holloran, James Babb, Xu Guo, and Andres J. P. Klein-Szanto. E2F-1: A Proliferative Marker of Breast Neoplasia. *Cancer Epidemiology Biomarkers & Prevention*, 9(4):395–401, 2000.

Ledu Zhou, Yun Huang, Jingdong Li, and Zhiming Wang. The mTOR pathway is associated with the poor prognosis of human hepatocellular carcinoma. *Medical Oncology*, 27(2):255–261, 2010.

Changjun Zhu, Jian Zhao, Marina Bibikova, Joel D. Leverson, Ella Bossy-Wetzel, Jian-Bing Fan, Robert T. Abraham, and Wei Jiang. Functional analysis of human microtubule-based motor proteins, the kinesins and dyneins, in mitosis/cytokinesis using RNA interference. *Molecular Biology of the Cell*, 16(7):3187–3199, 2005.

Vittoria Zinzalla, Daniele Stracka, Wolfgang Oppliger, and Michael N.

Hall. Activation of mTORC2 by Association with the Ribosome. *Cell*, 144(5):757 – 768, 2011.

Roberto Zoncu, Liron Bar-Peled, Alejo Efeyan, Shuyu Wang, Yasemin Sancak, and David M. Sabatini. mTORC1 Senses Lysosomal Amino Acids Through an Inside-Out Mechanism That Requires the Vacuolar H^+-ATPase. *Science*, 334(6056):678–683, 2011.