Understanding the Regulation of the Human Amino Acid Transporter ASCT2

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Abstract

Glutamine is a very versatile source of energy and a precursor for many molecules which are essential to the growth and survival of actively dividing cells. Numerous amino acid transporters are involved in the transport and homeostasis of glutamine and ASCT2 is one of the most important transporters expressed in mammals, due to its moderate affinity for glutamine (Km = 70 - 90 μM), its ubiquitous organ expression, and its correlation with aggressive forms of cancer. Yet, the regulation of ASCT2 in a non-pathological setting is poorly understood. In this study, I developed a new cell model for the study of ASCT2 by examining the molecular and functional characteristics of glutamine uptake in RWPE-1, an immortalized prostate epithelial human cell line.

The expression of the canonical isoform of ASCT2 (SLC1A5) mRNA in RWPE-1 cells was confirmed by quantitative PCR and ASCT2 protein by western blotting. Immunofluorescence microscopy confirmed ASCT2 to be abundant and primarily localized to the plasma membrane. Radio-labelled glutamine uptake in RWPE-1 cells determined that ASCT2 is the major contributor (45 %) of glutamine transport in this cell line. Additionally, I have confirmed that EGF regulates the glutamine uptake via ASCT2, by regulating, through an unknown mechanism, the trafficking of the transporter to the plasma membrane.

Thus, I conclude that the RWPE-1 cell-line is the first model to date used to study the detailed assessment of amino acid transporters' role in the physiology of the prostate; moreover, RWPE-1 is a physiologically relevant model for the study of ASCT2 regulation. Moreover, I performed a largescale MYTH screening to target ASCT2 and discover potential interactors of the transporter which may help my future studies to further investigate and understand the regulation of the transporter.

Dedication

This manuscript is the result of perseverance, commitment, and hard work. For these qualities, I must thank my life-partner Terry, my be-loved Zia Anna and above all, my parents.

Thank you.

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I will start by thanking Dr. Imogen Coe, who has been so kind to welcome me into her lab a long time ago. With no idea about who I was, she gave me this great opportunity to fulfill many dreams, not only academic ones. Above all, I want to thank her for being an inspiring mentor who always maintained a positive and supportive attitude throughout my degree and for providing valuable guidance, support and when needed, scolding.

I am unable to dignify with suitable words the deep gratitude I have toward Dr. Zlatina Naydenova, not only for providing professional support in the form of brainstorming, help, and shared knowledge, but also for providing advice and support on a personal level.

I am also very glad to have shared many pleasant moments with the other members of Dr. Coe's lab: Dr. Natàlia Grañé, Dr. Sila Baskoy, Dr. Alexandre Rodrigues, Dr. Alex Bicket, Maliha Zafar, Julia Mariglia, Katarina Stevanovic and, Bianca Scuric. I also would like to thank Dr. Pamela Plant and Dr. Caterina Di-Ciano (Li Ka Shing Knowledge Institute, Toronto) for their professional support in the arts of qPCR and confocal microscopy and Dr. Lara Console (University of Bari, Italy) for providing the hASCT2-FLAG construct.

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Table of Contents

Abstract ii

Dedication iii

Acknowledgements iv

Table of Contents v

List of Tables viii

Lists of Figures ix

Chapter 1 : Introduction 1

Glutamine, a Very Essential "Non-Essential" Amino Acid 1

Transporters for The Amino Acid Glutamine 5

Alanine Serine Cysteine Transporter 2 (ASCT2) 8

ASCT2 and Pathology 13

ASCT2 and its Regulators 16

EGFR and EGF, Roles in Cellular Regulation 18

mTOR Signalling 22

Objectives of the Study 26

Chapter 2 : The regulation of ASCT2-dependent glutamine uptake and its distribution at the PM are modulated by EGF 27

Introduction 27

Materials and Methods 30

Materials 30

ASCT2.RBD 31

Cell Line Choice Rationale 31

Cell Culture 33

EGF Treatments 33

RNA Extraction and cDNA Synthesis 34

Quantitative PCR 35

Protein Extraction and Western Blot Analysis 36

Densitometry and Normalization 37

In-situ Protein Biotinylation Assays and Immunoblot ... 37
Total [^{3}H]-glutamine Uptake Assays ... 38
Specific [^{3}H]-glutamine Uptake Assays ... 39
Immunofluorescence Microscopy of Endogenous hASCT2 ... 40
In-plate Flow cytometry ... 40
Statistical analysis ... 41
Results ... 42
Analysis of THLE-3 Cells Revealed Presence of Endogenous ASCT2 ... 42
Endogenous hASCT2 Expression and Localization Patterns in RWPE-1 Cells. ... 44
Endogenous hASCT2 is Fully Functional and Represents the Primary Contributor of Glutamine Transport in RWPE-1 Cells. ... 48
RWPE-1 Cells are Receptive to Epidermal Growth Factor (EGF) Stimulation. ... 50
EGF Increases ASCT2-dependent Glutamine Uptake in RWPE-1 With No Effect on Protein Abundance. ... 54
The EGF Stimulated Increase in ASCT2-dependent Glutamine Uptake in RWPE-1 is Due to an Increase of Transporter Presence at the Plasma Membrane. ... 58
Discussion ... 64
Chapter 3 : Interactors and regulators of ASCT2 ... 67
Introduction ... 67
Materials and Methods ... 69
Materials ... 69
Cell Culture ... 70
GPNA Treatments ... 70
Torin 1 and XL388 Treatments ... 70
Protein Extraction and Western Blot Analysis ... 70
Densitometry and Normalization ... 71
ASCT2-dependent [^{3}H]-glutamine Transport Assays in the Presence of GPNA ... 71
Total and ASCT2-dependent [^{3}H]-glutamine Transport in the Presence of mTOR Inhibitors ... 72
Immunofluorescence of Endogenous PDZK1 and hASCT2 ... 73
Generation of HA-ASCT2 Construct ... 74

RWPE-1 Transfection for Immunoprecipitation 76
Immunoprecipitation of HA Tagged ASCT2 76
MYTH Screening for hASCT2 77
Statistical analysis 77
Results 78
GPNA Inhibition of ASCT2-dependent Glutamine Uptake Effects on mTORC2 Phosphorylation. 78
Total and ASCT2-dependent Glutamine Transport is Not Affected by the Inhibition of mTOR 83
Analysis of Endogenous PDZK1 Confirms Expression and Localization Patterns in RWPE-1 cells. 86
ASCT2 and PDZK1 do not Co-immunoprecipitate nor Co-localize in RWPE-1 cells. 89
Bioinformatics Analysis of hASCT2 Potential Interactors 93
Discussion 100

Chapter 4 : Overview and General Considerations 115

Limitations 118
Future work 120

References 123

Appendix 145

Abbreviations 145
Solutions and Buffers 149

List of Tables

Chapter 1- Introduction

Table 1.1. Amino acid transporters involved in glutamine homeostasis............................. 5

Chapter 2- EGF Receptor-stimulated Regulation of hASCT2-dependent Uptake and Surface Distribution in RWPE-1 Cells

Table 2.1. Primers used in this study for qPCR. 36

Chapter 3- Interactors and regulators of ASCT2 in RWPE-1 cells

Table 3.1. Sequencing Primers used in this study. 75

Table 3.2. MYTH screening summary 93

Table 3.3. Plasma membrane hASCT2 putative interactors... 94

Table 3.4. ER-Golgi trafficking hASCT2 putative interactors... 95

Table 3.5. Enzyme-related putative hASCT2 interactors.. 96

Table 3.6. Transporter- related putative hASCT2 interactors. 97

Table 3.7. hASCT2 putative interactors with unknown or diverse function...................... 98

Table 3.8. Putative hASCT2 interactors with miscellaneous function identified in the Human Prostate Adenocarcinoma library. 99

Lists of Figures

Chapter 1 – Introduction

Figure 1.1. Chemical structure of the amino acid L- glutamine. 1

Figure 1.2. Biosynthetic fate and metabolism of glutamine 3

Figure 1.3. Network of transporters involved in glutamine homeostasis 7

Figure 1.4. hASCT2 putative topology. 9

Figure 1.5. Putative hASCT2 three-dimensional structure. 10

Figure 1.6. EGF-dependent signalling network 18

Figure 1.7. mTOR signalling pathway 22

Chapter 2 – The Regulation of ASCT2-dependent Glutamine Uptake and its Distribution at the PM are Modulated by EGF

Figure 2.1. THLE-3 cells express hASCT2 43

Figure 2.2. hASCT2 is expressed in RWPE-1 cells. 45

Figure 2.3. Cellular distribution of endogenous hASCT2 in RWPE-1. 46

Figure 2.4. Glutamine transport in RWPE-1. 49

Figure 2.5. EGF induces tyrosine phosphorylation of proteins in RWPE-1. 51

Figure 2.6. EGF-induced tyrosine phosphorylation of protein in RWPE-1 cells is inhibited by AG1478. 52

Figure 2.7. EGF receptor is tyrosine phosphorylated by extracellular EGF in RWPE-1 cells. 53

Figure 2.8. Total sodium-dependent [^{3}H]-glutamine uptake increases in the presence of EGF. 55

Figure 2.9. ASCT2-dependent [^{3}H]-glutamine uptake increases 3 fold after EGF treatment. 56

Figure 2.10. Effects of extracellular EGF on ASCT2 expression and protein levels in RWPE-1 cells. 57

Figure 2.11. EGF induces an increase of ASCT2 protein at the plasma membrane in RWPE-1 cells. 60

Figure 2.12. EGF induces an increase of surface ASCT2 transporter at the plasma membrane. 61

Figure 2.13. EGF induces an increase of ASCT2-related fluorescence at the plasma membrane. 63

Chapter 3 – Interactors and Regulators of ASCT2

Figure 3.1. ASCT2-dependent glutamine uptake is inhibited by GPNA in RWPE-1 cells. 79

Figure 3.2. Pharmacological inhibition of hASCT2-dependent glutamine uptake regulates the mTOR pathway in RWPE-1. 81

Figure 3.3 XL388 and Torin1 inhibit mTOR pathway in RWPE-1 cells. 84

Figure 3.4. Glutamine transport is not affected by the inhibition of mTOR in RWPE-1. 85

Figure 3.5. RWPE-1 express PDZK1. 87

Figure 3.6. PDZK1 does not co-immunoprecipitate with hASCT2 in RWPE-1. 90

Figure 3.7. ASCT2 and PDZK1 do not appear to co-localize in RWPE-1. 91

Figure 3.8. A cartoon depicting the putative downstream effects of ASCT2 inhibition on mTOR pathways. 105

Figure 3.9. Integrated hASCT2 interactome. 108

Figure 3.10. Sequence alignment between calcineurin B and CHP2. 110

Figure 3.11. Putative calcineurin B docking region on hASCT2. 111

Figure 3.12. Putative calmodulin binding site on ASCT2. 112

<u>Chapter 4 – Overview and General Consideration</u>

Figure 4.1. Putative hASCT2 topology showing putative predicted phosphorylation sites. 121

Figure 4.2. Putative hASCT2 topology showing putative docking sites and phosphorylation sites. 122

Chapter 1 : Introduction

Glutamine, a Very Essential "Non-Essential" Amino Acid

Most cells are able to produce glutamine (Figure 1.1) via the reaction catalyzed by the enzyme glutamine synthetase (GS) and for this reason, it has been classified as a "non-essential" amino acid; nevertheless, glutamine (gln) is one of the most abundant amino acids in the plasma (0.6 - 0.9 mmol/L) (Labow and Souba, 2000).

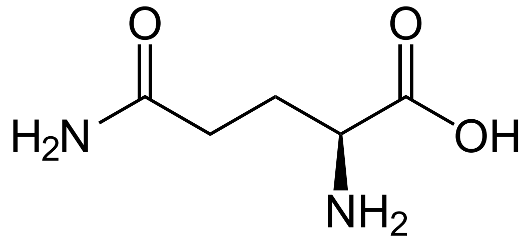


Figure 1.1. Chemical structure of the amino acid L- glutamine. Adapted from public domain image (https://en.wikipedia.org/wiki/Glutamine#/media/File:L-Glutamin_-_L-Glutamine.svg).

Regardless of its classification, glutamine has extensive cellular and physiological functions in mammalian cells which are summarized in Figure 1.2; particularly, these functions are related to the growth and survival of actively dividing cells such as enterocytes, fibroblasts, and lymphocytes (Brand, 1985, Ko *et al.*, 1993, Engström and Zetterberg, 1984).

Glutamine, enters the mitochondria via an unknown glutamine transporter, this is still a widely debated topic in the scientific community. The only available information was provided by the isolation of rat's kidney mitochondria and the extraction of an uncharacterized 24kDa glutamine transporter (Indiveri *et al.,* 1998). Possible candidates for glutamine transport in the

mitochondrion are thought to be the orphan transporters from the subfamilies SLC25A25, 39, and 16 (Palmieri, 2008).

However the mechanism of entry, glutamine is a major source of non-toxic ammonia which is utilized in the urea cycle, transamination reactions, gluconeogenesis, and production of energy in the form of ATP (Labow *et al.*, 2001). Glutamine is also utilized as a metabolic intermediate that contributes carbon and nitrogen for the synthesis of other amino acids, fatty acids, nucleic acids, and proteins (Neu *et al.*, 1996). Moreover, glutamine is the major source of cellular glutamate, and it can be the limiting step in the synthesis rate of glutathione, an antioxidant that protects cells from oxidative stress (Matés *et al.*, 2002, Roth *et al.*, 2002). Glutamine also coordinates protein translation, metabolic flux, and nitrogen balance to maintain tissue homeostasis and cell volume control (Dall'Asta *et al.*, 1990). In addition to contributing to anaerobic respiration in cancer, glutamine play a fundamental role the maintenance of the metabolic needs of rapidly proliferating cells such as activated lymphocytes and stem cells (Newsholme *et al.*, 1985).

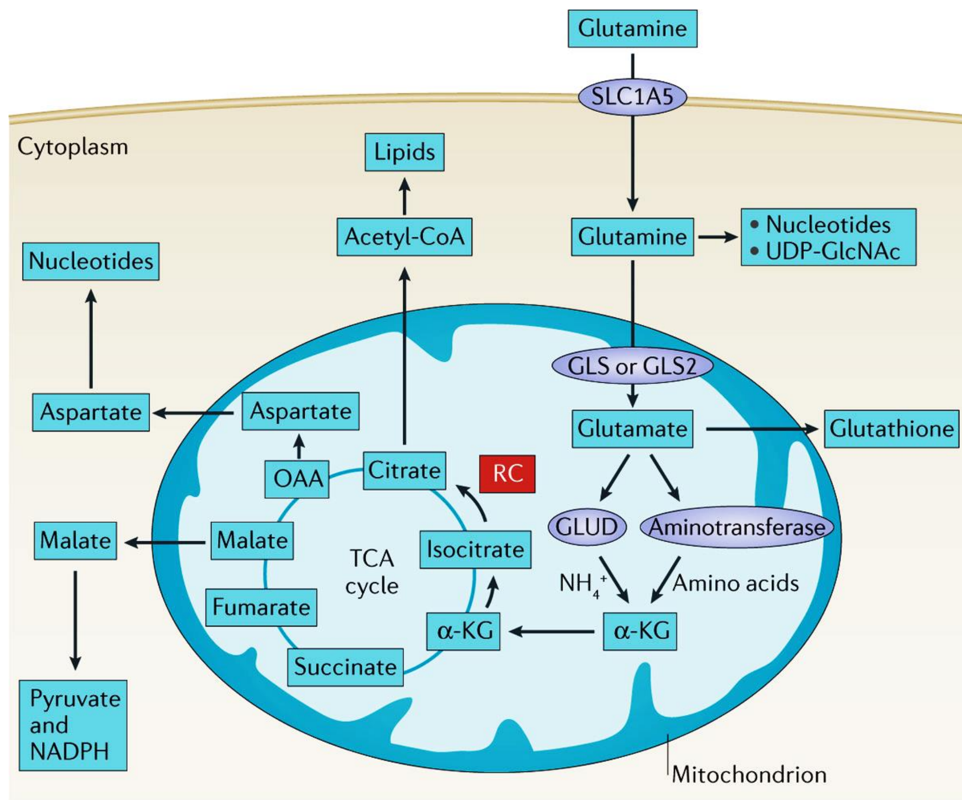


Figure 1.2. Biosynthetic fate and metabolism of glutamine (Altman *et al.*, 2016)

Over the last 20 years it has been recognized that the demand for glutamine exhibited during certain conditions has exceeded the cellular endogenous production capacity of this amino acid, so much so that in this context, glutamine has been reclassified as "conditionally essential" (Souba, 1993). In fact, cells typically derive energy via aerobic metabolism in which they metabolize glucose to pyruvate (glycolysis) which is then converted into energy (ATP and NADH) in the mitochondria by oxidative phosphorylation. Under anaerobic conditions, the same cells will reduce pyruvate into lactate instead and they will synthesize the necessary glutamine via nitrate reduction of glutamate which requires ATP (Baggetto, 1992). Somatic cells, due to genetic aberrations in key tumour suppressor genes such as p53 and/or to dysregulation of onco-related proteins such as PI3K, Akt, myc and Ras, undergo tumorigenic transformation resulting in

metabolism becoming anaerobic to support their greater need of glycolytic intermediates and glutamine for macromolecular synthesis and redox maintenance (Medina *et al.*, 1992, Bode, 2001, Cairns *et al.*, 2011). This gives them a major advantage, despite the low efficiency of their catabolic metabolism, for they are able to either bypass or disrupt all the check points responsible for metabolic homeostasis and growth regulation. Having glutamine metabolism provides a source of carbon utilized as an oxidative source of energy for the generation of ATP and other biosynthetic precursors (Vander Heiden *et al.*, 2011). This metabolic change is universally recognised as the phenotype of tumours or Warburg Effect (Cantor and Sabatini, 2012).

Given the metabolic and regulatory versatility of amino acids and particularly of glutamine, it is not a surprise that it has been reported as the main oxidizable substrate for actively dividing cells (Ohh, 2012, Reitzer *et al.*, 1979). *De novo* synthesis of glutamine is not sufficient to meet the increased demand in tumour cells. This results in an increase in the expression of transporters such as LAT1 (Macheda *et al.*, 2005) and ASCT2 (Fuchs and Bode, 2005), which are responsible for the exchange/uptake of glutamine and other amino acids. Interestingly, ASCT2 alone may play a key role in cell transformation by modulating amino acid uptake, most importantly glutamine, which is important for metabolic reprogramming by enabling cancer cells to survive and proliferate under hypoxia (Metallo *et al.*, 2012).

Transporters for The Amino Acid Glutamine

Amino acid homeostasis is a highly-regulated mechanism driven by many transporters and of particular interest are transport systems with specificity for glutamine, since these transporters have pivotal roles in cell growth, proliferation, and viability in both normal and abnormal cells (Bode, 2001, McGivan and Bungard, 2007, Pochini *et al.*, 2014). Unlike glucose, glutamine can be transported across the plasma membrane, with different affinities and mechanisms, by numerous amino acid transporters (Hensley *et al.*, 2013, Kanai and Endou, 2001, Kanai and Hediger, 2004, Scalise *et al.*, 2016).

The mammalian amino acid transporters known to be involved in the maintenance of glutamine homeostasis are denoted in Table 1.1, on the basis of their transport mechanism, by systems A (SLC38A1 and SLC38A2) and N (SLC38A3, SLC38A5, and SLC38A7), system L (SLC7A5, SLC7A8) and by the transporters B0AT1 (SLC6A19), ATB0$^+$ (SLC6A19), and ASCT2 (SLC1A5) (Kanai and Hediger, 2004, Pochini *et al.*, 2014, Bode, 2001, Kanai and Hediger, 2003, McGivan and Bungard, 2007, Utsunomiya-Tate *et al.*, 1996).

Table 1.1. Amino acid transporters involved in glutamine homeostasis in mammals.

Member	Alias	Variant	Transport mechanism	Km for Gln
SLC1A5	ASCT2	NP_005619.1	Na^+ - gln/ aa^0 antiport	0.07-0.09 mM
SLC6A14	ATB0$^+$	NP_009162.1	$2Na^+$ - $1Cl^-$/gln co-transport	0.6 mM
SLC6A19	B0AT1	NP_001003841 .1	Na^+ - gln co-transport	0.25 mM
System L				
SLC7A5	LAT1	NP_003477 .4	Gln/ large aa^0 antiport	0.5 mM
SLCA8	LAT2	NP_036376.2	Gln/ small aa^0 antiport	0.3-0.9 mM
System A				
SLC38A1	SNAT1	NP_001265317 .1	Na^+ - gln cotransport	498 mM
SLC38A2	SNAT2	NP_061849.2	Na^+ - gln cotransport	1.5 mM
System N				
SLC38A3	SNAT3	NP_006832.1	Na^+ - gln/H^+ antiport	0.7 -1 mM
SLC38A5	SNAT5	NP_277053.2	Na^+ - gln/H^+ antiport	0.7 -1 mM
SLC38A7	SNAT7	NP_060701 .1	Na^+ - gln/H^+ antiport	0.7 -1 mM

Systems A and System N (SLC38 or SNATs) are sodium-dependent facilitative transporters which co-transport amino acids (glutamine, methionine, proline, serine, asparagine, glycine, and histidine) inwardly by following the sodium electrochemical gradient (Mackenzie and Erickson, 2004, Hägglund *et al.*, 2011, Schiöth *et al.*, 2013, Bröer, 2014); they are set apart on the basis of their ability to be inhibited by the amino acid analogue metil-amino isobutyric acid (MeAIB) (Christensen *et al.*, 1965). SNATs are expressed broadly in the body, including the intestine and prostate (Schiöth *et al.*, 2013, Jenstad and Chaudhry, 2013, Kondou *et al.*, 2013).

System L members LAT1 (SLC7A5) and LAT2 (SLC7A8) are widely expressed in kidney, placenta, brain, prostate, and other tissues; their transport is usually sodium-independent and may only occur when in a heteromeric state with their co-expressed heavy chain unit 4F2hc (del Amo *et al.*, 2008). Their substrate specificity is very broad and includes tryptophan, phenylalanine, serine, leucine, histidine, alanine, glycine, and at low affinity, glutamine and threonine (Kanai *et al.*, 1998, Pineda *et al.*, 1999, Fraga *et al.*, 2005, Yoon *et al.*, 2005, Fotiadis *et al.*, 2013).

Transporter B0AT1 (SLC6A19) is a sodium-dependent electrogenic glutamine transporter expressed in the intestine, skin, stomach, liver, and prostate (Kleta *et al.*, 2004, Seow *et al.*, 2004, Böhmer *et al.*, 2005, Bröer and Palacín, 2011, Bröer, 2014). The transporter recognizes a wide range of substrates which includes leucine, cysteine, and glutamine; however, arginine, aspartate, glutamate, lysine, and the inhibitor MeAIB are not recognised (Bröer *et al.*, 2004, Camargo *et al.*, 2005).

Transporter ATB0^{+} (SLC6A14) is considered a wide spectrum amino acid transporter for its broad substrate selection which includes small and large neutral amino acids such as leucine and glutamine, cationic amino acids, and even carnitine (Bode, 2001, Nakanishi and Tamai, 2011, Hatanaka *et al.*, 2001). Human ATB0^{+} is expressed principally in colon, lung, eye and mammary

gland and it is inhibited by α-methyl-DL-tryptophan (α-MT) and Arginine (Sloan and Mager, 1999, Ganapathy and Ganapathy, 2005).

A brief overview of these transporters characteristics and other related transporters involved the homeostasis of glutamine, will enlighten the complexity and the challenges in elucidating the homeostasis of amino acids in mammalian tissues (Figure 1.3).

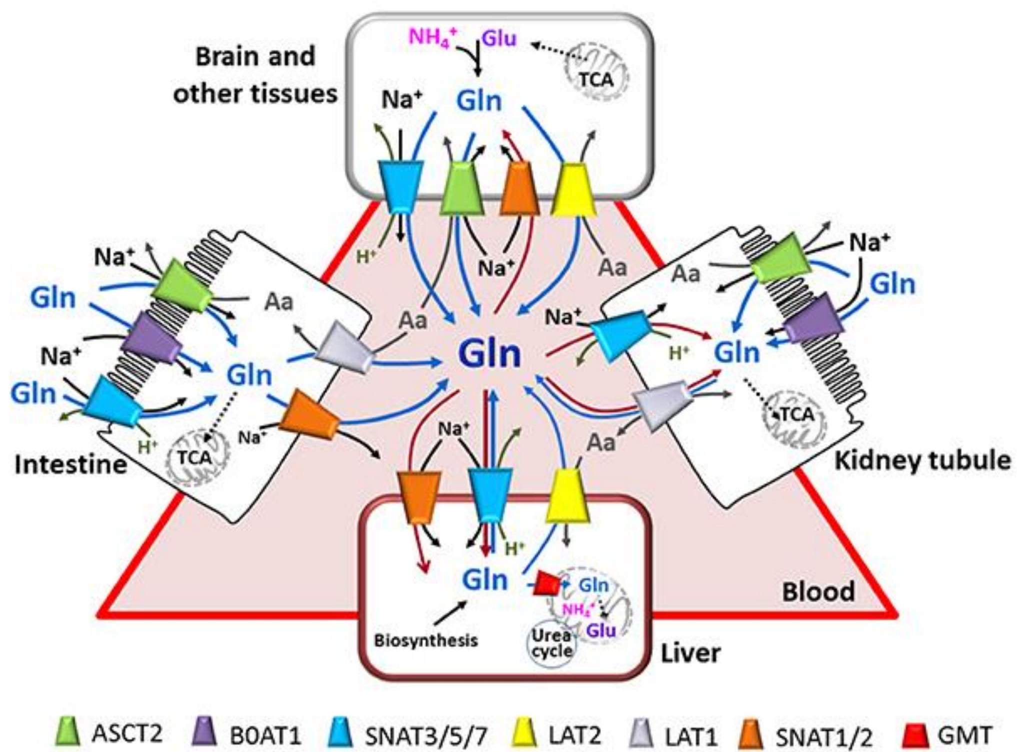


Figure 1.3. Network of transporters involved in glutamine homeostasis (Pochini *et al.*, 2014)

Alanine Serine Cysteine Transporter 2 (ASCT2)

The transporter ASCT2 (SLC1A5) is encoded by the gene NC_000019.10 annotated on chromosome 19q13.32; ASCT2 localization among mammalian tissues is broad and includes kidney, intestine, brain, lungs, placenta, pancreas, and prostate (Kekuda *et al.*, 1996, Utsunomiya-Tate *et al.*, 1996, Bröer *et al.*, 1999, Bröer and Brookes, 2001, Deitmer *et al.*, 2003, Gliddon *et al.*, 2009, Kanai and Hediger, 2004, Namikawa *et al.*, 2014). Although three different splice variants have been reported, only the variant NP_005619.1 has been characterized and is known to consist of 2873 nucleotides and 8 exons, producing a functional peptide of 541 amino acids (Kekuda *et al.*, 1996, Kanai *et al.*, 2013).

Two dimensional models of hASCT2 topology suggests that the transporter structure may be similar to the glutamate transporter 1 (GLT-1) (Jiang and Amara, 2011, Reyes *et al.*, 2009, Slotboom *et al.*, 1999) and consist of 10 transmembrane domains (TMD) and alternating intra and extra cellular loops. The C- and N-termini of the transporter are predicted to extend intracellularly (Figure 1.4). Important features of the transporter include two N-glycosylation sites, namely N163 and N212, which are located on the predicted large extracellular loop between TMD3 and TMD4. These two glycosylation sites were recently correlated with the ability of the transporter to reach the plasma membrane when transiently overexpressed in HEK-293 cells (Console *et al.*, 2015). Although both N-glycolic moieties are important for the correct translocation of the transporter from the ER to the PM, the N212 glycolic moiety seems to be crucial for the correct placement of the transporter within the bi-layer. Notably, glycosylation-deficient hASCT2 is not affected functionally when reconstituted into proteoliposomes nor when transiently expressed in HEK-293 cells (Console *et al.*, 2015), which suggests that the N-glycans are not correlated with the transporter functionality, but rather with its trafficking as previously described for other

transporters such as SLC26A3, intestinal ion exchanger and OCTN1, a carnitine transporter (Hayashi and Yamashita, 2012).

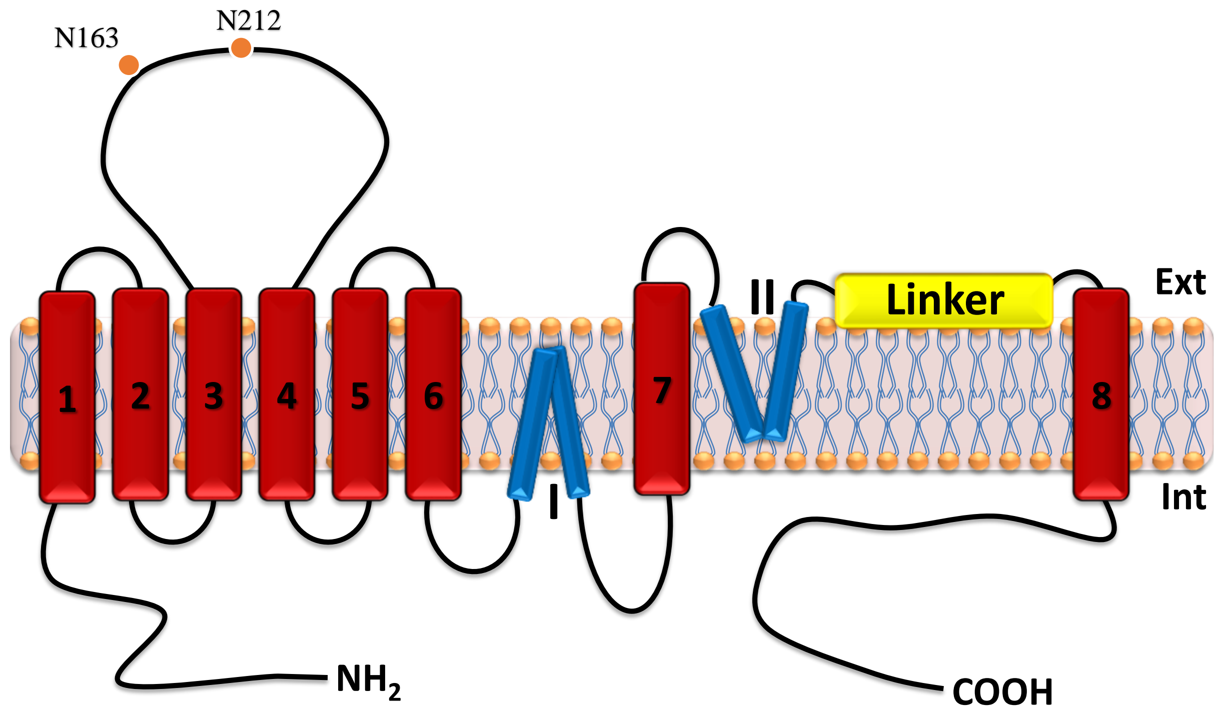


Figure 1.4. hASCT2 putative topology. Adapted from Jiang and Amara, 2011. The large loop between the 7th and 8th TMD, the two hairpins denoted with "I and II" (blue), the linker region (yellow), and the COOH-terminus are hypothesized to be involved in the transportation of the substrate. The two N-glycosylation sites, N163 and N212, are represented by the orange balls.

The three-dimensional structure of hASCT2 is unknown. However, some structural predictions were obtained by performing homology modeling on the known crystal structure (1XFH) of the archaeal glutamate transporter (Gltph) from *Pyrococcus horikoshii* (Yernool *et al.*, 2004), which suggest that the functional ASCT2 may be an oligomer (Figure 1.5). Furthermore, data obtained using cross-linking techniques support this hypothesis and provide evidence that the human ASCT2 transporter forms at least homodimers (Scalise *et al.*, 2014). Moreover, the hypothesis of a functional oligomeric hASCT2 is further supported by the recent unveiling of the trimeric crystal structures of the human excitatory amino acid transporter 1 (EAAT1), also a member of the SLC1 family (Canul-Tec *et al.*, 2017).

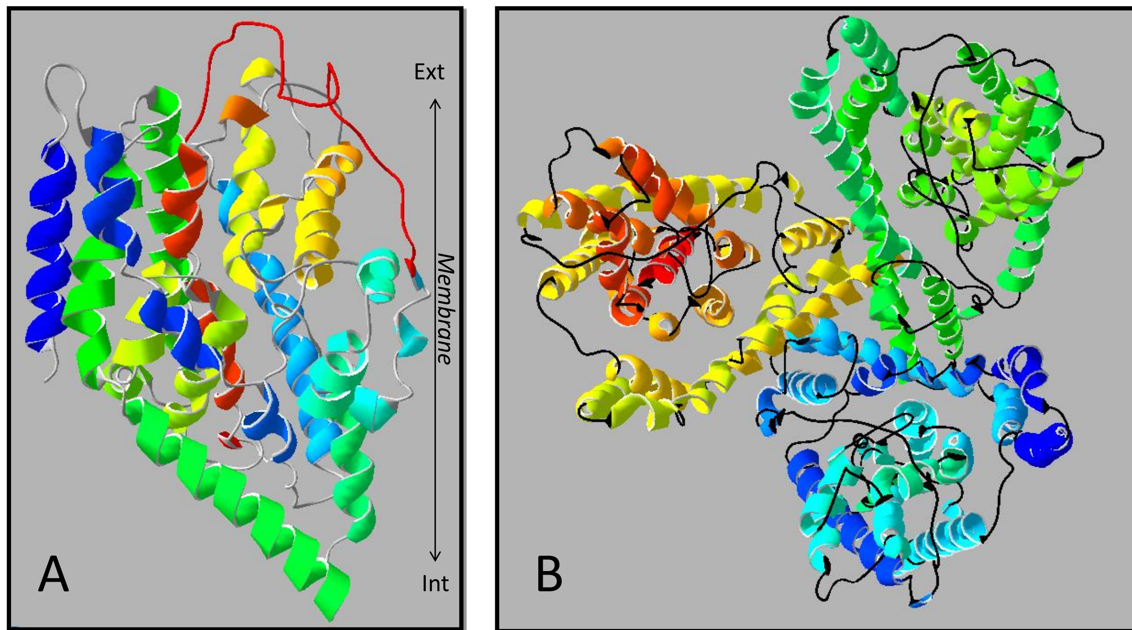


Figure 1.5. Putative hASCT2 three-dimensional structure. Monomeric (A) and oligomeric (B) hASCT2 structures generated with SWISS MODEL and readapted with SWISS-PDB viewer. The ribbon model was based on *P. horikoshii* (Gltph) crystal structure (1XFH) (Kanai and Hediger, 2004).

The integral membrane protein ASCT2 (AlaSerCys transporter 2) despite its name, is a predominant sodium dependent, neutral amino acid exchanger in mammalian cells with a moderate preference for glutamine (K_m= 70– 90 µM) and high preference for glutamine (K_m= 39 µM) in the proteo-liposome (Utsunomiya-Tate *et al.*, 1996, Torres-Zamorano *et al.*, 1998, Bode, 2001, Bröer *et al.*, 1999, Pingitore *et al.*, 2013, Pochini *et al.*, 2014, Scalise *et al.*, 2014, Zander *et al.*, 2013, Avissar *et al.*, 2008, Oppedisano *et al.*, 2007). ASCT2 is widely expressed in numerous tissues where it is responsible for glutamine delivery (Christensen *et al.*, 1967, Christensen, 1990, Pingitore *et al.*, 2013, Pochini *et al.*, 2014, Scalise *et al.*, 2014, Torres-Zamorano *et al.*, 1998, Zander *et al.*, 2013).

Studies on the functional characteristics of the murine and more recently, human isoforms of ASCT2 have all confirmed that the transporter catalyzes an obligatory antiport of other neutral amino acids with glutamine, and that the ability of ASCT2 to transport its substrate is strictly dependent on the presence of extracellular sodium (Na^+) which can’t be substituted with other ions such as K^+ and or Li^+ (Bröer and Palacín, 2011, Torres-Zamorano *et al.*, 1998).

The exact stoichiometry of the transport is still unknown, although some kinetic data obtained by studying the purified human isoform of ASCT2 into proteoliposomes suggested a 1:1 sodium to amino acid coupling ratio (Pingitore *et al.*, 2013, Scalise *et al.*, 2014).

Aspects of the electrical net ion flux of transport are yet to be clarified. Some studies have shown that the Na^+ transported inwardly should induce electrical currents therefore generating an electrogenic transport (Kekuda *et al.*, 1996, Pingitore *et al.*, 2013, Torres-Zamorano *et al.*, 1998, Grewer and Grabsch, 2004). Other studies, however, describe the net ion flux of the ASCT2-dependent transport to be zero, defining the transport as electroneutral (Bröer *et al.*, 1999, Utsunomiya-Tate *et al.*, 1996). A study focused on ASCT2-dependent transport cycle suggested

the possibility that more than one Na^+ ion may be transported along with the translocation of the amino acids across the plasma membrane. Therefore, even if the amino acid exchange is thought to be electroneutral, because of the movement of Na^+ ions across membrane, the overall transport cycle is definitely electrogenic (Zander *et al.*, 2013).

Recent studies of the kinetics of the hASCT2 reconstituted into proteoliposomes, have established that the influx of glutamine is sodium coupled (1:1 ratio) in exchange for an intracellular neutral amino acid such as Gln, Ser, Asp, or Thr. Instead, the intracellular sodium seems to bind to the protein allosterically, regulating its activity without being transported (Scalise et al. 2014).

Because the crystal structure of ASCT2 is not known, studies on the pharmacology of the transporter has been challenging; therefore, potential inhibitors of ASCT2 were designed on the basis of the structural homology of ASCT2 with the glutamate transporter Gltph (Grewer and Grabsch, 2004, Esslinger *et al.*, 2005, Albers *et al.*, 2012). For example, the competitive inhibitor serine biphenyl-4-carboxylate (BenSer), which is structurally similar to an inhibitor of the glutamate transporter, blocks ASCT2 with moderate affinity (Ki= 30 μM) (Albers *et al.*, 2012). Other studies have identified compounds designed on a (N/L) -γ-substituted glutamine, the most specific being L-γ-glutamil-ρ-nitroanilide (GPNA), characterizing a compound that inhibits with a low affinity (Ki= 250 μM) at 100 μM glutamine (Esslinger *et al.*, 2005). These inhibitors were successfully used in studies targeting the ASCT2-dependent transport pharmacologically and arrest the development of cell proliferation (Polet *et al.*, 2016, Wang *et al.*, 2014, Wang *et al.*, 2015).

ASCT2 and Pathology

ASCT2 is physiologically relevant in many tissues. For example, in the placenta, ASCT2 mediates the transport of glutamine used in the fetal liver to synthesize glutamate, an essential amino acid for the correct function of the fetus metabolism (Torres-Zamorano *et al.*, 1998). In the brain under ischemic conditions ASCT2 has a protective action by facilitating the efflux of glutamine from astrocytes in exchange for the protonated glutamate (glt^{+}) released in the synaptic cleft, thereby reducing the toxic activity of this neurotransmitter (Bröer *et al.*, 1999, Gegelashvili *et al.*, 2006). ASCT2 is also used as an anchoring "receptor" by human retroviruses allowing them to infect human cells (Antony *et al.*, 2011, Marin *et al.*, 2003).

Over the last decade, the expression and function of ASCT2 has been increasingly correlated with angiogenesis, which marked it as a potential target for antitumor drug treatment (Fuchs *et al.*, 2004, Fuchs *et al.*, 2007, Kaira *et al.*, 2015, Shimizu *et al.*, 2014, Nicklin *et al.*, 2009, Bode *et al.*, 1995, Bode, 2001). The shift toward anaerobic metabolism undertaken by cells during tumorigenesis, to meet the sudden need for glutamine to support the generation of ATP is very well known as the "Warburg effect" (Vander Heiden *et al.*, 2009, Liberti and Locasale, 2016a, Liberti and Locasale, 2016b). This metabolic shift causes an increased presence of membrane transporters involved in glutamine uptake (Cantor and Sabatini, 2012, Ohh, 2012). Among these, ASCT2 is one of the most highly represented glutamine transporters in tumours, which suggests that this transporter may play an important role in the maintenance of the cancer phenotype by modulating glutamine uptake in this context (Fuchs *et al.*, 2007, Kanai and Hediger, 2004, Palmada *et al.*, 2005, Wang *et al.*, 2014, Metallo *et al.*, 2012). In fact, microarray studies on both non-malignant and malignant prostate tissues have shown a correlation between hASCT2 expression and a more aggressive behaviour of adenocarcinoma (Li *et al.*, 2003).

A role for ASCT2 in angiogenesis is supported by the described cooperative mechanism of regulation between ASCT2 and the large neutral amino acid transporter-1 (LAT1), another major player involved in this process (Nicklin et al., 2009, Shimizu et al., 2014, Bothwell and Bode, 2017). Active LAT1, forming a heterodimer complex with 4F2 heavy chain (4F2hc), is a Na^{+}-independent antiporter which exchanges intracellular glutamine for extracellular essential amino acids such as arginine, phenylalanine, tyrosine, tryptophan, and most importantly leucine (Fuchs and Bode, 2005, Uchino *et al.*, 2002). Data suggest that ASCT2 utilizes the amino acids transported by LAT1 to transport glutamine back into the system both for energy purposes and to fuel LAT1 transport cycle. The alleged cooperation between ASCT2 and LAT1 should provide the necessary leucine for the regulation of mTOR, a key regulator of the cells also involved in uncontrolled cell proliferation (Fuchs and Bode, 2005, Fuchs *et al.*, 2007, Nicklin *et al.*, 2009, Ganapathy *et al.*, 2009).

The concept of ASCT2 being involved in the regulatory system of mTOR was introduced by studies in hepatoma cells, where silencing ASCT2 using RNA interference (RNAi) triggers a reduction in the activity of mTOR leading to cell apoptosis within 48 h (Fuchs *et al.*, 2004, Fuchs *et al.*, 2007), although the underlying mechanisms are unclear. Another report proposed a possible regulation of the transporter via EGF stimulated pathways involving mTOR, MAPK, PI3K, and Rho, though conducted without separating the ASCT2-dependent transport from the B0AT1/B0AT2-dependent transport (Avissar *et al.*, 2008). A similar regulation, involving EGF and the kinases SGK1, SGK3, and PKC which are activated by mTORC2, was reported in *Xenopus laevis* oocytes (Palmada *et al.*, 2005) although the ASCT2 transporter does not harbor the canonical consensus sequences for these kinases. Nevertheless, studies on ASCT2 expression in cancer tissues have shown a regulation by tumour suppressor pRb via the transcription factor E2F-

3 which is also involved in the regulation of other amino acid transporters (Reynolds *et al.*, 2014). These data reinforce the relevance of ASCT2 in this context and highlighting the possible role of mTOR in the regulation of the transporter.

ASCT2 and its Regulators

To understand the regulation of ASCT2, it is important to have knowledge of possible interactions occurring between the transporters and other proteins. Unfortunately, very few studies have been aimed at understanding the interactome of ASCT2. Very little information is available regarding the interaction and co-regulation of ASCT2 by and/or with other proteins. Among the few examples that have been described is a physical interaction between ASCT2 and the epidermal growth factor receptor (EGFR) (Lu *et al.*, 2016). The complex formation between ASCT2 and EGFR, was exploited as a pharmacological target with the anticancer agent cetuximab in the treatment of tumours overexpressing EGFR (Lu *et al.*, 2016). A similar study conducted in astrocytes, described an interaction between the serotonin transporter (SERT) and ASCT2 which leads to a downregulation of SERT's activity and a reduction of the functions mediated by the serotonergic system (Seyer *et al.*, 2016).

Another interaction which seemed to open a new door toward a further understanding of ASCT2 molecular regulatory mechanism was the description of the physical interaction between hASCT2 and the scaffold protein, PDZK1 (Scalise *et al.*, 2014). PDZK1 interacts with many other proteins forming complexes, thereby coordinating the interplay of multiple proteins at the cell membrane in response to growth factors such as EGF, in breast cancer (Kim *et al.*, 2013, Hu *et al.*, 2009). More examples of the regulatory nature of PDZK1 toward membrane transporters can be found in studies focused on the regulation of several members of the SLCs family such as OCTN1 (Kato *et al.*, 2005), OCTN2 and PEPT1 (Sugiura *et al.*, 2008) which results in change of transport activity rate and regulated recycling and/or trafficking (Kim *et al.*, 2013, Walther *et al.*, 2015). PDZK1's expression is limited to epithelial cells and it is overexpressed in a variety of carcinomas where they interact with MAP17, a membrane-associated protein involved in regulation of cell

proliferation (Ghosh *et al.*, 2000, Kocher *et al.*, 1998). Despite these findings, most of my understanding of the regulation of hASCT2 is either incomplete or unknown. For these reasons, a deeper insight into the basic biology of hASCT2 will fill an essential gap in the field relating to the mechanisms implicated in promoting and supporting angiogenesis. The identification of the key regulatory pathways that control hASCT2 activity may allow for the development of inhibitors which could be used as a valuable target for anticancer agents (Nakanishi and Tamai, 2011).

EGFR and EGF, Roles in Cellular Regulation

ASCT2 provides amino acids which are essential in cellular processes regulated by growth factors (Turner and Grose, 2010, Avraham and Yarden, 2011). Crucial for correct cellular functions is the Epidermal Growth Factor (EGF) which exerts its effects by binding to its membrane receptor EGFR (or ErbB1), therefore activating the intracellular signalling pathways controlling cell growth, proliferation, differentiation, migration, adhesion, and apoptosis (Yarden and Pines, 2012, Wong, 2003, Jorissen *et al.*, 2003, Ko *et al.*, 1993, Sibilia *et al.*, 1998, Wolfgang *et al.*, 2003). An overview of the EGF-dependent signalling cascades is depicted in Figure 1.6.

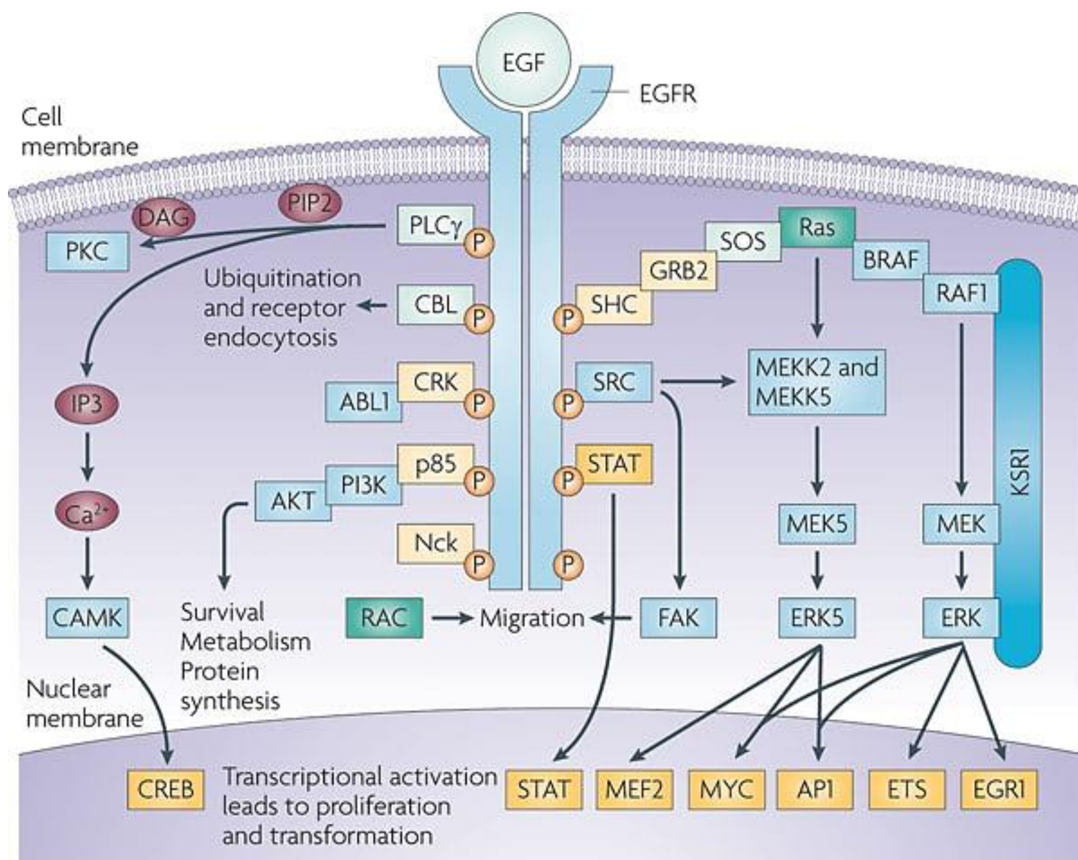


Figure 1.6. EGF-dependent signalling network (Kolch and Pitt, 2010).

Immediately after binding with its ligand, EGFR undergoes dimerization, this causes the activation of the kinase domains and the trans-autophosphorylation of intracellular tyrosine residues (Honegger *et al.*, 1989, Ferguson, 2008). At the same time, the EGF/EGFR complex is internalized very quickly via clathrin-mediated endocytosis which triggers a downregulation of the signalling cascade (Rappoport and Simon, 2009). The internalized receptor is separated from its ligand and de-phosphorylated by cellular phosphatases; then the EGFR is either recycled to the plasma membrane or sent to lysosome for degradation (Avraham and Yarden, 2011). These phosphorylation events induce the activation of many downstream cascades, starting with the formation and activation of the Shc-Grb2-Sos complex and its recruitment to the plasma membrane (Rozakis-Adcock *et al.*, 1992, Simon and Schreiber, 1995) which in turn recruits the downstream effectors of Ras (Raf) to the plasma membrane, and initiates a series of phosphorylation events which starts the Ras/MAPK/Erk 1/2 signalling cascade (Kyriakis, 2009, Sakaguchi *et al.*, 1998). This signalling cascade has many targets: (1) the activation of Ras/MAPK promotes the beginning of the cell cycle and cell proliferation (Avraham and Yarden, 2011) and stimulates cell migration (Jorissen *et al.*, 2003); (2) MAPK/Erk 1/2 can translocate to the nucleus and phosphorylate several transcription factors, therefore altering gene expression (McCubrey *et al.*, 2007); (3) Active Ras interacts with RalGEF, promoting the conversion of GDP to GTP and activation of RhoGTPases such as Cdc42, leading to cytoskeletal rearrangements and cell migration (Cantor *et al.*, 1995, Jullien-Flores *et al.*, 1995); (4) Activated Ras also targets the phospholipase C (PLCγ/ε) which hydrolyzes the phosphatidylinosidol 4,5- biphosphate (PIP2) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). This reaction activates the protein kinase C (PKC) which induces the release of actin-modifying proteins from the plasma membrane, which will act as a second messengers thus regulating (a) the reorganization of actin cytoskeleton and cell migration (Chen

et al., 1996), and (b) the regulation of intracellular calcium release (Rhee, 2001, Kelly and Levin, 2001).

Another major signalling cascade initiated by the activation of the EGFR is the PI3K/Akt pathway. Upon EGFR activation, PI3K is recruited to the plasma membrane where it binds and phosphorylates second messenger phosphatidylinositol 3,4-biphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3), which in turn recruits the phosphoinositide-dependent kinase 1 (PDK1) and the serine-threonine kinase Akt (Kyriakis, 2009, Chalhoub *et al.*, 2009). The activation of the PI3K/Akt pathway is a key regulator of cellular functions, including the promotion of antiapoptotic responses, the starting of the cell cycle, and cell survival (Jorissen *et al.*, 2003). For example, activation of Akt leads to: (1) the phosphorylation and inhibition of BAD, a member of the Bcl-2 family of pro-apoptotic factors (Datta *et al.*, 1997); (2) the phosphorylation and activation of κB inhibitor (IkB) which in turns activates the nuclear factor κ-light-chain-enhancer of B cells (NFκB), therefore increasing the transcription of anti-apoptotic genes (Bai *et al.*, 2009); (3) the promotion of protein synthesis via activation of mammalian target of rapamycin (mTOR) through the inhibition of the tuberous sclerosis proteins (TSC1 and TSC2) GTPase activity and activation of Rheb (Courtney *et al.*, 2010).

Due to its pivotal role in cell physiology, alteration in EGF signalling may be a key contributor to uncontrolled cell proliferation. Overexpression, deletion and mutation of the EGFR gene have been correlated with different types of cancer (Turner and Grose, 2010, Avraham and Yarden, 2011, Burgess, 2008, Casanova *et al.*, 2002, Klos *et al.*, 2006, Salomon *et al.*, 1995, Rajkumar and Greipp, 2001). For example, mutations occurring within the EGFR gene are known to contribute to a ligand-independent activation of the receptor with a strong enough signal to cause a constant activation of the downstream signalling cascade but insufficient to target the EGF

receptor for the natural post-activation degradation (Avraham and Yarden, 2011, Normanno *et al.*, 2006, Lemmon and Schlessinger, 2010). Studies on oral, lung, and prostate cancer, have reported a correlation between the earlier development of these tumours and the overexpression of EGFR, caused by an increase in both gene amplifications and transcription (Grandis and Sok, 2004). Most importantly, increased EGF receptor levels have been associated with shorter survival and increased presence of metastasis (Veale *et al.*, 1993, Radinsky *et al.*, 1995).

Therefore, due to the significant role of EGF and EGFR during development and its correlation with neoplastic transformation which also involves downstream kinases such as mTOR (Nomura *et al.*, 2003, Dibble and Cantley, 2015), its involvement in regulating nutrient transporters associated with tumour development and survival has been subject of many studies (Aggarwal *et al.*, 2011, Ray *et al.*, 2005, Tsuchihashi *et al.*, 2016, Zelenaia *et al.*, 2000).

mTOR Signalling

Mechanistic target of rapamycin (mTOR) (Figure 1.7) is a widely conserved serine/threonine kinase which integrates and regulates many signals of cell growth including growth factors, stress, and nutrients including amino acids (Wullschleger *et al.*, 2006, Foster and Fingar, 2010, Nobukuni *et al.*, 2005).

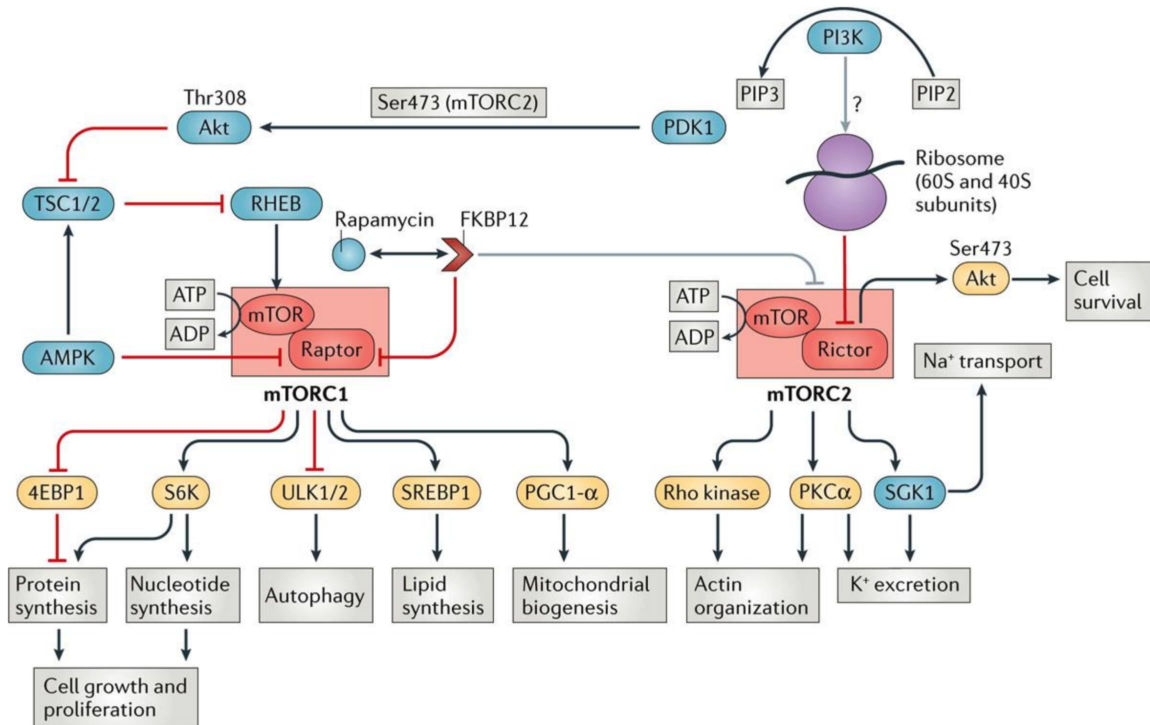


Figure 1.7. mTOR signalling pathway (Fantus *et al.*, 2016).

Mammalian TOR exists in two structurally different large complexes (mTOR complex 1 and mTOR complex 2) which share some interrelated functions within the cell: mTORC1 is exclusively associated with the regulatory-associated protein of TOR (RAPTOR) and the proline-rich Akt substrate 40 kDa (PRAS40) (Avruch *et al.*, 2009b, Hara *et al.*, 1998, Hara and Mizushima, 2006, Kim *et al.*, 2011, Thedieck *et al.*, 2007, Vander Haar *et al.*, 2007, Wang *et al.*, 2007, Sancak *et al.*, 2007, Sancak *et al.*, 2010); whereas, mTORC2 is exclusively associated with rapamycin-

insensitive associated protein of TOR (RICTOR), mammalian stress-activated map kinase-interacting protein 1 (mSin1) and rictor-associated proteins (protor 1/2) (Frias *et al.*, 2006, Jacinto *et al.*, 2004, Jacinto *et al.*, 2006, Pearce *et al.*, 2007, Pearce *et al.*, 2011, Sarbassov *et al.*, 2004). Both complexes share the catalytic mTOR subunit (TOR), the mammalian lethal with SEC thirteen 8 (mLST8 or GbL), DEP domain containing TOR-interacting protein (DEPTOR), and the Tti1/Tel2 complex (Jacinto *et al.*, 2004, Kaizuka *et al.*, 2010, Kim *et al.*, 2002, Kim *et al.*, 2003, Peterson *et al.*, 2009).

mTORC1 has many functional roles, one of which is to regulate cell growth by phosphorylating the 70 kDa ribosomal protein S6 kinase (S6K1), which in turn regulates the eukaryotic initiation factor 3 (eIF3) translation complex (Holtz *et al.*, 2005, Ma *et al.*, 2008). Also, mTORC1 increases mRNAs translation by hyper-phosphorylating the eukaryotic translation initiation factor 4E (eIF4E) binding protein (4E-BP1), therefore causing it to release the eIF4E (Gingras *et al.*, 2004).

mTORC1 is also considered a signal integrator for amino acid availability which leads to protein turnover and autophagy, but the origin of the signal and the exact mechanism via which mTOR senses intracellular amino acids is still ambiguous (Avruch *et al.*, 2009a, Durán *et al.*, 2012, Heublein *et al.*, 2010, Jewell *et al.*, 2013, Jewell and Guan, 2013, Kim *et al.*, 2011, Wullschleger *et al.*, 2006, Yu *et al.*, 2010, van der Vos and Coffer, 2012). Some theories suggest that intracellular amino acid sensors are working together with transporters such as ASCT2 and LAT1 to support the regulation of the sensing mechanism exerted by mTOR (Kim, 2009). Other data suggest that some amino acid transporters, such as PAT1, are translocated from the plasma membrane to the lysosome/endosome where they aid the modulation of mTOR activity in response to a growth-

dependent stimulus by importing amino acids from the extracellular environment into the cell (Sagné *et al.*, 2001).

mTORC2 is a newly discovered complex and very little is known about its function; nevertheless, its primary role appears to include the regulation of cytoskeletal remodeling and cell migration by phosphorylating many kinases such as PKA, PKG, and PKC (Jacinto *et al.*, 2004, Sarbassov *et al.*, 2004, Gan *et al.*, 2012, Thomanetz *et al.*, 2013, Li *et al.*, 2014) and the modulation of the trafficking of some nutrient transporters (Hara *et al.*, 2002, Kim *et al.*, 2002, Edinger, 2007). However, the most important role of mTORC2 is to modulate cell survival, proliferation, and growth by phosphorylating and activating the Akt Ser/Thr kinase at serine 473 and promoting Rheb GTP-loading; however, the underlying mechanism of regulation of mTORC2 is yet to be clarified (Guertin and Sabatini, 2007, Jacinto *et al.*, 2004, Sarbassov *et al.*, 2004, Sarbassov *et al.*, 2006, Wullschleger *et al.*, 2006). Finally, mTORC2 activity has been also correlated with the regulation of ion transport via the phosphorylation of the SGK1 kinase (García-Martínez and Alessi, 2008).

Many studies over the last decade support the important role of mTOR signalling pathways in pathogenesis. Some mutagenic events such as loss of p53 and dysregulation of the PI3K signalling pathway promote constitutive mTORC1 activation (Averous and Proud, 2006, Feng *et al.*, 2005, Fingar *et al.*, 2004). The exact mechanism and contribution to oncogenesis caused by the dysregulation of the protein synthesis machinery downstream of mTORC1, is unclear (Dowling *et al.*, 2010, Hsieh *et al.*, 2010, Yang and Guan, 2007).

mTORC2 has been also associated with cancer proliferation, mainly due to its regulation of Akt phosphorylation. In many gliomas, the overexpression of mTORC2 has been reported to further promote the constitutive assembly and activation of mTORC2 leading to an increase in

further cell proliferation and invasion potential (Hietakangas and Cohen, 2008, Masri *et al.*, 2007). The loss of the tumour suppressor PTEN is a crucial event in the development of prostate cancer in mice, caused by a dysfunction of the mTORC2 activity (Guertin *et al.*, 2009). These data support the important role of mTORC1 and mTORC2 in promoting the development of cancer and suggest that designing of effective pharmacological agent aimed at reducing the activity of either complex is essential for successful anticancer therapies. To date, however, there is no pharmacological agent selective enough to target and inhibit mTORC2 without also affecting mTORC1, therefore the prospects of a short-term solution are small.

Objectives of the Study

To date, our basic understanding of the biochemistry of ASCT2 is not sufficient to allow us to make use of this transporter as a target for drug therapy. Furthermore, questions remain unanswered on the fundamentals of ASCT2 regulation and the identity of its interactors. Because of the broad distribution, activity, and major role in cell metabolism, a deeper understanding of hASCT2 is crucial. Moreover, even the limited information I have was primarily obtained in models which are not representative of a normal tissue, such as immortalized cell lines derived from primary tumours such as HEPG2, Caco-2 and BeWo cells, or embryonic cell lines such as HEK-293 or oocytes derived from *Xenopus laevis*. Therefore, the overall objective of my research is to develop better tools to gain a holistic understanding of the basic cell biology of hASCT2 and its regulatory mechanisms, primarily in the context of cellular transformation. In addition, the aim was to develop and characterize an appropriate cellular model for the study of hASCT2 and confirm the EGF-dependent regulation of hASCT2 in an appropriate model which is representative of a normal tissue such as RWPE-1 cells. Finally, I will identify mechanisms involved in EGF-dependent regulation of ASCT2. Taken together these data will fill essential gaps in our understanding of the underlying regulatory mechanisms of endogenous human isoform of ASCT2.

Chapter 2 : The regulation of ASCT2-dependent glutamine uptake and its distribution at the PM are modulated by EGF

Introduction

Homeostasis in mammalian cells is a highly complex process which is regulated by many pathways including uptake and metabolism of amino acids. Of particular interest are transport systems with specificity for glutamine since these transporters have pivotal roles in cell growth, proliferation and viability in both normal and abnormal cells (Bode, 2001, Pochini *et al.*, 2014, McGivan and Bungard, 2007). Glutamine has a major role in cellular proliferation and unlike glucose, it may be transported with different affinities and mechanisms by diverse, non-homologous amino acid transporters (Bungard and McGivan, 2004, Kanai and Endou, 2001, Hensley *et al.*, 2013). The mammalian amino acid transporters known to be involved in the maintenance of glutamine homeostasis are denoted, on the basis of their transport mechanism, by systems A, N and, L and by transporters B0AT1, $ATB0^{+}$, and ASCT2 (Christensen *et al.*, 1967, Christensen, 1990, McGivan and Bungard, 2007, Pochini *et al.*, 2014).

The integral membrane protein ASCT2 is expressed ubiquitously in numerous mammalian tissues and is a sodium dependent neutral amino acid exchanger with a moderate preference for glutamine (Km= 70 – 90 μM) in cell systems (Bröer *et al.*, 1999, Pingitore *et al.*, 2013, Pochini *et al.*, 2014, Scalise *et al.*, 2014, Torres-Zamorano *et al.*, 1998, Utsunomiya-Tate *et al.*, 1996, Zander *et al.*, 2013). Over the last decade, it has been increasingly apparent that the expression and function of ASCT2 correlates with angiogenesis in many tissues which marked it as a potential target for antitumor drug treatment (Albers *et al.*, 2012, Fuchs and Bode, 2005, Fuchs *et al.*, 2007, Kaira *et al.*, 2015, Nicklin *et al.*, 2009, Shimizu *et al.*, 2014, Namikawa *et al.*, 2014). Together,

these data highlight the important role of ASCT2 in both physiological and pathophysiological settings. Therefore, a more in depth understanding of the regulatory mechanisms of the endogenous human isoform of ASCT2, in a model which is representative of a non-tumorigenic tissue, will facilitate targeted approaches to be used in those clinical settings where uncontrolled cellular proliferation needs to be halted. Nevertheless, the majority of what is currently known about ASCT2 structure and function has been obtained by heterologous expression of the protein and subsequent analysis in transformed cell lines, in *Xenopus laevis* oocytes or by reconstitution of the transporter into proteoliposomes (Console *et al.*, 2015, Fuchs *et al.*, 2007, Palmada *et al.*, 2005, Shimizu *et al.*, 2014). Currently, there is no well-established, fully characterized cellular model for the study of ASCT2 and its regulation. Thus, to address this gap, I have characterized the human epithelial prostate cell line, RWPE-1 (Bello *et al.*, 1997, Webber *et al.*, 2001). RWPE-1 cells are non-tumorigenic (in nude mice), which have been immortalized with HPV18 allowing them to mimic both the physiological morphology and androgen response of the tissue of origin. Indeed, RWPE-1 cells express the functional markers of the prostate tissue such as PSA and AR, the markers identifying their epithelial origin such as CK18 and CK18, and the tumour suppressor markers such as the p53 gene and the Rb protein (Bello-DeOcampo *et al.*, 2001a, Webber *et al.*, 1997). Furthermore, RWPE-1 cells have been adopted as a negative control in many studies investigating features indicative of tumorigenesis such as increased cell mobility, invasiveness, and metastasis (Aggarwal *et al.*, 2011, Fernández-Martínez *et al.*, 2010, Giannoni *et al.*, 2009). Based on these properties, I characterized RWPE-1 cells to be used as a physiologically relevant model to have a better understanding of the basic biology and of the regulatory mechanisms of the endogenous human glutamine transporter, ASCT2. The effects on glutamine uptake induced by EGF have been previously reported (Iannoli *et al.*, 1997, Ray *et al.*, 2003, Ray *et al.*, 2005). Some

studies investigating the regulation of ASCT2 have suggested that the transporter's activity may be EGF-induced (Avissar *et al.*, 2008, Palmada *et al.*, 2005, Rosario *et al.*, 2013). Therefore, I have investigated the effects of EGF on the regulation of the transporter in RWPE-1 cells.

Materials and Methods

Materials

Keratinocyte serum free medium (K-SFM- 17005042), Dulbecco's modified Eagle's medium (DMEM), bovine pituitary extract (BPE), fetal bovine serum (FBS), supplemental epidermal growth factor (EGF), Hank's balanced salt solution (HBSS), and PureLink^{TM} RNA Mini isolation kit were purchased from Life Technologies (Burlington, ON). The SuperScript® III RT kit, Superscript First-Strand Synthesis System for RT-PCR, fluorescently labelled antibodies, plasma membrane and nuclear dyes were purchased from Invitrogen (Carlsbad, CA). Quantitative PCR reactions were performed with the EvaGreen 2X qPCR MasterMix-ROX from ABM inc. (Richmond, BC) using StepOne Plus thermo-cycler by Applied Biosystems (Burlington, ON). Goat HRP-conjugated anti-rabbit antibodies were purchased from BioRad (Mississauga, ON). The chemiluminescent substrate for immunoblot analysis was purchased from Mandel Scientific (Guelph, ON). Labelled glutamine ([^{3}H]-glutamine) was purchased from Moravek Biochemicals (Brea, CA). The cellular grade EGF, amino acids, and EGFR inhibitor (tyrphostin AG1478) used in this study were purchased from Sigma Aldrich (Oakville, ON). Antibodies against tyrosine phosphorylated protein (4G10 Platinum-#05-1050), pan EGFR (#06-847), and hASCT2 (#ABN73) were purchased from Millipore (Billerica, MA). The hASCT2.rbd-antibody-conjugated was purchased from Metafora Biosystems (Evry cedex, France). Antibodies against β-actin (#4967) and hASCT2 (#5345) were purchased from Cell Signalling Technologies (Danvers, MA). The cocktails of protease and phosphatase inhibitors (cat#11836153001) were purchased from Roche Diagnostics (Mississauga, ON). All other chemicals were purchased from BioShop (Burlington, ON) unless otherwise specified.

ASCT2.RBD

ASCT2 is utilized by human retroviruses as an anchoring "receptor" to infect the host (Antony *et al.*, 2011, Marin *et al.*, 2003). This characteristic was exploited by Metafora Biosystems (Evry cedex, France) in designing an anti-ASCT2 retroviral binding domain (RBD) that could bind specifically to the protein residing at the plasma membrane. The ligand is constituted of an undisclosed isolated viral glycoprotein fused to a fragment antigen binding (Fab) of a rabbit grown antibody where the a fluorescently labelled secondary antibody will bind to.

Cell Line Choice Rationale

Human epithelial prostate cells (RWPE-1) were chosen based on hASCT2 expression pattern data obtained from the European Bioinformatics Institute (http://www.ebi.ac.uk/). Furthermore, these cell lines are derived from primary human tissues and they are physiologically relevant for my studies in that they are reported as non-tumorigenic in mice (Bello *et al.*, 1997, Webber *et al.*, 2001). RWPE-1 cells are immortalized with human papilloma virus 18 (HPV18) DNA containing the adenoviral SV40 large T antigen gene, and the adenoviral E1a and E7 protein genes. This genetic combination allows the modulation of pRb and p53 proteins levels instead of their inactivation. This allows RWPE-1 cells to retain all the characteristics of the tissue of origin such as prostate specific antigen (PSA) and androgen receptor (AR) expression, growth factor and androgen response, expression of cytokeratin 18 and 8, and expression of p53. Moreover, an extensive knowledge on the morphology and tissue-specific response of metabolic pathways stimulated by growth factors in RWPE-1 cells is available and it could be involved in the regulation of hASCT2 (Reynolds and Kyprianou, 2006, Giannoni *et al.*, 2009). Furthermore, the presence of characterized sister cell lines derived by RWPE-1 with different grades of neoplasticity (RWPE-2 and RWPE-3 cells) may be the ultimate tool in studying the regulation of hASCT2 at different

stages of the disease in comparable models (Bello *et al.*, 1997, Bello-DeOcampo *et al.*, 2001a, Bello-DeOcampo *et al.*, 2001b). RWPE-1 cells represent a clinically relevant model since the Canadian Cancer Society reported that ¼ of the total deaths of male cancer patients are due to prostate cancer and that previous data correlate hASCT2 expression with a more aggressive prostate adenocarcinoma (Li *et al.*, 2003).

Human liver cells (THLE-3) (Pfeifer *et al.*, 1993) were chosen as negative control, due to the data obtained from the European Bioinformatics Institute, reporting very low mRNA expression of ASCT2 in the liver. However, I confirmed that these cells do express abundant amount of functional hASCT2, therefore are no longer suitable as a negative control for my studies.

Human embryonic kidney cells (HEK-293), were chosen as a positive control for technical assays as they are a very well characterized model which has been used in previous studies investigating ASCT2 (Scalise *et al.*, 2014, Zander *et al.*, 2013).

Cell Culture

Immortalized human prostate epithelial RWPE-1 cells were purchased from ATCC (CRL-11609) and cultured per ATCC's guidelines. RWPE-1 monolayers are kept in proliferative conditions in Keratinocyte SEM (Gibco) serum free medium supplemented with 0.05 mg/mL of bovine pituitary extract and 5ng/mL of human recombinant epidermal growth factor at a temperature of 37ºC in a humidified incubator and a 5% (v/v) CO_2 atmosphere. HEK-293 were purchased from ATCC and were cultured in DMEM supplemented with 10% (v/v) FBS at a temperature of 37 ºC in a humidified incubator and a 5% (v/v) CO_2 atmosphere. Transformed human liver epithelial THLE-3 cells, purchased from ATCC, were chosen as a low/no expressing tissue for (SLC1A5) ASCT2 and tested as potential negative control to be used in parallel for my studies. THLE-3 cells were purchased from ATCC and were kept in proliferative conditions on pre-coated plates in BEGM basal medium supplemented with 5 ng/mL EGF, 70 ng/mL phosphoethanolamine, and 10% (v/v) FBS at a temperature of 37ºC in a humidified incubator and a 5% (v/v) CO_2 atmosphere.

EGF Treatments

RWPE-1 cells were cultured as previously described, incubated in sodium uptake buffer (5hr, 37 °C) and exposed to extracellular EGF (300 ng/mL, 3, 5, and 10 min, 37 °C) in the presence or absence of EGFR inhibitor, Tyrphostin AG1478 (20, 40, and 60 µM, 10 min, 37 °C). Cells were then lysed, and protein analysis obtained via immunoblotting. RWPE-1 and HEK-293 cells were cultured as described above, treated with extracellular EGF (300 ng/mL, 30 min, 37 °C) and used for the study of ASCT2's mRNA expression and ASCT2 protein analysis via Immunoblot. RWPE-1 cells cultured as described above and exposed to extracellular EGF (300 ng/mL, 3 min, 37°C) were used to study both ASCT2's functionality via transport assays and ASCT2's cellular

distribution via surface protein biotinylation assays, immunofluorescence, and in-plate flow cytometry.

RNA Extraction and cDNA Synthesis

RWPE-1 and HEK-293 monolayers (90% confluence) were washed twice with PBS (4°C) and total RNA was isolated using the PureLink^{TM} RNA mini kit as per manufacturer's instruction. Briefly, lysis buffer supplemented with 1% (v/v) mercaptoethanol was added to the cells and they were scraped off the plate. The suspension was then homogenised by syringing (10 times) using a 26-Gauge needle. Total RNA (~400 ng/µl yield) was obtained via column elution in RNAse free water and stored at -80 °C. Purity, integrity and concentration of the extracted RNA were verified with Agilent 2100 Bioanalyzer (Santa Clara, CA). Total RNA which met quality criteria was converted to cDNA using SuperScript® III RT kit as per manufacturer's instructions. Briefly, first strand cDNA was synthesized using the Superscript First-Strand Synthesis System for RT-PCR. For each sample in a volume of 10 µl, total RNA (1µg), 1 µl (10mM) dNTPs Mix, 1 µl (50 µM) Oligo20 (dTs), and DEPC water were combined and heated to 65 °C for 5 min then chilled on ice for 1 min. A second mixture containing 2 µl (10mM) DTT, 2 µl 10X RT buffer, 4 µl (25 mM) $MgCl_2$, 1µl (40 units/µl) RNAse OUT recombinant RNAse inhibitor and 1 µl (200 units/µl) of SuperScript III enzyme was added to each sample except the negative control, followed by incubation at 50 °C for 50 min, then 85 °C for 5 min. The tubes were chilled on ice for 5 min before the addition of 1 µl of *E. coli* RNase H (2 units/ µl) and incubation at 37 °C for 20 min. The cDNA was stored at -20 °C.

Quantitative PCR

Detection and quantification of hASCT2 (SLC1A5) expression levels in RWPE-1 cells was performed via qPCR using the EvaGreen 2X qPCR MasterMix-ROX in a total volume of 20 µl (10 µl EvaGreen Master Mix, 1 µg of cDNA and a final concentration of 250 nM of each of 5' and 3' primers). Gene specific primers sequences for hASCT2 including the reference genes GAPDH, HPRT1 and HMBS are reported in Table 2.1. The reaction was performed with a standard thermal profile, followed by melting curve analysis to ensure the amplification of a single and unique product per reaction. Briefly, the reaction was conducted in three steps: (1) enzyme activation: 1 cycle of 10 min at 95 °C; (2) denaturing, annealing and extension: 15 sec at 60 °C, 40 cycles of 1 min at 95 °C; (3) melting curve: 15 sec at 95 °C, 1 min at 60 °C, 15 sec at 95 °C and 15 sec at 60 °C. All samples were run in technical triplicate and each experiment included a no-template negative control (NTC). Each experiment was repeated at least 3 times. To verify the amplification efficiency, standard curves for the cDNA target and each set of primers were established; qPCR assays were performed on total cDNA from RWPE-1 cells. Relative gene expression between hASCT2 and the reference genes was calculated using the geometric averaging of multiple internal control genes method and by comparing it to the data obtained from HEK-293 cells (Vandesompele *et al.*, 2002).

Table 2.1. Primers used in this study for qPCR. hASCT2 (SLC1A5) primers were designed using the Clone Manager 9 software and they were analyzed again via the IDT- Oligo design and analysis tool. The remaining primers sequences were provided by Dr. Pamela Plant (Li Ka Shing Knowledge Institute).

Gene	Primer sequences 5'-3'		Slope	*Eff= 10^ (1/slope)
	Forward	Reverse		
hASCT2 (NP_005619.1)	AGCTGCTTATCCGCTTCTTCAA	AGCAGGCAGCACAGAATGTA	-3.39	1.97
GAPDH (NM_002046)	CAATGACCCCTTCATTGACC	GACAAGCTTCCCGTTCTCAG	-3.48	1.93
HPRT1 (NM_000194)	CCTGGCGTCGTGATTAGTGAT	AGACGTTCAGTCCTGTCCATAA	-3.33	1.99
HMBS (BC019323.1)	TGCAACGGCGGAAGAAAA	AGCTGGCTCTTGCGGGTAC	-3.37	1.98

** Efficiency of the primers as defined by (Pfaffl, 2001).*

Protein Extraction and Western Blot Analysis

THLE-3 and RWPE-1 cells monolayers were cultured as described above and were washed twice with PBS (4°C) and protein extraction was performed using 1 % (v/v) NP-40 lysis buffer. Total cell lysates were incubated on ice for 5 min, subjected to syringing with a 26-Gauge needle to solubilize the plasma membrane and then centrifuged (19000 g, 5 min, 4 °C). Protein quantification of the supernatant was done using Bio-Rad PC DC Protein Assay kit based on modified Lowry assay. The absorbance was read at 750 nm and the concentration of the samples was calculated using a standard curve made of increasing concentrations of BSA. Total cell lysate (10 or 20 µg) was resolved by 12 % (w/v) SDS-PAGE (Bio Rad Mini-PROTEAN Tetra System) (90 min, 150 V) and the protein was transferred on transfer buffer-activated nitrocellulose membrane (Bio-Rad – 1620090 - 0.45 microns) using a Bio-Rad semi-dry apparatus (60 min, 20 V).

The membranes were blocked (5% (w/v) milk/ TBST, 1 h, 25 °C) and incubated overnight at 4 °C with antibodies against hASCT2 (1:1000, 1% (w/v) milk/TBST), antibodies against tyrosine phosphorylated protein (1:2000) or β-actin (1:2000) (4 °C, 1 % (w/v) milk/TBST). Total lysate of EGF-treated HeLa cells (Millipore 20-168) was used as a positive control. The following day, membranes were washed 3 times for 10 min each in TBST, then incubated with goat-anti-rabbit horseradish peroxide conjugated secondary antibodies (1:3000, 1% (w/v) milk/ TBST, 1 h, 25 °C). The membrane was then washed (3 times for 10 min each in TBST) and incubated in chemiluminescent substrate solution for 1 minute, exposed to an X-ray film and developed.

Densitometry and Normalization

Densitometry of the bands detected via Immunoblot was obtained using Image J. The Normalization was obtained using either β-actin or for phosphorylated kinases, their equivalent pan. Relative normalizing value was obtained by dividing all the normalizing controls values (NC) by the highest NC density value. The data was normalized by subtracting the densities of my protein of interest (PI) and that of the relative normalizing control (rNC) in their respective lanes. The untreated control is set at 1 and all treatments are calculated accordingly.

In-situ Protein Biotinylation Assays and Immunoblot

RWPE-1 cells were grown to 90% confluence as described above, treated in the presence or absence of EGF (300 ng/mL, 3 min, 37 °C) and immediately moved to an iced-water bath to stop trafficking. All the biotinylation steps were performed at 4 °C unless indicated otherwise. Cells were then washed 3 times with PBS and incubated for 1 h with 2 mL of Sulfo-NHS-SS-Biotin (1 mg/mL, in PBS; Pierce, Rockford, Illinois). Cells were rinsed three times with PBS containing 100 mM glycine followed by a 15 min quenching step followed by three quick washes in PBS. RWPE-1 cells were scraped in 300 µL of NP-40 lysis buffer, and let lyse for 10 min on ice. Lysates were

syringed 10 times with a 26-Gauge needle and centrifuged at 19000 g for 5 min at 4 °C. Equal concentrations of lysate were incubated with 30μL of streptavidin-conjugated beads per sample (15 h at 4°C). After rinsing beads three times with NP-40 buffer, protein was eluted at 90°C for 10 min in standard 1X protein loading buffer without DTT (to minimize IgG contamination). Protein was resolved on a 12% (w/v) SDS-PAGE for 90 min at 15 V. The nitrocellulose membrane obtained after semi-dry transfer was blocked with 5% milk and probed with antibodies against hASCT2 (Millipore, 1:1000), phosphorylated EGFR (1:2000), and pan-EGFR (1:2000) in 1 % (w/v) milk/TBST at for 15 h followed by probing with secondary HRP-conjugated antibodies (1:4000, 1 % (w/v) milk/TBST, 25°C, 1 h). Protein signals were detected via enhanced chemiluminescence (ECL) solution, developed on film and analyzed by densitometry using ImageJ software.

Total [^{3}H]-glutamine Uptake Assays

To measure the kinetics of glutamine transport in RWPE-1 cells, radio-labelled glutamine uptake was measured. RWPE-1 cells were grown in Keratinocyte SFM medium (GIBCO) in 5 % CO2 at 37 °C and plated at ~1.2 x 10^6 cells/well in 6-wells plates or at ~0.5 x 10^4 cells/well in a 24-wells plate and allowed to adhere for 24 h. Cells were rinsed twice with sodium (Na^+) uptake buffer. Transport assays were performed at 37 °C. The transport was started by adding 50 μM of [^{3}H]-glutamine (2 μCi/mL, 25 °C) and stopped at the indicated times by washing with x sodium-free buffer (4°C). The cells were lysed (2 N NaOH, 1 h, 25 °C) and protein concentration was determined with a modified protein Lowry Assay (BioRad; Mississauga, ON). [^{3}H]-glutamine uptake was measured by standard liquid scintillation counting. Transport is expressed as picomoles of substrate per mg of protein (pmols/mg).

Specific [^{3}H]-glutamine Uptake Assays

To identify only the ASCT2-dependent glutamine transport, distinguish between different amino acid transport systems and assess the sodium-dependent/independent glutamine transport mechanisms in RWPE-1, cells were treated for 15 min at 37°C in the presence or absence of 2 mM competitive amino acids inhibitors (threonine to inhibit specifically hASCT2, histidine to inhibit system N, arginine to inhibit ATB0$^+$, phenylalanine to inhibit B0AT1, and MeAIB to inhibit System A). In this context, the uptake of radio-labelled glutamine (50 µM [^{3}H]-glutamine, 2 µCi/mL) was measured in RWPE-1 monolayers via the modified cluster-tray method (Gazzola *et al.,* 1981). Cells were plated at ~5 x 10^4 cells/well in 24-wells culture plates and allowed to adhere for 24 h. Cells were rinsed twice with either sodium (Na^+) or choline (Ch^+) uptake buffer. Transport assays were performed at 37 °C in the presence or absence of sodium via the Gazzola's modified cluster-tray method (Gazzola et al. 1981) using buffer containing [^{3}H]-glutamine (50 µM, 2 µCi/mL) and in the presence or absence of competitive amino acids and inhibitor. The transport was stopped at 3 min by washing the cells sodium-free buffer (4°C). The cells were lysed in 2 N NaOH, protein concentration was determined with a modified protein Lowry Assay and [^{3}H]-glutamine uptake was measured by standard liquid scintillation counting. Following transport assays, cells were lysed (4 °C, 1 h, shaking) with 2 N NaOH. Protein concentrations of cell lysates were determined with a modified protein Lowry Assay (BioRad; Mississauga, ON) and [^{3}H]-glutamine uptake was measured by standard liquid scintillation counting. Transport is expressed as picomoles of substrate per mg of protein (pmols/mg) at 3 min. Na^+-independent glutamine uptake was defined in presence of choline chloride. The Na^+-dependent glutamine uptake was calculated by subtracting the transport in presence of choline chloride from the total transport uptake in the presence of sodium. Each transporter contribution was obtained by subtracting the

residual uptake after the inhibition of each transporter from the Na^+-dependent uptake (systems A, N and transporters ASCT2, B0AT1 and $ATB0^+$) and from the choline uptake (system L only).

Immunofluorescence Microscopy of Endogenous hASCT2

RWPE-1 cells were grown as to 65 % confluence on microscopy cover slips (1.5 µm thickness-Carl Zeiss) as described above and then transferred in serum-free media for 15h. Cells at 70 % confluence were kept in sodium buffer (5 h, 37 °C). All further steps were performed at 25°C unless otherwise specified. Cells were rinsed 3 times with warm (37 °C) HBSS, exposed to EGF (300 ng/mL, 37 °C, 3 min), and fixed (2 % (v/v) PFA (in HBSS) for 20 min) immediately after treatment. Successively, RWPE-1 cells were incubated in 50 mM NH_4Cl in PBS for 10 min, to reduce auto fluorescence and washed once with 95 mM glycine and 3 times with PBS. Cells were treated with 10 % (w/v) BSA for 1h to block non-specific antibody binding and probed with anti-hASCT2.rbd (1:100, Metafora Biosystems, 3 % (w/v) BSA/PBS, 30 min, 37 °C). Cells were washed, probed with donkey anti-rabbit IgG (H+L) antibody AlexaFluor555 (1:2000, 3 % (w/v) BSA/PBS, 1 h, 37 °C) and the membrane was stained with Alexa488- conjugated WGA (1:3000 PBS). Cells were then washed 3 times with PBS, permeabilized for 5 min with 0.1 % (v/v) TritonX-100 in PBS (supplemented with 3 % (w/v) BSA), and treated with 10 % (w/v) BSA for 1h. Cells were then, re-probed with anti-hASCT2.rbd and secondary antibodies Alexa555 as described above. Nuclei were stained with DAPI (1:30000) and the cells were mounted on microscopy slides (0.5 mm thickness – Carl Zeiss) with DAKO mounting media and left to dry in the dark 15h.

In-plate Flow cytometry

RWPE-1 cells were cultured as a monolayer to 65 % confluence in 12-well plate (Costar #DWG00674) as described above and then in serum-free basal media for 15 h. Cells at 70 % confluence were kept in sodium buffer (5 h, 37 °C), rinsed 3 times with warm (37 °C) HBSS,

exposed to EGF (300 ng/mL, 37 °C, 3 min) and fixed in 2 % (v/v) PFA (in HBSS) for 20 min immediately after treatment. To reduce auto fluorescence, cells were incubated in 50mM NH_4Cl in PBS for 10 min. Cells were blocked with 10 % (w/v) BSA for 30 min and probed with hASCT2.RBD (1:50-metafora Biosystems, 3 % (w/v) BSA/PBS, 1 h, 37 °C). Cells were washed, probed with donkey anti-mouse IgG (H+L) antibody AlexaFluor594 (1:2000, 3 % (w/v) BSA/PBS, 1 h, 37 °C), and the nuclei were stained with DAPI (1:30000) to allow cell localization with ImageXpress microscope. After probing and staining, the cells were kept in 1mL of PBS and scanned with the ImageExpress flow cytometer. Analysis of the surface fluorescence emitted by labelled hASCT2 was performed using Acuity Meta express 6.

The software, detects the fluorescence emitted by labelled ASCT2 to identify the boundaries of the plasma membrane, this will provide a value of the fluorescence emitted by all the positively probed cells present in the well and automatically removing the background if any, before and after the treatment. The data obtained is similar to that obtained with a flow cytometer, with the difference that the cells are not being detached from the plate. Five independent experiments were conducted.

Statistical analysis

Data are reported as mean ± SEM, unless otherwise specified. Statistical analysis on the data was performed by paired two tailed t-test with Graph Pad Prism 6 statistical software package.

Differences were considered significant at $P < 0.05$ and $P < 0.001$ with a confidence interval of 99% (as indicated). Each value represents the mean of at least triplicate experiments with measures at least 6 replicate measurements per condition, unless otherwise indicated.

Results

Analysis of THLE-3 Cells Revealed Presence of Endogenous ASCT2.

After performing a bioinformatic analysis on the expression of ASCT2 in various tissues, THLE-3 cells were chosen as a possible negative control. However, I confirmed that THLE-3 cells express ASCT2 protein abundantly (Figure 2.1a). The visualization of ASCT2 protein appears to be different than that previously described in the literature (Console *et al.*, 2015, Marin *et al.*, 2003). Indeed, a smear located between 57 and 75k Da was detected which could represent different stages of glycosylated ASCT2 transporter. Also, a defined band visible at 150 kDa may suggest formation of ASCT2 complexes which were not resolved by the denaturing conditions.

ASCT2-dependent [^{3}H]-glutamine uptake in the presence of sodium is responsible for about 55 % of the total glutamine uptake (Figure 2.1b).

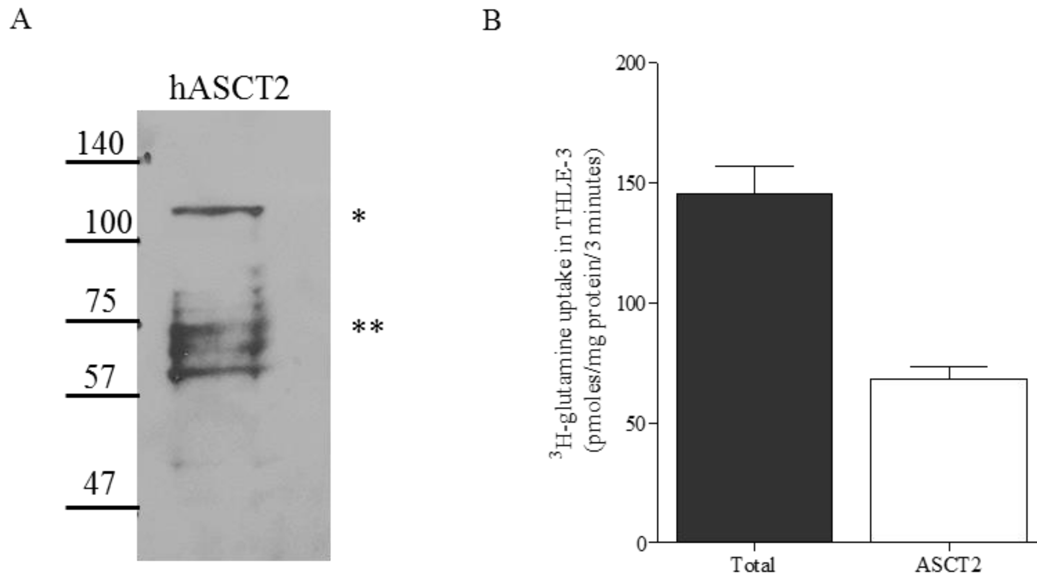


Figure 2.1. THLE-3 cells express hASCT2. (A) Detection of hASCT2 protein detection in THLE-3 cells. THLE-3 cells were grown and extracted proteins were resolved by SDS-PAGE as described in Materials and Methods. Proteins were analyzed via immunoblot using antibodies against ASCT2. A smear (**) is visible between 57 and 75 kDa, representing endogenous non/glycosylated hASCT2 respectively and a single band (*) visible at 150 kDa represents putative hASCT2 oligomers. Representative immunoblot of 3 independent experiments. **(B) ASCT2 is responsible for 55 % of the total sodium-dependent glutamine uptake in THLE-3.** THLE-3 cells were grown and treated as described in Materials and Methods. The transport was performed in the presence of sodium buffer containing [^{3}H]-glutamine plus or minus 20 μM Nimesulide, 2mM Arginine, Histidine, and Phenylalanine. Data presented as mean ± SEM (N=3 independent experiments).

Endogenous hASCT2 Expression and Localization Patterns in RWPE-1 Cells.

I confirmed RWPE-1 as a model for the study of endogenous hASCT2 by using validated primers and qPCR. The canonical isoform of ASCT2 is encoded by the gene SLC1A5 and is endogenously expressed in RWPE-1 cells (Figure *2.2*a). ASCT2 mRNA expression is significantly less abundant than that observed in HEK-293 cells when measured against the same endogenous reference genes (HPRT1, HMBS, and GAPDH). Previous studies (Marin *et al.,* 2003) and more recently by Console and colleagues (2015), have shown that ASCT2 undergoes N-glycosylation. My data confirm that ASCT2 is present in RWPE-1 cells and can be identified with apparent molecular mass of 75 and 57 kDa which are likely to represent the monomeric glycosylated and non-glycosylated forms of ASCT2 respectively (Figure 2.2b). In addition, a third band at 150kDa is present suggesting the presence of a putative complex (which can occur during these types of analysis with membrane proteins, due to their highly hydrophobic nature, despite the presence of denaturing conditions). Although the glycosylation of ASCT2 was not confirmed experimentally via PGNase enzymatic reaction, the distribution of putative glycosylated forms and complexes of ASCT2 I have examined in RWPE-1 cells, is consistent with data previously shown in HEK-293 (Pingitore *at al.,* 2013; Scalise *et al.,* 2014).

Having confirmed the presence of ASCT2 mRNA and protein in RWPE-1 cells I performed immunofluorescence assays to determine the endogenous distribution of ASCT2 in these cells using two different antibodies. Using the Metafora Biosystems-produced ASCT2.rbd-conjugated antibody, I observed that ASCT2 protein is distributed, as expected, at the plasma membrane (Figure 2.3); however, ASCT2 seems to be distributed more abundantly and in a punctate manner, in the cytoplasm just adjacent the plasma membrane and around the ER area (Figure 2.3).

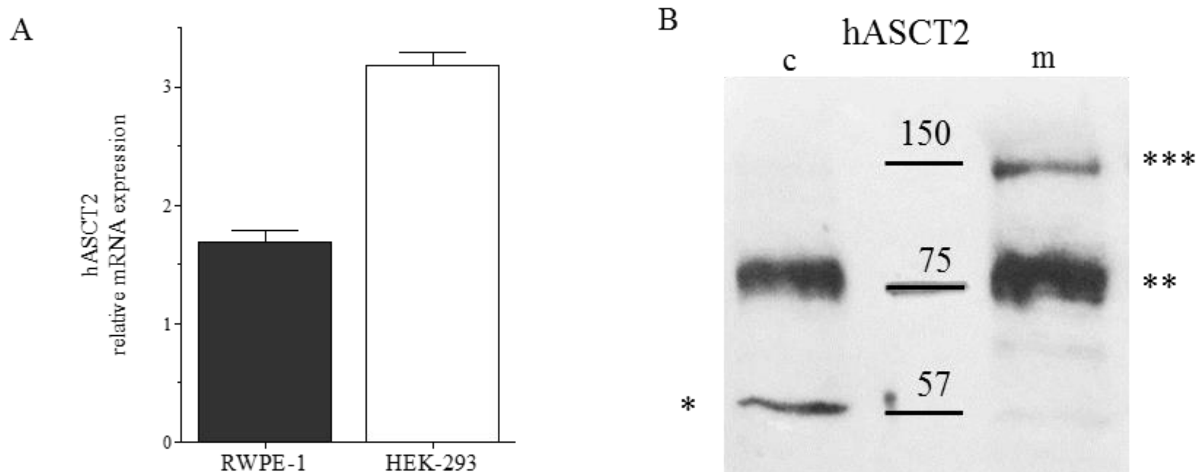
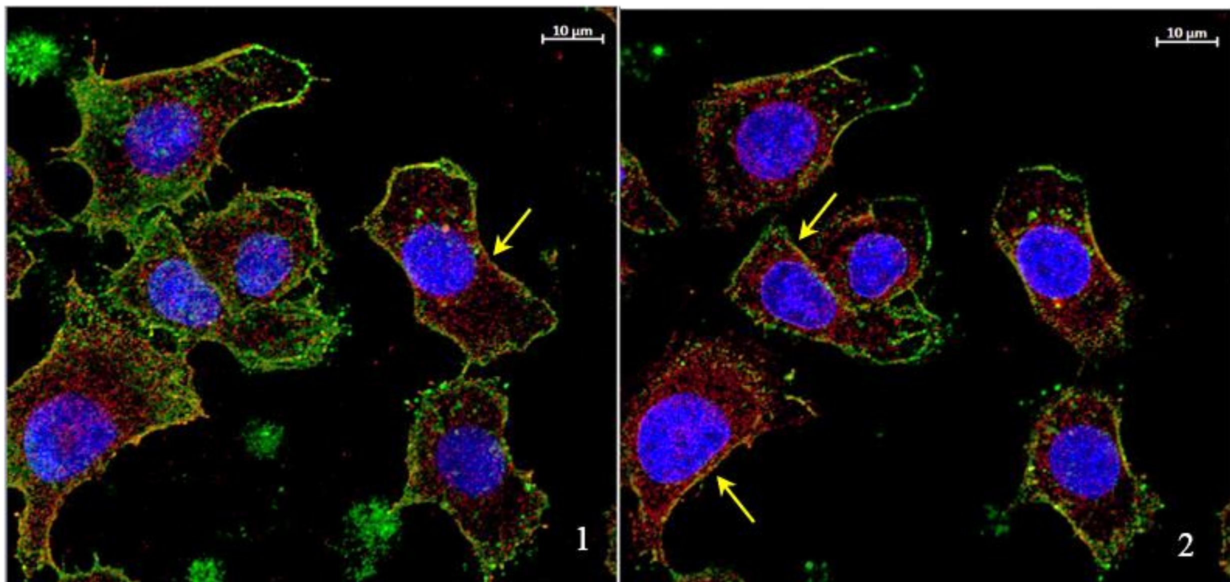
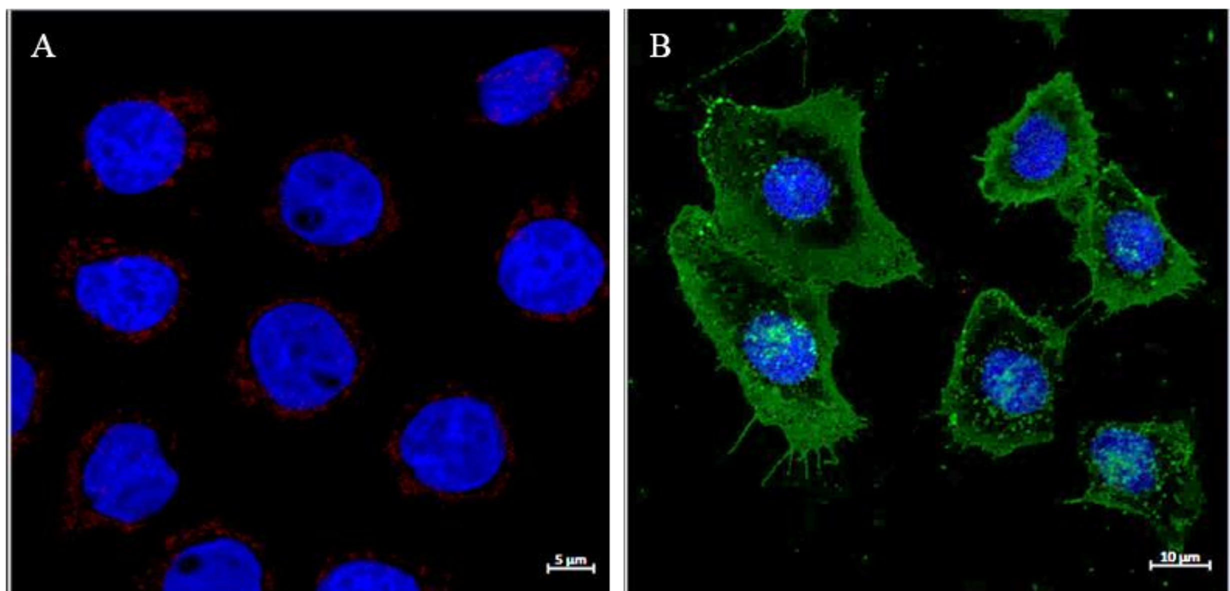


Figure 2.2. hASCT2 is expressed in RWPE-1 cells. (A) Relative mRNA expression of hASCT2 in RWPE-1 compared to HEK-293 cells. RWPE-1 cells were grown in Keratinocyte SFM medium (GIBCO) in 5 % CO_2 at 37 °C at 0.5 x 10^6 cells/60 mm dish. RNA extracted from HEK-293 cells was used as a control. Data are presented as the mean ± SEM (N=4 HEK-293 cells and N=6 RWPE-1 cells). **(B) hASCT2 protein detection in RWPE-1 cells.** RWPE-1 total cell lysate was analysed via immunoblotting using two different antibodies against ASCT2 (Cell Signalling (c) and Millipore (m). Two specific bands can be seen with both antibodies, presumably representing endogenous glycosylated (**) and non-glycosylated (*) hASCT2 at 75 and 57 kDa respectively. A band at 150 kDa (***) representing putative hASCT2 oligomers was also detected.

hASCT2.RBD (Metafora Biosystems)





A. Control. 2° antibody (Alexa 594)

B. Membrane stain WGA (Alexa 488-conjugated)

Figure 2.3. Cellular distribution of endogenous hASCT2 in RWPE-1. RWPE-1 cells were grown, fixed, and permealized as described in Materials and Methods. Cells were probed with hASCT2.RBD (Metafora Biosystems) and with secondary antibodies Alexa Fluor® 555 donkey anti-mouse IgG. ASCT2 (red) is distributed in clusters at the plasma membrane, some protein

seems to reside just underneath the bilayer (yellow arrow), while the remaining the protein is spread within the cytoplasm in a punctate manner. Nuclei (blue) were stained with DAPI. Cell membranes (green) were stained with WGA-Alexa488. Scale bar – 10 µm. Images are representative of 3 independent experiments. Images (1 and 2) are a Z stack (24 slices) collected at 0.28 µm intervals with a Zeiss laser scanning confocal fluorescent microscope-64X. Controls - RWPE-1 cells labelled with secondary antibodies only (A) and WGA only (B) are shown. Scale bar – 5 µm.

Endogenous hASCT2 is Fully Functional and Represents the Primary Contributor of Glutamine Transport in RWPE-1 Cells.

The nature of the glutamine transport in RWPE-1 cells was investigated and the optimal transport time was determined to be within the 3 to 5 min' range, which is well within the linear phase of uptake (Figure 2.4a). Subsequently, functional studies in RWPE-1 were performed to investigate the contribution of ASCT2 to the overall uptake of glutamine in these cells. I performed transport assays which selected each individual transporter and/or system (where individual transporters could not be differentiated) and used a combination of specific amino acids and pharmacological compounds based on their known inhibition of each targeted transporter. I established that, as predicted, the major contributor (45 %) to sodium dependent glutamine uptake in RWPE-1 is hASCT2 (Figure 2.4b). The remaining uptake is due to transporters: B0AT1 (18 %), $ATB0^{+}$ (7 %) and transporter system N (13 %). Sodium independent uptake (14 %) is due almost exclusively to system L transporters. I did not detect any transport from system A transporters.

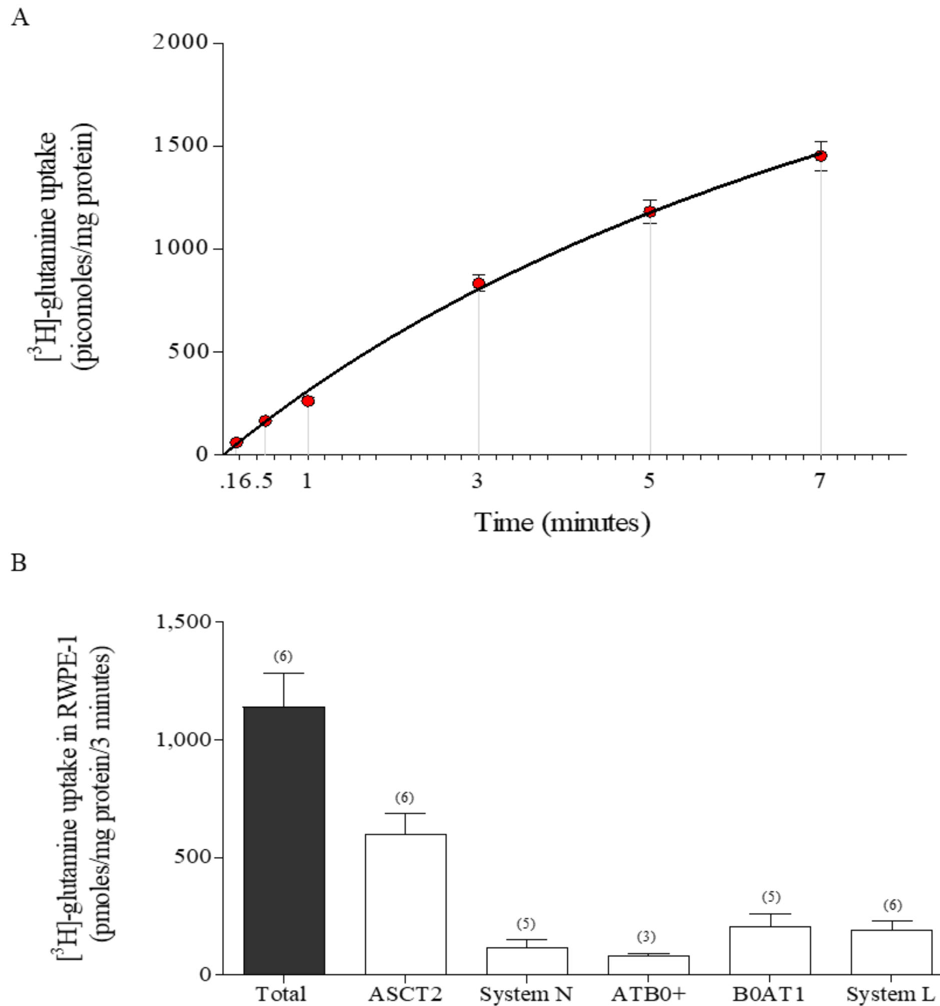
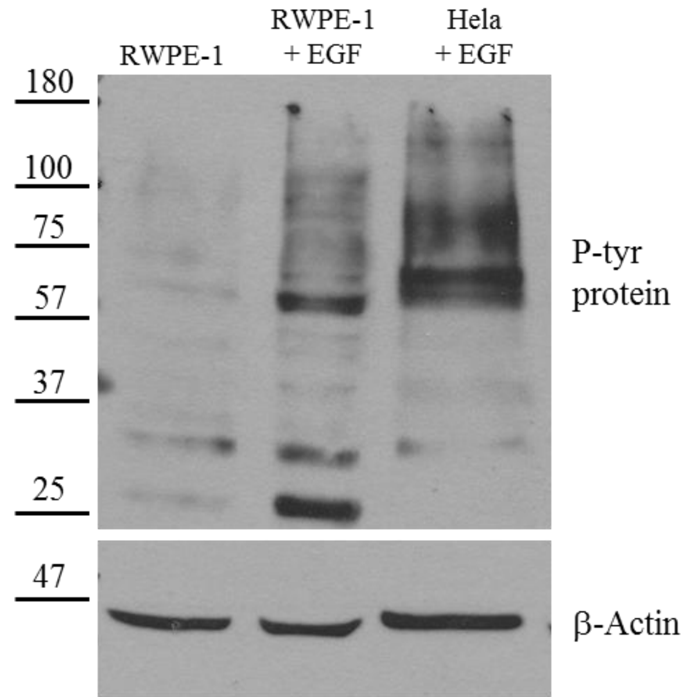


Figure 2.4. Glutamine transport in RWPE-1. (A) Time course of [^{3}H]-glutamine uptake in RWPE-1. Data are presented as mean ± SD (each data point represents six replicates). **(B) ASCT2 is the major contributor of [^{3}H]-glutamine uptake in RWPE-1 cells.** Cells were pre-treated (30 min, 37 °C) in the presence or absence of 2 mM competitive amino acids inhibitors threonine, histidine, phenylalanine or arginine to inhibit hASCT2, System N, B0AT1/system L and ATB0$^+$ respectively. The transport was performed in the presence or absence of sodium using buffer containing [^{3}H]-glutamine and in the presence or absence of amino acid inhibitors. Data are presented as mean ± SEM. The numbers in brackets represents the number of independent experiments per condition.

RWPE-1 Cells are Receptive to Epidermal Growth Factor (EGF) Stimulation.

To study the regulation of ASCT2, and validate my model, I determined the sensitivity of RWPE-1 to EGF. Cells were treated with EGF as described in Materials and Methods. Immunoblot analysis (Figure 2.5) shows that RWPE-1 cells possess a phospho-tyrosine pathway which is activated in response to EGF. My data show that RWPE-1 cells are responsive to EGF within 10 min of exposure and this effect is blocked in the presence of the EGF receptor inhibitor AG1478 (Figure 2.6) confirming the activation is due to EGF. I also confirmed that the EGF-dependent activation of the cells leads to the phosphorylation of the EGFR receptor and is inhibited as expected by the inhibitor. Data (Figure 2.7), show that phosphorylation of the receptor is correlated with EGF exposure in a time dependent manner. Noteworthy is the line showing the status of panEGFR in the presence of the inhibitor AG1478; in this context, the EGF receptor undergoes dimerization without the consequent activation of the downstream pathway, this causes an aggregation of non-functional dimeric receptor which in turn causes the detection of a more intense band on the immunoblot (Figure 2.7).



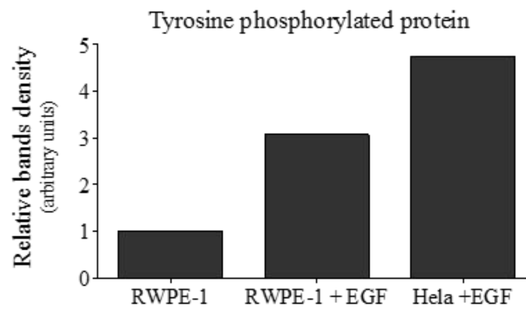
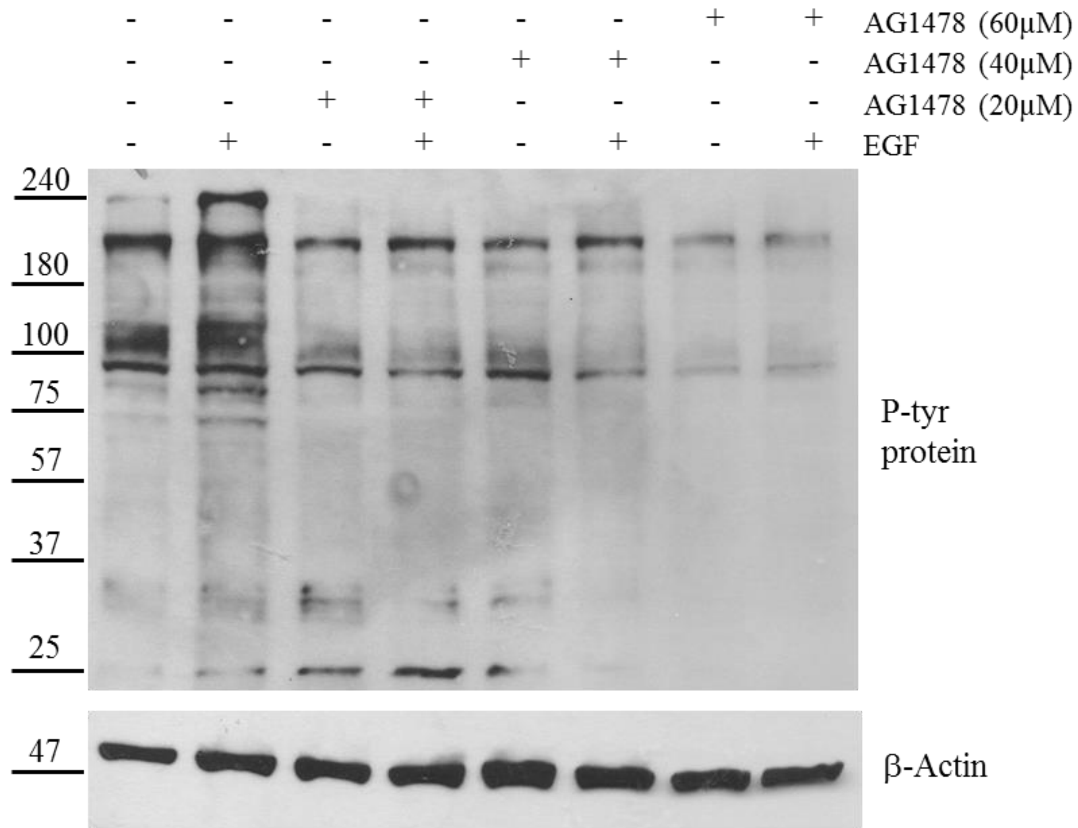


Figure 2.5. EGF induces tyrosine phosphorylation of proteins in RWPE-1. RWPE-1 cells were grown as described in Materials and Methods and were treated in the presence or absence of EGF. The membranes were probed with antibodies against phosphorylated tyrosine protein (P-tyr) and β-actin. Total lysate of EGF-treated HeLa cells was used as a positive control. Exposure time of the film ranged between 1 and 5 min. Densitometry of the immunoblot obtained with ImageJ is shown. Representative blot of 2 independent experiments.



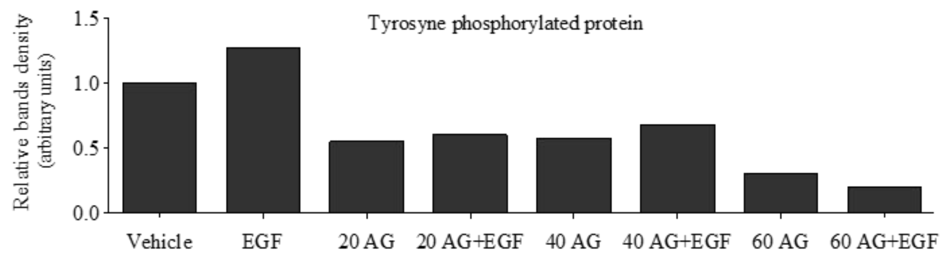


Figure 2.6. EGF-induced tyrosine phosphorylation of protein in RWPE-1 cells is inhibited by AG1478. RWPE-1 cells were grown as described in Materials and Methods and were treated in the presence or absence of EGF and in the presence or absence of EGF-R inhibitor AG1478 (20, 40, and 60 µM). The membranes were probed with antibodies against phosphorylated tyrosine protein and β-actin. Densitometry of the immunoblot obtained with ImageJ is shown. Representative immunoblot of 3 showing similar results.

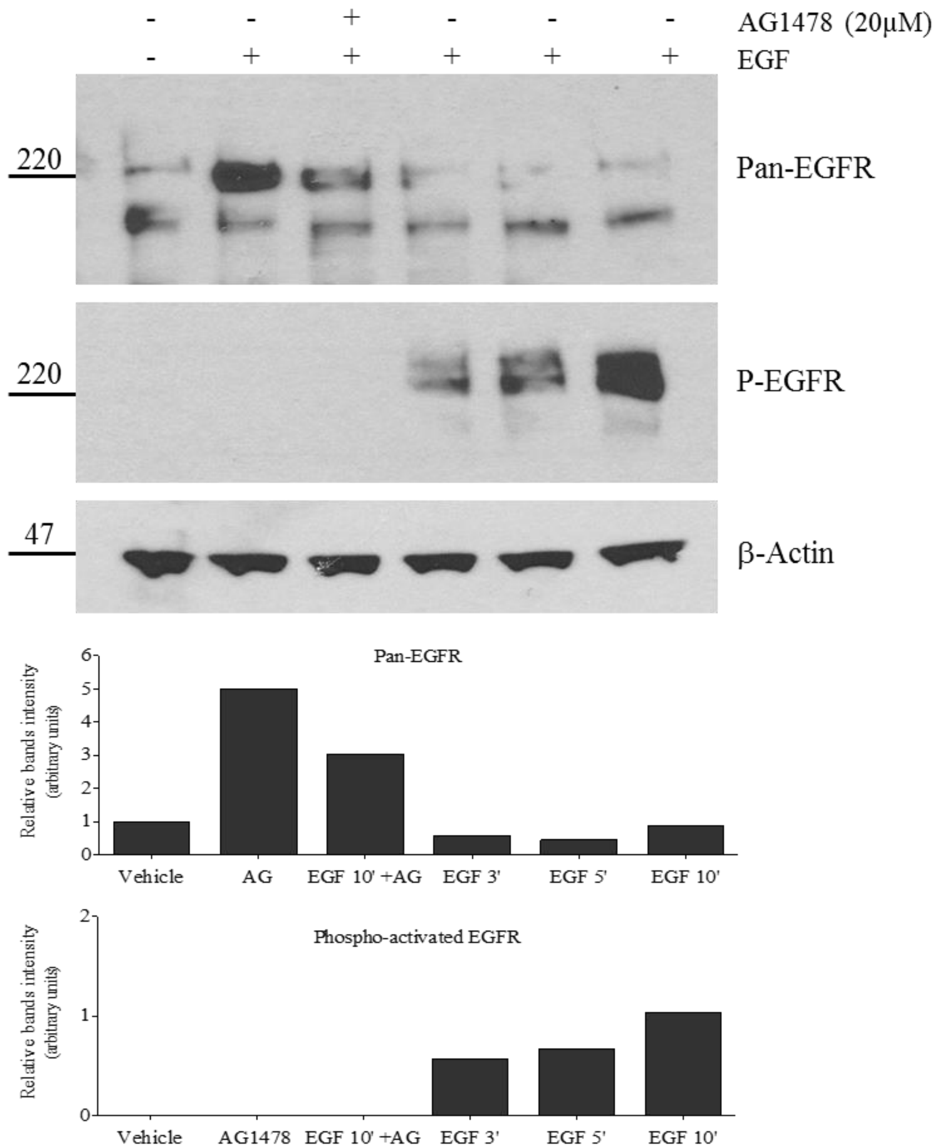


Figure 2.7. EGF receptor is tyrosine phosphorylated by extracellular EGF in RWPE-1 cells. RWPE-1 cells were treated in the presence or absence of EGF (300 ng/mL, 3, 5, and 10 min), and in the presence or absence of EGFR inhibitor (AG1478, 10 min, 20 µM). Cell were collected, and the extracted protein prepared for immunoblotting as described in Materials and Methods. Membranes were probed with antibodies against phosphorylated EGFR (P-EGFR), total EGFR (Pan-EGFR), and β-actin. Densitometry of the immunoblot obtained with ImageJ is shown. Representative immunoblot of 3.

EGF Increases ASCT2-dependent Glutamine Uptake in RWPE-1 With No Effect on Protein Abundance.

Based on previous findings (Ribeiro *et al.*, 2007, Lee *et al.*, 2010, Jones *et al.*, 2012), I hypothesized that activation of the EGFR-stimulated signalling pathway would lead to the regulation of glutamine uptake in RWPE-1. To confirm this proposed model of ASCT2 regulation, I treated the cells with EGF and measured [^{3}H]-glutamine uptake. My data confirm that EGF stimulation increases the overall uptake of glutamine in RWPE-1 by 23 % (Figure *2.8*).

Based on the observations that ASCT2 is very abundant in RWPE-1 and it makes a significant contribution to the overall glutamine transport in RWPE-1 cells, I hypothesized that ASCT2-dependent uptake will be regulated by EGF leading to an EGF-stimulated increase in glutamine transport. To confirm this, I conducted functional assays in the presence or absence of EGF while targeting only the ASCT2-dependent [^{3}H]-glutamine uptake. My data confirm that EGF-stimulation leads to a more than doubling of the ASCT2-dependent glutamine uptake (Figure *2.9*).

EGF-stimulated increases in ASCT2-dependent uptake could be due to several mechanisms including, but not limited to: increases in ASCT2 protein abundance, increases in transporter activity, downregulation of protein turn-over, trafficking and so on. Given the short time frame of regulation (3 min), I hypothesized that increases in ASCT2 protein expression were not likely to be involved in the observed increase in glutamine uptake. I confirmed that, following EGF treatment, there was no change in ASCT2 relative mRNA expression (Figure 2.10 a) or overall protein levels in RWPE-1 (Figure 2.10b).

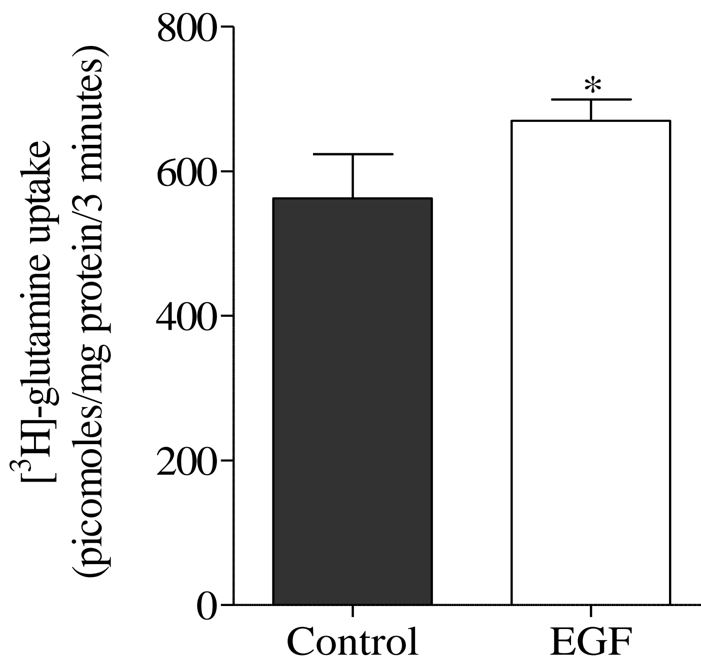


Figure 2.8. Total sodium-dependent [^{3}H]-glutamine uptake increases in the presence of EGF. RWPE-1 cells were kept in sodium buffer (5 h, 37 °C). Sodium dependent [^{3}H]-glutamine transport was measured in the presence or absence of EGF. and is expressed as picomoles of substrate per mg of protein at 3 min (pmols/mg/3min). Each value represents the mean ± SEM (n= 5). (*) $p<0.05$.

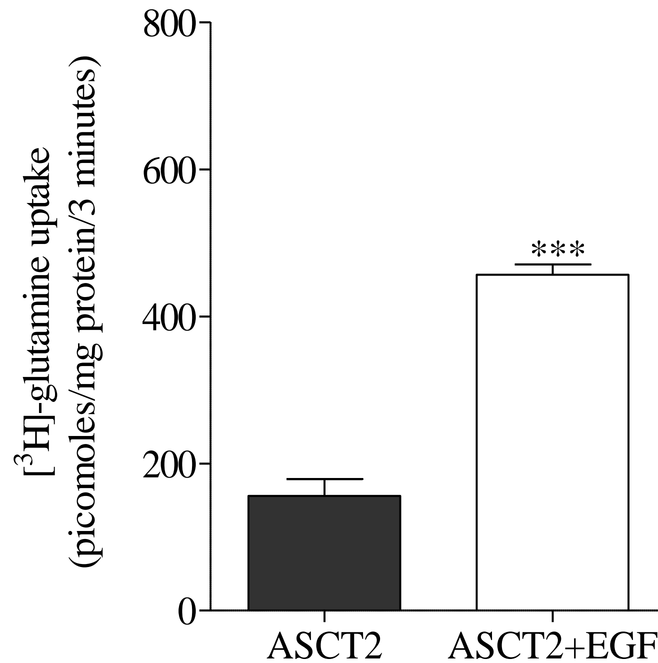


Figure 2.9. ASCT2-dependent [^{3}H]-glutamine uptake increases 3-fold after EGF treatment. RWPE-1 cells were grown and treated as described in Materials and Methods. hASCT2-dependent uptake was selected by inhibiting the other transporters present in RWPE-1 with 2 mM arginine, histidine, phenylalanine, and 20 µM nimesulide to inhibit ATB0^{+}, system N, system L, and B0AT1 respectively. The ASCT2-dependrnt [^{3}H]-glutamine transport was measured in the presence or absence of EGF and expressed in picomoles per milligrams of protein at 3 min (picomoles/mg protein/3min). Each value represents the mean ± SEM (n= 3). (***) $p<0.001$.

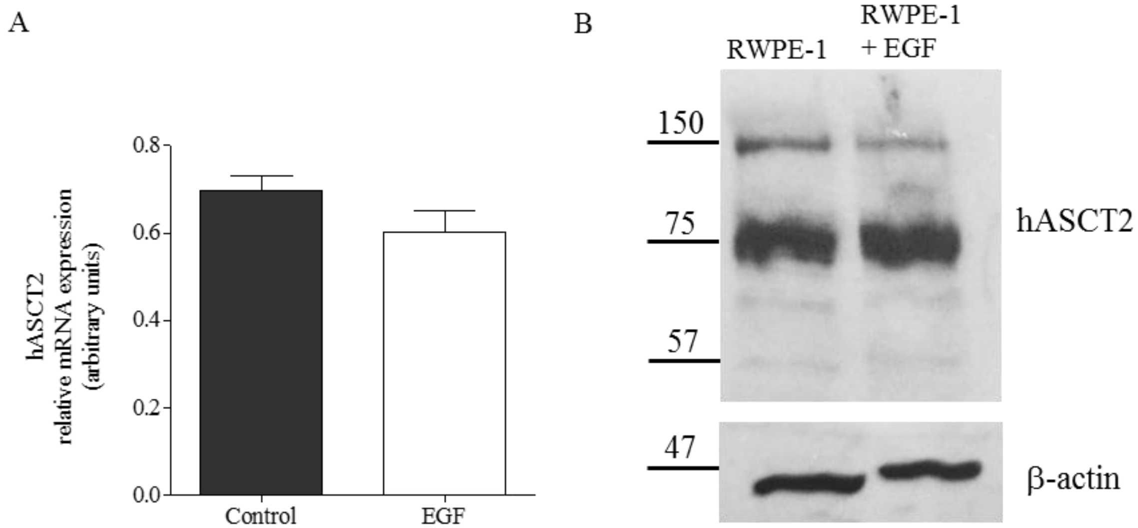


Figure 2.10. Effects of extracellular EGF on ASCT2 expression and protein levels in RWPE-1 cells. (A) EGF does not affect hASCT2 mRNA expression. RWPE-1 cells were grown as described in Materials and Methods and kept in sodium buffer (5 h, 37 °C). ASCT2 mRNA expression levels in the presence or absence of EGF (30 min, 37 °C) is shown. Each value represents the mean ± SEM (n= 5). P>0.05. **(B) EGF does not affect ASCT2 protein levels in RWPE-1.** Membranes were probed with antibodies against hASCT2 (Millipore) and β-actin. Representative immunoblot (n= 4).

The EGF Stimulated Increase in ASCT2-dependent Glutamine Uptake in RWPE-1 is Due to an Increase of Transporter Presence at the Plasma Membrane.

Following confirmation that ASCT2 is responsible for the EGF-stimulated increase in glutamine uptake and that this is not due to rise in ASCT2 protein levels, I hypothesized that the underlying mechanism of regulation was likely to be regulated trafficking and/or oligomerization of the transporter at the membrane. Conformational changes of ASCT2 due to either de/phosphorylation of interacting proteins or of the transporter itself could also lead to regulated changes in function (Ribeiro *et al.*, 2007, Lee *et al.*, 2010, Angonno, 2012). I found that EGF-stimulated increase in ASCT2-dependent glutamine uptake is due to an increase in ASCT2 at the cell membrane. Biotinylation assays confirmed that EGF-stimulation leads to an increase (30 %) in ASCT2 at the plasma membrane compared to the untreated biotinylated control (Figure 2.11). Detection of cytosolic phosphorylated EGF receptor and biotinylated total EGF receptor before and after the treatment are also shown. This control is essential in showing the EGF-dependent activation of the receptor. When EGF binds to the receptor, a self-phosphorylation event occurs and the receptor gets internalized (Tomas et al., 2014). Furthermore, analysis of untreated RWPE-1 cells via fluorescence confocal microscopy confirmed that approximately 60 % of the fluorescently labelled endogenous ASCT2 is localized within the bi-layer (Figure 2.12a), as expected for a membrane transporter, while the remaining protein appears to be distributed either underneath the plasma membrane or intracellularly (possibly undergoing post-translational processing).

Analysis of EGF treated RWPE-1 cells via fluorescence confocal microscopy suggested a change in the distribution of ASCT2, such that 70 - 80 % of endogenous ASCT2 was re-distributed to the plasma membrane (Figure 2.12b). RWPE-1 cells stained with secondary antibody only were used as a negative control to show the absence of non-specific binding and artefacts (Figure 2.12c).

Since confocal microscopy provides somewhat qualitative data, I confirmed these observations using a quantitative approach; in-plate flow cytometry assays data suggest a 25 % increase in ASCT2 presence at the plasma membrane stimulated by EGF (Figure 2.13).

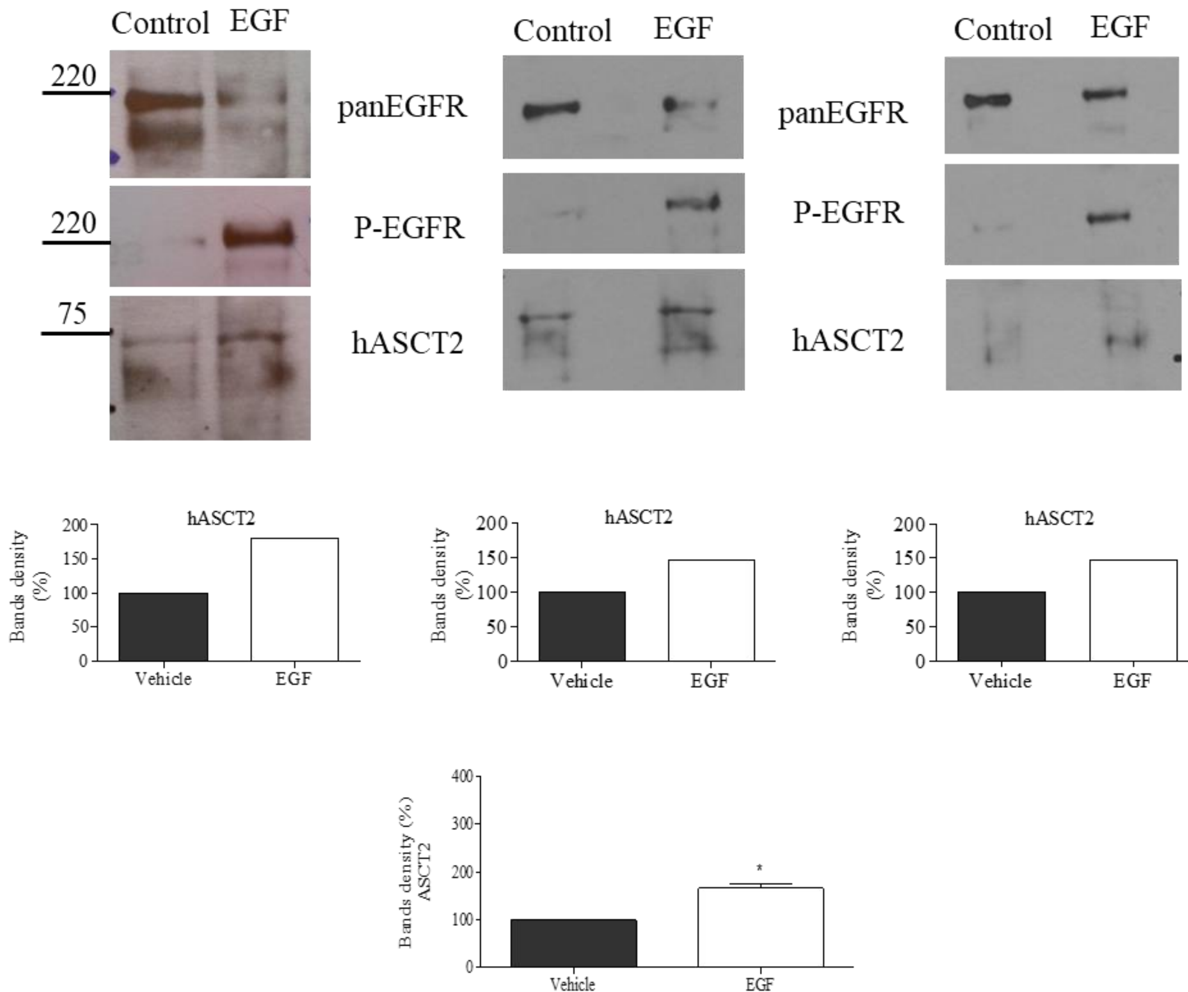
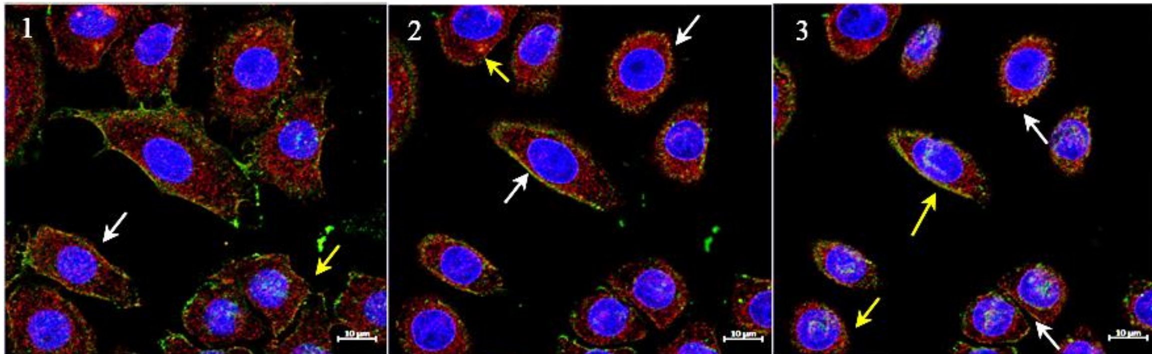
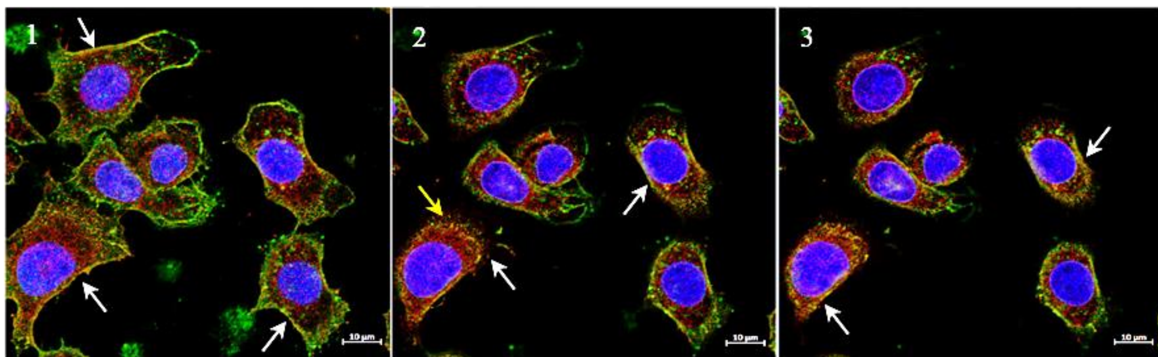


Figure 2.11. EGF induces an increase of ASCT2 protein at the plasma membrane in RWPE-1 cells. RWPE-1 cells were grown and treated in the presence or absence of EGF in as described in Materials and Methods. In the figure, immunoprecipitated surface ASCT2 is represented by a band (75 kDa) which increases in intensity in the presence of the EGF treatment Equal loading of sample protein was ensured by using the same concentration of Sulfo-NHS-SS-Biotin (1 mg/mL) in all samples and by incubating an equal amount of cell lysate (500 µg) with streptavidin-conjugated beads (60 µL) during the purification step (IP). Densitometry of surface biotinylated hASCT2 obtained with ImageJ software, is shown. Data is presented as mean ± SEM (n= 3). P<0.05. Raw data is also shown

A Control - Untreated RWPE-1 cells



B EGF - Treated RWPE-1 cells



C Secondary antibody only + membrane stain (Alexa555 + WGA488)

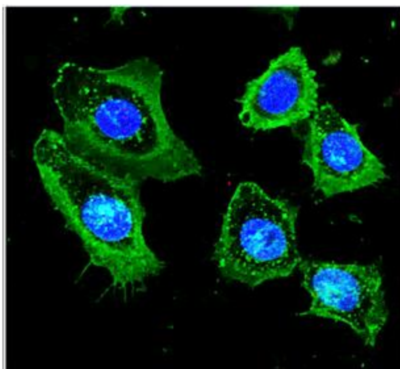


Figure 2.12. EGF induces an increase of surface ASCT2 transporter at the plasma membrane. RWPE-1 cells were treated as described in Materials and Methods. In the absence of the treatment (A) ASCT2 (red) is distributed at the plasma membrane in small clusters with a yellow/orange signal (white arrows). Most of the signal seems to be distributed just underneath the bilayer (yellow arrows), with punctate signal around the nuclei. In the presence of external

EGF (B) an increase in ASCT2 at the plasma membrane (yellow/orange signal) can be detected, while the punctate signal around the cytoplasm decreases. Nuclei (blue) were stained with DAPI and cells membrane (green) were stained with WGA Alexa488. Scale bar – 10 µm. Images (A) and (B) 1 to 3, are Z stacks collected at 0.25 µm intervals (26 slices) with a Zeiss laser scanning confocal fluorescent microscope at 64X. Control cells (C) probed with secondary antibodies and membrane stain only are shown. Images are representative of 3 independent experiments.

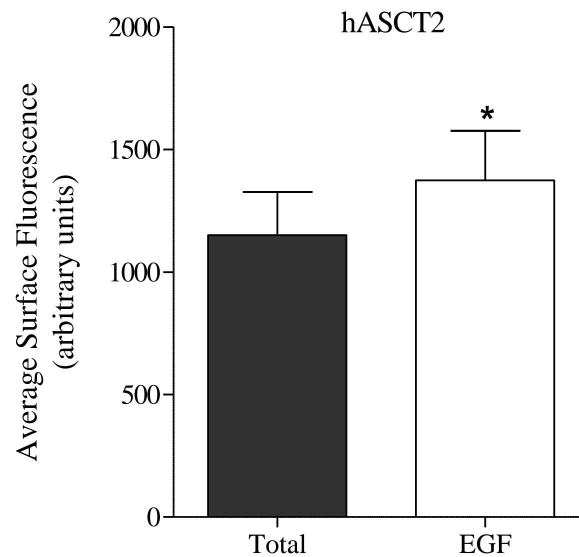


Figure 2.13. EGF induces an increase of ASCT2-related fluorescence at the plasma membrane. RWPE-1 cells were treated as described in Materials and Methods Analysis of the surface fluorescence emitted by labelled hASCT2 was performed using Acuity Meta express 6 software and it shows a 25 % increase in surface fluorescence in the presence of EGF when compared to untreated cells. Statistical analysis was performed with Graph Pad Prism and data represent the mean ± SEM (N= 5). $p<0.05$ (*).

Discussion

In the last decade, numerous studies have highlighted the importance of the ASCT2 transporter in the development and proliferation of tumours (Oka *et al.*, 2012). In prostate adenocarcinoma, a correlation between the expression of ASCT2 with the aggravated form of the disease has been revealed (Li *et al.*, 2003). In another study, a correlation between the inhibited activity of the transporter and the reduced ability of the prostate tumour cells to grow and develop has been reported (Wang *et al.*, 2015). However, our current understanding of the biology of human ASCT2 is very limited, in particular, the physiological role and mechanisms of regulation of the transporter are yet to be elucidated in a non-disease context. Hence, the objective of this study was to identify and characterize a cell model that can be used as a tool in enhancing my understanding of the biological function and regulation of endogenous human ASCT2, in a physiologically relevant environment

I propose that RWPE-1 cells are a suitable model for the study of hASCT2 transporter and a novel tool in the context of investigating the expression and functionality of glutamine transporters in the prostate. The expression of the endogenous canonical isoform of the human ASCT2 transporter (SLC1A5) that I identified in RWPE-1 cells, correlates with previous studies where it is widely distributed and overexpressed (Bungard and McGivan, 2005, Li *et al.*, 2003, Wang *et al.*, 2015).

The endogenous ASCT2 protein detected in RWPE-1 is consistent with that described in studies conducted with yeast overexpressed and purified human ASCT2 (Pingitore *et al.*, 2013, Scalise *et al.*, 2014) and with that described in studies concerning the glycosylation of heterologously expressed hASCT2 in HEK-293 cells (Console *et al.*, 2015, Marin *et al.*, 2003). Furthermore, in my study I show the first evidence of the distribution of the endogenous hASCT2

protein in RWPE-1 which is very similar to that observed in 3T3Ll cells and in several breast cancer derived cell lines as it has the same punctate pattern mostly concentrated at the plasma membrane and around the endoplasmic reticulum (van Geldermalsen *et al.*, 2015, Takahashi *et al.*, 2015). My functional studies have confirmed that hASCT2 is the major contributor to the uptake of glutamine in RWPE-1. This reflects earlier results in the literature, using similar techniques, in which ASCT2 was also found to be the major contributor of glutamine uptake in C2 cells (Avissar *et al.*, 2008), human hepatocytes, and liver-derived cells (Bode *et al.*, 1995, Bode, 2001). Along with having shown the first evidence of amino acid transport in RWPE-1 by ASCT2, I was also able to detect the individual contribution to the transport of both B0AT1 and $ATB0^+$ which has not been described before. Other transporters involved in maintaining the glutamine homeostasis within RWPE-1 were identified in systems N and L; however, I had no means to distinguish between the individual members of these two families (Avissar *et al.*, 2008). Nevertheless, on the basis of the information available in various tissue-specific gene expression databases, I came to the conclusion that the most probable contributors may be SNAT1 and SNAT2 (system N) and, LAT1 and LAT2 (system L). I have not detected any contribution from system A.

Studies conducted *in vivo* have shown a correlation between exposure to growth hormones and amino acids uptake (Iannoli *et al.*, 1997, Ray *et al.*, 2003). Some studies have also correlated ASCT2 transporters' activity with EGF suggesting that this transporter may be essentially involved in the cellular metabolic processes beyond the disease context (Avissar *et al.*, 2008, Palmada *et al.*, 2005, Wang *et al.*, 2015, Rosario *et al.*, 2013). Therefore, I have investigated the effects of EGF on the regulation of the transporter in RWPE-1 cells. I have confirmed that EGF stimulates ASCT2-dependent glutamine uptake confirming previous studies (Avissar *et al.*, 2008, Palmada *et al.*, 2005). I found a discrepancy between the small increase in total glutamine uptake (23%)

induced by EGF and the noticeable tripling of the ASCT2-dependent glutamine uptake in the presence of EGF. This could be due to the fact that, in a non-manipulated environment, the presence of EGF may induce an upregulation of some transporters' activity and a downregulation of others. On the other hand, when all other transporters but ASCT2 are inhibited, this causes an even more dramatic preference toward ASCT2, in response to the sudden need for glutamine stimulated by EGF.

Nevertheless, we confirmed the EGF-dependent stimulation of the ASCT2-dependent uptake acts via regulated trafficking of the transporter to the plasma membrane as reported for other transporters and receptors (Jorissen *et al.*, 2003, Jones *et al.*, 2012). In conclusion, I have shown that RWPE-1 cells are an excellent research tool in which to undertake further studies of the biology and the regulation of endogenous hASCT2. Furthermore, I have shown that ASCT2-dependent glutamine uptake and cellular distribution can be regulated by EGF.

Chapter 3 : Interactors and regulators of ASCT2

Introduction

Human ASCT2, a member of the solute carrier 1 (SLC1) family, is a Na^+-dependent neutral amino acid exchanger widely distributed in many organs. The ubiquitous presence of ASCT2 highlights the important physiological role of this transporter (Kanai and Hediger, 2003, Kanai and Hediger, 2004, Albers *et al.*, 2012, Oppedisano *et al.*, 2007, Oppedisano *et al.*, 2010, Utsunomiya-Tate *et al.*, 1996). Moreover, ASCT2 expression is frequently upregulated in human cancers, and silencing of ASCT2 results in mechanistic target of rapamycin (mTOR) downregulation, a decrease in glutamine uptake, and subsequent cell death, which suggests a possible role in the maintenance of the cancer phenotype (Bungard and McGivan, 2004, Bungard and McGivan, 2005, Fuchs *et al.*, 2007, Gegelashvili *et al.*, 2006, Wang *et al.*, 2014). Studies on the substrate interaction site of ASCT2 have led to the development of more effective competitive inhibitors such as H-ser(Bzl)-OH (BenSer) (Grewer and Grabsch, 2004) and l-γ-glutamyl-ρ-nitroanilide (GPNA) (Esslinger *et al.*, 2005) which have permitted more targeted pharmacological studies of ASCT2 and more specific analysis of the effects that the inhibition of ASCT2-dependent uptake has on cell metabolism (Wang et al., 2015, Marshall et al., 2017). Studies conducted in primary human trophoblasts have shown that mTOR is involved in the regulation of transporters by modulation of their trafficking and amino acid uptake (Rosario *et al.*, 2013). Studies in *Xenopus laevis* oocytes suggest a direct link between the glutamine uptake by hASCT2 and signalling to or via mTOR (Li *et al.*, 2003, Nicklin *et al.*, 2009). Similarly, a study conducted in colon adenocarcinoma cells (C2 and Caco-2), found a correlation between EGF signalling and the regulation of ASCT2/B0AT1-B0AT2 which may be regulated in an mTOR dependent manner (Avissar *et al.*, 2008). Despite

these findings, my knowledge of the underlying components and mechanisms of regulation of ASCT2 are still unclear, particularly in a non-pathological environment.

Transporters and receptors can be regulated by several different mechanisms including phosphorylation events and interaction with accessory proteins (Ribeiro *et al.*, 2007, Lee *et al.*, 2010, Anggono and Huganir, 2012, Jones *et al.*, 2012). Interestingly, an interaction between ASCT2 and the scaffold protein PDZK1 has been recently described (Scalise *et al.*, 2014). PDZK1 is involved in the formation of multi-protein complexes, which are involved in the coordination and regulation of several solute carriers such as OCTN1 (Kato *et al.*, 2005), OCTN2, and PEPT1 (Sugiura *et al.*, 2008), resulting in changes of transport's activity and regulated recycling and/or trafficking, often in response to growth factors (Ghosh *et al.*, 2000, Hu *et al.*, 2009, Kim *et al.*, 2013, Walther *et al.*, 2015). Therefore, I hypothesized that PDZK1 may also be involved in the regulation of hASCT2 with a similar complex formation mechanism.

Furthermore, to fully understand the regulation of ASCT2, it is necessary to identify putative protein-protein interactions (PPI) beyond PDZK1. For example, screenings for the EGF receptor via tandem mass spectrometry studies (Lu *et al.*, 2016) and for the serotonin transporter SERT via affinity purification (AP) and mass spectrometry (MS) studies (Seyer *et al.*, 2016) have identified a physical interaction with ASCT2, although the physiological relevance is unclear. Nevertheless, identifying members of the interactome of ASCT2 could help explain factors and mechanisms involved in the transporter regulation. To investigate this, I performed a large-scale membrane two hybrid (MYTH) screening which has been previously successful in the identification of relevant interactors for other membrane proteins such as ABC transporters and ENTs (Snider *et al.*, 2013, Stasi *et al.*, 2014, Thaminy *et al.*, 2003, Yao *et al.*, 2017, Petschnigg *et al.*, 2017).

Materials and Methods

Materials

Keratinocyte serum free medium (K-SFM- 17005042), Dulbecco's modified Eagle's medium (DMEM), bovine pituitary extract (BPE), fetal bovine serum (FBS), supplemental epidermal growth factor (EGF), and Hank's balanced salt solution (HBSS) were purchased from Life Technologies (Burlington, ON). Fluorescently labelled secondary antibodies, plasma membrane and nuclear dyes were purchased from Invitrogen (Carlsbad, CA). Goat HRP-conjugated anti-rabbit antibodies were purchased from BioRad (Mississauga, ON). Primary antibodies against β-actin (#4970), RICTOR (#9476), SGK1 (#12103), 4E-BP1 (#9644) and phospho-4E-BP1(#9459), S6K1 (#2708) and phospho-S6K1 (#9206), Akt (#8596) and phospho-Akt (#4060) were purchased from Cell Signalling Technologies (Danvers, MA). The chemiluminescent substrate for immunoblot analysis was purchased from Mandel Scientific (Guelph, ON). Labelled glutamine ([^{3}H]-glutamine) was purchased from Moravek Biochemicals (Brea, CA). and the amino acids used in this study were purchased from Sigma Aldrich (Oakville, ON). GPNA inhibitor for ASCT2 was purchased from Sigma Aldrich (#G1135-1G). Antibodies against phospho-RICTOR (#07-1331) were purchased from Millipore (Billerica, MA). Human ASCT2.rbd-antibody-conjugated was purchased from Metafora Biosystems (Evry cedex, France). Primary antibodies against human PDZK1 (ab121230) and Phospho-SGK1 (ab55281) were purchased from Abcam Inc. (Cambridge, MA). mTOR inhibitors Torin 1 was purchased from Millipore (Billerica, MA) and Xl388 was purchased from Santa Cruz Biotechnology (Dallas, TX). All other chemicals were purchased from BioShop (Burlington, ON) unless otherwise specified.

Cell Culture

Immortalized human prostate epithelial RWPE-1 cells were purchased from ATCC (CRL-11609) and cultured per ATCC's guidelines. RWPE-1 monolayers are kept in proliferative conditions in keratinocyte basal medium supplemented with 0.05 mg/mL of bovine pituitary extract and 5 ng/mL of human recombinant epidermal growth factor at a temperature of 37 ºC in a humidified incubator and a 5 % (v/v) CO_2 atmosphere.

GPNA Treatments

RWPE-1 cells were cultured as previously described, incubated in sodium uptake buffer (5 h, 37 °C) and exposed to extracellular GPNA (L-glutamic Acid γ-(ρ-Nitroanilide) (400 µM, 10 and 30 min, 37 °C). Cells treated thus were either used for functionality assays or lysed for protein kinase analysis via immunoblotting.

Torin 1 and XL388 Treatments

Cells were grown as described above and kept in Na^+-uptake for 5 hr, treated (15 min, 37 °C) with either Torin1 (100 nM) or XL388 (700 nM) and in the presence or absence of EGF (300 ng/µL, 3 min, 37 °C). Cells treated thus were either lysed for protein kinases analysis via immunoblotting or used to perform transport functionality assays.

Protein Extraction and Western Blot Analysis

RWPE-1 monolayers were grown as described above, washed twice with PBS (4°C), and protein extraction was performed using NP-40 lysis buffer. Cell lysates were incubated on ice for 5 min, syringed with a 26-Gauge needle to solubilize the plasma membrane and then centrifuged (19000 g, 5 min, 4 °C). Protein quantification of the supernatant was done using Bio-Rad PC DC Protein Assay kit based on modified Lowry assay. The absorbance was read at 750 nm and sample concentration was calculated using a standard curve of increasing concentrations of BSA. RWPE-

1 total cell lysate (10, 20 or 100 µg) containing endogenous protein kinases was resolved by SDS-PAGE (Bio Rad Mini -PROTEAN Tetra System). The proteins were transferred to a nitrocellulose membrane (Bio Rad - 1620090 - 0.45 microns) and activated in transfer buffer. The transfer was performed by using a Bio-Rad semi-dry apparatus (20 V, 60 min). The membranes were blocked (5 % (w/v) milk/TBST, 1 h, 25 °C) and incubated overnight at 4°C with antibodies against RICTOR (1:2000), phospho-RICTOR (1:500), Akt and phospho-Akt (1:3000), SGK1 (1:3000), phospho-SGK1 (1:500), S6K1 (1:3000), phospho-S6K1 (1:1000), 4E-BP1 (1:50000), and phospho 4E-BP1 (1:10000) in 1 % (w/v) milk/TBST. The following day, membranes were washed 3 times for 10 min each in TBST, then incubated with goat-anti-rabbit horseradish peroxide conjugated secondary antibody (1:3000, 1 % (w/v) milk/ TBST, 1 h, 25 °C). The membranes were then washed (3 times for 10 min each in TBST) and incubated in chemiluminescent substrate solution for 1 minute.

Densitometry and Normalization

Densitometry of the bands detected via Immunoblot was obtained using Image J. The Normalization was obtained using either β-actin or for phosphorylated kinases, their equivalent pan. Relative normalizing value was obtained by dividing all the normalizing controls values (NC) by the highest NC density value. The data was normalized by subtracting the densities of my protein of interest (PI) and that of the relative normalizing control (rNC) in their respective lanes. The untreated control is set at 1 and all treatments are calculated accordingly.

ASCT2-dependent [^{3}H]-glutamine Transport Assays in the Presence of GPNA.

Inhibition of ASCT2-dependent glutamine uptake in RWPE-1 cells was measured in the presence or absence of the inhibitor GPNA *(400 µM, 10 and 30 min, 37 °C)*. RWPE-1 monolayers were plated at ~5 x 10^4 cells/well in 24-wells culture plates and allowed to adhere for 24 h. Cells were

rinsed and kept in sodium buffer for 5 h. Total [^{3}H]-glutamine uptake was measured in the presence and absence of sodium. ASCT2-dependent glutamine transport only was measured by pre-treating the cells with 2 mM of competitive amino acids inhibitors histidine to inhibit system N, arginine to inhibit ATB0$^+$, and 20 µM of competitive inhibitor Nimesulide to inhibit B0AT1 for 15 min at 37 °C, then cells were immediately exposed to GPNA. Sodium-independent was also measured and subtracted by the total glutamine uptake to obtain the Na$^+$-dependent fraction and eliminate the system L-dependent glutamine transport. Radio-labelled glutamine transport *(50 µM* [^{3}H]-glutamine*, 2 µCi/mL, 3 min, 25 °C)* was measured via the modified cluster-tray method (Gazzola *et al.*, 1981) in uptake buffer at 3 min. Transport assays were performed at 37 °C. Following transport assays, cells were lysed (4 °C, 1h, shaking) with 2 N NaOH. Protein concentrations of cell lysates were determined with a modified protein Lowry Assay (BioRad; Mississauga, ON) and [^{3}H]-glutamine uptake was measured by standard liquid scintillation counting. Transport was expressed as picomoles of substrate per mg of protein at 3 minutes (pmols/mg/3 min).

Total and ASCT2-dependent [^{3}H]-glutamine Transport in the Presence of mTOR Inhibitors

Total and ASCT2-dependent glutamine uptake was measured in the presence of the mTOR inhibitors Torin1 and XL388. RWPE-1 monolayers were plated at ~5 x 10^4 cells/well in 24-wells culture plates and allowed to adhere for 24 h. Cells were rinsed and kept in sodium buffer as previously described. Total [^{3}H]-glutamine transport was measured in the presence and absence of sodium. ASCT2-dependent glutamine transport only was measured as described in Materials and Methods and in the presence or absence of either extracellular Torin 1 (100 nM, 30 min, 37 °C) or XL388 (700 nM, 30 min, 37 °C). Radio-labelled glutamine transport (50 µM [^{3}H]-glutamine, 2 µCi/mL; 37 °C) was measured via the modified cluster-tray method in sodium uptake buffer at 3 min. Following transport assays, cells were lysed (4 °C, 1 h, shaking) with 2N NaOH. Protein

concentrations of cell lysates were determined with a modified protein Lowry Assay (BioRad; Mississauga, ON) and [^{3}H]-glutamine uptake was measured by standard liquid scintillation counting. Transport was expressed as picomoles of substrate per mg of protein (pmols/mg/3 min).

Immunofluorescence of Endogenous PDZK1 and hASCT2

To determine the intracellular distribution of endogenous PDZK1, RWPE-1 cells were cultured as a monolayer in Keratinocyte complete medium to 65 % confluence on microscopy cover slips (1.5 µm thickness- Carl Zeiss) and then kept in serum-free media for 15 h. All further steps were performed at 25°C unless otherwise specified. Cells were rinsed 3 times with warm (37 °C) HBSS and fixed in 2 % (v/v) PFA/HBSS for 20 min. To reduce auto fluorescence, the cells were incubated in 50mM NH_4Cl in PBS for 10 min then they were washed once with 100mM glycine and three times with PBS. Cells were permeabilized for 5 min with 0.1 % (v/v) TritonX-100 in PBS (supplemented with 3 % (w/v) BSA), blocked with 10 % (w/v) BSA for 1h and then probed with antibodies against PDZK1 (1:500, 3 % (w/v) BSA/PBS, 1 h, 25 °C). Cells were washed three times with PBS and probed with AlexaFluor488 donkey anti-rabbit IgG (H+L) secondary antibodies (1:2000, 3 % (w/v) BSA/PBS, 1 h, 37 °C). Nuclei were stained with DAPI (1:30000, 5min), cells were rinsed three times with PBS and were mounted on microscopy slides (0.5 mm thickness - Carl Zeiss) with DAKO mounting media and left to dry in the dark overnight.

To confirm the co-distribution of endogenous ASCT2 and PDZK1, cells were cultured and treated as described above; cells were then probed with antibodies against hASCT2.rbd (1:100, 5 % (w/v) BSA/PBS, 30 min, 37 °C), washed three times and probed with AlexaFluor555 goat anti-mouse (H+L) secondary antibodies (1:2000, 3 % (w/v) BSA/PBS, 1 h, 25 °C). Afterward, cells were probed with antibodies against PDZK1, washed, the nuclei were stained and mounted as

described above. All samples were then scanned and analysed using a Zeiss scanning confocal fluorescence microscope.

Generation of HA-ASCT2 Construct

Plasmid DNA for human HA tagged ASCT2 was purchased from DNA 2.0 (Newark, CA) and received in a generic Pj201 vector in a dry form. The gene was designed with unique restriction sites at each end of the gene with no alteration of the protein sequence: a HindIII restriction site was placed before the Pj201-HA-hASCT2 start codon, and a KpnI restriction site was placed just after the stop codon, following the last 10 amino acids of the HA-ASCT2 C-terminus. A short HA tag (YPYDVPDYASLGGP) positioned on the protein putative extracellular loop between the 3rd and 4th transmembrane domains. The clone (2 µg) was extracted from the filter paper disk in 50µL of RNAse free water via 2 centrifugations at 19000g, 5 min each at 25 °C. Both pj201-HA-hASCT2 clone and pCDNA3.1 vector, were transformed into DH5α competent cells (Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines. The Gel Extraction Kit (Qiagen Inc., Canada), was used to isolate and purify HindIII/KpnI digested fragments from both the pCDNA3.1vector (Clontech Laboratories Inc., Mississauga, Canada) and pj201-HA-hASCT2insert. Purified fragments, HA-hASCT2 insert and pCDNA3.1 vector, were then ligated using T4 DNA ligase (New England BioLabs; Pickering, Canada). Ligation reactions were incubated for 2 h at 37 °C and then transformed into DH5α competent cells (Invitrogen, Carlsbad, CA). Aliquots of 50 and 100 µL were plated on LB-agar supplemented with 50 µg/mL kanamycin and allowed to grow for 15 h at 37 °C. Single colonies were selected and grown in LB medium supplemented with kanamycin for 8h (8000 rpm) at 37 °C. Clone pCDNA3.1-HA-hASCT2, was isolated from bacterial cultures via Genelute Plasmid Mini Prep Kit (Sigma-Aldrich, Oakville, Canada); plasmid DNA was either linearized or double digested with restriction enzymes HindIII-

HF and KpnI-HF (New England BioLabs; Pickering, ON, Canada) and analyzed on a 1 % (w/v) agarose gel to confirm a successful ligation. Positive clones were then sequenced in both forward and reverse directions at The Centre for Applied Genomics (Peter Gilgan Centre for Research and Learning, The Hospital for Sick Children, Toronto, Canada) and those showing correct and in-frame sequence were further amplified and purified using NucleoBond Xtra Midi EF (Macherey-Nagel GmbH & Co.KG, Germany). Sequencing primers are shown in Table 3.1.

Table 3.1. Sequencing Primers used in this study. hASCT2 (SLC1A5) primers were designed using the Clone manager 9 software and analyzed again via the IDT- Oligo design and analysis tool. The remaining primer sequences were provided by The Centre for Applied Genomics (The Hospital for Sick Children, Toronto, Canada).

hASCT2 sequencing primers - 5'-3'	
Forward	TAT CAA GCT TAT GGT GGC CGA TCC TCC TCG AGA C
Reverse	CGT GGT ACC CAT GAC TGA TTC CTT CTC AGA GG
Int-Forward	GGT CTC CTG GAT CAT GTG
Int-Reverse	CTA CCA AGC CCA GGA TGT TC
pCDNA3.1 vector sequencing primers - 5'-3'	
pCDNA3.1_T7	TAA TAC GAC TCA CTA TAG GG
pCDNA3.1_BGHR	TAG AAG GCA CAG TCG AGG

RWPE-1 Transfection for Immunoprecipitation

RWPE-1 cells were seeded in 60mm plates, allowed to attach for 24 h and reach a density of 85 % in Keratinocyte complete medium as previously described. Cells were transiently transfected with plasmid HA-tagged human ASCT2 using X-tremeGENE HP DNA transfection reagent (Roche Diagnostics, cat#06366244001) according to the manufacturer's protocol. Transfection with GFP empty vector was used as a positive control to monitor the transfection efficiency using a fluorescence microscope. The transfection reagent and Keratinocyte serum free medium were warmed at 25°C. HA-ASCT2 plasmid DNA (5 µg) and 15 µl of X-tremeGENE reagent (1:3 ratio) were added to 500 µl medium in an Eppendorf tube with gentle pipetting. The transfection mix was incubated for 30 min at 25°C. In this time, the medium of the cells was changed to 5 mL fresh complete medium. At the end of the incubation time the transfection mix was added to the plates dropwise. The medium was changed 24h later with fresh complete medium and 15h later the cells were used for immunoprecipitation.

Immunoprecipitation of HA Tagged ASCT2

RWPE-1 cells transiently transfected with pCDNA3.1-HA-hASCT2 vector were washed with PBS (4°C) and lysed in NP-40 buffer. For immunoprecipitation, 500 µg protein of the total lysate (in a final volume of 200 µl) was incubated with 20µl of anti-HA agarose beads (HA-Tag IP/Co-IP Application Set, Thermo Scientific cat#26180Y) for 2 h at 25°C in a spin column with gentle end-over-end mixing. At the end of the incubation time, the flow through samples were collected by pulse centrifuge for 10 sec and the beads were washed 3 times with 0.5 ml of TBST. Immuno-purified HA-hASCT2 proteins bound to the beads were eluted in 25 µl of 2 X Non-Reducing Sample Buffer at 95°C for 5 min and resolved by SDS-PAGE.

MYTH Screening for hASCT2

Membrane yeast two-hybrid (MYTH) screening assays were performed as previously described (Snider *et al.*, 2010, Snider *et al.*, 2013, Fetchko and Stagljar, 2004, Lam *et al.*, 2015, Stagljar *et al.*, 1998). The membrane yeast two-hybrid (MYTH) system is based on the concept of split ubiquitin developed by (Johnsson and Varshavsky, 1994). Human ASCT2 was cloned into MYTH vectors pB102 (N-STE2-hASCT2-Cub-LexA-VP16-C) and pB101 (N-LexA-VP16-Cub-hASCT2-C) which allow for the bait protein (hASCT2) to be fused to the *N*-terminal and C-terminal region of the split-ubiquitin (Cub) transcription factors, respectively. These constructs were then used to perform the screening against the available Human Prostate Adenocarcinoma and the Human Adult Colon (NubG-x) commercial libraries. Bait cloning and validation, bait transformation into undisclosed yeast strains with all controls (negative and positive) and vectors self-activation checks, library screening, and results delivery of the positive prey proteins were performed by Hybrigenics Services SAS (Paris, France).

Statistical analysis

Data are reported as mean ± SEM, unless otherwise specified. Statistical analysis on the data was performed by paired two tailed t-test with Graph Pad Prism 6 statistical software package. Differences were considered significant at $P < 0.05$ and $P < 0.001$ with a confidence interval of 99% (as indicated). Each value represents the mean of at least triplicate experiments with measures at least 6 replicate measurements per condition, unless otherwise indicated.

Results

GPNA Inhibition of ASCT2-dependent Glutamine Uptake Effects on mTORC2 Phosphorylation.

Previous studies have shown that ASCT2 silencing leads to a downregulation of mTOR followed by cell apoptosis within 48h (Avissar *et al.*, 2008, Fuchs *et al.*, 2007). Therefore, I hypothesized that pharmacological inhibition of ASCT2 would affect the phosphorylation of the canonical read-outs of the mTOR downstream pathway. I first confirmed that GPNA (Wang *et al.*, 2015) inhibits ASCT2 uptake in RWPE-1 cells, by measuring ASCT2-dependent glutamine transport in the presence or absence of the inhibitor. I confirmed that GPNA inhibits ASCT2-dependent glutamine uptake by ~70 % (Figure 3.1).

ASCT2 inhibition and silencing leads to a downregulation of mTOR and eventually to cell death (Avissar *et al.* 2008). mTORC2 is involved in the regulation of Rho dependent protein-trafficking to the plasma membrane (García-Martínez and Alessi, 2008, Gulhati *et al.*, 2011, Avissar *et al.*, 2008). Therefore, I hypothesized that the pharmacological inhibition of ASCT2-dependent transport would result in a decrease in intracellular glutamine and this would result in an increase of mTORC2 activity. I found that following prolonged inhibition of the ASCT2-dependent transport, mTORC2 activity decreases (

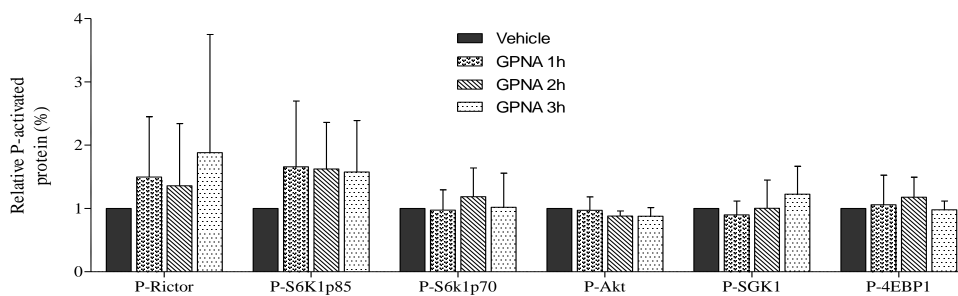


Figure ***3.2***). Within one hour, Rictor phosphorylation increases while p-Akt and p-SGK1 are unchanged. At the same time, the activity of 4E-BP1 decreases due to its hyper-phosphorylation, while the activity of p70 S6K1 increases due to its hyper-phosphorylation. The

less understood S6K1 isoform, p85, shows a decrease in phosphorylation which may highlight an unknown regulation by this kinase.

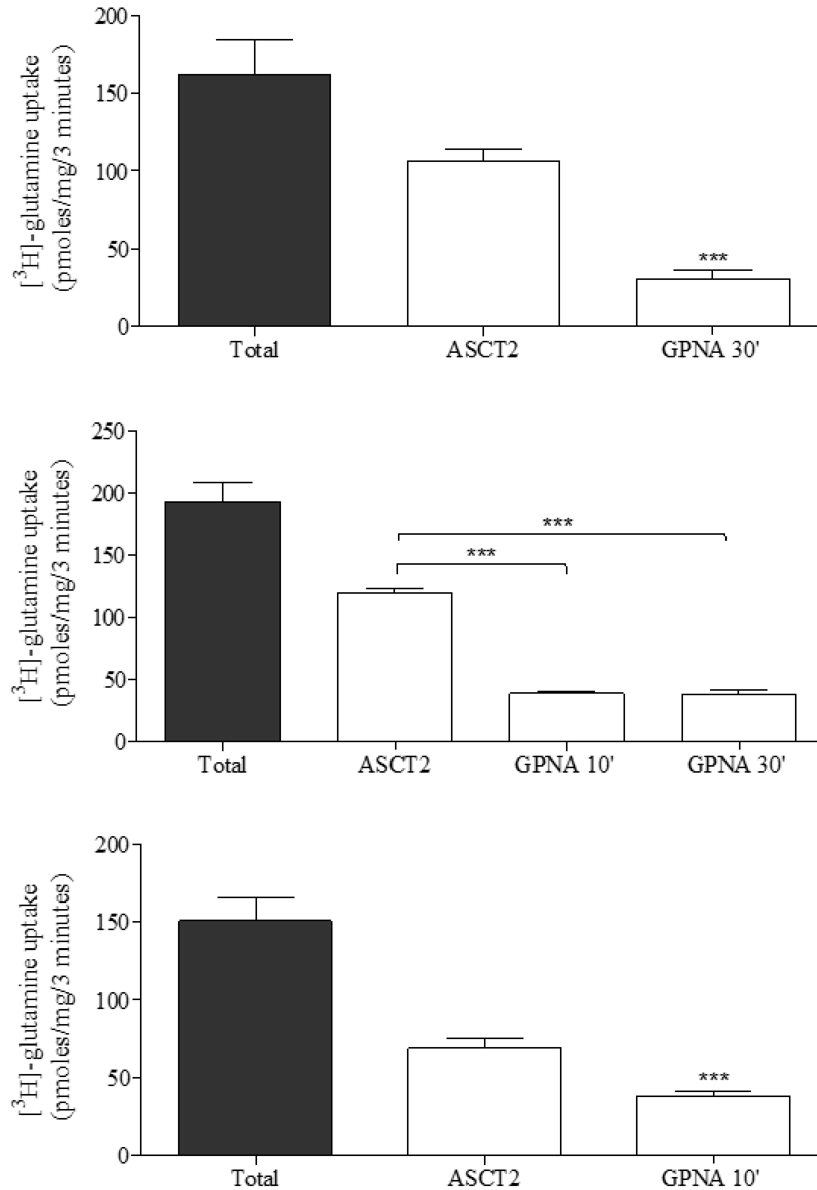


Figure 3.1. ASCT2-dependent glutamine uptake is inhibited by GPNA in RWPE-1 cells. RWPE-1 cells were grown as described in Materials and Methods, treated in the presence or absence of competitive amino acid inhibitors and in the presence or absence of GPNA. and transport is expressed as picomoles of substrate per mg of protein at 3 min (pmols/mg/3min). Three

independent experiments are presented (each condition was conducted in six replicates) Data are presented as the average ± SD. (***) $p<0.001$.

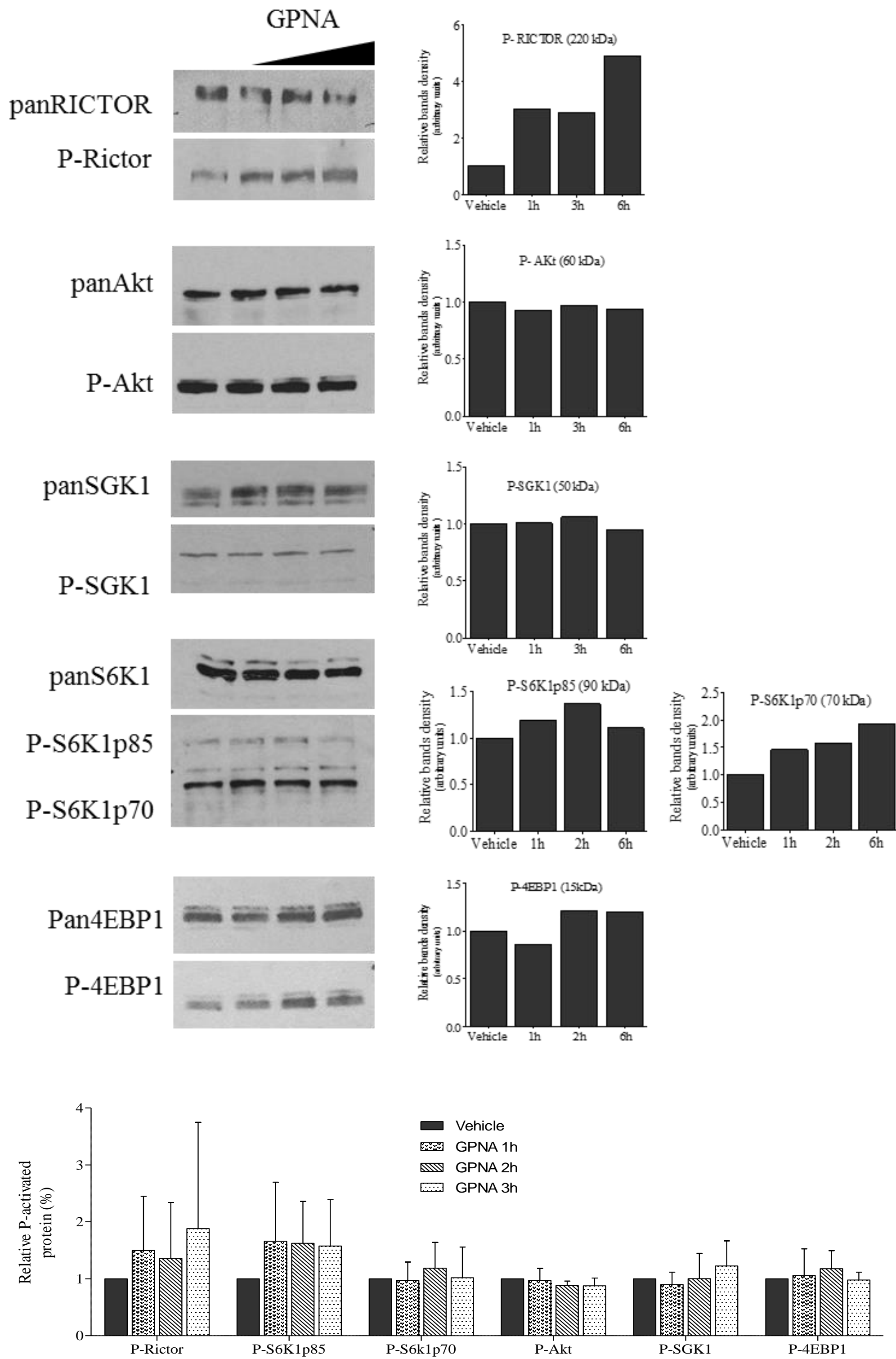


Figure 3.2. Pharmacological inhibition of hASCT2-dependent glutamine uptake regulates the mTOR pathway in RWPE-1. RWPE-1 cells were grown as described in Materials and

Methods in the presence or absence of GPNA. The membranes were probed with antibodies against pan and phosphorylated kinases Representative data of 3 independent experiments is shown. Densitometry obtained with ImageJ is shown. Data is shown as average ±SEM. $P>0.05$.

Total and ASCT2-dependent Glutamine Transport is Not Affected by the Inhibition of mTOR

I found that mTORC2 activity is decreased following prolonged inhibition of ASCT2 while the activity of mTORC1 seems to increase. Because of cross-talk between the two mTOR pathways, it is very difficult to draw conclusions regarding the involvement of either complex in the regulation of ASCT2. Therefore, I investigated the effects of mTOR inhibition on the total and ASCT2-dependent glutamine transport in RWPE-1 using widely used mTOR inhibitors such as Torin1 and XL388 (Liu *et al.*, 2009, Liu *et al.*, 2010, Takeuchi *et al.*, 2013, Thoreen *et al.*, 2009). I confirmed that, in the presence of Xl388, both Akt and 4EBP1 are inhibited (hypo-phosphorylated) even in the presence of EGF therefore confirming the inhibition of both mTORC1 and mTORC2 complex (Figure 3.3). On the other hand, the inhibition of mTOR by Torin 1 resulted in a slightly different outcome: Akt is hypo-phosphorylated and therefore downregulated even in the presence of EGF but 4EBP1 seems to be hyper phosphorylated in the presence of Torin, suggesting that mTORC1 is not being successfully inhibited (Figure 3.3). I hypothesized that inhibition of mTOR, would result in an overall decrease in glutamine uptake due to a decrease of ASCT2 availability at the plasma membrane. However, my data show that in RWPE-1, the inhibition of mTOR by either XL388 and of mTORC2 by Torin1, had no e effect on either the total glutamine transport (Figure 3.4a) or the ASCT2-dependent glutamine uptake (Figure 3.4b). Taken together, these data suggest that mTOR may not be involved in the regulation of ASCT2.

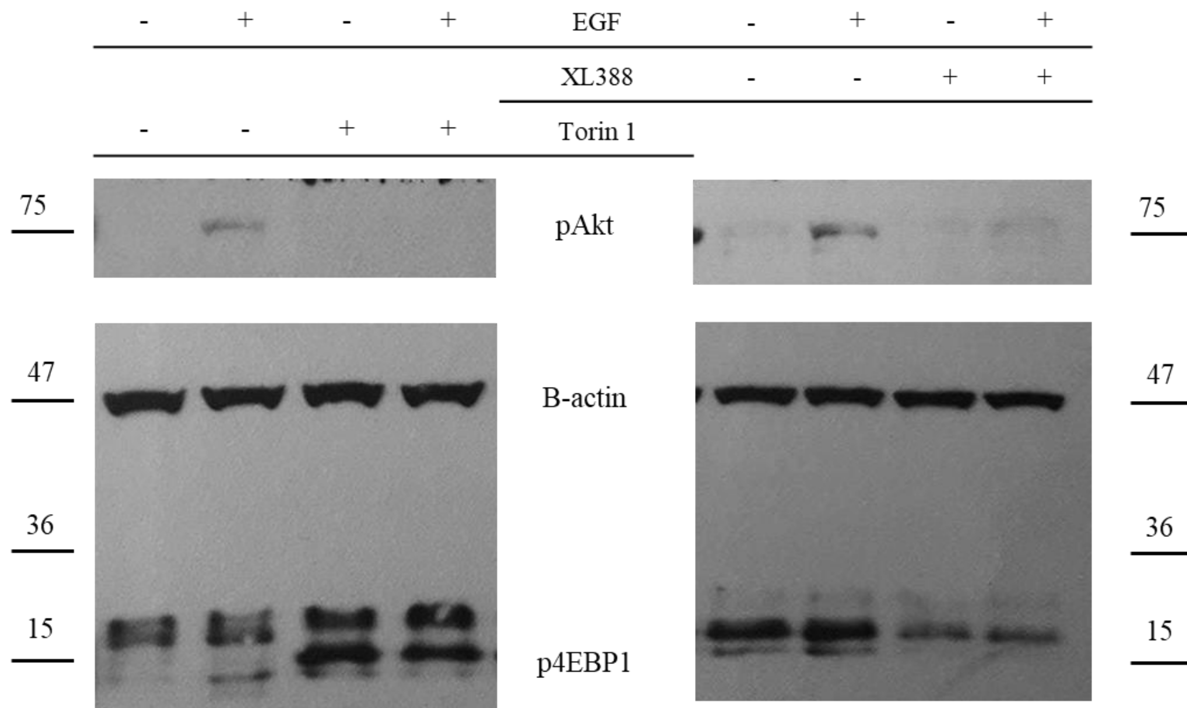
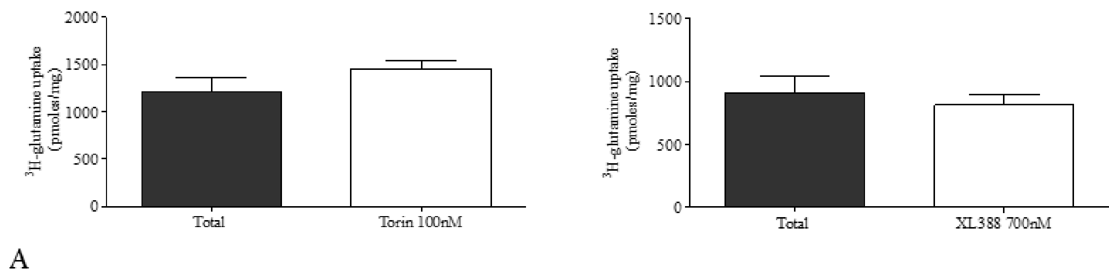


Figure 3.3 XL388 and Torin1 inhibit mTOR pathway in RWPE-1 cells. Cells were grown as described in Materials and Methods and treated with either Torin1 or XL388 and in the presence or absence of EGF. The membranes were probed with antibodies against pAkt, p4E-BP1, β-actin. Representative data of 2 independent experiments.



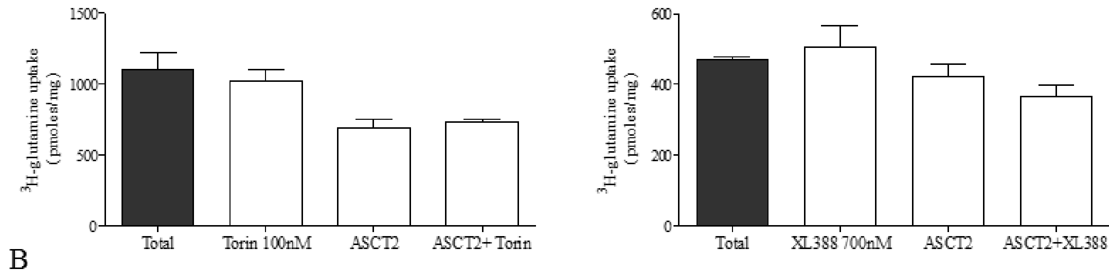


Figure 3.4. Glutamine transport is not affected by the inhibition of mTOR in RWPE-1. RWPE-1 cells were grown as described in Materials and Methods. **A) Total glutamine transport.** [^{3}H]-glutamine uptake measured in the presence or absence of Torin1 and XL388is shown. **B) ASCT2-dependent glutamine transport**. ASCT2-dependent [^{3}H]-glutamine uptake measured in the presence or absence of the inhibitors (2 mM His and Arg, and Nimesulide) and in the presence or absence of Torin1 or XL388 is shown. Data are presented as picomoles of substrate per mg of protein (pmols/mg). Each value represents the mean ± SD (n= 1 experiment; n= 6 technical replicates per condition).

Analysis of Endogenous PDZK1 Confirms Expression and Localization Patterns in RWPE-1 cells.

I hypothesized that regulation of ASCT2 in response to EGF may be due to an interacting protein or a complex of proteins involved in short-term trafficking which may be regulated via mTOR. Therefore, I investigated the possible role of PDZK1, a scaffold protein involved in regulating the surface expression of plasma membrane proteins by coordinating their regulatory components in epithelial cells (Silver, 2002, D'Amico *et al.*, 2010, Zheng *et al.*, 2014). PDZK1 was previously found to interact with ASCT2 (Scalise *et al.*, 2014). I confirmed the presence of PDZK1 protein in RWPE-1 cells (Figure 3.5a); which is consistent with previous findings (Luo *et al.*, 2017, Scalise *et al.*, 2014, Sugiura *et al.*, 2008, Walther *et al.*, 2015).

Having confirmed the presence of the PDZK1, I looked at the intracellular localization of PDZK1 and determined that PDZK1 is mainly distributed within the cytoplasm of the cells in a punctate manner (Figure 3.5b). This pattern is slightly different from the one shown in previous studies conducted in transiently transfected cells, where protein abundance may affect protein distribution (Choi *et al.*, 2011, Kim *et al.*, 2012).

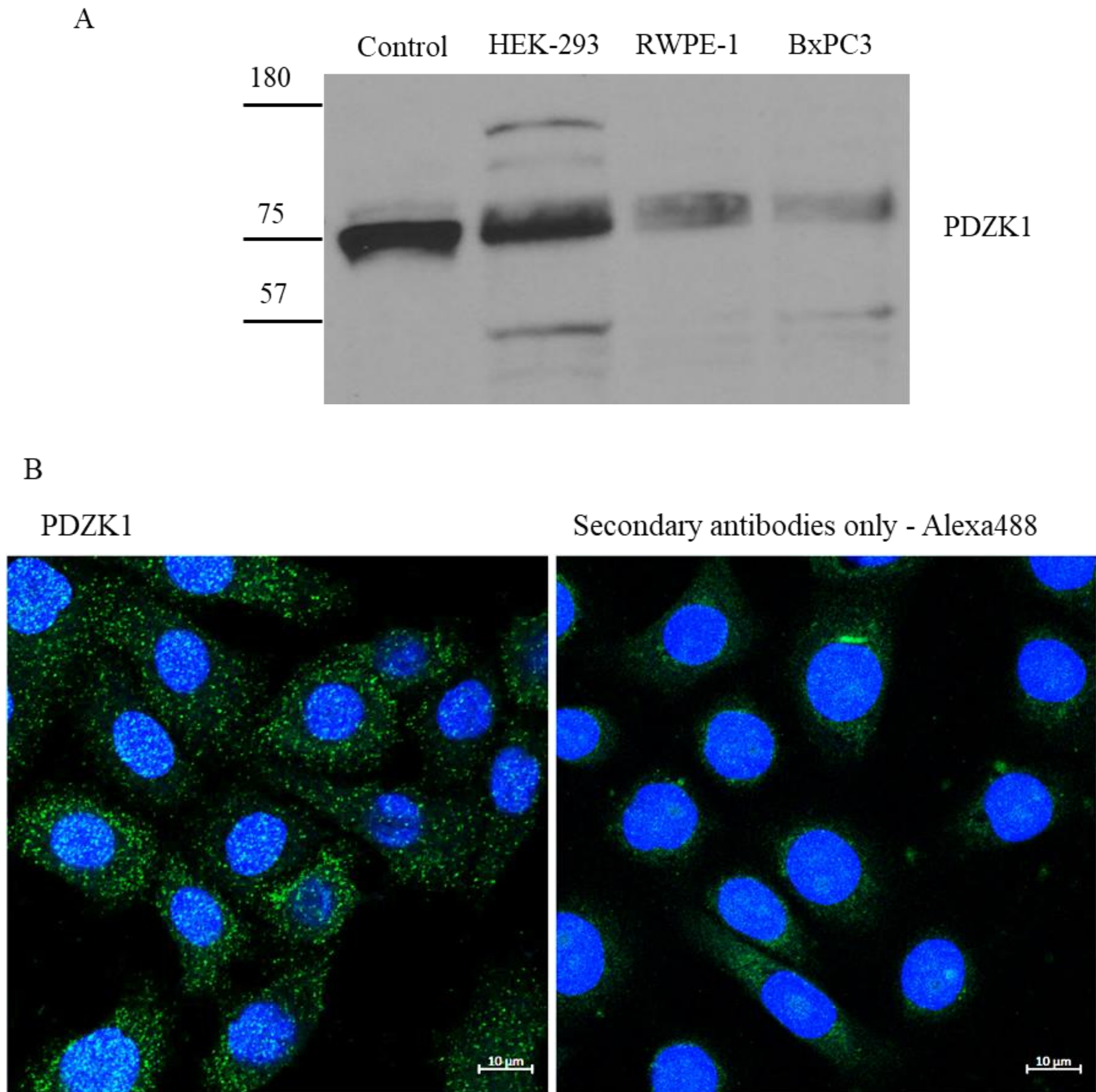


Figure 3.5. RWPE-1 express PDZK1. (A) PDZK1 protein is present in RWPE-1. RWPE-1 cells were prepared as described in Materials and Methods. Membrane was probed with antibodies against PDZK1. T47D whole cell lysate (ab14899 ABCAM) was used as a positive control. A band (75 kDa) representing endogenous PDZK1 detected in all samples is clearly visible. **(B) Distribution of PDZK1 protein in RWPE-1 cells.** RWPE-1 cells were handled as described in Material and Methods. Cells were probed with antibodies against PDZK1and fluorescent

secondary antibody Alexa Fluor® 488 goat anti-rabbit IgG. Nuclei (blue) were stained with DAPI. PDZK1(green) is widely distributed within the cytoplasm in a punctate manner. Scale bar – 10 μm. Images were collected with a Zeiss laser scanning confocal fluorescent microscope at 64X and they are representative data from 3 independent experiments. RWPE-1 cells probed with secondary antibody only are shown.

ASCT2 and PDZK1 do not Co-immunoprecipitate nor Co-localize in RWPE-1 cells.

A recent study by Scalise and colleagues (2014) revealed an interaction between ASCT2 and PDZK leading us to speculate that PDZK1 could have a role in the EGF-dependent ASCT2 trafficking.

To investigate this, I first determined whether co-immunoprecipitation of ASCT2 and PDZK1 occurs in RWPE-1 and HEK-293 cells. However, I could not confirm the existence of an interaction between the two proteins in either cell model as indicated by Co-IP (Figure 3.6). Moreover, there is no co-localization nor co-occurrence between ASCT2 and PDZK1 in RWPE-1: each protein seems to be distributed in small red or green clusters independently from one another and no signal overlapping can be detected by immunofluorescence (Figure 3.7).

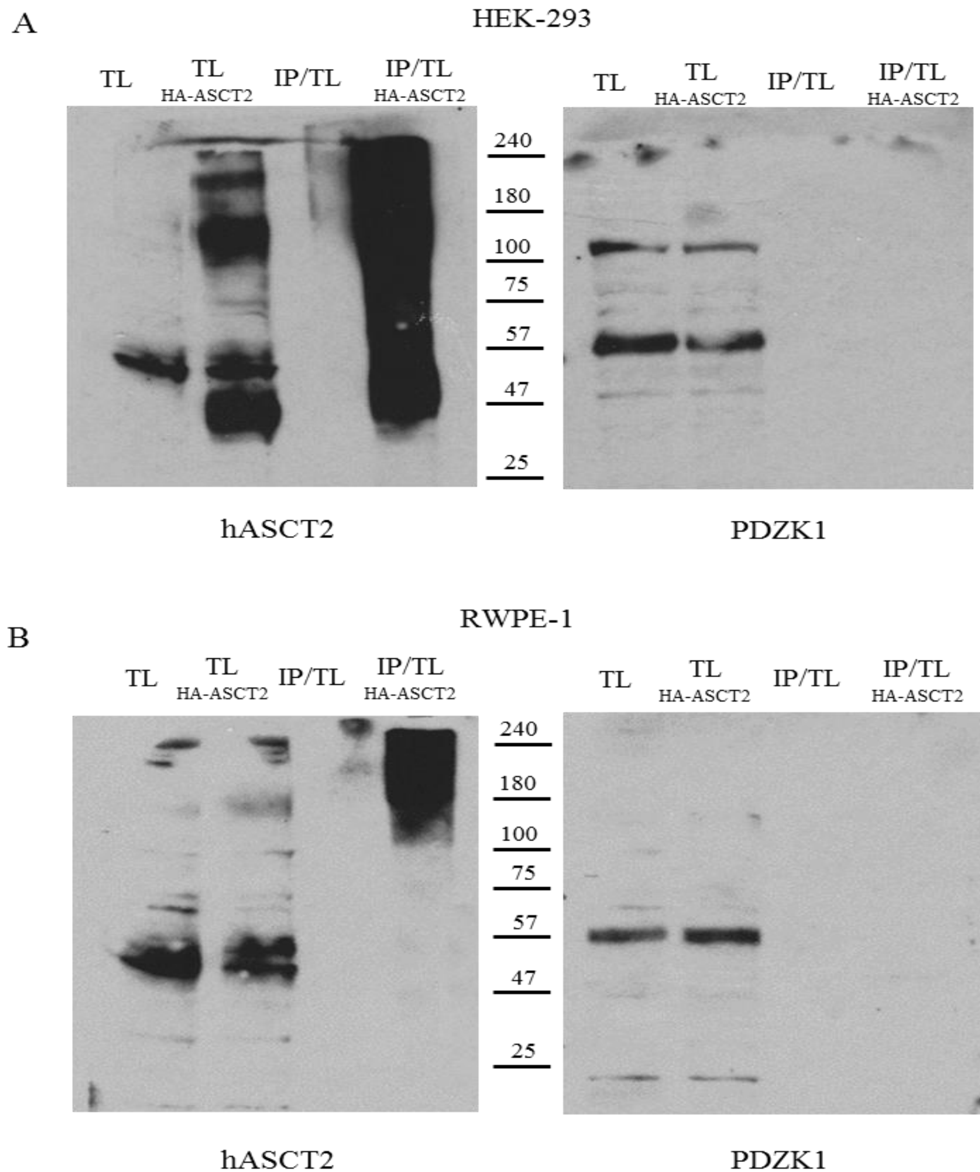


Figure 3.6. PDZK1 does not co-immunoprecipitate with hASCT2 in RWPE-1. HEK-293 and RWPE-1 cells were grown and transiently transfected with the HA tagged ASCT2 construct as described in Materials and Methods. Membranes were probed with antibodies against (A) hASCT2 and (B) PDZK1. Samples: TL, Non-transfected RWPE-1 or HEK-293 cells total lysate. $TL_{HA-ASCT2}$, HA-ASCT2 transfected RWPE-1 or HEK-293 cells total lysate. IP/TL, IP elution of non-transfected total lysate. $IP/TL_{HA-ASCT2}$, IP-elution of HA-ASCT2 protein. Representative immunoblot of 3 independent experiments.

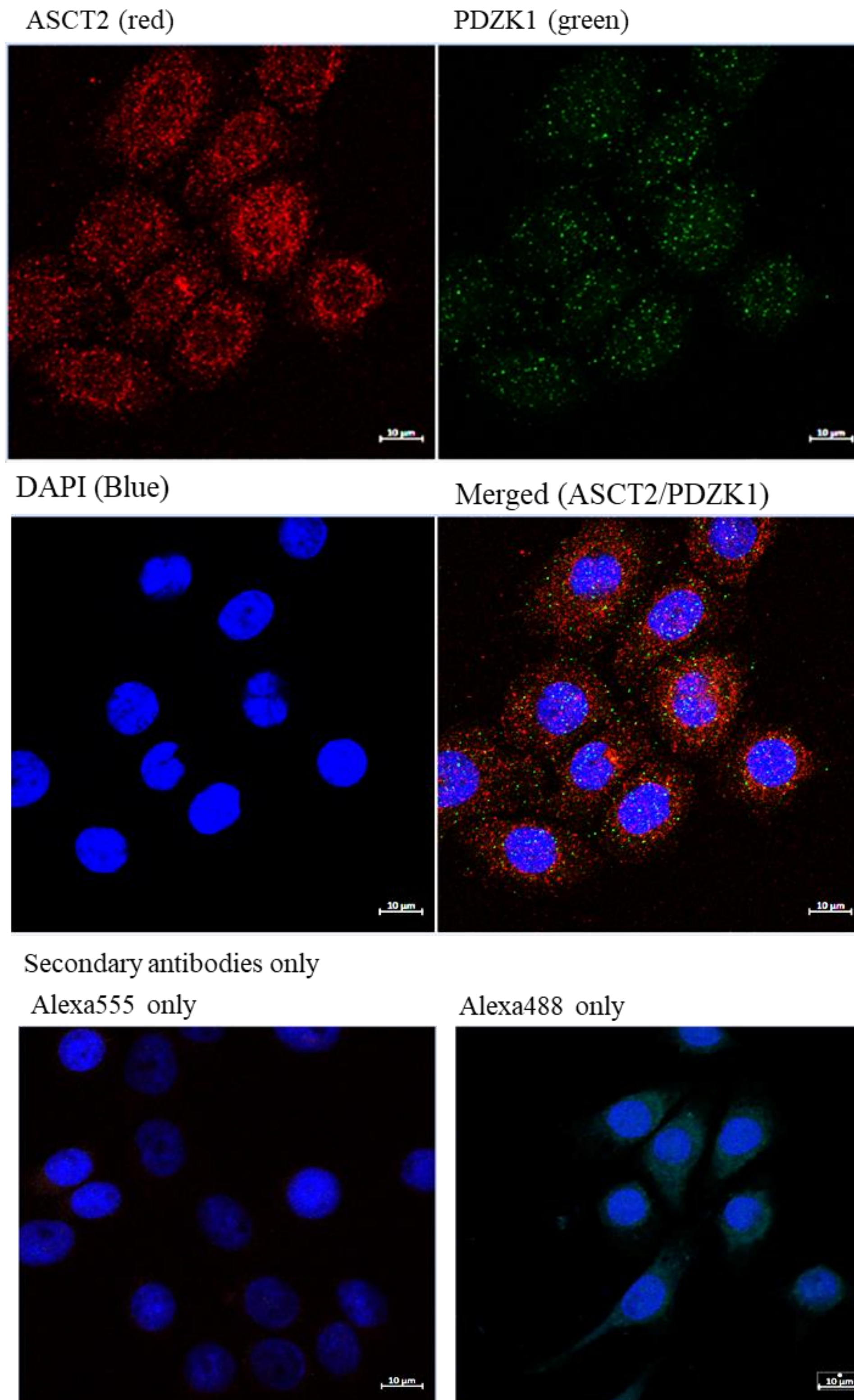


Figure 3.7. ASCT2 and PDZK1 do not appear to co-localize in RWPE-1. RWPE-1 cells were grown, fixed, and permeabilized described in Materials and Methods. Cells were probed with

ASCT2.rbd-conjugated antibodies, PDZK1, and secondary antibodies Alexa Fluor® 555 donkey anti-mouse IgG and Alexa Fluor® 488 goat anti-rabbit IgG. Nuclei (blue) were stained with DAPI. Both ASCT2 (red) and PDZK1 (green) are distributed in a punctate manner within the cells and no overlapping signal (yellow-orange) can be detected, which suggests no co-localization. Images are representative data of 5 independent experiments and are Z-stacks taken at 28µm intervals (27 slices) with a Zeiss laser scanning confocal fluorescent microscope-64X. Scale bar - 10µm. Negative control of RWPE-1 cells probed with secondary antibodies only is shown.

Bioinformatics Analysis of hASCT2 Potential Interactors

I confirmed that PDZK1 and ASCT2 do not co-localize or interact in RWPE-1. Therefore, to proceed in the understanding of the molecular mechanism regulating ASCT2's regulation I attempted to identify other putative interactors by performing the first large-scale MYTH screening targeted at hASCT2. Human ASCT2 was used as a prey in two independent MYTH screening in which 60 million interactions were tested, yielding 58 potential interactors which I sorted by localization and function, as shown in *Table 3.2*.

Table 3.2. MYTH screening summary.

hASCT2 vs. Human Adult Colon library	
Membrane - associated proteins	7
ER-Golgi trafficking proteins	8
Proteins with enzymatic activity	9
Proteins with transporter function	8
Proteins with diverse or unknown functions	16
False positive	2

hASCT2 vs. Human Adenocarcinoma library	
Proteins with diverse or unknown functions	7
False positive	1

Their function and possible involvement in hASCT2 regulation, is described in the following tables (3.3 to 3.8).

Table 3.3. Plasma membrane hASCT2 putative interactors.

hASCT2 vs. Human Adult Colon NubG-x library		
Gene	**Protein**	**Function**
ATP13A1	Manganese transporting ATPase	Involved in the manganese transport homeostasis of the endoplasmic reticulum.
CHP2	Calcineurin B homologous protein 2	It has a Na/H exchange activity. Potential activator of the calcineurin/NFAT signalling pathway. Binds to and activates SLC9A1/NHE1 receptor. Plays a role in the regulation of cell proliferation and tumour growth by increasing the calcium-dependent phosphatase activity of PPP3CA.
ADGRE5	Protein ADGRE5	CD97/ADGRE5 is a membrane protein of the epidermal growth factor- 7 transmembrane family (EGF-TM7) that belongs to adhesion G protein-coupled receptors (GPCR).
ATP6AP2	Renin receptor	Functions as a renin and pro-renin cellular receptor. May mediate renin-dependent cellular responses by activating ERK1 and ERK2.
MS4A8	Membrane-spanning 4 domains subfamily A, member 8	May be involved in signal transduction as a component of a multimeric receptor complex
TMC4	Transmembrane channel-like protein 4	Probable ion channel
TMC5	Transmembrane channel-like protein 5	Probable ion channel
TMCO3	Transmembrane and coiled-coil domain-containing protein 3	Probable Na^+/H^+ antiporter. TMCO3 belongs to the monovalent cation protein antiporter 2 (CPA2) transporter family.
SSTR2	Somatostatin receptor type 2	It is a G-protein coupled receptor for somatostatin-14 and -28. Inhibits cell growth through enhancement of MAPK1 and MAPK2 phosphorylation and up-regulation of CDKN1B.

Table 3.4. ER-Golgi trafficking hASCT2 putative interactors.

hASCT2 vs. Human Adult Colon NubG-x library		
Gene	**Protein**	**Function**
BCAP29	B-cell receptor-associated protein 2 (Bap29)	Involved in ER to Golgi vesicle mediated transport. Forms a heterodimer with BCAP31. It assists or regulate BCAP31 function.
BCAP31	B-cell receptor-associated protein 31 (Bap31)	It is a chaperone protein. Plays role in the export of secreted proteins from and to the ER. Helps in the recognition of misfolded proteins and aids their targeting to the ER associated degradation.
EMC7	ER membrane protein complex subunit 7 (C15orf24)	It is member of EMC which is implicated in ER-associated degradation, lipid transport and tethering between the ER and mitochondrial outer membranes, and assembly of multi-pass ER-membrane proteins.
TVP23C	Golgi apparatus membrane protein TVP23, homolog C (FAM18B2)	Probably involved in vesicle mediated transport and protein secretion
GPR89A	Golgi pH regulator A	Works as a voltage dependent anion channel which modulates Golgi functions through the regulation of acidification.
HERPUD1	Homocysteine responsive endoplasmic reticulum resident ubiquitin-like domain member 1 protein	Component of ER-associated degradation system. Involved in ubiquitin-dependent protein degradation.
SERP1	Stress associated endoplasmic reticulum protein 1	Protects unfolded target proteins against degradation during ER stress. SERP1 interacts with the chaperone calnexin, thus controlling early biogenesis of membrane protein.
SSR3	Translocon-associated protein subunit gamma (TRAP-gamma)	TRAP (signal sequence receptor complex) is required for the translocation of secretory and membrane proteins in the endoplasmic reticulum.

Table 3.5. Enzyme-related putative hASCT2 interactors.

hASCT2 vs. Human Adult Colon NubG-x library		
Gene	**Protein**	**Function**
HMOX2	Heme oxigenase 2	Catalyze the degradation of heme to carbon monoxide (CO), ferrous iron, and biliverdin. It is a defensive mechanism against oxidative stress.
OACT2	Lysophospholipid acyltransferase 5 (LPCAT3)	Involved in lipid and phospholipid biosynthesis and metabolism.
RNF5	E3 ubiquitin-protein ligase RNF5	It is involved in protein ubiquitination. Has E2-dependent E3 ubiquitin-protein ligase activity.
TMEM195	Alkylglycerol monooxygenase (AGMO)	Glyceryl-ether monooxygenase that cleaves the O-alkyl bond of ether lipids.
SPCS2	Signal peptidase complex subunit 2	Component of the microsomal signal peptidase complex which removes signal peptides from nascent proteins as they are translocated into the lumen of the ER.
TECR	Very-long-chain enoyl-CoA reductase	It is involved in the fatty acid biosynthesis pathway.
UBIAD1	UbiA prenyltransferase domain-containing protein 1	Prenyltransferase that mediates the formation of menaquinone-4 (MK-4) and coenzyme Q10.
ELOVL1	Elongation of very long chain fatty acids protein 1	Catalyzes the first reaction of the long-chain fatty acids elongation cycle. Exhibits activity toward saturated C18 to C26 acyl-CoA substrates, with the highest activity towards C22:0 acyl-CoA.

Table 3.6. Transporter- related putative hASCT2 interactors.

hASCT2 vs. Human Adult Colon NubG-x library		
Gene	**Protein**	**Function**
ABCA8	ATP-binding cassette sub-family A member 8	Transporters characterized by their ability to transport lipids across cellular membrane and to regulate lipid homeostasis.
AQP7	Aquaporin 7	Member of aquaglyceroporins which transport glycerol as well as water. Adipocyte glycerol permeation through AQP7 was appointed as a novel regulator of adipocyte metabolism and whole-body fat mass.
SLC13A2	Solute carrier family 13 member 2 (NaDC1)	Low affinity Na^+/dicarboxylate transporter.
SLC23A1	Solute carrier family 23 member 1 (SVCT1)	Na^+-dependent vitamin C transporter. Mediates electrogenic uptake of vitamin C. Highly expressed in adult small intestine, kidney, thymus, ovary, colon, prostate and liver
SLC35A3	UDP-N-acetylglucosamine transporter	Is the major UDP-N-acetylglucosamine transporter in mammals. It forms a heterologous complex with UDP-galactose transporter in the Golgi's membranes.
SLC5A1	Sodium/glucose cotransporter 1 (SGLT1)	It actively cotransports glucose/ Na^+ into cells and is completely functional as a monomer. Expressed in the intestine, kidney, heart, trachea, and prostate.

Table 3.7. hASCT2 putative interactors with unknown or diverse function.

hASCT2 vs. Human Adult Colon NubG-x library		
Gene	**Protein**	**Function**
ARL6IP5	PRA family protein 3 (JWA)	Novel microtubule-associated protein, which regulates cancer cells differentiation and chemically inducted apoptosis.
C18orf32	UPF0729 protein C18orf32	It has signal transducer activity and it may activate the NF-kappa-B signalling pathway
OCIAD1	OCIA domain containing protein	Endosome. Isoform 1 is highly expressed in many tissues, including testis, brain, placenta, ovary, prostate and mammary gland.
CCL2	C-C motif chemokine 2	-
ACKR1	Atypical chemokine receptor 1, (DARC)	Belongs to the G-protein coupled receptor 1 family. Localization: early endosome, recycling endosome, cell membrane.
ACKR4	Atypical chemokine receptor 4 (CCR11)	Belongs to the G-protein coupled receptor 1 family. Localization: early endosome, recycling endosome, cell membrane.
FUNDC2	FUN14 domain-containing protein 2	Mitochondrial outer membrane, nucleus
FIS1	Mitochondrial fission 1 protein	Is involved in the fragmentation of the mitochondrial network and its perinuclear clustering.
TM4SF20	TM4 L6 protein 20	Transmembrane protein 20
ITGB1	Integrin beta-1	Belongs to the integrin beta chain family
TMEM128	GRB2, (Growth factor receptor-bound protein 2)	Adapter protein that provides a critical link between cell surface growth factor receptors and the Ras signalling pathway.
TMEM19/	TM protein 19	Transmembrane protein 19
TMEM237	TM protein 237	Component of the transition zone in primary cilia.
TMEM242	TM protein 242	Transmembrane protein 242
TMX2	Thioredoxin-related TM protein 2	Involved in cell redox homeostasis.
TMEM35B	TM protein 35B	Transmembrane protein 35B

Table 3.8. Putative hASCT2 interactors with miscellaneous function identified in the Human Prostate Adenocarcinoma library.

Gene	Protein	Function
C14orf1	Probable ergosterol biosynthetic protein 28	Endoplasmic reticulum multi-pass membrane protein.
CNIH4	Protein cornichon homolog 4	Involved in G protein-coupled receptors (GPCRs) trafficking from the ER to the cell surface; it promotes the exit of GPCRs from the early secretory pathway, likely through interaction with the COPII machinery.
EBP	3-beta-hydroxysteroid-Delta (8), Delta (7) -isomerase	It is involved in the pathway cholesterol biosynthesis, which is part of the steroid biosynthesis. ER multi-pass membrane protein. Nucleus envelope, Cytoplasmic vesicle.
EMC4	ER membrane protein complex subunit 4	May mediate anti-apoptotic activity.
SERP1	Stress-associated endoplasmic reticulum protein 1	Interacts with target proteins during their translocation into the lumen of the ER. Protects unfolded target proteins against degradation during ER stress. May facilitate N-glyc of proteins after termination of ER stress.
TMCO3	TM and coiled-coil domain-containing protein 3	Probable Na^+/H^+ antiporter.
TMEM14A	TM protein 14A (PTD011)	Inhibits N-(4-hydroxyphenyl) retinamide-induced apoptosis through the stabilization of mitochondrial membrane potential
HDAC6	Histone deacetylase 6, nucleus	**Common false positive.**

Discussion

I previously confirmed that EGF stimulates an increase in glutamine uptake mainly due to an increase of hASCT2 protein at the plasma membrane which is consistent with the regulation described for other transporters (Lee *et al.*, 2010, Ribeiro *et al.*, 2007, Jones *et al.*, 2012). The underlying mechanism of regulation of redistribution of ASCT2 appears to involve mTOR; however, it is not known whether the signal is transmitted via mTORC1 or mTORC2 pathway (Shimizu *et al.*, 2014, Takahashi *et al.*, 2015). Here I evaluated the downstream effects of short-term pharmacological inhibition of ASCT2-dependent uptake on the downstream targets of both mTOR complexes.

The serine/threonine kinase mTOR is conserved across all eukaryotes and its key role within cells is an amino acid sensor and regulator of cell metabolism (Malik *et al.*, 2013, Shimobayashi and Hall, 2014). The mTOR complex 1 mTORC1, is regulated by amino acids, oxidative stress, insulin and growth factor signalling (Hay and Sonenberg, 2004). These stimuli promote cell growth and proliferation by controlling the activity of protein S6K1 and the eukaryotic initiator factor 4E-BP1, which in turn increases protein synthesis, regulating mitochondrial metabolism and autophagy (Gingras *et al.*, 1999, Kim and Guan, 2015, Ruvinsky and Meyuhas, 2006, Wang and Proud, 2006, Klionsky, 2010). In contrast, mTORC2 is strictly controlled by growth factors and, via its downstream targets which are SGK1 protein, PKC and Akt kinases, it is thought to coordinate cytoskeleton assembly, cell size, and sodium transport (Alessi *et al.*, 2009, Fingar and Inoki, 2012, Oh and Jacinto, 2011).

My data show that in RWPE-1 cells, inhibition of ASCT2 using GPNA (Esslinger *et al.*, 2005) leads to a decrease in activity of mTORC2 due to hyper-phosphorylation of the complex protein RICTOR and an increase in activity of mTORC1 determined via detection of the hyper-

phosphorylation of S6K1 p70; this is unexpected since previous findings have shown that long term inhibition and/or silencing of ASCT2, in certain cancers where its expression is very abundant, leads to a downregulation of mTORC1 activity (Bruhat *et al.*, 1997, Wang *et al.*, 2014, Wang *et al.*, 2015). Also, studies on ASCT2 silencing have shown that the absence of ASCT2 results in decreased amino acid uptake, mTOR downregulation, and finally cell death (Fuchs and Bode, 2005, Fuchs *et al.*, 2007, Kudo and Boyd, 2002).

The activation of mTORC1 results in the hyper-phosphorylation and downregulation of 4E-BP1 and in the hyperphosphorylation and activation of p70 S6K1 (Shimobayashi and Hall, 2014). As expected, I found that p70 S6K1 and 4E-BP1 are both hyper-phosphorylated. The inhibition of 4E-BP1 due to its hyperphosphorylation is the first step in the activation of the cap-dependent mRNA translation (Brunn *et al.*, 1997, Gingras *et al.*, 1998, Gingras *et al.*, 1999, Pause *et al.*, 1994). This event, effected by the complex FRAP/mTOR at Thr37 and Thr46, serves only as a primer for 4E-BP1 which is to be followed by phosphorylation at Ser65 and Thr70, which seems to be cell-specific and due to many extracellular inputs (Fadden *et al.*, 1997, Gingras *et al.*, 1999). Because, as previously described (chapter 2), I did not detect an increase in the expression of ASCT2 mRNA following the treatment of RWPE-1 cells with EGF, but only a redistribution of the transporter ASCT2 at the plasma membrane, I can conclude that the phosphorylation of 4EPB1is the first step toward the metabolic readjustment of the cell in response to the decrease in glutamine availability. Instead of starting translation of new ASCT2 right away, which may be energetically costly, the cells prepare for the possibility of protein translation should the lack of amino acids continue. The role of mTOR as a contributor to the regulation of many receptors and transporters is well established (Rosario *et al.*, 2013, Almilaji *et al.*, 2012, Roos *et al.*, 2009, Shojaiefard *et al.*, 2006). Previous studies have shown that mitogenic stimulation and

phosphorylation by mTORC1 leads to activation of ribosomal protein S6 kinase (S6K1) which results in negative feedback on mTORC2 by phosphorylating the complex protein RICTOR and therefore inhibiting the Akt1 downstream signalling (Dibble *et al.*, 2009, Dufner and Thomas, 1999, Julien *et al.*, 2010, Sun *et al.*, 2016, Holz *et al.*, 2005, Pullen and Thomas, 1997).

My data show that inhibition of ASCT2, leads to a decrease in mTORC2 activity, which could be due to the previously reported S6K1-dependent negative feedback to PI3K which will arrest the signalling cascade (Dancey, 2010, Rahman and Haugh, 2017); this is confirmed by the detection of the hyper-phosphorylated and therefore hyper-activated p70 S6K1. Nevertheless, I also detected hypo-phosphorylated p85 S6K1 isoform. This may suggest a previously unreported regulation of the mTORC2-dependent pathway in response to ASCT2 inhibition, which could be specific to RWPE-1 cells. The possible regulative role of p85 S6K1 isoform has not been investigated, few studies have focused only its subcellular localization and its association with enhanced cellular proliferation and metastasis (Ismail *et al.*, 2013, Rosner *et al.*, 2013, Tavares *et al.*, 2015, Amaral *et al.*, 2016). Given the lack of information on p85 S6K1, further studies using techniques such as silencing and phosphorylation analysis of this kinase and RICTOR, in relation to the EGF-dependent activity and regulation of ASCT2, are needed to confirm this novel putative regulation.

Previous data have shown that mTOR is involved in regulating membrane transporters (Edinger and Thompson, 2002, Edinger *et al.*, 2003, Edinger, 2007, Liu *et al.*, 2004, Peng *et al.*, 2002, Peyrollier *et al.*, 2000, Roos *et al.*, 2009, Roos *et al.*, 2007). Specifically, mTORC2 has been found to be a crucial regulator of the activity and trafficking of systems A and L in human trophoblast cells (Rosario *et al.*, 2013). However, in all these cases the molecular mechanisms of this form of regulation are still unclear.

A possible growth factor-dependent regulation of ASCT2 through SGK1 has been previously suggested in *Xenopus laevis* oocytes (Palmada *et al.*, 2005). This led us to presume that the EGF-dependent regulation of ASCT2's activity and trafficking could involve SGK1 and signal transduction through mTORC2. I predicted that, if SGK1 was involved in the regulation of ASCT2, the inhibition of ASCT2 activity would result in an increase of mTORC2 activity following an upregulation SGK1 and mechanisms involving protein trafficking through the early endosome pathway. However, in RWPE-1 the inhibition of ASCT2 did not have any consequences on the phosphorylation of SGK1 or Akt. This led us to the conclusion that either I was unable to detect a very finely tuned regulation or that simply neither SKG1 nor Akt are involved in the short-term regulation of ASCT2 in these cells.

To exclude the possibility of a lack of sensitivity in detecting changes in the kinases phosphorylation on my part, I inhibited mTOR using pharmacological inhibitors Torin1 and XL388 (Takeuchi *et al.*, 2013, Thoreen *et al.*, 2009, Xiong *et al.*, 2017). Data suggest that short term inhibition of mTOR activity does not affect the overall glutamine uptake in RWPE-1 cells or downregulate the ASCT2-dependent glutamine transport. This is in contrast to previously published data showing a reduction of mTOR activity due to silencing of ASCT2 (Avissar *et al.*, 2008) and reduction of ASCT2 functionality due to mTOR silencing and inhibition with rapamycin in hepatoma cells (Fuchs *et al.*, 2007). This could be due to the fact that: (a) RWPE-1 cells are not as sensitive to the inhibitors as other cell lines are, therefore longer (chronic) treatments are required to induce a detectable regulation of the transporter by mTOR; (b)The regulation could be so subtle that is undetectable in non-tumorigenic cell line; (c) The regulation between ASCT2 and mTOR previously described in other tumour-derived cell lines, may be a manifestation of a dysregulated system which may not occur in a non pathological environment To better understand

this mechanism of regulation, further studies are required. A summary of my findings is depicted in Figure 3.8. Overall, I don't have enough information to confirm that a link between ASCT2 inhibition and mTOR activity exists; this is mostly due to biological variability and to the fact that the regulation we are looking for may occur differently in my model, compared to overstimulated systems which are dysregulated where any changes are detected promptly and abundantly.

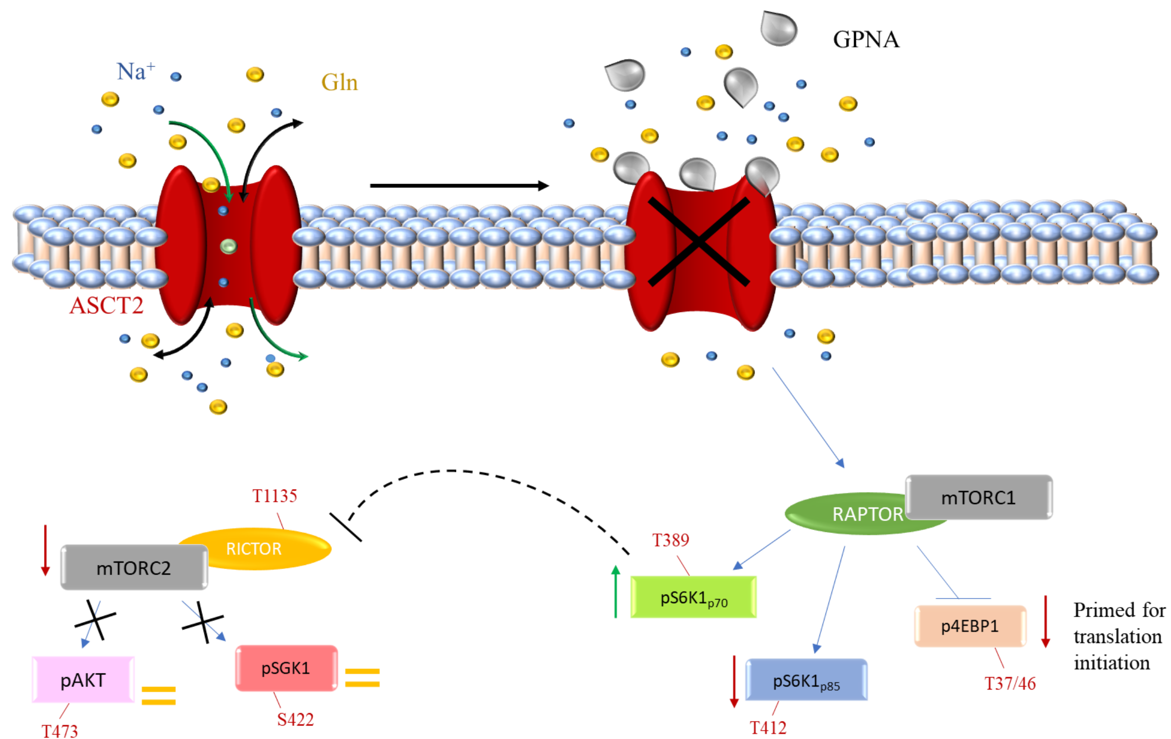


Figure 3.8. A cartoon depicting the putative downstream effects of ASCT2 inhibition on mTOR pathways. The inhibition of ASCT2 due to GPNA results in a lack of glutamine uptake which causes a decrease in 4E-BP1 activity due to its hyper-phosphorylation on tyrosine 37 and 46. This phosphorylation primes the kinase which initiates signalling for protein translation and stops the regulation of mTORC1 on the downstream pathways involving SGK1 and Akt. At the same time, p70 S6K1 is hyper-phosphorylated and activated which may be the cause of the detected downregulation of RICTOR, while isoform p85 S6K1 is hypo phosphorylated and its activity decreased. Upregulation (green arrow); downregulation (red arrow); no change in activity (yellow parallel). Dotted black arrows represent unverified regulation.

There was no information on ASCT2 interactome until very recently, when the scaffold protein PDZK1 was described as an *in vitro* interactor of the transporter (Scalise *et al.*, 2014). PDZK1 is involved in the regulation of many members of the SLCs family such as OCTN1 (Kato *et al.*, 2005), OCTN2, and PEPT1 (Sugiura *et al.*, 2008).Its regulation involves change of transport activity rate, regulated recycling and/or trafficking (Kim *et al.*, 2012, Walther *et al.*, 2015). PDZK1 is expressed exclusively in epithelial cells and it is overexpressed in various carcinomas where it interacts with MAP17, a membrane-associated protein involved in regulation of cell proliferation (Kocher *et al.*, 1998, Ghosh *et al.*, 2000). In breast cancer, PDZK1 is also part of many complexes where it interacts with and coordinates, in response to growth factors such as EGF, the interplay of multiple proteins at the cell surface (Hu *et al.*, 2013, Kim *et al.*, 2012, Kim *et al.*, 2013). Hence, given the regulatory nature of PDZK1 I presumed this protein may be a potential candidate in having a role in the regulation of ASCT2.

Furthermore, given the rapid response of the transporter to the EGF stimuli, I predicted that the mechanism may occur via selective and/or regulated recycling of the transporter residing just beneath the membrane, which is recruited through an unknown signalling pathway stimulated by EGF, which could include PDZK1.

I have confirmed the presence and the distribution of PDZK1 in RWPE-1 cells and my data are similar to those previously reported (Choi *et al.*, 2011, Scalise *et al.*, 2014, Walther *et al.*, 2015). Since I could not confirm the interaction between ASCT2 and PDZK1, I decided to look at other ASCT2-interacting proteins which could be involved in the EGF-dependent regulation of the transporter. I used the membrane yeast two-hybrid system to perform a large-scale screening of ASCT2 putative interactions.

The membrane yeast two-hybrid (MYTH) system is based on the concept of "split ubiquitin" developed by Johnsson and Varshavsky, (1994) which was re-adapted and used as a sensor for the detection of protein-protein interactions in *S. cerevisiae* (Fetchko and Stagljar, 2004, Lam *et al.*, 2015, Snider *et al.*, 2010, Snider *et al.*, 2013, Stagljar *et al.*, 1998).

MYTH is a widely utilized technique to get insight into membrane protein's interactome, however it poses some challenges that may affect the interpretation of the data such as the presence of high numbers of false negative/positive interactions due to unnatural aggregation of the overexpressed bait and preys, false positive may be due to interactions of the protein in yeast that are not typically co-expressed in a mammalian cell; also, some interactions which are typical of mammalian systems, may not occur in yeast, therefore overlooking biologically relevant interactions which go undetected. Despite its limitations, the MYTH system offers a powerful tool for the detection of interactions between membrane-associated proteins and possible regulators *in vivo*. Furthermore, the interaction network I present here is the first ever large output screening performed for hASCT2 and is a powerful resource which will help researchers move forward in understanding of the regulation of hASCT2.

I performed a bioinformatic analysis of the results using the STRING database of predicted functional association between proteins (Szklarczyk *et al.*, 2015). I found that hASCT2 is associated with various proteins involved in a surprisingly diverse variety of functions some of which are already part of complexes with specific roles (Figure 3.9). Some of the MYTH-detected interactions are unexpected but they may indicate yet undiscovered roles for ASCT2 along with unidentified regulatory pathways (Ratushny and Golemis, 2008, Serebriiskii and Golemis, 2001). Here, I highlight ASCT2 putative protein interactions which show potential for future studies on regulation, along with some interaction which may have physiological relevance.

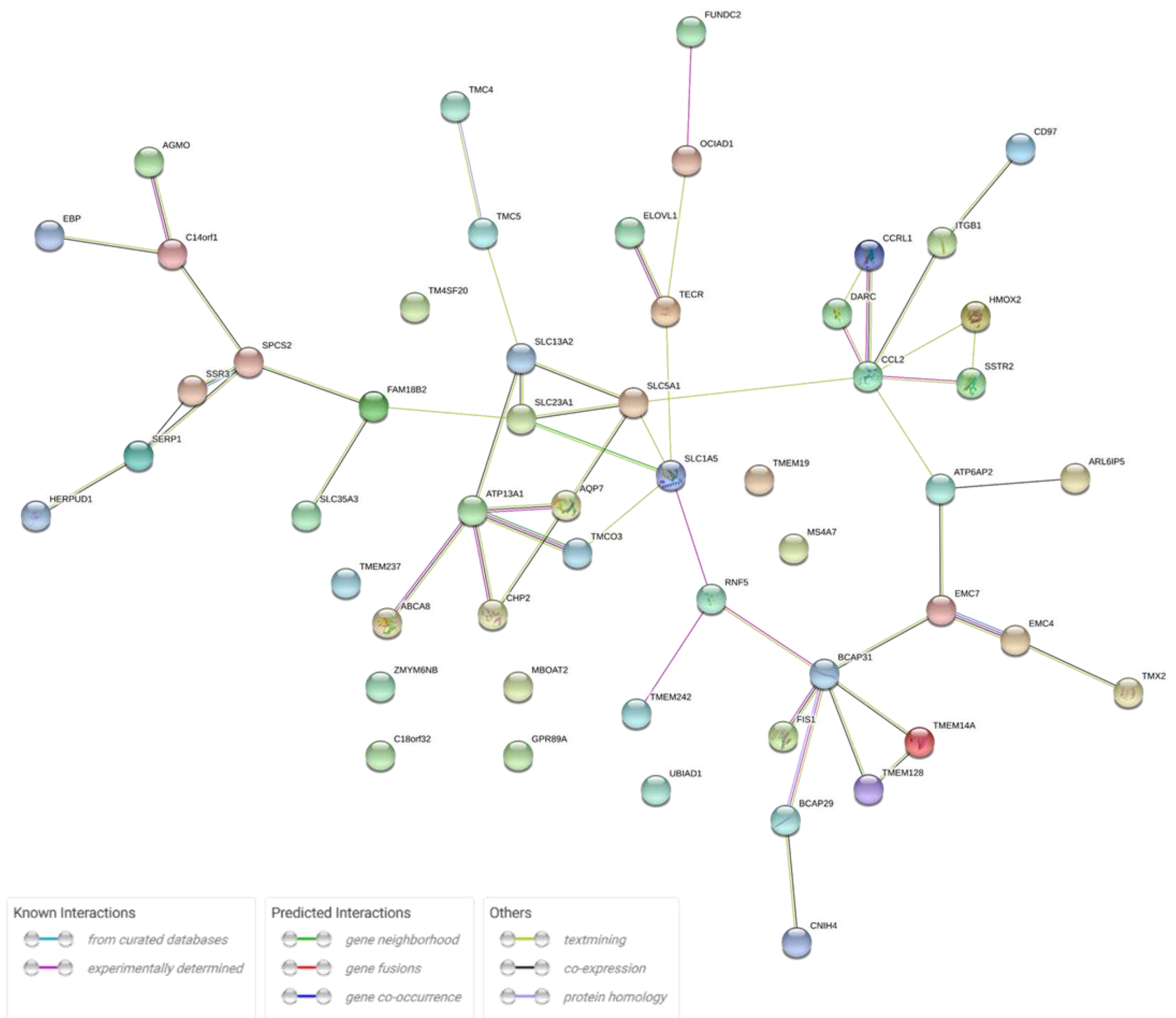


Figure 3.9. Integrated hASCT2 interactome. Novel interactions identified by MYTH screening are represented by the coloured spheres and ASCT2 (SLC1A5) is used as a central node. The links shown represent interactions of various nature previously reported in the BioGRID database. Known false positive interactions were excluded from this analysis. The interactome was generated using STRING software.

Among the ASCT2 interactors I detected via MYTH, there are some which possess possible biologically relevant and/or regulatory roles such as the UDP-N-acetylglucosamine transporter (SLC35A3). SLC35A3 is a membrane protein localized in the Golgi apparatus which transports uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) (Ishida *et al.*, 1999). SLC35A3 was correlated with the development of autism spectrum disorder, epilepsy and arthrogryposis (Edvardson *et al.*, 2013). The gene encoding SLC35A3 is downregulated in patients with pancreatic adenocarcinoma along with another putative ASCT2-interacting protein I found in my screening, the Golgi apparatus membrane protein TVP23 homolog C (TVP23C) (Liang *et al.*, 2012) which is involved in vesicle-mediated trafficking (Matsuda *et al.*, 2003).

My screening also confirmed an interaction of ASCT2 with the Ring Finger Protein 5 (RNF5) which has been previously described in breast cancer (Jeon *et al.*, 2015). Upon paclitaxel-induced ER stress in BCa cells, RNF5 associates with ASCT2 and SNAT2 (SLC38A2), facilitating the ubiquitination and degradation of these two transporters. This reduces glutamine uptake, decreases TCA cycle components levels and mTOR signalling while promoting cell death (Jeon *et al.*, 2015). Moreover, RNF5 receives a signal from the ER stress-associated response complex, two of which proteins were also found as putative interactors of ASCT2 in my MYTH screening: the translocon-associated signal receptor gamma (SSR3) and the stress associated ER protein 1 (SERP1).

The interaction between ASCT2 and members of the ER-stress associated complex could have a correlation with the alleged protective action carried out by ASCT2 on brain cells under ischemic conditions (Gegelashvili *et al.*, 2006); it seems that at low pH, ASCT2 acts as a scavenger for the protonated glutamate (glt^+), thereby reducing the toxic activity of this neurotransmitter in the brain.

One very interesting putative interactor of ASCT2 found in my MYTH screening is the calcineurin B homologous protein 2 (CHP2). This protein is required for the localization and stabilization of the Na^{+}/H^{+} exchanger (SLC9A1/NHE1) at the plasma membrane (Pang *et al.*, 2001, Pang *et al.*, 2004) and in the regulation of transporters in breast cancer (Amith and Fliegel, 2013).

CHP2 shares 63% sequence similarity and 32% sequence identity with Calcineurin B (CnB) (Figure 3.10), the regulatory subunit of calcineurin, and it can interact with the catalytic subunit of calcineurin (CnA) as CnB would (Li *et al.*, 2008).

```
P63098 CANB1_HUMAN      1    MGNEASY--------PLEMCSHFDADEIKRLGKRFKKLDLDNSGSLSVEEFMSLPELQQN     52
O43745 CHP2_HUMAN       1    MGSRSSHAAVIPDGDSIRRETGFSQASLLRLHHRFRALDRNKKGYLSRMDLQQIGALAVN     60
                             **..:*:          :.  : *.  .: ** :**: ** ::.* **  :: .:  *  *

P63098 CANB1_HUMAN     53    PLVQRVIDIFDTDGNGEVDFKEFIEGVSQFSVKG----------------DKEQKLRFAF     96
O43745 CHP2_HUMAN      61    PLGDRIIESFFPDGSQRVDFPGFVRVLAHFRPVEDEDTETQDPKKPEPLNSRRNKLHYAF    120
                             ** :*:*: *  **. .***  *:. :::*                     .:.:**::**

P63098 CANB1_HUMAN     97    RIYDMDKDGYISNGELFQVLKMMVGNNLKDTQLQQIVDKTIINADKDGDGRISFEEFCAV    156
O43745 CHP2_HUMAN     121    QLYDLDRDGKISRHEMLQVLRLMVGVQVTEEQLENIADRTVQEADEDGDGAVSFVEFTKS    180
                             ::**:*:** **. *::***::*** ::.: **::*.*:*: :**:**** :** **

P63098 CANB1_HUMAN    157    VGGLDIHKKMVVDV--                                                170
O43745 CHP2_HUMAN     181    LEKMDVEQKMSIRILK                                                196
                             :  :*:.:** : :
```

Figure 3.10. Sequence alignment between calcineurin B and CHP2. Calcineurin B and CHP2 share 32% sequence identity and two of the four calcium binding sites necessary for its activation (blue). The alignment was performed using CLUSTALO.

Calcineurin is a Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase which takes part in many cellular processes including calcium-dependent signal transduction pathways involving calmodulin (Rusnak and Mertz, 2000, Shibasaki *et al.*, 2002, Aramburu *et al.*, 2004, Li *et al.*, 2011). This could be of interest due to data showing the interaction between ASCT2 and the

serotonin transporter (SERT) in HEK-293 (Seyer *et al.*, 2016). It seems that the co-expression of these two proteins in HEK-293 cells affects the glycosylation and cell-surface localization of SERT causing a decrease in serotonin uptake, while depletion of ASCT2 via RNAi causes an increase in serotonin uptake in primary mesencephalon neurons. SERT activity and trafficking are finely modulated by a complex network of kinases and phosphatases including the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (Seimandi *et al.*, 2013). Several studies have shown how phosphorylation can regulate membrane proteins by inducing conformational changes and altering protein-protein interactions, activity and trafficking (Mohapatra *et al.*, 2007). As previously described, CHP2 can bind to the catalytic subunit of calcineurin (CnA) and being so similar to its homologue (CnB), it could potentially use the same docking consensus sequence of calcineurin B, to bind target protein which is represented by the conserved motif LxVP, usually localized on the C-terminus of target proteins (Li *et al.*, 2011). Therefore, I conducted *in silico* bioinformatic analysis of the ASCT2 sequence to detect putative binding sites for calcineurin B and I confirmed the presence of the putative binding site, as predicted, is located intracellularly on the C-terminus of hASCT2 at residues 510-513 (Figure 3.11).

```
hASCT2            451 DHISLILAVDWLVDRSCTVLNVEGDALGAGLLQNYVDRTESRSTEPELIQ
Consensus CHP2        --------------------------------------------------

hASCT2            501 VKSELPLDPLPVPTEEGNPLLKHYRGPAGDATVASEKESVM
Consensus CHP2      1 ---------LXVP----------------------------
```

Figure 3.11. Putative calcineurin B docking region on hASCT2. I detected the motif (LxVP) located at the C-terminus of human ASCT2 (Q15758) sequence at residues 510-513.

The presence of the putative docking site in the sequence of hASCT2 suggest that, similar regulatory processes involving de-phosphorylation of ASCT2 by calcineurin are plausible and they could open a new and very exciting line of research which was unknown until now.

I went further in my analysis and I considered the possibility of an interaction between ASCT2, calcineurin, and calmodulin. As previously elucidated, calcineurin is dependent on calcium ions and calmodulin to be fully activated (Li *et al.*, 2011) and calmodulin has been previously described as a modulator of the equilibrative nucleoside transporters (ENTs) (Bicket *et al.*, 2016). Therefore, I conducted *in silico* analysis of the human (Q15758), mouse (P51912), and rabbit (019105) ASCT2 protein sequences using the Calmodulin Target Database (Yap *et al.*, 2000). I detected one high probability putative binding region represented by the motif CxxxxLxxxL, located intracellularly at the N-terminus between residues 41 and 62, conserved among all three homologs of ASCT2 (Figure 3.12).

```
Q15758 AAAT_HUMAN    1   MVADPP-RDSKGLAAAEPTANGGLALASIEDQGAAAGGYCGSRDQVRRCLRANLLVLLTV    59
P51912 AAAT_MOUSE    1   MAVDPPKADPKGVAVDS--SRRCPALGSREDQSAKAGGCCGSRDRVRRCIRANLLVLLTV    58
O19105 AAAT_RABIT    1   MVADPPKGDPKGLAAVEPTANGAPAQDPLEDSGAAVGRCCSSRDQVRRCLRANLLVLLTV    60
                          *..***  * **:*. .  :.   *    **..* .*  *.***:****:**********

Q15758 AAAT_HUMAN   60   VAVVAGVALGLGVSGAGGALALGPERLSAFVFPGELLLRLLRMIILPLVVCSLIGGAASL   119
P51912 AAAT_MOUSE   59   AAVVAGVGLGLGVSAAGGADALGPARLTRFAFPGELLLRLLKMIILPLVVCSLIGGAASL   118
O19105 AAAT_RABIT   61   VAVVAGVALGLAVSGAGGALALGPARLIAFAFPGELLLRLLKMIILPLVVCSLVGGAASL   120
                          .******.***.**.**** **** **  *.**********:***********:******
```

Figure 3.12. Putative calmodulin binding site on ASCT2. I analyzed the human (Q15758), mouse (P51912), and rabbit (019105) ASCT2 protein sequences using the Calmodulin Target Database (Yap *et al.* 2000). One high probability putative binding site (black bar) was found, located at the N-terminus of ASCT2, between residues 41 and 62. The bioinformatic software examines the sequence and scores the motifs on potential binding to large hydrophobic residues such as Phe, Val, Ile, Leu, or Cys (Rhoads and Friedberg, 1997).

As previously described, ASCT2 and the EGFR receptor physically interact and they are co-regulated (Lu *et al.*, 2016). In my MYTH screening for hASCT2 I found proteins which are related to EGFR such as the transmembrane and coiled-coil domain-containing protein 3 (TMCO3), a member of the monovalent cation protein antiporter 2 transporter family, which is considered a putative Na^+/H^+ antiporter. A previous MYTH screening for EGFR revealed TMCO3 as a yet unconfirmed interactor of the receptor (Deribe *et al.*, 2009).

Another putative interactor of ASCT2 is the growth factor receptor-bound protein 2 (GRB2), which is an adapter protein which acts as a link between receptors located at the plasma membrane and Ras signalling pathway associated with EGFR .This isoform inhibits the EGF-induced transactivation of the RAS-responsive element by suppressing proliferative signals, and triggers programmed cell death (Lowenstein *et al.*, 1992, Dharmawardana *et al.*, 2006, Giubellino *et al.*, 2008, Belov and Mohammadi, 2012, Ahmed *et al.*, 2013).

The Adhesion G Protein-Coupled Receptor E5 (ADGRE5) was also detected as a putative ASCT2 interactor. ADGRE5 is a mediator of cell-cell interactions and its expression is increased by the epidermal growth factor (EGF), this leads the progression and invasion of thyroid carcinoma cells (Hölting *et al.*, 1995, Steinert *et al.*, 2002, Ward *et al.*, 2013). These interactions, if confirmed with biochemical and functional assays, may be potential targets for future investigation of the regulation of ASCT2.

Other interesting ASCT2 interactors found in my MYTH screening include: the renin receptor (ATP6AP2) (Nguyen, 2010), the somatostatin receptor type 2 (SSTR2) (Grant *et al.*, 2004, Grant *et al.*, 2008), and the Na^+/dicarboxylate cotransporter 1 (SLC13A2) (Pajor, 1996, Okamoto *et al.*, 2007).

Given what has been reported to date about ASCT2, it is natural to assume that it is likely to be a very versatile protein, which transcends its role as mere amino acid transporter, used widely in different tissues as co-regulator of transporters and receptors.

Chapter 4 : Overview and General Considerations

The canonical isoform of human Alanine Serine Cysteine Transporter 2 (hASCT2) plays an important role in the physiology of many tissues by providing the cells with neutral amino acids (Bröer *et al.*, 1999, Gegelashvili *et al.*, 2006, Torres-Zamorano *et al.*, 1998) and acting as an anchoring “receptor” for human retroviruses (Antony *et al.*, 2011, Marin *et al.*, 2003). In the last decade, the interest toward this transporter has changed and many studies focused on the importance of the ASCT2 in the so called “Warburg effect” (Vander Heiden *et al.*, 2009, Liberti and Locasale, 2016b). An increasing number of published investigations have highlighted ASCT2 as one of the most important glutamine transporters involved in the support of the shift toward anaerobic metabolic pathways by cancerous cells, to meet the consequent need for more glutamine to support the generation of ATP thereby maintaining the cancer phenotype (Fuchs *et al.*, 2007, Kanai and Hediger, 2004, Palmada *et al.*, 2005, Wang *et al.*, 2013, Wang *et al.*, 2014, Vander Heiden *et al.*, 2011). An example of this role can be found in prostate adenocarcinoma, where a correlation between the increased expression of ASCT2 is correlated with highly aggressive form of the disease (Li *et al.*, 2003). Another example of this role is highlighted by correlation between the inhibited activity of the ASCT2 in prostate tumour cells and their consequent reduced ability to grow and develop (Wang *et al.*, 2015). Also, some data have been reported on the possible regulation of ASCT2 by mTOR and growth factors suggesting that this transporter may be essentially involved in the cellular metabolic processes beyond the disease context (Avissar *et al.*, 2008, Palmada *et al.*, 2005, Rosario *et al.*, 2013).

While these studies provide some contributions to our knowledge of the regulatory mechanisms of ASCT2, our current state of understanding of the biology and the mechanisms of regulation of human ASCT2 are still unclear due to contradicting findings and by the fact that most

of our data derives either from *in vitro* models or from disease contexts, where the physiology is altered (Palmada *et al.*, 2005, Wang *et al.*, 2015, Avissar *et al.*, 2008, Pingitore *et al.*, 2013, Scalise *et al.*, 2014, Rosario *et al.*, 2013). Hence, the main objective of this study was to fill this gap by identifying and characterizing a cell model which can be used as a tool in increasing my understanding of the biological function of ASCT2 and the proteins contributing to the regulation of the endogenous human isoform of the transporter in a physiologically relevant environment.

I characterized RWPE-1 cells as a physiologically relevant model, suitable for the study of endogenous hASCT2. This cell line represents a novel tool in the context of investigating the expression and functionality of glutamine transporters in the prostate. My findings on the expression, distribution, and functionality of the canonical isoform of the endogenous human ASCT2 (SLC1A5) in RWPE-1 cells correlate with previous studies in other cell lines (Bröer *et al.*, 1999, Bungard and McGivan, 2005, Li *et al.*, 2003, Marin *et al.*, 2003, Pingitore *et al.*, 2013, Scalise *et al.*, 2014, Takahashi *et al.*, 2015, van Geldermalsen *et al.*, 2015, Wang *et al.*, 2015). Most importantly, my functional studies of endogenously expressed ASCT2 provide the first evidence of selective transport by ASCT2 relative to other amino acid transporters in RWPE-1 cells. I have confirmed that hASCT2 is the major contributor to the uptake of glutamine in RWPE-1, reflecting earlier results obtained in C2 cells (Avissar *et al.*, 2008), human hepatocytes, and liver-derived cells (Bode *et al.*, 1995, Bode, 2001). Of great interest was the identification of the individual contribution of B0AT1 and $ATB0^{+}$ to the glutamine transport, which has not been described before and specifically not in RWPE-1 cells. Other transporters involved in maintaining the glutamine homeostasis within RWPE-1 cells were identified in systems N and L. Although it was not possible to distinguish between the uptake of the individual members of these two families, by using information available in various organ-specific gene expression databases, I concluded

that the most probable contributors may be SNAT1 and SNAT2 (system N) and LAT1 and LAT2 (system L). The lack of knowledge on the individual members of these systems limits our understanding of the glutamine homeostasis at the molecular level in the body and more in-depth are needed to shed light on this matter.

As described extensively in chapter 2, studies conducted *in vivo* have shown a correlation between exposure to growth hormones and amino acids uptake (Iannoli *et al.*, 1997, Ray *et al.*, 2003, Ray *et al.*, 2005). My data show that ASCT2-dependent glutamine transport and its cellular distribution can be regulated by extracellular EGF, with no effects on overall protein level within the cell, therefore confirming previous studies on ASCT2 regulation (Avissar *et al.*, 2008, Palmada *et al.*, 2005). This mode of regulation is similar to the modulation of other transporters and receptors by growth factors (Almilaji *et al.*, 2012, Roos *et al.*, 2007, Rosario *et al.*, 2013, Shimizu *et al.*, 2014, Takahashi *et al.*, 2015, Lee *et al.*, 2010, Jones *et al.*, 2012, Ribeiro *et al.*, 2007, Jorissen *et al.*, 2003).

The role of mTOR as a contributor to the regulation of many receptors and transporters has been previously reported (Shojaiefard *et al.*, 2006, Almilaji *et al.*, 2012, Roos *et al.*, 2009, Rosario *et al.*, 2013, Shimizu *et al.*, 2014, Takahashi *et al.*, 2015). Therefore, I hypothesized that the EGF-dependent regulation of endogenous human ASCT2 regulation might occur via mTOR. My data (chapter 3) show that in RWPE-1, the pharmacological inhibition of ASCT2 using GPNA leads to a decrease of mTORC2 activity due to hyper-phosphorylation of the complex protein RICTOR and to an increase of mTORC1 activity as detected through hyper-phosphorylation of p85 S6K1; this finding was unexpected compared with previous findings in which long term inhibition and/or silencing of ASCT2 leading to a decrease of mTORC1 activity (Wang *et al.*, 2014, Wang *et al.*, 2015). Inhibition of mTOR using Torin1 and Xl388, had no effect on the overall glutamine uptake

or the ASCT2-dependent glutamine transport in RWPE-1. This is in contrast with previously published data showing a reduction of mTOR activity due to silencing of ASCT2 (Avissar *et al.*, 2008) and reduction of ASCT2 functionality due to mTOR silencing and inhibition with rapamycin in hepatoma cells (Fuchs *et al.*, 2007).

Therefore, I can not confirm nor deny the possible role of mTOR in the regulation of the EGF-dependent regulation of ASCT2 and, given the complexity and the cross-talk between the two mTOR pathways, further studies are required to better understand this mechanism of regulation. Nevertheless, I may have uncovered a previously undescribed regulation of mTORC2 via the p85 S6K1 isoform in response to ASCT2 inhibition, which could be specific to RWPE-1 cells and may affect the regulation of the trafficking of ASCT2. p85 S6K1 is an overlooked kinase, which was never included amongst the possible targets of mTOR. Few studies have focused on its localization and only recent data have associated this isoform overexpression with enhanced cellular proliferation (Amaral *et al.*, 2016, Ismail *et al.*, 2013, Rosner *et al.*, 2013, Tavares *et al.*, 2015).

Limitations

RWPE-1 cells were chosen for my studies for the characteristics outlined in Chapter 2; however, they are not normal, therefore some of the regulatory mechanism involved in cell metabolism may be altered although in smaller measure, when compared to totally dysregulated systems, such as tumour derived-cell lines. To eliminate this issue, primary cell lines should be used in the future, possibly obtained from the same source to guaranty repeatability.

In determining the mRNA expression of ASCT2, a limiting factor was highlighted by the use of housekeeping genes (HKGs). It has been widely reported that even the expression of such

genes can be altered in response to many treatments. This was overcome by utilizing three of the most stable most stable genes HKGs and normalizing according to Vadesompele and colleagues (2004).

It is important to point out that my Immunofluorescence protocol was not designed to highlight the subcellular localization of ASCT2 other than its distribution at the plasma membrane and general subcellular localization. To obtain further insight, a combination of standard SDS-PAGE immunoblotting of cell lysates fractionated over a sucrose gradient to separate the plasma membrane fraction from the other membranous fractions within the cell (i.e. hASCT2 present in vesicular pools rather than at the membrane) can be used. Also, immunofluorescence with appropriate markers (Early endosomes-GFP dye; lysotracker for lysosomes; ER-tacker from the endoplasmic reticulum…) should be used to determine the proportion of hASCT2 in different cell compartments.

Although an established technique, immunoblot can be limiting when trying to quantify changes in kinase phosphorylation or exact proteins' molecular weights are needed. In the first case, alterative technique could be used in the future to better understand and quantify the changes in kinases' activity in response to changing cellular environment such as in cell kinase assay or crisps. For what concerns antibodies specificity, I am convinced that the antibodies used in my studies are indeed detecting the correct proteins; however, there is a chance of non-specific binding occurring for PDZK1, and this could be why no co-localization between ASCT2 and PDZK1 was detected in RWPE-1. This could be overcome completely by utilizing a more quantitative approach such as high-resolution mass spectrometry.

The use of ASCT2.rbd "antibody" for the detection of the protein results in a highly specific labelling; however, because the ligand binds to ASCT2 facing extracellularly this poses some

technical challenges when utilizing Immunofluorescence. ASCT2 is glycosylated and WGA, membrane stain, binds to the available sugar at the plasma membrane therefore masking the epitope utilized by the ASCT2.rbd ligand to bind to the transporter. This was overcome by labelling for ASCT2 first and then staining the membrane with WGA. However, this caused the loss of some of the membrane stain.

Future work

This dissertation describes very early stages in the understanding of the regulation of ASCT2. Looking forward, to continue this work a more refined and sustained approach to ASCT2 and mTOR inhibition in RWPE-1 cells needs to be developed and compared to a highly cancerogenic sister cell line such as RWPE-3 cells, to better understand the differences occurring in the regulation of ASCT2 and the triggers of the metabolic changes.

Furthermore, studies on the putative regulation of mTORC2 via p85 S6K1 and of the proteins involved in ASCT2's trafficking (Yang *et al.*, 2017), could be used to identify connections between these events and expand our knowledge of the regulation of the transporter (chapter 2).

Additionally, studies on the phosphorylation of ASCT2 could be extremely beneficial to my understanding of the transporter regulation. Some of the most basic regulatory processes are undertaken following de/phosphorylation of target sites present on transporters by kinases such as PKC and PKA, therefore inducing conformational changes, altering protein-protein interactions, activity, sorting and degradation, and trafficking (Mayati et al., 2017, Xu et al., 2017, Grimsrud et al., 2010, Mohapatra et al., 2007, Reyes et al., 2011). For example, the *in-silico* analysis of ASCT2's sequence using both NetPhos 2 and NetPhosK1 prediction servers revealed the presence of phospho-sites for PKA, CaMKII, and CKII (Figure 4.1). Of particular interest is the phosphorylation site for CaMKII, which is a Ca^{2+}/calmodulin regulated kinase.

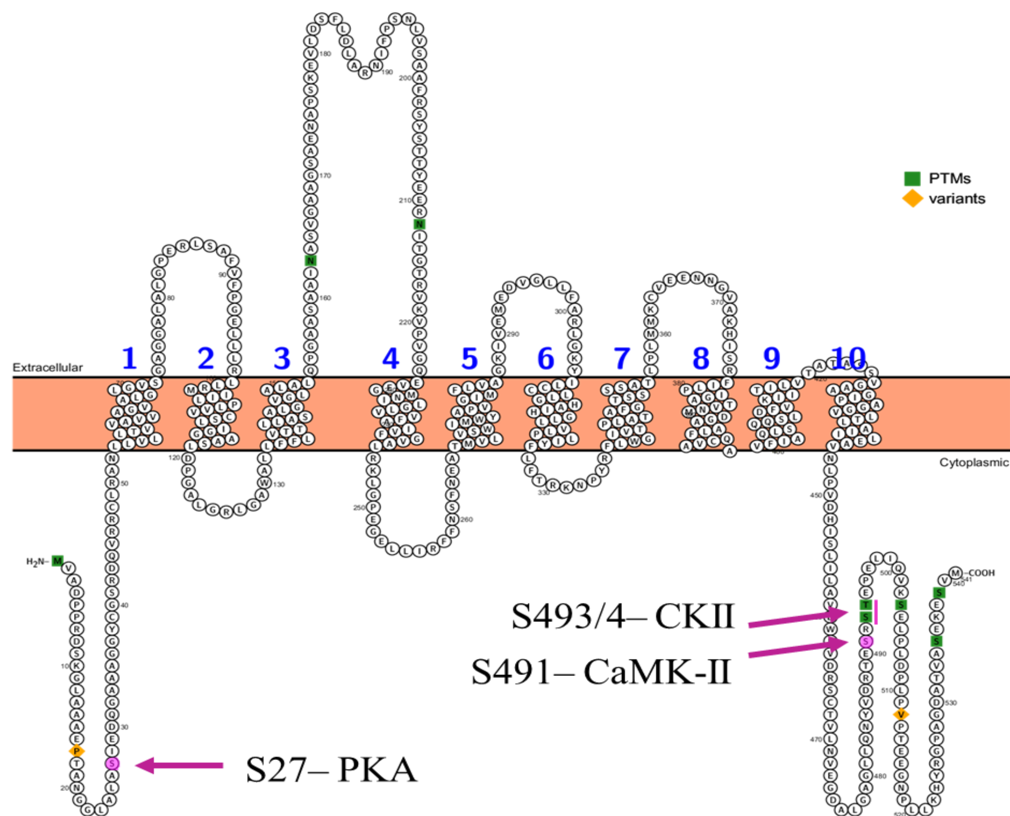


Figure 4.1. Putative hASCT2 topology showing putative predicted phosphorylation sites. hASCT2 putative topology was designed using Protter online tool. (Omasits *et al.,* 2013).

The information obtained from studies of the phosphorylation of ASCT2, will give us a more in-depth understanding on how the cells respond to metabolic changes and EGF growth signalling via ASCT2 regulation, which may include a contributive regulation via mTOR; this could provide interesting potential therapeutic target for cancer and other conditions.

Another knowledge gap in the study of ASCT2 regulation is represented by the lack of information on the ASCT2 interactome. For example, the investigation of the interaction between ASCT2 and human calcineurin B homologous protein 2 (CHP2) (chapter 3) and the localization

of all the regulatory sites necessary for calmodulin, CHP2 and CamKII to regulate hASCT2 in a Ca^{2+}/calmodulin-dependent manner (Figure 4.2)

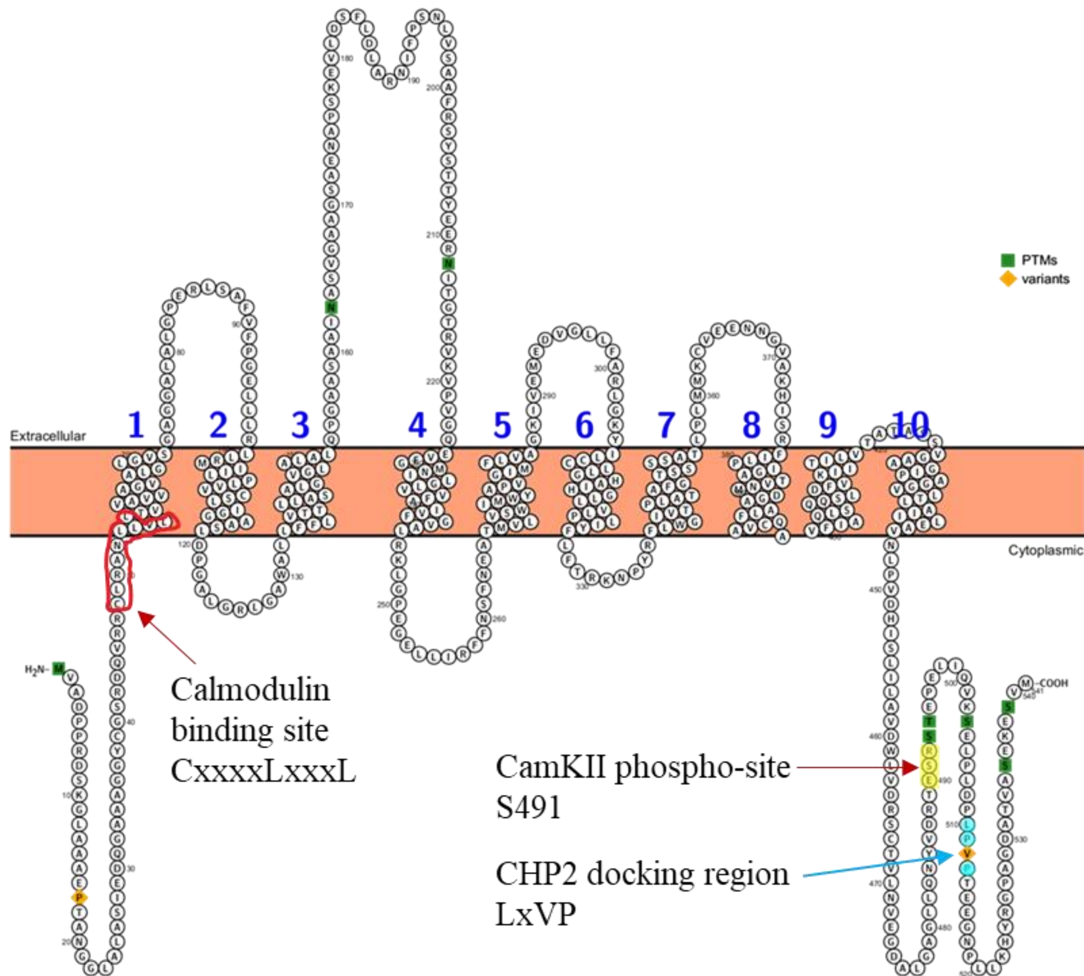


Figure 4.2. Putative hASCT2 topology showing putative docking sites and phosphorylation sites. hASCT2 putative topology was designed using Protter online tool. (Omasits *et al.,* 2013).

Interacting proteins are likely to be involved in the regulation of ASCT2, as described for other transporters such as ENTs (Bicket *et al.*, 2016) and could open a new and very exciting line of research unknown until now for hASCT2.

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Appendix

Abbreviations

- AG1478 – Tyrphostin (EGF-R inhibitor)
- Alexa Fluor 488 – Donkey Anti-Rabbit IgG (H+L) Antibody
- Alexa Fluor 555 – Goat Anti-Mouse IgG (H+L) Antibody
- Alexa Fluor 594 – Donkey Anti-Rabbit IgG (H+L) Antibody
- ASCT2 – Alanine Serine Cysteine Transporter 2
- ATCC – American Type Culture Collection
- BSA – Bovine serum albumin
- Caco-2 – human Colorectal Adenocarcinoma
- Ch^+ – Choline
- Co-IP – Co-immunoprecipitation
- DAPI – 4.6-diamidino-2-phenylindole
- DMEM – Dulbecco modified Eagle's medium
- DoMo – Donkey anti mouse antibody
- DoRb – Donkey anti Rabbit antibody
- EAAC1 – Excitatory amino acid carrier 1
- EBI – European Bioinformatics Institute
- EGF – Epidermal growth factor
- EGFR – Epidermal growth factor receptor
- ENT – Equilibrative nucleoside transporter
- FBS – fetal bovine serum
- FLAG tag – FLAG-octapeptide

- Gln – glutamine
- GoRb – Goat anti Rabbit antibody
- GPNA – L-Glutamic Acid - γ- ρ -Nitroanilide
- HA tag – Hemagglutinin tag
- hASCT2 – Human Alanine Serine Cysteine Transporter 2
- HBSS – Hank's balanced salt solution
- HEK-293 – Human embryonic kidney cells
- HEPG2 – Human hepatocellular carcinoma
- HMBS – Hydroxymethylbilane synthase
- HPRT1 – Hypoxanthine phosphoribosyl transferase 1
- ICC – Immunocytochemistry
- IF – Immunofluorescence
- IP – Immunoprecipitation
- iRNA – RNA interference
- LAT1 - L-type amino acid transporter 1
- MAPK – mitogen activated protein-kinase
- MeAIB – Methil-aminoisoburitic acid
- MesNa – MercaptoEthane Sulfonate sodium
- mRNA – Messenger ribo nucleic acid
- mTOR – Mechanistic target of rapamycin
- MYTH – Membrane yeast Two Hybrid
- NP-40 – (Nonident P-40) octylphenoxypolyethoxyethanol
- O-Benzyl-L-serine – (S)-2-Amino-3-benzyloxypropionic acid

- PBS – Phosphate Buffered Saline
- PDZK1– Na+/H+ exchange regulatory cofactor NHE-RF3
- PFA – Paraformaldehyde
- PI3K – Phosphatidyl inositol 3 kinase
- PKB/Akt – Serine/threonine protein kinase B
- PM – Plasma membrane
- qPCR – Quantitative polymerase chain reaction
- RBD – Retroviral binding domain
- Rho GTPase –Guanosine triphosphate hydrolase
- RT-PCR – Reverse transcriptase polymerase chain reaction
- RWPE-1 – Human epithelial prostate cells
- SDS-PAGE – Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis
- SGK1/3 – Serum and glucocorticoid-regulated kinase 1/3
- SLC1 – Solute carrier 1
- Sulfo-NHS-SS-biotin – sulfosuccinimidyl-2-(biotinamido) -ethyl-1,3'-dithiopropionate
- TBST– Tris Buffered Saline, 0.1% Tween 20
- THLE-3 – Human epithelial liver cells
- TMD – Trans-membrane domain
- Torin1–1-[4-[4-(1-Oxopropyl)-1-piperazinyl]-3-(trifluoromethyl) phenyl]-9-(3-quinolinyl)-benzo[h]-1,6-naphthyridin-2(1H)-one
- Na_3VO_4 – Sodium orthovanadate
- WGA – Wheat germ agglutinin

- XL388 – [7-(6-Amino-3-pyridinyl)-2,3-dihydro-1,4-benzoxazepin-4(5H)-yl]-[3-fluoro-2-methyl-4-(methylsulfonyl)phenyl]-methanone

Solutions and Buffers

- Plate coating: 0.01 mg/mL fibronectin, 0.03 mg/mL bovine collagen type I, and 0.01 mg/mL bovine serum albumin.
- Sodium Uptake buffer: 145mM NaCl, 5mM glucose, 1mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES-Tris and, 3mM K_2HPO_4, pH 7.4.
- Sodium-free uptake buffer: 145mM choline chloride, 5mM glucose, 1mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES-Tris and, 3mM K_2HPO_4, pH 7.4.
- PBS: NaCl 140mM, KCl 2.7mM, KH_2PO_4 1.5mM, and Na_2HPO_4 8.1mM.
- NP-40 lysis buffer: 50mM Tris-HCl, 150mM NaCl, 1% (v/v) nonident P-40 detergent, 10mM Na_3VO_4, and a cocktail of protease and phosphatase inhibitors (Roche) at pH8.
- Transfer buffer: 25mM Tris, 192mM glycine, 20% (v/v) methanol.
- 10X RT buffer: 200mM Tris-HCL, 500mM KCl, (pH8.4).
- Kanamycin 1000x: Kanamycin 50 mg/ml in autoclaved ddH_20.
- Luria-Broth (LB) liquid medium: Bacto-tryptone 1% (w/v), yeast extract 0.5% (w/v), and NaCl 1% (w/v) (pH 7). Autoclaved.
- LB-agar medium: Bacto-tryptone 1% (w/v), yeast extract 0.5% (w/v), and NaCl 1% (w/v) (pH 7). Agar 1.5% (w/v) added. Autoclaved.
- Protein Loading Buffer 5X (PLB 5X): Tris-HCl 60 mM (pH 6.8), glycerol 25% (v/v), SDS 2% (w/v), β-mercaptoethanol 0.05% (v/v), and Bromophenol Blue 0.1% (v/v).
- Running buffer 10X: Tris-HCl 25mM (pH 8.3), glycine 192mM, and SDS 0.1% (w/v).
- TBS 1X: 25mM Tris-HCl, 150mM NaCl (pH 7.5).
- TBST: TBS 1X and Tween-20 0.5% (v/v).