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FLUORESCENCE-BASED IMAGING OF CELLULAR DEFECT IN LYSINURIC PROTEIN INTOLERANCE (LPI)

by

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To my family

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Fluorescence-based imaging of cellular defect in lysinuric protein intolerance (LPI)

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ABSTRACT

Lysinuric protein intolerance is an autosomal recessive disease of Finnish disease heritage characterized by cationic amino acid transport defect in kidney and small intestine epithelium basolateral cell membranes. The primary defect leads to a variety of symptoms, such as failure to thrive, osteoporosis, growth failure, nausea and postprandial hyperammonemia.

LPI gene *SLC7A7* (solute carrier family 7, member 7) codes for y^+LAT1, the light chain of basolateral transporter for cationic amino acids, which dimerizes with the heavy subunit 4F2hc. Today, over 50 LPI-causing mutations have been identified in *SLC7A7* gene.

In this study, a selection of LPI causing mutations and nine C-terminal truncating deletions of y^+LAT1 were expressed and the resulting transporters were visualized in mammalian cells using GFP (green fluorescent protein) fusions of y^+LAT1. The results on the LPI mutants confirmed the observations obtained from non-mammalian system: y^+LAT1 requires the 4F2hc for membrane trafficking. Equally to wild type transporter, the point mutant G54V is localized to the plasma membrane, but the frameshift and nonsense mutant transporters remain cytoplasmic. In contrast to these observations, the truncated constructs lacking less than or equal to 50 amino acids also localized to the plasma membrane.

To study, whether the reason for the trafficking defect is the inability of frameshift mutant y^+LAT1 to dimerize with 4F2hc, fluorescence resonance energy transfer (FRET) method was utilized. ECFP (cyan) and EYFP (yellow) fusion proteins of the heterodimer subunits were expressed in mammalian cells, and FRET was measured using flow cytometry (FACS) FRET. When mutant y^+LAT1 protein interaction with 4F2hc was analyzed using FACS-FRET, all the studied mutants dimerized at equal efficiency. Thus, the inability of 4F2hc to interact with mutant y^+LAT1 proteins is not causing the trafficking defect.

Throughout the series of experiments, all the mutant y^+LAT1 proteins were expressed in lower rate compared to wild type y^+LAT1. The frameshift and nonsense mutant y^+LAT1 protein expressing cells had higher mortality than non-expressing cells in the same sample. In contrast, wild type and G54V point mutant positive cells had lower mortality, demonstrating the different effects of the mutant proteins on cell viability and proliferation independently or via the interaction with 4F2hc.

The LPI_{Fin} *SLC7A7* mRNA levels did not differ significantly from wild type mRNA levels in fibroblasts and lymhoblasts. The analysis of the *SLC7A7* promoter suggested regulatory elements in the 5' non-coding region as well as in the first two exons.

The most significant factor in LPI pathogenesis is the primary amino acid transport failure, causing processes dependent on its' substrates to function deficiently. However, the clinical significance of the mistargeting of the y^+LAT1/4F2hc complex needs further studies.

Keywords: lysinuric protein intolerance, amino acid transporter, GFP (green fluorescent protein), protein localization, FRET (fluorescence resonance energy transfer), dimerization

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Fluoresenssiperusteiset kuvantamismenetelmät lysinurisen proteiini-intoleranssin (LPI) soluhäiriön tutkimuksessa
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TIIVISTELMÄ

Lysinurinen proteiini-intoleranssi on suomalaiseen tautiperintöön kuuluva autosomaalisesti peittyvästi periytyvä sairaus, jonka aiheuttaa kationisten aminohappojen kuljetushäiriö munuaisten ja ohutsuolen epiteelisolujen basolateraalikalvolla. Aminohappojen kuljetushäiriö johtaa moniin oireisiin, kuten kasvuhäiriöön, osteoporoosiin, immuunijärjestelmän häiriöihin, oksenteluun ja runsasproteiinisen ravinnon nauttimisen jälkeiseen hyperammonemiaan.

LPI-geeni *SLC7A7* (solute carrier family 7 member 7) koodaa y^+LAT1 proteiinia, joka on basolateraalinen kationisten ja neutraalien aminohappojen kuljettimen kevyt ketju, joka muodostaa heterodimeerin raskaan alayksikön 4F2hc:n kanssa. Tällä hetkellä *SLC7A7*-geenistä tunnetaan yli 50 LPI:n aiheuttavaa mutaatiota.

Tässä tutkimuksessa erityyppisiä y^+LAT1:n LPI-mutaatiota sekä yhdeksän C-terminaalista polypeptidiä lyhentävää deleetiota kuvannettiin nisäkässoluissa y^+LAT1:n GFP (green fluorescent protein) -fuusioproteiineina. Tulokset vahvistivat muissa soluissa tehdyt havainnot siitä, että 4F2hc on edellytyksenä y^+LAT1:n solukalvokuljetukselle, G54V-pistemutantti sijaitsee solukalvolla samoin kuin villityyppinen proteiini, mutta lukukehystä muuttavia ja proteiinia lyhentäviä mutantteja ei kuljeteta solukalvoon. Lisäksi havaittiin, että poikkeuksena tästä säännöstä ovat y^+LAT1-deleetioproteiinit, joista puuttui korkeintaan 50 C-terminaalista aminohappoa. Nämä lyhentyneet kuljettimet sijaitsevat solukalvolla kuten villityyppiset ja LPI-pistemutanttiproteiinit.

Dimerisaation osuutta kuljetushäiriön synnyssä tutkittiin käyttämällä fluorescence resonance energy transfer (FRET) menetelmää. Heterodimeerin alayksiköistä kloonattiin ECFP (cyan) ja EYFP (yellow) fuusioproteiinit, joita ilmennettiin nisäkässoluissa, ja FRET mitattiin virtaussytometri-FRET -menetelmällä (FACS-FRET). Tutkimuksissa kaikkien mutanttien havaittiin dimerisoituvan yhtä tehokkaasti. Kuljetushäiriön syynä ei siten ole alayksiköiden dimerisaation estyminen mutaation seurauksena.

Tutkimuksessa havaittiin, että kaikki mutantti-y^+LAT1-transfektiot tuottavat vähemmän transfektoituneita soluja kuin villityyppisen y^+LAT1:n transfektiot. Solupopulaatioissa, joihin oli tranfektoitu lukukehystä muuttava tai stop-kodonin tuottava mutaatio havaittiin suurempi kuolleisuus kuin saman näytteen transfektoitumattomissa soluissa, kun taas villityyppistä tai G54V-pistemutanttia tuottavassa solupopulaatiossa oli pienempi kuolleisuus kuin saman näytteen fuusioproteiinia ilmentämättömissä soluissa. Tulos osoittaa mutanttiproteiinien erilaiset vaikutukset niitä ilmentäviin soluihin, joko suoraan y^+LAT1:n tai 4F2hc:n kautta aiheutuneina.

LPI_{Fin} *SLC7A7* lähetti-RNA:n määrä ei merkittävästi poikennut villityyppisen määrästä fibroblasteissa ja lymfoblasteissa. *SLC7A7*:n promoottorianalyysissä oli osoitettavissa säätelyalueita geenin 5' ei-koodaavalla alueella sekä ensimmäisten kahden intronin alueella.

LPI-taudin tautimekanismin kannalta keskeisin tekijä on kuitenkin aminohappokuljetuksen häiriö, jonka vaikutuksesta näistä aminohapoista riippuvaiset prosessit elimistössä eivät toimi normaalisti. Havaittu virheellinen y^+LAT1/4F2hc kuljetuskompleksin sijainti edellyttää lisätutkimuksia sen mahdollisen kliinisen merkityksen selvittämiseksi.

Avainsanat: Lysinurinen proteiini-intoleranssi, aminohappokuljetin, GFP (green fluorescent protein), proteiinin sijainti, FRET (fluorescence resonance energy transfer), dimerisaatio

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LIST OF ORIGINAL PUBLICATIONS

This doctoral thesis is based on the following original articles referred to in the text by their Roman numerals I-III

I Toivonen M, Mykkänen J, Aula P, Simell O, Savontaus ML, Huoponen K. Expression of normal and mutant GFP-tagged y^+L amino acid transporter-1 in mammalian cells. Biochem Biophys Res Commun. 2002 Mar 15;291(5):1173-9.

II Minna Toivonen, Maaria Tringham, Johanna Kurko, Perttu Terho, Olli Simell, Kaisa M. Heiskanen and Juha Mykkänen. Interactions of y^+LAT1 and 4F2hc in the y^+l amino acid transporter complex: consequences of lysinuric protein intolerance-causing mutations Gen Physiol Biophys. 2013 Aug 12; 4, Vol. 32: 479-488

III Juha Mykkänen, Minna Toivonen, Maaria Kleemola, Marja-Liisa Savontaus, Olli Simell, Pertti Aula and Kirsi Huoponen: Promoter analysis of the human *SLC7A7* gene encoding y^+L amino acid transporter-1 (y^+LAT1). Biochem Biophys Res Commun. 2003 Feb 21;301(4):855-61.

In addition, some unpublished data have been included in this thesis.

ABBREVIATIONS

(h)4F2hc	(human) surface antigen 4F2 heavy chain
aa	amino acid
AARE	amino acid response element
AGT-1	aspartate-glutamate transporter 1
AM	alveolar macrophage
APC	amino acid –polyamine –choline
asc-1	system asc amino acid transporter 1
$B^{0,+}$AT-1	system B amino acid transporter 1
BBB	blood brain barrier
$b^{0,+}$AT	system $b^{0,+}$ amino acid transporter
bp	base pair
BP	band pass detector
CAT	cationic amino acid transporter
cDNA	complementary deoxyribonucleic acid
CFP	cyan fluorescent protein
cRNA	complementary ribonucleic acid
CMV	cytomegalovirus
Cys	cysteine
ED	ectodomain
E_F	FRET efficiency
EG/C/YFP	enhanced green/cyan/yellow fluorescent protein
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
FDH	Finnish disease heritage
FRAP	fluorescence recovery after photobleaching
FRET	fluorescence resonance energy transfer (also: Förster resonance energy transfer)
GFP	green fluorescent protein
GHD	growth hormone deficiency
HAT	heteromeric amino acid transporter
HEK293	human embryonic kidney cell line 293
HSHAT	heavy subunit of heteromeric amino acid transporter
Igf1	insulin-like growth factor 1
Igfbp 1	insulin-like growth factor 1 binding protein 1
IgG	immunoglobulin class G
IUGR	intra-uterine growth restriction
kDa	kilo Dalton

LAT1(2)	system L amino acid transporter 1 (2)
LPI	lysinuric protein intolerance
LPI_{Fin}	Finnish founder mutation c.895-2A>T; p.Thr299IlefsX10
LSHAT	light subunit of heteromeric amino acid transporter
MDCK	Madin-Darby canine kidney -cell line
MLPA	multiplex ligation probe amplification
OMIM	Online Mendelian Inheritance in Man
OTC	ornithine transcarbamylase
PAP	pulmonary alveolar proteinosis
PCR	polymerase chain reaction
PI	propidium iodide
PIA	primary inherited aminoaciduria
rBAT	related to $b^{0,+}$ amino acid transporter
SBT	spectral bleed-trough
SLC3	solute carrier family 3
SLC7	solute carrier family 7
Ste T	serine-threonine exchanger transporter
SPMR1	Schistosome permease 1
TAT-1	T-type amino acid transporter 1
TEM	transmission electron microscopy
TMD	trans-membrane domain
YFP	yellow fluorescent protein
y^+LAT1 (2)	system y^+L amino acid transporter 1 (2)
xCT	system x_c^- amino acid transporter
Å	Ångström; 1nm=10Å

1. INTRODUCTION

Lysinuric protein intolerance (LPI), a disorder that belongs to the Finnish disease heritage, is an autosomal recessive defect of cationic amino acid transport in the small intestine and kidney tubule epithelium. The symptoms of LPI include intolerance to dietary protein, leading to spontaneous protein aversion, failure to thrive, poor growth rate, hepatosplenomegaly and hyperammonemic episodes after high protein ingestion. The plasma concentration of lysine, arginine and ornithine is low, whereas the excretion of these amino acids is increased to the urine. LPI is a rare disease, with some 50 patients to date in Finland and approximately 250 patient diagnosed worldwide.

Lauteala *et al.* (Lauteala *et al.* 1997b) mapped the LPI locus to the long arm of chromosome 14 using linkage analysis in Finnish LPI families and excluded known cationic amino acid transporter genes as LPI candidates. The underlying molecular defect of LPI was discovered by Torrents *et al.* (Torrents *et al.* 1998), who reported a novel cationic amino acid transporter y^+LAT1, which associates with 4F2 heavy chain. Subsequently in 1999 Torrents *et al.* (Torrents *et al.* 1999) also reported LPI specific mutations in the *SLC7A7* gene coding for the transporter y^+LAT1. Of the 61 LPI-causing mutations reported in the literature, 14 have been functionally verified *in vitro* (Borsani *et al*. 1999, Torrents *et al*. 1999, Mykkänen *et al*. 2000, Sperandeo *et al*. 2000, Sperandeo *et al*. 2005a, Sperandeo *et al*. 2005b), and the subcellular localization of seven has been analyzed in *Xenopus laevis* oocytes or MDCK cells (Mykkänen *et al*. 2000, Sperandeo *et al*. 2005b).

The current study was aimed at identifying methods to visualize the cellular defect in LPI and to study the target tissue expression levels and regulation of y^+LAT1. The project started with localization studies of y^+LAT1 using green fluorescent protein (GFP) tagged y^+LAT1 and 4F2hc fusions in mammalian cells. It was further expanded to characterize the observed mutant y^+LAT1 targeting defect by FRET analysis and the effect of truncated mutant protein expression effect on y^+LAT1/4F2hc transporter subunit recognition. The regulation of *SLC7A7* gene was studied by identifying epithelial cell specific transcription factor binding sites.

2. REVIEW OF THE LITERATURE

2.1 Lysinuric protein intolerance

2.1.1 General aspects of LPI

LPI (OMIM #222700), also named hyperdibasic aminoaciduria type 2 or familial protein intolerance is an autosomal recessive disorder in which the absorption of cationic amino acids in the intestine and their reabsorption in the kidney tubules is defective. In 1965, Perheentupa and Visakorpi described a new inborn error of amino acid metabolism in three familial Finnish patients who all were suffering from dietary protein intolerance and deficient transport of basic amino acids (Perheentupa, Visakorpi 1965). The patients all suffered from vomiting and diarrhea appearing at weaning, refused to eat animal proteins, had growth failure and had had hyperammonemic episodes. In early 1980's the transport defect in LPI was shown to be located basolaterally at the epithelium of small intestine and kidney tubuli both *in vitro* (Desjeux *et al*. 1980) and *in vivo* (Desjeux *et al*. 1980, Rajantie, Simell & Perheentupa 1981). In the work by Desjeux *et al*. the defect was detected as reduction of net influx of lysine in jejunal biopsies. Rajantie and coworkers discovered that after an orally administrated dose of lysine-glycine dipeptide, the plasma concentration of glycine increased in LPI patients whereas the lysine concentration remained low. Thus, the luminal intake of the dipeptide is intact, but the basolateral export of monomeric lysine is affected in LPI (Rajantie, Simell & Perheentupa 1981).

Approximately 250 LPI patients have been reported to date (Norio 2003b), of which ~50 are of Finnish origin; thus, LPI belongs to the Finnish disease heritage along with some 40 other monogenic, mostly autosomally recessively inherited disorders (Norio 2003b). In Finland, the incidence of LPI is around 1:60 000 newborns (Simell 2001) with carrier frequencies varying between 1:194 in Helsinki and 1:91 in Oulu region (Pastinen *et al.* 2001). Sporadic cases have been reported worldwide, but clustering of LPI is reported in two geographical regions. In Northern Japan in the Iwate area the local LPI incidence reaches 1 in 57 000 newborns with a carrier frequency of 1:119 of the founder mutation c.1228C>T (Koizumi *et al.* 2000). Also, several families with LPI have been identified in a restricted region in Southern Italy (Borsani *et al.* 1999). However, it has been suggested, that the disease is mis- or underdiagnosed due to the variable phenotype (Ogier de Baulny, Schiff & Dionisi-Vici 2012, Sperandeo, Andria & Sebastio 2008), which may contribute to the generally low prevalence.

2.1.2 LPI as a primary inherited aminoaciduria (PIA)

LPI belongs to the group of primary inherited aminoacidurias (PIAs, Table 1), conditions in which the aminoaciduria is caused by a defect in amino acid transporters rather than as an aberrant end product of defective metabolic pathways, such as for example phenylketonuria (PKU). Other PIA conditions include cystinuria (OMIM #220100), hyperdibasic aminoaciduria type 1 (OMIM #222690), Hartnup disorder (OMIM #234500), renal familial iminoglycinuria (OMIM #242600) and dicarboxylic aminoaciduria (OMIM #222730) (Palacin *et al.* 2005) (Figure 1). The most common PIA is cystinuria with the highest incidence in Libyan Jews (1: 2500) and the lowest in Swedes (1:100 000) (Barbosa *et al.* 2012).

LPI and cystinuria, in which the disease gene and multiple pathogenic mutations of the corresponding genes have been identified, are disorders linked to heteromeric amino acid transporters, HATs. HATs are heterodimeric amino acid transporters comprised of a heavy subunit, HSHAT of ~80 aa and a light subunit LSHAT of ~40 aa, covalently linked via a cysteine bond (Pfeiffer *et al.* 1998). Together, the seven currently known LSHAT transporters form the solute carrier family 7 (SLC7).

The genes mutated in Hartnup disorder (*SLC6A19*) (Kleta *et al.* 2004, Seow *et al.* 2004) and dicarboxylic aminoaciduria (*SLC1A1*) (Peghini, Janzen & Stoffel 1997, Smith *et al*. 1994, Bailey *et al*. 2011) belong to solute carries families 6 and 1, respectively, and associating transporter systems (B^0 and X^-_{AG}, respectively) have been identified. In the case of dicarboxylic aminoaciduria direct mutation evidence in humans is still lacking (Palacin *et al*. 2005, Verrey *et al*. 2004), but *SLC1A1* null-knockout mice present phenotype identical to the human disease (Peghini, Janzen & Stoffel 1997). The transport system underlying the autosomal dominant hyperdibasic aminoaciduria type 1 remains to be found along with the gene involved in the disorder.

Table 1. Primary inherited aminoacidurias (PIAs). AD-autosomal dominant; AR-autosomal recessive.

PIA	MIM	Gene	Transporter	Reported mutations	Transport system	Mode of inheritance	Prevalence
Cystinuria type A	220100	*SLC3A1*	rBAT	142	$b^{0,+}$	AR	1:7000
Cystinuria type B		*SLC7A9*	$b^{0,+}$AT	103	$b^{0,+}$	AD	
Hyperdibasic aminoaciduria I	222690	-	-		-		
Lysinuric protein intolerance	222700	*SLC7A7*	y^+LAT1	53	y^+L	AR	
Hartnup disorder	234500	*SLC6A19*	B^0AT-1	24	B^0	AR	1:30 000
Iminoglycinuria, digenic	242600	*SLC6A20*	XT3	1	IMINO	AR	
Iminoglycinuria, digenic		*SLC36A2*	PAT2	2			
Dicarboxylic aminoaciduria	222730	*SLC1A1*	EAAT3	2	X^-_{AG}	AR	

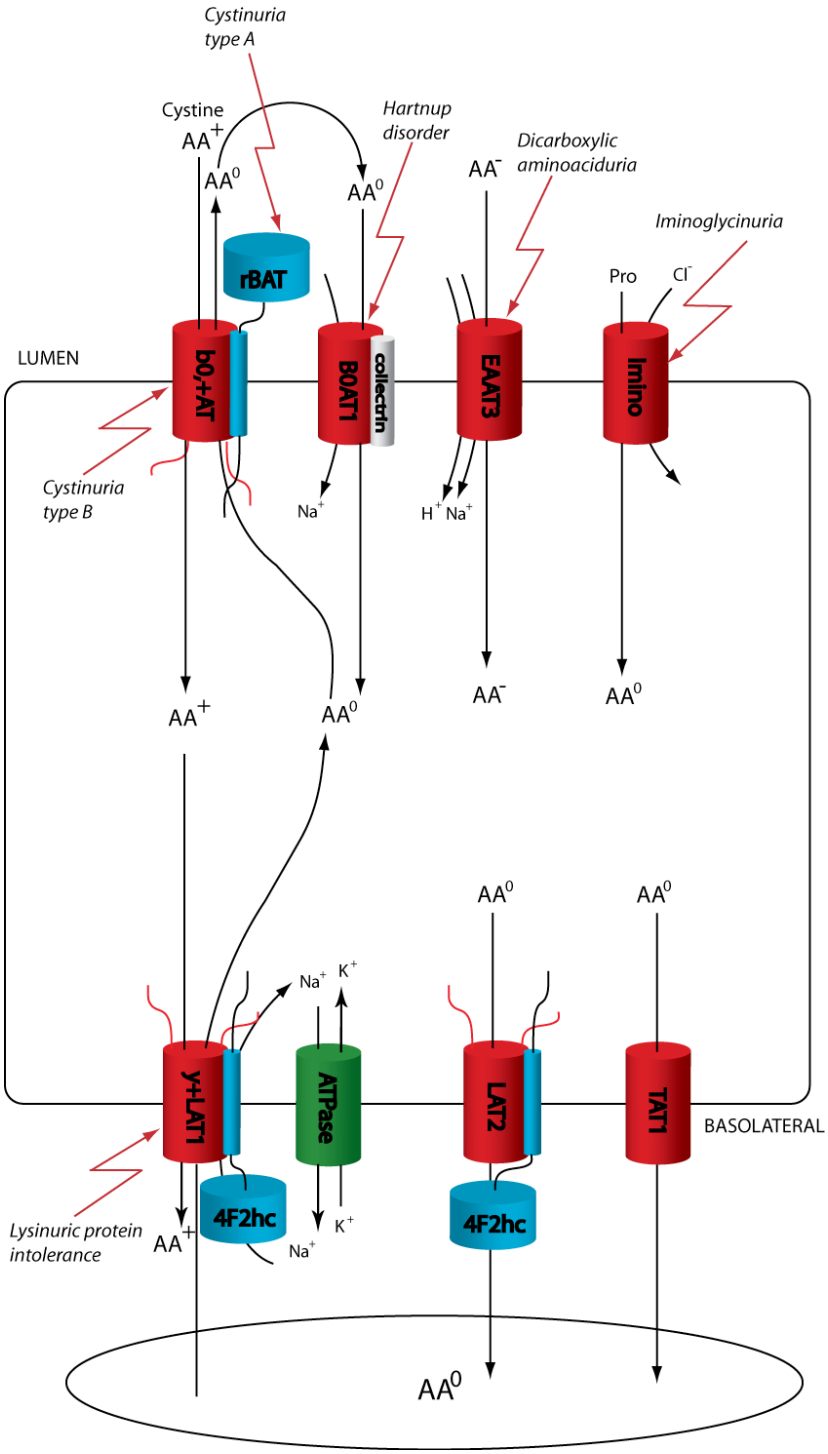

Figure 1. Primary inherited aminoacidurias (PIAs) and their associated transporters in epithelial cells. AA0: neutral amino acids; AA+: cationic amino acids; AA-: anionic amino acids. B0+AT1 auxiliary protein collectrin is present in the kidney and is replaced by ACE2 in the intestine. Imino: the transport system rather than the two separate transporters is depicted for clarity.

2.1.3 Clinical picture and treatment

Patients with LPI are normal at birth and during early development. The first symptoms, nausea, vomiting and diarrhea typically appear after weaning when the dietary protein intake is increased. The LPI infants thrive poorly, manifest muscle hypotonia and very often develop spontaneous aversion to protein rich foods at young age. The liver and spleen are enlarged and skeletal maturation is delayed (Simell 2001).

The poor growth rate commonly leads to short adult stature. The growth failure, osteopenia and osteoporosis are likely to be at least partly consequent of protein deprivation and deficiency of cationic amino acids due to both restricted diet and protein malabsorption (Parto *et al.* 1993a). The chronic deficiency of lysine alone inhibits protein synthesis and thus manifests as slowing of bone growth leading to general growth failure and osteoporosis (Svedstrom *et al.* 1993). In an LPI patient, severe growth failure associated with LPI was connected to growth hormone deficiency (GHD). The patient responded well to growth hormone replacement therapy. However, the study failed to confirm whether GHD is generally a feature of LPI phenotype or only coincidentally associated in the single patient (Esposito *et al.* 2006). More recently in a study by Niinikoski and coworkers, growth hormone therapy was reported to benefit the group of LPI patients in improved growth even though three patients in the group of four had normal growth hormone levels (Niinikoski *et al.* 2011). In addition, two earlier studies reported normal GH secretion in LPI patients (Awrich *et al*. 1975, Goto, Yoshimura & Kuroiwa 1984), suggesting the protein malnutrition as a cause for the growth failure in LPI.

After ingestion of protein rich food LPI patients suffer from hyperammonemia, manifesting from mild drowsiness to hyperammonemic coma. However, hyperammonemic crises are rare in the patients, possibly due to the spontaneous protein aversion that protects them from excess intake of dietary protein. The mental development of LPI patients is usually normal, but recurrent or prolonged hyperammonemic episodes may cause damage to the central nervous system and affect mathematical and other cognitive skills (Simell 2001). The mechanism underlying hyperammonemia is the functional deficiency of urea cycle intermediates arginine and ornithine (Palacin *et al*. 2004, Sebastio, Sperandeo & Andria 2011); the urea cycle enzyme activities in LPI patients are at normal levels (Kekomäki, Räihä & Perheentupa 1967). LPI patients are predisposed, especially in childhood, to a severe complication, namely pulmonary alveolar proteinosis (PAP). In PAP proteinous material accumulates in the lung alveoli along with abnormal macrophages causing a potentially lethal acute pulmonary insufficiency. PAP can also be associated with life threatening multiple organ dysfunction. The exact mechanisms leading to the development of PAP are currently unknown (Parto *et al.* 1993b). In a study done on patients with interstitial lung disease Rotoli and coworkers found system y^+L to operate most of arginine influx in alveolar macrophages (AM). Both y^+LAT1 and y^+LAT2 are expressed in AM, but y^+LAT1 is the predominant isoform. Since PAP, as a complication of LPI, is often associated with AM

impairment, y^+LAT1 activity loss in human AM might contribute to the pathogenesis of PAP in LPI (Rotoli *et al.* 2007).

The LPI patients' immune response functions inefficiently against infections and vaccinations, and the patients suffer from recurrent and more severe common viral and bacterial infections compared to their non-affected siblings. LPI patients have subnormal serum IgG3 and IgG4 suggesting an impaired humoral immune response. The low serum concentration of IgG3 leads to the decreased production of antibodies against vaccines. Since the neutralization of viruses and antibody-dependent cellular cytotoxicity in viral infections is dependent on IgG3, viral infections may be complicated and prolonged in LPI patients. Especially, patients have developed severe, generalized varicella infections, resembling those of immunocompromised patients (Lukkarinen *et al.* 1999).

In LPI, the laboratory findings include markedly increased excretion of lysine and moderate over-excretion of arginine and ornithine in the urine, while the plasma concentrations of the same amino acids are normal to subnormal. Hyperammonemia and orotic acid uria occur after dietary protein load due to malfunction of the urea cycle, caused by functional deficiency of arginine and ornithine. In addition to the abnormal amino acid concentrations and markers of urea cycle dysfunction, the patients often have increased plasma concentrations of cholesterol and triglycerides. The combined hyperlipidemia can only partly be explained by the high carbohydrate and fat diet. The highest triglyceride concentrations were associated with renal failure (Tanner *et al.* 2010).

All the diagnosed Finnish LPI patients are on low protein diet to control their blood ammonia levels. The medical treatment in LPI focuses on avoiding hyperammonemia by oral citrulline supplementation dosed according to patients' protein intake. Citrulline is a neutral amino acid that is efficiently absorbed and is further metabolized into ornithine and arginine by the urea cycle enzymes in the hepatocytes. The increased efficiency of the urea cycle prevents the development of hyperammonemia and helps the patients to tolerate more dietary protein and thus avoid unnecessary strict protein restriction and receive at least near adequate protein amount in their daily nutrition. However, the otherwise beneficial citrulline therapy fails to normalize the growth rate of the patients: even though the protein tolerance is improved, citrulline therapy does not correct the lysine deficiency (Simell 2001). The explanation for the growth failure is unclear, but most of the citrulline treated children have developed renal insufficiency. This may contribute to growth failure, even though the growth failure is often diagnosed before the symptoms of impaired renal function appear [Lukkarinen *et al.* (unpublished)].

Intravenous infusion of L-lysine caused a transient increase in plasma lysine concentration without signs of hyperammonemia or increased orotic acid excretion, probably due to sufficient citrulline supplementation to preserve urea cycle function (Lukkarinen *et al.* 2000). Also, low

dose oral L-lysine-HCl (0.05 mmol/kg) supplementation succeeded in normalizing the patients' plasma lysine concentrations without induction of hyperammonemia (Lukkarinen *et al.* 2003). Following these results, the well-tolerated treatment was started for most of the Finnish LPI patients and, after a follow up of six to 60 months of lysine supplementation, the plasma lysine concentrations of the patients were improved. The long-term effects of the therapy for example on the growth are difficult to estimate and thus remain unclear (Tanner *et al.* 2007).

2.1.4 The LPI gene

The LPI gene locus was mapped to chromosome 14q11 using linkage studies in 11 Finnish LPI families (Lauteala *et al.* 1997a). Torrents and coworkers (1998) isolated the LPI gene *SLC7A7* in the mapped chromosomal region. The gene codes for a cationic amino acid transporter subunit y^+LAT1 (y^+L amino acid transporter 1). The described *SLC7A7* cDNA was 2245 bp long and with a 1536 bp open reading frame coding for a 511 amino acid polypeptide. The hydrophobicity analysis of the predicted protein revealed a typical transporter or pore structure with 12 putative transmembrane domains. Due to the chromosomal localization of the *SLC7A7* gene to the previously mapped LPI locus, and functional identification of the gene product y^+LAT1 as a transporter of cationic amino acids, *SLC7A7* was suggested as a candidate gene for LPI (Torrents *et al.* 1998).

The discovery of LPI specific mutations in *SLC7A7* and the functional testing of the LPI mutant y^+LAT1s confirmed *SLC7A7* as the LPI gene (Torrents *et al.* 1999, Borsani *et al.* 1999). The *SLC7A7* gene consists of 11 exons, the first two of which are not translated into the protein (Noguchi *et al*. 2000, Mykkänen *et al*. 2000, Sperandeo *et al*. 2000) (GenBank original reference: NM_003982.3, updated reference: NM_001126106). Currently, 57 individual *SLC7A7* mutations have been reported in LPI patients according to HGMD® Professional 2013.1 database. In addition, at least two unpublished LPI mutations have been found in patients of European origin (Virpi Laitinen, personal communication).

2.1.4.1 The Finnish founder mutation, LPI_{Fin}

A prominent feature of the LPI mutation spectrum is the Finnish founder mutation, LPI_{Fin}, which is detected homozygous in all Finnish LPI patients. The LPI_{Fin} mutation is named 1181-2A>T, IVS6-2A-T and 1136-2A>T in the literature**.** According to HGVS nomenclature (den Dunnen, Antonarakis 2001), the mutation is described in cDNA and protein level as follows: c.895-2A>T; p.Thr299IlefsX10. LPI_{Fin}, located at the 3' end of intron 6 is a point mutation of the splicing acceptor site AG to TG, a substitution which destroys the conserved splicing signal. The mutation results in cryptic splicing at 10 bp downstream of the following exon 7, causing a 10 bp deletion in the coding sequence. This results in frameshift and a premature stop codon after ten amino acid residues (Torrents *et al.* 1999, Borsani *et al.* 1999). The LPI_{Fin} mutation, present in all Finnish LPI chromosomes, has only been found

in homozygous state in one non-Finnish LPI patient of known Finnish ancestry, resident in northern Norway (unpublished data). The LPI_{Fin} carrier frequency is estimated to be 1:125 in the early settlement areas of Finland, *i.e.* southern and south-western parts (Lauteala *et al.* 1997a, Lauteala *et al.* 1997b) or even 1: 194 (Pastinen *et al.* 2001), but higher in late settlement areas of eastern and northern parts of Finland (1:91-1:94), which were populated by settlers from southern parts of Savo in the 17th century (Pastinen *et al.* 2001).

2.1.4.2 The non-Finnish LPI mutations

Today, LPI cases have been reported in over 20 countries worldwide. The non-Finnish LPI mutations are variable in both position and type, including amino acid substitutions, truncating mutations as well as larger rearrangements resulting in missing exons (Sperandeo, Andria & Sebastio 2008). Despite the fact that most non-Finnish LPI mutations are unique, a local cluster of LPI cases in Iwate region in northern Japan shows a mutational founder effect. The nonsense mutant p.Arg410Ter is found homozygous in five families and a mass screening of the newborns revealed a mutation carrier rate of 1:119 in the area. According to the original article in year 2000, the p.Arg410Ter mutation had not been found outside of the Tohoku region or in the Caucasian population (Koizumi *et al.* 2000). Coincidentally, the same mutation has been found as a heterozygous mutation in a French LPI patient (unpublished). Furthermore, another LPI cluster is located in southern Italy, where 12 LPI mutant alleles are reported from 18 families. The founder mutation c.1384_1385insACTA was found as a homozygous mutation in five patients from four families originating from a single defined region. Also, the p.Trp242Ter mutation is enriched locally and is found homozygous in four families (Borsani *et al.* 1999, Sperandeo *et al.* 2000, Sperandeo *et al.* 2007). Thus, the local LPI clusters show some mutational founder effect, but not as extensively as in the Finnish LPI cohort, where a single disease allele contributes to all LPI chromosomes.

Using the MLPA method (multiplex ligation probe amplification) Font-Llitjós and coworkers (Font-Llitjós *et al.* 2009) detected five large genomic rearrangements of the *SLC7A7* genomic region, which represent a new mutation mechanism among the LPI mutations reported so far. The two largest deletions, spanning from exon 4 to exon 11 and exon 6 to exon 11, respectively, originate from crossing-over events between two common *Alu* repeats, located at intron 3 and 3' UTR or intron 5 and 3' UTR, respectively. The mutant transcripts lack both 3' UTR and poly-A signals most likely leading to rapid degradation of the mRNA. Consistent with the explained mutant mRNA depletion hypothesis, these gross deletions were associated with the most severe phenotypes in the study cohort. However, mutations leading to premature stop codon, namely c.625+1G>C and p.Arg468Ter, causing nonsense mediated mRNA decay, are associated with generally less severe phenotypes. Two frameshift mutations, c.1185_1188delTTCT and c.820dupT result also in milder phenotypes, suggesting a residual transport activity. The mildest phenotype in the study was described from a patient bearing three heterozygotic polymorphisms, but the possibility of an existing intronic LPI mutation

remained open. Thus, the described mutations provide further material for the ongoing studies on LPI pathophysiology, but fail to constitute a genotype-phenotype correlation in the disease. Generally, specific *SLC7A7* mutations do not lead to specific clinical manifestations as the patients in the LPI clusters carrying identical mutations (Finland, northern Japan and southern Italy) show high variability in phenotypes that has not been explained by genetic or environmental factors (Torrents *et al*. 1999, Mykkänen *et al*. 2000, Borsani *et al*. 1999).

2.1.5 Functional defect of y⁺LAT1 in LPI

The functional studies on *SLC7A7* mutations causing LPI are based on myc-tagged y⁺LAT1 coding cRNA microinjected in *X. laevis* oocytes (Mykkänen *et al*. 2000, Torrents *et al*. 1999, Sperandeo *et al*. 2005b, Sperandeo *et al*. 2005a). All the studied mutations so far (p.Met1Leu, p.Glu36del, p.Met50Lys, p.Gly54Val, p.Thr188Ile, p.Trp242Ter, p.Leu334Arg, p.Ser386Arg, p.Tyr457Ter, p.Phe335LeufsX15, p.Ser396LeufsX122, p.Pro421ArgfsX98, p.Thr299IlefsX10) with the exception of p.Phe152Leu completely failed to induce cationic amino acid transport in injected cells. The p.Phe152Leu mutation was detected heterozygous in a pediatric patient together with the p.Glu36del mutation in the other allele. The p.Phe152Leu expression resulted in moderately reduced transport activity and the transporter was localized in the basolateral membrane of MDCK cells. A more benign effect was observed in other LPI mutations, maybe due to the fact that Phe152 is not conserved in evolution but it is replaced by leucine in some mammals and glycine in other vertebrates, indicating a non-critical position in the polypeptide. Despite the residual transport activity induced by the p.Phe152Leu mutant, the patient had classical LPI features, indistinguishable from patients with totally abolished y⁺LAT1 transport activity (Sperandeo *et al.* 2005b).

The subcellular localization of mutated y⁺LAT1 protein is determined by mutation type. Frameshift mutations and nonsense mutations generating a putative truncated polypeptide have intracellular localization, whereas point mutations and the in-frame single amino acid deletion (p.Glu36del) result in correct plasma membrane targeting. The inability of a falsely targeted, truncated protein to convey the transport function is obvious. However, the correctly trafficked point mutant transporters are also equally nonfunctional, and the disease phenotype is comparable to the patients with a transport-inactivating mutation and even with the patients homozygous for completely y⁺LAT1 abolishing mutation p.Met1Leu (Sperandeo *et al.* 2007). According to HGMD® database (2013.1) out of the 25 recorded LPI point mutations 20 change a highly conserved amino acid residue. Only few point mutations have been tested *in vitro*, but since the non-tested mutations associate with the disease phenotype, the transport defect can be assumed to be caused by the mutations, including the non-conserved amino acid substitution p.Phe152Leu with residual activity.

Even though the y⁺L transporter is mainly expressed in the epithelial tissues, the symptoms of LPI affect also in a number of other cell types and tissues. Many of the common LPI symptoms

affecting non-epithelial tissues cannot be explained to be directly caused by the primary transport defect of cationic amino acids, but are instead caused indirectly via affected pathways. These include hepatosplenomegaly, PAP and susceptibility to viral infections such as varicella.

Little is known about the control of y^+L system transport in tissue or organ level when y^+LAT1 activity is abolished due to a LPI causing mutation. In fibroblasts (Dall'Asta *et al.* 2000) and erythrocytes (Boyd *et al.* 2000) derived from LPI patients the y^+L transport is not altered, most probably because y^+LAT2 is normally expressed. It has been suggested that due to the difference in tissue expression pattern between the y^+L transporters 1 and 2 the transport activity can be compensated in a limited number of tissues but probably only to a restricted degree. Despite the lack of direct evidence this compensatory mechanism cannot be ruled out as a factor in the clinical phenotype in LPI. The regulation of the y^+L transporter gene expression has been suggested to be interrelated at least in cultured lymphoblasts in which the LPI patient derived cells the *SLC7A7* mRNA amount was low as expected, but the *SLC7A6* mRNA levels were statistically higher than in control cells (Shoji *et al.* 2002). However, the unaffected y^+LAT2 expression does not save the transporter defect phenotype caused by the mutated, non-functional y^+LAT1, at least in the tissues where y^+LAT1 expression predominates over y^+LAT2, *i.e.* kidney and intestinal epithelium. In addition, Sperandeo and coworkers concluded that the p.Glu36del LPI mutant y^+LAT1 also has a dominant negative effect, *i.e.* the mutant y^+LAT1 interferes with the y^+LAT2/4F2hc transporter complexes. This abolishes the activity of the latter and thus suggests that the transporter unit exists as a dimer of heterodimers in which both y^+LAT1 and y^+LAT2 are involved. More generally, the hypothetical multi-heterodimeric complexes consisting of both y^+LAT1/4F2hc and y^+LAT2/4F2hc could be affected by association of a mutant light subunit, resulting in a more severe phenotype in a compound heterozygote or even a partial absorption defect in carriers (Sperandeo *et al.* 2005b). A genome wide expression analysis was more recently published on the Finnish LPI cohort (Tringham *et al.* 2012). In the transcriptome of the LPI patients obtained from peripheral whole blood samples *SLC7A7* was down-regulated by 80%. However, the average *SLC7A6* (y^+LAT2) expression level was not up- or down-regulated although the mRNA levels showed significant variation both ways between individual patients. Thus, in contrast to both the previous works, the *SLC7A6* expression does not compensate the severe *SLC7A7* down-regulation at least in the sample tissue used in the mentioned study.

2.1.6 SLC7A7 deficient mouse: animal model of LPI

In a work by Sperandeo and coworkers (Sperandeo *et al.* 2007) the phenotype of *SLC7A7*$^{-/-}$ mouse, the animal model of LPI is described. Curiously, it seems to be much more severe in some aspects than LPI in humans typically is, including fetal growth retardation and neonatal lethality: only two *SLC7A7*$^{-/-}$ deficient mice survived. However, after protein-rich feeding, the mice presented with identical metabolic derangement compared to human LPI. The difference in the phenotype compared to human LPI is in contrast in the results

obtained in the type A and B cystinuria mouse models (Peters *et al.* 2003, Feliubadalo *et al.* 2003), respectively, which mimic the phenotype in humans and thus provide a valid model in further studies on the etiology and treatment of cystinuria (Font-Llitjos *et al*. 2007, Ercolani *et al*. 2010, Goldfarb 2011). The reason for the difference in the outcome in the mouse models is not known but implicates an indispensable function of y^+LAT1 in mouse.

The insulin-like growth factor 1 (*Igf1*) and Igf1 binding protein 1 (*Igfbp1*) genes were significantly down-regulated in the fetal liver explaining the intra-uterine growth restriction observed in all *SLC7A7*$^{-/-}$ mouse fetuses. Furthermore, the down-regulation rate correlated with the decrease severity in fetal growth. Since arginine stimulates the production of growth hormone by increasing the Igf1 secretion, the reduced efflux of intracellular arginine due to absence of *SLC7A7* might cause the growth failure in mouse fetuses.

The absence of functional *SLC7A7* resulted in the change of expression in 400 mRNAs in the intestine and 500 mRNAs in the liver, the largest category of differentially expressed mRNAs corresponding to genes involved in transport (Sperandeo *et al.* 2007). The widespread effect of *SLC7A7* depletion in the mouse model reflects, albeit indirectly, the various symptoms associating with the human LPI. However, while the mouse model of LPI as such is not a duplicate of the human disease, it has provided new insights in the network of genetic interactions of *SLC7A7*.

2.2 Amino acid transport systems

The known molecular transporters are classified into a hierarchical family structure according to their substrate specificity, structure and functional properties. One of the groups is the solute carrier (SLC) family which includes the known amino acid transporters and consists of a total of 52 subfamilies of transporters, among them the SLC7 transporter group. Originally, the SLC7 amino acid transporters were characterized and named on the basis of their substrate spectrum and co-transported ion dependence. The current SLC nomenclature provides specific information on the properties of the transporters but has not completely replaced the original names (Table 2). The SLC7 group is further divided into CAT (SLC7A1-4) and LAT (LSHAT; SLC7A5-11) clusters. CAT transporters from CAT1 to CAT3 correspond for the system y^+ (cationic amino acid import) and are encoded by *SLC7A1-SLC7A3* genes, respectively. Also a fourth CAT isoform, CAT4 (*SLC7A4*) exists, but it is not known to exhibit any transport activity (Wolf *et al.* 2002). The predicted structure model of all CAT polypeptides suggests a 14 transmembrane domain (TMD) polypeptide, including a C-terminal extension of 100 amino acids comprising of two transmembrane helices lacking in the LAT family (Verrey *et al.* 1999, Torrents *et al.* 1999). The first 12 TMD regions of CATs share approximately 25% amino acid sequence identity with the vertebrate LAT family members that share a 12 TMD structure.

The LAT subfamily was named after the first transporter in the group, LAT1. The LAT cluster transporters are responsible for systems L, y^+L, x^-_c, asc and $b^{0,+}$, functioning as obligatory

exchangers, and sharing higher than 40% identity level (Verrey *et al.* 1999). SLC7A12 and SLC7A13 are structurally similar to LSHAT transporters, but the associating heavy chain is unidentified (Chairoungdua *et al.* 2001, Matsuo *et al.* 2002). In contrast, the newest SLC7 member, designated *14* that has 14 TMDs, has a structure more closely related to the CAT subfamily than the LSHATs (Sreedharan *et al.* 2011). Functionally the most significant differences between LAT and CAT groups exist in the structure of the transporter unit: CAT proteins form the transporters independently and are N-glycosylated, whereas the non-glycosylated, highly hydrophobic LAT transporters require a heavy subunit of the SLC3 family for surface expression and transport function (Closs *et al.* 2006, Bergeron *et al.* 2008).

Table 2. Amino acid transport systems of the SLC7 family

System	Definition	Transporter(s)
y^+	Na^+-independent cationic amino acid uptake (Arg, Lys, Orn)	CAT-1, CAT-2 (A and B isoforms), CAT-3
L	Na^+-independent exchange of neutral amino acids (Ala, Asn, Cys, Gln, Gly, Ile, Leu, Phe, Ser, Thr, Trp, Tyr, Val)	LAT1, LAT2
y^+L	Na^+-independent export of cationic amino acids (Arg, Lys, Orn), Na^+-dependent uptake of neutral amino acids (Gln, Ile, Leu)	y^+LAT1, y^+LAT2
asc	Na^+-independent exchange of small neutral amino acids (Ala, Cys, Gly, Ser, Thr)	Asc-1
x^-_c	Cys/Glu exchange	xCT
$b^{0,+}$	Na^+-independent exchange of neutral/cationic amino acids; Arg, Cys, Lys, Orn (re)absorption	$b^{0,+}$ AT

2.2.1 Heteromeric amino acid transporters (HATs)

The two currently known HSHATs, 4F2hc (4F2 cell surface antigen heavy chain or CD98(hc)) and rBAT (related to $b^{0,+}$ amino acid transporter; also NBAT) are type II single transmembrane domain N-glycoproteins with a cytoplasmic amino terminus and a bulky C-terminal domain showing homology with bacterial and insect glycosidases (Palacin *et al*. 2005, Fort *et al*. 2007). A HAT complex comprises of a heavy subunit from the SLC3 family and a light subunit from the SLC7 family covalently linked by a disulphide bond (Figure 2). The cysteine residue in the HSHAT (Cys109) forming the disulphide bridge between the subunits is located four amino acids from the transmembrane domain (Fort *et al.* 2007), and its location is conserved in both of the currently known HSHATs (Franca *et al.* 2005). The disulphide bond is not required for the plasma membrane trafficking of the transporter complex, and the resulting transporter has same transport characteristics as a covalently bound holotransporter, but the maximal transport rates are reduced by 30-80% (Pfeiffer *et al.* 1998, Nakamura *et al.* 1999, Fort *et al.* 2007). In LSHATs, the corresponding cysteine residue at the second extracellular loop is highly conserved, existing both in amphibian and mammalian transporter homologs, and is also found in the platyhelminth *Schistosoma mansoni* transporter SPRM1 (Pfeiffer *et al.* 1998). Since the

non-covalent interaction between the subunits is relatively weak and not sufficient to maintain consistent transporter capacity alone, the Cys residues clearly are of importance in the composition of HAT complexes: either in increasing the efficiency of the heterodimer formation or stabilizing the holotransporter at the plasma membrane (Torrents *et al.* 1998). In a study by Fernandez and coworkers (Fernandez *et al.* 2006) the analysis of functional expression of HATs reveals that the functional unit of a HAT is a heterodimer formed by light and heavy subunit. In the case of rBAT, the heterodimers exist as a dimeric complex forming a heterotetramer, but the transport function is still performed by the heterodimer subunit within the complex. Whether the same dimer-of-heterodimers composition applies to the y^+LAT1/4F2hc complex is unclear, since its native tissue expression mode of has not been resolved yet.

In the amino acid transporter complex, the heavy subunit is responsible for the plasma membrane trafficking and, in the case of 4F2hc, cellular domain selectivity of the transporter by associating with β-integrins (Fenczik *et al.* 2001). The light subunit is the catalytic part of the complex, forming the actual channel structure(s) and defining the substrate specificity of the transporter in question (Table 3). The LSHAT remains cytoplasmic in the absence of the corresponding HSHAT whereas the HSHAT is carried to the plasma membrane when expressed alone in *X. laevis* oocytes (Mastroberardino *et al.* 1998, Kanai *et al.* 1998, Nakamura *et al.* 1999). Since 4F2hc expressed alone in oocytes both localizes correctly and activates amino acid transport, it has been assumed to associate with the endogenous LSHATs of the oocyte, even though it is of human origin. The interaction between the HAT subunits is highly conserved: also the platyhelminth transporter SPRM1 can functionally associate with human 4F2hc forming a transporter with substrate specificity defined by the LSHAT (Pfeiffer *et al.* 1998, Mastroberardino *et al.* 1998). Three of the HAT subunits, rBAT, $b^{0,+}$AT and y^+LAT1 are involved in amino acid transport defects; the first two in cystinuria type A and B, respectively, and the last in LPI (Table 1).

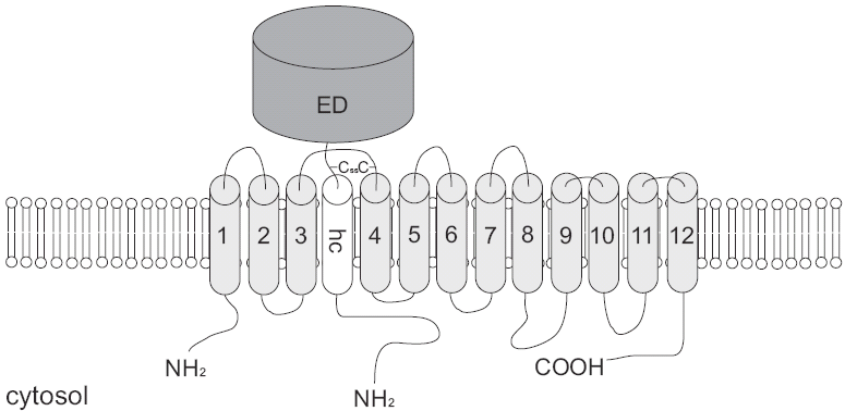

Figure 2. The predicted membrane topology of HSHAT complex. The cysteine residue at the second extracellular loop of the LSHAT forming a sulphide bond with Cys residue at the position 109 of the heavy subunit (4F2hc) is shown. The narrow numbered cylinders represent the LSHAT TMDs. ED: the glycosidase-like ectodomain of 4F2hc. hc: HSHAT TMD. (Adapted from: Pfeiffer *et al.* 1998, Fort *et al.* 2007)

Table 3. Heteromeric amino acid transporters in human: LSHATs and corresponding HSHATs. [Adapted from (Kanai, Endou 2001)]

Heavy subunit (HSHAT)	Light subunit (LSHAT)	Gene	Size (aa)	Transport system	Tissue/localization	Locus	Reference
4F2hc (CD98hc)		*SLC3A2*	529		ubiquitous	11q13	(Haynes *et al*. 1981, Hemler, Strominger 1982, Bertran *et al*. 1992a)
	LAT1	*SLC7A5*	507	L	placenta, spleen, thymus, liver, small intestine, kidney, brain, blood-brain barrier, tumour cells	16q24.3	(Kanai *et al.* 1998, Mastroberardino *et al.* 1998, Prasad *et al.* 1999)
	LAT2	*SLC7A8*	535	L	brain, placenta, kidney (proximal tubule epithelium), small intestine (epithelium), testis, skeletal muscle/ basolateral	14q11.2	(Pineda *et al.* 1999, Rossier *et al.* 1999, Segawa *et al.* 1999, Bassi *et al.* 1999)
	y^+LAT1	*SLC7A7*	511	y^+L	kidney (proximal tubule epithelium), leucocytes, small intestine, spleen , placenta, lung, heart, testis /basolateral	14q11.2	(Torrents *et al.* 1998)
	y^+LAT2	SLC7A6	515	y^+L	non-epithelial tissues, ubiquitous	16q22.1	(Torrents *et al.* 1998)
	asc-1	SLC7A10	523	asc	brain, lung, small intestine, placenta, kidney, heart	19q12-13	(Nakauchi *et al.* 2000, Fukasawa *et al.* 2000)
	xCT	SLC7A11	523	x_c^-	brain, spinal cord, activated macrophages, epithelial cell lines	4q28-q32	(Sato *et al.* 1999)
rBAT		*SLC3A1*	685		liver, epithelium of proximal kidney tubules and small intestine/apical	2p16.3	(Bertran *et al.* 1992b)
	$b^{0,+}$AT	*SLC7A9*	487	$b^{0,+}$	liver, epithelium of proximal kidney tubules and small intestine/ apical	19q12-13	(Feliubadalo *et al.* 1999, Pfeiffer *et al.* 1999a)
?	AGT-1 (XAT2)	*SLC7A13*	470		proximal kidney tubule epithelium/ basolateral	8q21.3	(Matsuo *et al*. 2002, Blondeau 2002)

2.2.1.1 Heavy subunits of heteromeric amino acid transporters: 4F2hc and rBAT

4F2hc (SLC3A2) was originally described as the heavy subunit of an early activated T- and B-lymphocyte and monocyte surface antigen recognized by the monoclonal antibody 4F2 (Haynes *et al*. 1981, Hemler, Strominger 1982). They reported a 125 kDa antigen composed of two subunits, an N-glycosylated heavy subunit of ~85 kDa and a non-glycosylated light subunit of ~40 kDa, of which the former is referred 4F2hc or CD98 (CD98hc). Soon after the protein characterization the human 4F2hc gene, *SLC3A2* was mapped in chromosome 11 (Peters *et al*. 1982, Francke, Foellmer & Haynes 1983). The *SLC3A2* gene consists of nine exons spanning 8 kb of genomic DNA (Lumadue, Glick & Ruddle 1987, Teixeira, Di Grandi & Kuhn 1987, Quackenbush *et al*. 1987) (Gen Bank accession number NM_002394) and has a 5' upstream region with housekeeping gene promoter -like properties. *SLC3A2* has sequence homologies with several other inducible T-cell genes such as interleukin-2 and interleukin-2 receptor α chain (Gottesdiener *et al.* 1988).

The 4F2hc protein is a 529 amino acid polypeptide with a single transmembrane domain (Teixeira, Di Grandi & Kuhn 1987) and a large extracellular N-glycosylated domain at C-terminus homologous with bacterial α-glycosidases and α-amylases (Chillaron *et al.* 2001) (Figure 2). 4F2hc shares ~27% amino acid sequence identity (50% similarity) with glycoprotein rBAT (related to $b^{0,+}$ amino acid transport) (Palacin, Kanai 2004) which is mutated in cystinuria type A (Palacin 1994). Comparison of rBAT and 4F2hc structures revealed that they have almost identical hydrophobicity profiles with one transmembrane domain, they both lack a leader sequence and share four highly conserved amino acid sequences of 10-18 aa in their extracellular domains (Palacin 1994). *SLC3A2* expression is highly regulated in T-cells, but its 5' sequence has features typical for constitutively active housekeeping gene promoters: the GC-rich core 5' promoter does not have TATA- or CCAAT-sequences and has four Sp1-transcription factor binding sites. The low expression rate of *SLC3A2* in resting T cells is not due to inactive 5' promoter but instead results from transcript elongation inhibition at the first exon and intron. Three conserved enhancer elements residing in the first intron, namely NF-4FA, NF-4FB, and AP-1 are all required for high expression levels detected in several cell types including malignant T cells (Karpinski *et al.* 1989, Leiden *et al.* 1989).

Human rBAT (SLC3A1), along with its mammalian homologues, was the first identified HSHAT. They were detected by expression cloning by several groups in rabbit (Bertran *et al.* 1992b), rat (Bertran *et al*. 1992b, Tate, Yan & Udenfriend 1992, Kanai *et al*. 1992) and in human kidney (Lee *et al.* 1993). The human homolog of rBAT is a 685 amino acid polypeptide and it is characterized by its ability to induce amino acid transport of system $b^{0,+}$ -type when injected in *X. laevis* oocytes, *i.e.* cystine, dibasic and neutral amino acid transport (Table 2). Functional assays on 4F2hc in the same experimental system indicated amino acid transport activity of the polypeptide, thus characterized as a HSHAT by its association with transport system y^+L (Palacin 1994, Torrents *et al*. 1998, Pfeiffer *et al*. 1999b) and L (Kanai *et al.* 1998)

(Table 2, Table 3). In concordance with the transporter association of 4F2hc its subcellular localization is strictly regulated by integrin association at the basolateral membranes of proximal kidney tubule (Quackenbush *et al.* 1987, Fenczik *et al.* 1997) and small intestine epithelium (Palacin 1994) where amino acid absorption occurs.

Although 4F2hc is ubiquitously expressed among proliferating cell types including all established human tissue culture cell lines tested, the highest expression level is detected in malignant cell lines (Hara *et al*. 1999, Papetti, Herman 2001) and lectin- or antigen-mediated activated T-cells in rapid proliferation (Cotner *et al.* 1983). Cotner and coworkers suggested a role for 4F2hc in the onset of cell proliferation and activation, which was later supported by the characterization of the *SLC3A2* promoter. In addition, 4F2hc has been found to be involved in cell differentiation, adhesion, growth and tissue repair (Toyooka *et al.* 2008, Warren *et al.* 1996, Feral *et al.* 2005), autoimmune disease pathogenesis (Cantor *et al.* 2011) as well as malignant transformation (Hara *et al.* 2000, Yoon *et al.* 2003, Nawashiro *et al.* 2002, Hara *et al.* 1999). As a confirmation of its function in tumor formation, anti-4F2hc antibodies restricted cancer cell growth (Papetti, Herman 2001) whereas overexpression of 4F2hc resulted in malignant transformation of several cell types (Hara *et al.* 1999, Campbell *et al.* 2000, Storey *et al.* 2005, Kaira *et al.* 2010, Kaira *et al.* 2011b, Kaira *et al.* 2011c). Following the *in vitro* observations of the malignant cell growth promoting function of 4F2hc it was identified as a marker of poor prognosis and proposed as a cancer treatment target molecule in a high throughput tissue microarray-based study on a large cohort of breast cancer patients (Esseghir *et al.* 2006). Also, in several other cancer types 4F2hc expression rate has been reported to associate with more aggressive progression of cancer as well as poor survival rates (Kaira *et al.* 2011a, Kaira *et al.* 2010, Furuya *et al.* 2012). The overexpression of 4F2hc, along with associating light chain LAT1, is thought to be necessary for the tumor growth by delivering nutrients for the continuously dividing cancer cells. This hypothesis is supported by the observation of positive correlation between 4F2hc overexpression and nodule size in rat liver tumor (Ohkame *et al.* 2001) as well as tumor size in triple negative breast cancer tumor size (Furuya *et al.* 2012).

Human 4F2hc became the first HAT with its structure partially solved when Fort and coworkers (Fort *et al.* 2007) published their protein crystallography-based analysis on the 4F2hc ectodomain (ED). Despite the homology with bacterial enzymes 4F2hc-ED has major differences in the active sites of the enzymes and thus does not have similar enzymatic activity. Also, the major hydrophobic interaction with the plasma membrane is not mediated via the ectodomain of 4F2hc but instead via the transmembrane domain. This leaves the less hydrophobic ectodomain to interact with the extracellular loops of associating light subunits, which also enhances the attachment of 4F2hc-ED to the plasma membrane (Fort *et al.* 2007). The 4F2hc ectodomain is monomeric in solution and therefore the complex formation most likely depends on interaction between the intracellular or the transmembrane domains of the transporter subunits (Turnay *et al.* 2011).

Since HSHATs are not able to form a channel across the plasma membrane their role was concluded to be transport regulation or activation (Palacin 1994). The most obvious task of a HSHAT is to determine the cellular localization of the transporter complex. Therefore, 4F2hc heterodimers are all basolateral in polarized cells, determined by the β1 integrin interaction (Fenczik *et al.* 1997, Zent *et al.* 2000) which is mediated by the N-terminal cytoplasmic tail of 4F2hc (Cai *et al.* 2005), whereas the $b^{0,+}$AT/rBAT complex is apical. Mutations in rBAT causing cystinuria type A all result in a trafficking defect *i.e.* the mutant transporter complex remains cytoplasmic. In contrast, the $b^{0,+}$AT mutations causing cystinuria type B result in non-functional yet correctly trafficked transporters (Feliubadalo *et al.* 1999). The mechanism of the apical targeting of the $b^{0,+}$AT/rBAT complex has not been reported, but the plasma membrane targeting of the $b^{0,+}$AT/rBAT complex is mediated by $b^{0,+}$AT, as truncating mutations of the light chain C-terminus halt the complex in the ER, also preventing the glycosylation maturation of rBAT (Sakamoto *et al.* 2009). Thus, the transporter complex is assembled in the ER, similarly to the y^+LAT1/4F2hc dimer formation (Kleemola *et al.* 2007). In the case of $b^{0,+}$AT/rBAT transporter complex, the assembly of the transporter also controls heavy chain life time: rBAT monomers are rapidly degraded after synthesis in the ER if they remain unassembled (Bartoccioni *et al.* 2008). Differing from cystinuria-causing mutations, the LPI-causing mutations of y^+LAT1 can be either trafficking or transport-inactivating defects, depending on the mutant type (Mykkänen *et al*. 2000). In addition, the LSHATs are dependent on the corresponding HSHATs for the plasma membrane trafficking (Mastroberardino *et al.* 1998, Kanai *et al.* 1998, Nakamura *et al.* 1999, Bartoccioni *et al.* 2008) and maintaining transporter function. C-terminally truncated 4F2hc lacking 15 to 404 amino acids completely abolished y^+LAT2 transporter function in spite of the correct plasma membrane localization of the heterodimer, indicating that the C-terminus of the heavy chain is not needed for heterodimer formation but is involved in maintaining the transporter functional (Chubb *et al.* 2006). 4F2hc domains contribute to separate functions: the cytoplasmic and transmembrane domains are essential for the integrin interaction while the large extracellular domain contributes to the light chain binding. The two functional units are separate: 4F2hc lacking the extracellular domain can still regulate integrin function, and in spite of missing the integrin recognition site the polypeptide is able to promote amino acid transport (Fenczik *et al.* 2001). Furthermore, 4F2hc recognition of the several different light chains is shown to require separate domains at the extracellular domain (Broer *et al.* 2001). Currently the most unclear regulating task of HSHAT is at the level of gene expression. The up-regulation of one of the 4F2hc-associating light chains, namely LAT1, results in excessive expression of 4F2hc suggesting a coordinated regulation of the transporter complex subunit transcription or translation (Storey *et al.* 2005). The up-regulation of the LAT1/4F2hc dimer is observed also in several cancer cell cultures and cancer tissues (Yoon *et al.* 2003, Kaira *et al.* 2011a, Kaira *et al.* 2010, Kaira *et al.* 2009, Nawashiro *et al.* 2006).

Altogether six individual 4F2hc light subunits corresponding four transport systems (L, y^+L, asc and x^-_c) have been characterized (Chillaron *et al.* 2001). In contrast to the multiple associating light chains of 4F2hc, rBAT has only one currently known LSHAT, $b^{0,+}$AT. Thus, rBAT is functionally more limited than 4F2hc, inducing only system $b^{0,+}$ amino acid transport. This feature made it a candidate gene for cystinuria (OMIM #220100), the most common primary aminoaciduria, with an average worldwide incidence of 1: 7000. In 1994, rBAT was connected to cystinuria by Calonge and coworkers (Calonge *et al.* 1994), who reported rBAT point mutations causing cystinuria type 1 (later named cystinuria type A), the autosomal recessive form of the disease. At the moment, no genetic diseases have been associated with 4F2hc. 4F2hc deficiency by genetic knockout results in early embryonic lethality in mouse (Tsumura *et al.* 2003), suggesting multiple indispensable functions for the polypeptide.

2.2.1.2 Light subunits of the heteromeric amino acid transporters (LSHAT)

The human family SLC7 of catalytic subunits or light chains of heteromeric amino acid transporters (LSHATs) comprises of eight structurally similar, non-glycosylated polypeptides sharing 85-98% amino acid sequence identity (Table 3) (Chillaron *et al.* 2001, Fernandez *et al.* 2005). All LSHATs are approximately 500 aa polypeptides and have 12 transmembrane domains as well as intracellular C- and N- termini. Also, a re-entrant loop between transmembrane segments 2 and 3 has been suggested (Gasol *et al.* 2004), but the structure of the SLC7 family transporters has not yet been fully resolved. In the transporter complexes, their function is to correspond of the substrate specificity and transport activity by associating with membrane spanning glycoproteins 4F2hc and rBAT. Although the three-dimensional structure of HAT complexes remains elusive at the moment, the predictions of HAT subunits based on hydrophobicity suggest that the transport pore or pores are solely constituted of the transmembrane domains of the light chain (Pfeiffer *et al.* 1998). This hypothesis is further supported by first piece of direct functional as well as structural evidence on a prokaryotic LAT-cluster transporter, SteT (serine/threonine exchanger transporter), which was visualized in proteoliposomes using freeze-fracture and transmission electron microscopy by Reig and coworkers (Reig *et al.* 2007). SteT is 30% identical in amino acid sequence to human LSHATs and is expressed in bacterial membrane as a monomer unlike the heterodimeric LSHATs. TEM images revealed an elliptical structure with a central depression of size 5 to 6 nm, resembling the similar sized prokaryotic lactase permease, also consisting of 12 transmembrane domains. So far, this remains the only direct visualization attempt of a LAT transporter nanostructure, but the sequence similarity between SteT and LSHATs suggests that the SteT structure might be applicable also to other LAT cluster transporters.

Six of the light subunits (LAT1, LAT2, y^+LAT1, y^+LAT2, xCT, asc-1) heterodimerize with 4F2hc (Broer, Palacin 2011), whereas $b^{0,+}$AT associates with rBAT primarily as a dimer of

heterodimers (Fernandez *et al.* 2006). In addition to the transporter subunits mentioned at least one heavy subunit associating with human and mouse AGT-1 LSHATs remains to be identified (Matsuo *et al.* 2002).

L-type transporters (LAT1 and LAT2)

Both of the LAT transporters 1 and 2 exhibit system L activity, the sodium independent obligatory exchange of neutral amino acids, preferring aromatic (histidine, phenylalanine, tryptophane, tyrosine) and branched substrates (valine, leucine, isoleucine). However, LAT1 and LAT2 deviate greatly in their substrate affinities and cellular as well as tissue distribution, suggesting different physiological functions of the transporters (Pineda *et al.* 1999). LAT1 (SLC7A5), the first glycoprotein associated amino acid transporter was identified by expression cloning in the rat C6 glioma cell line (Kanai *et al.* 1998). In functional tests in *X. laevis* oocytes it was observed to induce system L amino acid transport together with 4F2hc (Kanai *et al.* 1998, Mastroberardino *et al.* 1998). LAT1 expression regulation has been shown to be dependent on amino acid availability in rat and mouse placenta (Campbell *et al.* 2000, Chrostowski *et al.* 2010) and the *SLC7A5* gene has sequence elements similar to AARE motifs (amino acid response elements) in the upstream region. AARE elements have been described in the promoter region of genes that have a transcription activation response to amino acid deprivation (Guerrini *et al.* 1993). In the case of LAT1, however, the regulatory mechanism is not mediated by these AARE-resembling sequence motifs (Diah *et al.* 2001).

LAT1 overexpression is detected in most malignant cell lines and tumors, together with 4F2hc overexpression. The high level of LAT1 mRNA in cancer cells seems to be consistent while the 4F2hc mRNA levels vary at least in leukemia cell lines (Yanagida *et al.* 2001). High expression level of LAT1/4F2hc transporter has been found to positively correlate to the nodule size of rat metastatic liver tumors, most likely due to the increased nutrition demand of the tumor (Ohkame *et al.* 2001). It has also been suggested to serve as a marker for more aggressive disease and poor prognosis in several cancer types (Furuya *et al.* 2012, Kaira *et al.* 2011c, Kaira *et al.* 2010, Esseghir *et al.* 2006). Due to its prominent role in malignant development of cells LAT1 has been proposed as a therapeutic target for cancer (Nawashiro *et al.* 2006).

Subsequently, mouse and human LAT2 (SLC7A8) were cloned on the basis of their homology with LAT1 (Pineda *et al.* 1999, Segawa *et al.* 1999, Rossier *et al.* 1999). Differing from LAT1, LAT2 prefers small and medium sized amino acids as substrates. LAT2 is highly expressed in the basolateral membranes of epithelial cells, most prominently in the kidney proximal tubule where LAT1 is absent, and to a smaller extent in the placenta, brain and small intestine. In all tissues, LAT2 localization is restricted to the basolateral membrane of the epithelium. This suggests a function in the net flow of cystine and neutral amino acid reabsorption (kidney), absorption (small intestine), and transfer from the placenta to the fetus.

y^+L type transporters (y^+LAT)

y^+LAT1 (SLC7A7) was first identified on the basis of its homology to LAT1. It was shown to induce amino acid transport of system y^+L (Table 2) in *X. laevis* oocytes when co-injected with 4F2hc. In the same time, the closely related LSHAT y^+LAT2 (SLC7A6) was characterized. The y^+LAT-transporters 1 and 2 exhibit higher pairwise sequence identity (72%) than other LAT cluster transporters (Verrey *et al.* 1999). They were also found to be functionally almost identical, including the Na^+ requirement which is the hallmark of the y^+L transport system. However, they differ significantly in tissue expression pattern: while y^+LAT2 is almost ubiquitously expressed in epithelial and non-epithelial tissues, y^+LAT1 is strictly restricted to the epithelial tissues, the highest expression levels found in kidney tubules, leucocytes and lung but also in small intestine, spleen and placenta among others (Torrents *et al.* 1998, Pfeiffer *et al.* 1999b). Both transporters mediate system y^+L-type transport, but y^+LAT2 has higher affinity for glutamine and more narrow substrate specificity than y^+LAT1, suggesting a physiological transport mode of y^+LAT2 to be the exchange of intracellular arginine to glutamine in brain and other non-epithelial tissues (Broer *et al.* 2000).

In polarized epithelium system y^+L is restricted to the basolateral membrane as the β-integrin interaction of 4F2hc dictates the localization of the transporter complex. The obligatory exchange function of y^+L transporters provides neutral amino acids for the intracellular pool which serves as a substrate source for LAT2 and the apical $b^{0,+}$AT/rBAT complex export function (Wagner, Lang & Broer 2001) (Figure 1). Also, arginine transport and release by y^+LAT1 and 2 has an important physiological role in many tissues and cell types, e.g. intestinal and kidney epithelial cells which provide arginine supply to other organs and endothelial cells of the blood-brain barrier transporting arginine for the brain. As y^+LAT1 is massively expressed in kidney epithelium it is responsible, together with the apical $b^{0,+}$AT/rBAT transporter, for cationic amino acid uptake from the primary filtrate.

Strong evidence of the significant function of y^+LAT1 for the cationic amino acid (re) absorption in human physiology was discovered when it was found to be defective in LPI where the primary defect is the malabsorption of cationic amino acids (Borsani *et al.* 1999, Torrents *et al.* 1999). Functional analysis of LPI-causing mutations revealed, that all but one of the studied mutations abolished the y^+L transport activity totally independent on the mutant type, the exception of the point mutant p.Phe152Leu expressing residual activity.

Recently *SLC7A7* up-regulation was reported to be a marker of poor prognosis marker in glioblastoma patients (Fan *et al.* 2013), widening the range of the clinical significance of the y^+L transporter system.

asc-type transporter (Asc1)

In 2000 Fukasawa and coworkers isolated a cDNA encoding a small neutral amino acid transporter functioning in a Na^+ independent mode from mouse brain, which they

designated Asc-1 for asc-type transporter-1 (Fukasawa *et al.* 2000). In 2000, Nakauchi and coworkers characterized the human homolog for Asc1 (Nakauchi *et al.* 2000). In contrast to the limited expression pattern in mouse (brain, lung, placenta and small intestine), human Asc1 is expressed in various tissues including brain, heart, placenta, skeletal muscle and kidney. Human Asc1, like the mouse homolog is highly expressed in the brain and was detected to transport not only L-isomers of neutral amino acids but also D-isomers. Its high affinity transport of D-serine suggests that it plays a significant role in D-serine transport in the brain (Nakauchi *et al.* 2000). The isolated bacterial homologue of Asc1 transporter, despite showing only 35% sequence homology to the human transporter, has a typical structure of the SLC7 transporters harboring 12 putative membrane spanning domains and consisting of almost completely of α-helices (Wang *et al.* 2010).

Cystine-glutamate exchanger (xCT)

xCT amino acid transporter was identified by Sato *et al.* (1999) by expression cloning in mouse activated macrophages (Sato *et al.* 1999). The transporter is responsible for x_c^--type transport, the electroneutral exchange of extracellular cystine for intracellular glutamate, using concentration gradient of the substrates as the driving force (Bannai 1986). x_c^--type transport is almost ubiquitous among cultured mammalian cell lines and its expression is highly regulated (Shih, Murphy 2001). Human xCT, encoded by *SLC7A11* gene, was identified as a typical LAT cluster transporter. Human xCT is localized intracellularly when expressed alone in oocytes but is trafficked to the plasma membrane when co-expressed with 4F2hc (Bassi *et al.* 2001). In HEK293 cells xCT-GFP fusion protein localized in a clustered pattern when co-transfected with 4F2hc hypothetically residing at sites where glutathione synthesis takes place or at cell-cell interaction sites (Shih, Murphy 2001). In the brain xCT has a neuroprotective role via glutathione synthesis pathway: cultured neurons with high expression levels of the transporter were found to be more tolerant against oxidative glutamate toxicity than fibroblast with naturally low expression levels (Shih *et al.* 2006). Similar to LAT1 overexpression in malignant cells, *SLC7A11* gene has been reported to be up-regulated in certain malignant cells and is suggested to be a marker of poor prognosis in hepatocellular carcinoma (Kinoshita *et al.* 2013). The transcription regulation of *SLC7A11* is directly influenced by amino acid concentrations in the environment. The gene has two highly conserved palindromic AARE-motifs at approximately 100 bp upstream of the transcription initiation site. Unlike *SLC7A5* associated AARE motifs, the distal AARE motif binds the ATF4 transcription factor activating *SLC7A11* transcription upon amino acid starvation in mice (Sato *et al.* 2004).

b0,$^+$ amino acid transporter (b0,$^+$AT)

$b^{0,+}$AT (SLC7A9) is currently the only known light chain of rBAT. It was cloned in 1999 (Chairoungdua *et al.* 1999, Feliubadalo *et al.* 1999, Rajan *et al.* 1999, Pfeiffer *et al.* 1999a) based on its linkage to type B cystinuria (previously named non-type I cystinuria).

According to mutation database HGMD®professional (version 2012.4), 103 cystinuria specific mutations have been reported in *SLC7A9* to date with varying allele frequencies between populations (Font-Llitjos *et al.* 2005, Shigeta *et al.* 2006). As expected, the *SLC7A9* deficient mice develop type B cystinuria with mimicking features of the human cystinuria (Feliubadalo *et al.* 2003). Interestingly, the *SLC7A9* knockout mice express rBAT protein in significant amounts, and it is detected in heterodimeric form with unidentified light subunit(s). Also, according to the hypothesis presented by Feliubadalo *et al.* (2003) the amount of $b^{0,+}$AT controls the functional expression of the $b^{0,+}$AT/rBAT complex and the heavy chain expressed in excess in kidney is degraded in the absence of $b^{0,+}$AT (Feliubadalo *et al.* 2003). So far, no studies on the co-regulation of 4F2hc and its LSHATs have been published, and the question whether the $b^{0,+}$AT/rBAT regulation pattern applies to them as well remains to be answered.

After the discovery of $b^{0,+}$AT, it has been extensively studied both structurally and functionally. It is a 487 aa polypeptide with distinctly similar structural features with other LSHATs, including the cysteine residue in the second extracellular loop. Interestingly, Fernandez and coworkers (Fernandez *et al.* 2006) reported that the complex formed by $b^{0,+}$AT and rBAT is further assembled into tetramers. The functional unit of the $[b^{0,+}AT/rBAT]_2$ transporter tetramer is the heterodimer, identically to 4F2hc transporter complexes (Fernandez *et al.* 2006). rBAT controls the oligomerization process, as shown by Fernandez and coworkers who were able to express an $[rBAT/xCT]_2$ tetramer but not a stable $[4F2hc/xCT]_2$ or $[4F2hc/LAT2]_2$ tetramer. rBAT determines the localization of the transporter tetramer to the apical membrane but many studies have shown that both subunits are required for the cell surface expression of the $b^{0,+}$AT/rBAT complex (Reig *et al.* 2002, Feliubadalo *et al.* 1999) and the $b^{0,+}$-transport activity induction (Feliubadalo *et al.* 1999, Pfeiffer *et al.* 1999a, Chairoungdua *et al.* 1999, Font *et al.* 2001). More recently, evidence for another function of $b^{0,+}$AT in transporter subunit assembly was presented by Rius and Chillaron, who reported on the oxidative folding involving multiple sulphide bond formation within the rBAT ectodomain that requires $b^{0,+}$AT association. The incompletely folded rBAT subunits lacking the critical cysteine bonds remained monomers and were degraded (Rius, Chillaron 2012).

The subcellular localization of only one *SLC7A9* mutation causing cystinuria, p.Pro482Leu, has been studied in cultured cells. The missense mutant was expressed as GFP fusion in HEK293 and MDCK cells and was found to reside normally on the apical plasma membrane when rBAT was co-expressed. Thus, the defect caused by the mutation abolishes only the transport function leaving the cellular sorting intact (Shigeta *et al.* 2006). In addition, the protein expression level of six cystinuria type B missense mutant *SLC7A9*s was studied by Font and coworkers (Font *et al.* 2001). Five of them were present in higher-than-wild-type levels in transfected HeLa cells, but one was detected in very low amounts in Western blot. The mutants expressed in high amounts were hypothesized to cause trafficking or transport inactivating defects, in contrast

to the low expression level mutant that might produce an instable mRNA or protein. The subcellular localization of the mutants was not reported (Font *et al.* 2001).

2.3 Fluorescent fusion protein based cell imaging

The green fluorescent protein, GFP of the jellyfish *Aequorea victoria* was first discovered by Shimomura and coworkers during studies concerning the bioluminescent protein aequorin (Shimomura, Johnson & Saiga 1962). *In vitro* aequorin emits blue fluorescence peaking at 470 nm in the presence of Ca^{2+}. However, in the close vicinity of GFP in the photogenic organ of the jellyfish some of the luminescence energy of aequorin is transferred to GFP resulting in green shifted fluorescence. *In vitro* studies on the bioluminescence system of *Aequorea* suggested a "Förster-type transfer mechanism" *i.e.* FRET (fluorescence resonance energy transfer) as the mode of fluorescence energy transfer from aequorin to GFP. The study involved the purification and crystallization as well as the definition of GFP emission/ excitation spectra and quantum yield of 0.72 (Morise *et al.* 1974). The wt *Aequorea* GFP (avGFP) has a complex spectrum: the major excitation peak is at 398 nm and minor at 475 nm, the former resulting in emission at 508 nm and the latter at 503 nm (Morise *et al*. 1974, Ward, Bokman 1982, Heim, Prasher & Tsien 1994). The spectral characteristics of GFP are due to a chromophore which is formed of tripeptide Ser-dehydroTyr-Gly at amino acid positions 65-67 of the polypeptide upon cyclization of the residues (Cody *et al.* 1993). The chromophore formation requires oxygen, proceeds in a constant, temperature-dependent rate and does not require any cofactors or enzymes (Heim, Prasher & Tsien 1994).

The avGFP gene was cloned and sequenced by Prasher and coworkers (Prasher *et al.* 1992) (GenBank accession M62653.1). The gene codes for a 238 aa polypeptide with the mass of 26.9 kDa. Since GFP does not require any *Aequorea*-originating enzymes to emit fluorescence, it was soon discovered to have the same emission spectrum when expressed in other organisms such as *E. coli* and *Caenorhabditis elegans* (Inouye, Tsuji 1994, Chalfie *et al*. 1994). This proved that it could be utilized as a gene expression and protein localization marker. However, avGFP has several disadvantageous properties, such as relatively slow fluorochrome maturation rate and low brightness. To overcome these challenges, avGFP has been subjected to mutagenesis (Heim, Prasher & Tsien 1994, Delagrave *et al*. 1995) in order to improve the fluorescence intensity and development as well as alter the emission and excitation spectra *i.e.* to create fluorescent proteins of various colors (Cubitt *et al.* 1995).

Heim and coworkers published the first spectral variants of the original avGFP sequence pruduced by random mutagenesis, among them the blue shifted GFP variant BFP containing the p.Tyr66His substitution (Heim, Prasher & Tsien 1994). The resulting amino acid substitution variants provided GFPs with improved fluorescence intensity and a single excitation peak in contrast to the double excitation maxima of avGFP. Later, Heim and Tsien

reported four additional mutant GFPs, all with an amino acid substitution at Ser65 and each increasing the brightness of the fluorochromes (Patterson *et al*. 1997, Heim, Tsien 1996). The p.Ser65Thr GFP variant also accelerates fluorochrome maturation and is incorporated in the enhanced GFP (EGFP) leading to 35 fold fluorescence brightness compared to avGFP. Its spectral variants, which also carry the p.Phe64Leu substitution, are enhanced in the polypeptide folding process in +37°C (Patterson *et al.* 1997).

GFP protein structure was first solved independently by both Yang and coworkers as well as Ormö and coworkers in 1996 (Figure 3) (Ormö *et al*. 1996, Yang, Moss & Phillips 1996). In the globular basic form of GFP, the 11 β-sheets of the polypeptide form a cylinder in which the α-helices are located in the center. The chromophore is a ring structure composed of three amino acids, Ser65, Tyr66 and Gly67. It is protected by the α-helices in the interior of the β can which is ~30 Å of diameter and ~40 Å of length. Polar amino acid residues and water molecules closely surround the chromophore, hence embedding it into the core of the GFP molecule. There it is resistant to quenching resulting from singlet oxygen observed by Rao and coworkers (Rao, Kemple & Prendergast 1980) and the protein-unfolding factors such as heat or various denaturants (Yang, Moss & Phillips 1996).

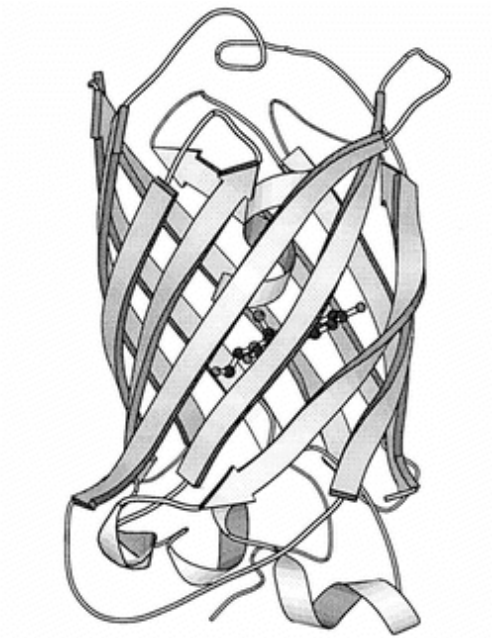

Figure 3. GFP structure showing the 11-beta-can structure embedding the chromophore visualized in a ball-and-stick model (From: Tsien 1998; original figure courtesy of SJ Remington, University of Oregon, USA).

Since GFP does not require addition of cofactors for fluorescence it has become widely used in live cell imaging. In addition to basic localization observation of one or several GFP spectral variant tagged proteins, time-lapse microscopy can be used to follow their

synthesis, trafficking and disposal. Fluorescence recovery after photobleaching (FRAP) is an imaging technique in which a specified cell compartment is photobleached using a focused laser beam and thus the GFP variant within is destroyed. Following this, the region is imaged using lower energy laser and the time required for recovery of GFP fluorescence is measured (Axelrod *et al.* 1976). The recovery results from the diffusion of the fluorescent fusion protein; if the fluorescence is recovered fast, the studied protein has a high mobility. In contrast, if the fluorescence shows no recovery, the studied protein is immobile and attached to a fixed structure. Thus, FRAP can be used to detect for example lateral membrane diffusion as well as protein trafficking between cellular compartments or organelles. Another imaging approach to study protein movement in cell is fluorescence loss in photobleaching (FLIP). In a FLIP experiment a region of interest containing a fluorescent protein tagged target protein is continuously bleached to destroy the protein. Simultaneously, the entire cell is observed for compartments which lose their fluorescence due to diffusion of the protein to replace the destroyed protein in the photobleached area. FLIP has been used in studies concerning e.g. Golgi membrane (Cole *et al.* 1996) and inner nuclear membrane protein dynamics (Ellenberg *et al.* 1997).

In 2008, Osamu Shimomura, Martin Chalfie and Roger Y. Tsien received the Nobel Prize in chemistry as recognition for their achievements on GFP research.

2.3.1 EGFP and its spectral variants

Generally, fluorescent proteins absorb higher energy wavelength light than they emit as energy is lost between the absorption and the emission events. This phenomenon is called the Stokes shift. The amount of lost energy depends onto the surrounding conditions. In the case of fluorescent proteins (FPs) the immediate environment is the protein structure surrounding the fluorochrome which affects the fluorescence properties of an FP along with the chromophore itself. Since the characterization of the original avGFP it has been modified by improving the fluorescence intensity and stability as well as excitation and emission spectra. The spectral variants of GFP cover the whole spectrum of visible light, from blue fluorescent proteins in 440-470 nm to orange and red FPs emitting at 551-575 nm and 576-670 nm, respectively. In addition to their spectra, the properties of the GFP spectral variants differ substantially. To be an ideal marker useful in bioimaging, an FP or a fluorochrome should first have a distinct one-peaked emission and excitation spectra. Second, it should have high quantum yield or quantum efficiency ϕ, which is the number of emitted photons divided by the number of absorbed photons by a given fluorochrome; therefore, the highest quantum yield is 1. Third, it should have a high molar extinction coefficient or molar absorptivity ε, a term which describes how well a fluorochrome absorbs light in a specific wavelength. The two latter define the intrinsic brightness (*I*) of an FP (Table 4):

$$I = \boldsymbol{\phi}^{*}\boldsymbol{\varepsilon}.$$

The first generation spectral variants of EGFP, namely enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP), were generated by modifying amino acid residues of the chromophore itself (ECFP) or affecting its conformation and stability locally (EYFP). ECFP carries the amino acid substitution p.Tyr66Trp and has excitation and emission maxima at 439 and 476 nm, respectively, whereas EYFP has amino acid substitution p.Thr203Tyr and has excitation at 514 nm and emission at 527 nm. ECFP has been further engineered to enhance its fluorescence and speed the maturation. For example, Cerulean has three additional substitutions resulting in a 2.5 fold fluorescence intensity compared to ECFP (Rizzo *et al.* 2004). Lately a cyan FP, mTurquoise, was developed. The engineering was based on the accumulated knowledge of the ECFP family architecture resulting in an FP that has a quantum yield of 0.93 vs. the 0.31 of ECFP making it a better candidate for FRET experiments (Goedhart *et al.* 2012). In contrast to ECFP and other blue shifted GFP variants, YFPs generally have high intensive brightness values. In fact, ECFP has both lower quantum yield and molar absorptivity than EYFP, resulting in ~4 fold reduction in fluorescence (Table 4). However, EYFP is more susceptible for photobleaching than ECFP, a disadvantage for its use in bioimaging, it can also be utilized as an acceptor in photobleaching FRET experiments.

Table 4. Properties of a selection of fluorescent proteins.

Fluorescent protein	Amino acid substitutions	λAbs nm	λEm, nm	molar extinction coefficient ε (x10^{3}M^{-1}cm^{-1})	Quantum yield ϕ	Intrinsic brightness *I*=ϕ*ε	reference
avGFP	-	475 (395)	508	25	0,79	19,75	**(Ormö *et al.* 1996, Patterson *et al.* 1997)**
EGFP	Phe64Leu, Ser65Thr	484	507	53	0,6	31,8	**(Heim, Prasher & Tsien 1994, Patterson *et al.* 1997)**
EBFP	Phe64Leu, Tyr66His, Tyr145Phe	383	445	31	0,25	7,75	**(Heim, Prasher & Tsien 1994)**
ECFP	Ser65Ala, Tyr66Trp, Ser72Ala, Asn146Ile, Met153Thr, Val163Ala	434	474	26	0,4	10,4	**(Heim, Prasher & Tsien 1994)**
mTurquoise		434	474	30	0,93	23,9	**(Goedhart *et al.* 2010)**
EYFP	Ser65Gly, Val68Leu, Ser72Ala, Thr203Tyr	514	527	84	0,61	51,24	**(Ormö *et al.* 1996, Wachter *et al.* 1998)**
DsRed	-	558	583	75	0,79	59,25	**(Matz *et al.* 1999, Gross *et al.* 2000, Shaner *et al.* 2004)**

2.4 FRET analysis using fluorescent proteins in studying protein-protein interactions

2.4.1 FRET theory

One of the applications using ECFP (cyan) and EYFP (yellow) is in FRET studies since these fluorochromes can be attached to a variety of proteins without affecting the function or trafficking of the target protein or the host cell. The fluorescence properties of ECFP and EYFP fulfill the requirements for FRET experiments but are only one among many FRET pairs used; these include also other fluorescent molecules than fluorescent proteins discussed in this review. FRET (also Förster energy transfer) is a quantum mechanical process which occurs when two fluorophores are at less than 10 nm (100 Å) distance from each other in a favorable angle. The excited donor fluorophore emission excites the acceptor fluorophore which defines the process as radiationless transfer of energy. The FRET efficiency (E) is defined in the Förster equation (Förster 1948):

$$E = R_0^6 / (R^6 + R_0^6),$$

in which R is the distance between donor and acceptor fluorophore centers, and R_0 is the distance at which energy transfer is 50% from maximum. R_0 depends on the 1) quantum yield (emission efficiency) of the donor, 2) the extinction coefficient (molar absorptivity) of the acceptor, 3) the overlap of the emission and excitation spectra of the FRET partners as well as the 4) relative orientation of the donor and acceptor. When the distance R increases, E rapidly becomes small (Tsien 1998).

The basic requirement for FRET is that the excitation spectrum of the acceptor overlaps the emission spectrum of the donor, but the spectra must be distinct enough to be separated in the imaging process. Also, to avoid the false FRET signal or contamination named spectral bleed through (SBT), the donor and acceptor excitation spectra as well as emission spectra should have minimum overlap. SBT occurs when donor emission spectrum overlaps that of the acceptor or when acceptor excitation spectrum overlaps donor excitation spectrum, both resulting in acceptor fluorescence that may be misinterpreted as FRET signal (Wallrabe, Periasamy 2005, Piston, Kremers 2007).

A widely used FRET pair of fluorochromes is CFP and YFP that have suitable emission and excitation spectra for the purpose (Figure 4 and Figure 5).

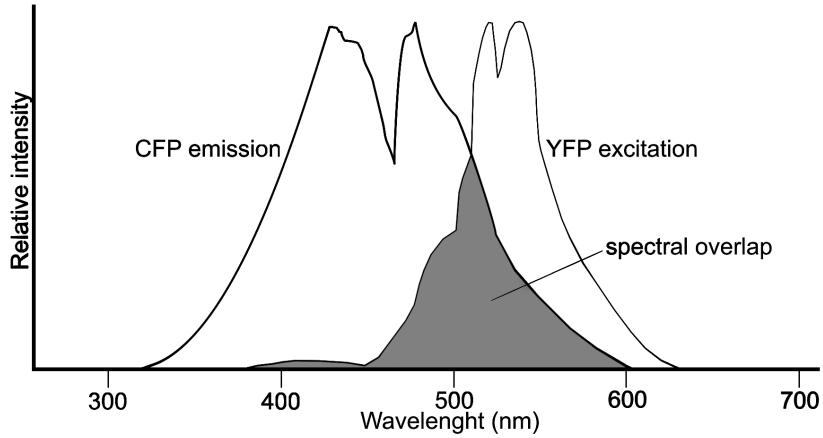

Figure 4. Emission spectrum of CFP partially overlaps the excitation spectrum of YFP, which is required for FRET to occur.

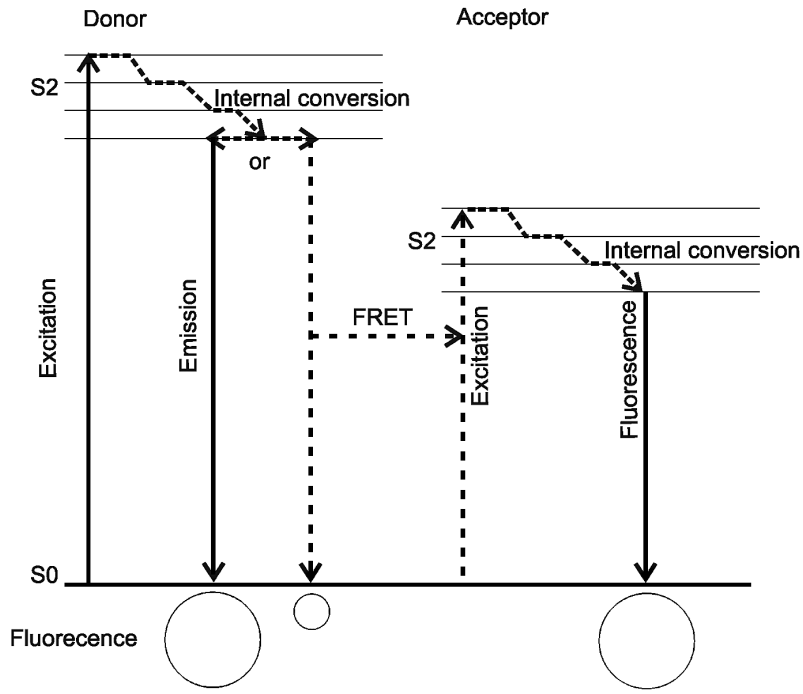

Figure 5. A schematic representation of FRET. The donor is excited by photon whose energy is absorbed and converted either into an emitted photon (fluorescence) or transferred to an acceptor fluorochome within short distance (FRET). If FRET occurs, the donor fluorescence is quenched. Acceptor absorbs the transferring fluorescence energy and after internal conversion emits fluorescence on its characteristic wavelength. Circles indicate the amount of emitted fluorescence.

FRET is observed as emission of acceptor fluorescence when only the donor is excited. Simultaneously, the donor emission is quenched. Chimeric fluorescent proteins are widely

used as FRET counterparts (e.g. CFP and YFP) since they do not compromise cellular structures and co-factors or substrates are not required thus enabling the measurements to be performed in live cells. (Pollok, Heim 1999, Sekar, Periasamy 2003).

2.4.2 FRET in practice

In basic protein-protein interaction studies when one interacting counterpart is labeled with a donor fluorochrome and the other with an acceptor, FRET imaging is used to study the interaction or complex forming of the proteins of interest. In such experimental settings FRET can be measured directly by monitoring donor fluorescence lifetime. FLIM-FRET (fluorescence lifetime imaging) is an imaging approach which can be used to measure the donor fluorescence lifetime in the presence or absence of an acceptor. If FRET occurs, the donor lifetime decreases due to energy transfer to the acceptor.

In acceptor photobleaching FRET imaging, the donor fluorescence intensity is compared between values obtained before and after the complete photobleaching of the acceptor fluorochrome. If FRET occurs between the donor and acceptor it is observed as an increase in the donor fluorescence intensity after photobleaching when the donor-emitted photons are detected as donor fluorescence rather than acceptor emission via FRET. Also, FRET can be detected without microscopy in cell population level using fluorescence activated cell sorting (FACS) (Chan *et al.* 2001). In the initial description on the method the authors demonstrate the interaction of a plasma membrane localizing receptor and a cytoplasmic signaling protein but state that the method can be used in FRET analyses of proteins localizing in any subcellular compartment. In addition, this method gives quantitative FRET data in a cell population which is not provided by basic microscopy FRET approaches.

FRET-based methods have been utilized in research on protein-protein associations, receptor-ligand interactions and demonstrating protease action (Wallrabe, Periasamy 2005, Rizzo *et al*. 2006). Also, conformational changes in some proteins and nucleic acids can be detected using FRET biosensors (Liu, Lu 2006) as well as gene expression and mRNA localization detection in living cells (Santangelo *et al.* 2004). Instead of using a donor-acceptor pair, a group of multiple fluorochromes can be utilized as FRET biosensors (Pauker *et al*. 2012, Kim, Gunther & Katzenellenbogen 2010) enabling the visualization of a three or four molecule complex formation dynamics, respectively.

As FRET is by definition restricted to occur between a donor-acceptor pair within a very short distance, it is easily influenced by minor changes in distance and orientation of the FRET counterparts (Piston, Kremers 2007). When a FRET approach is utilized to study protein complexes, additional unlabeled endogenous proteins interacting with one or several complex subunits can block or weaken the interaction between the donor and acceptor. False negative FRET can also occur, if the donor and acceptor fluorochromes reside in the opposite ends of a protein complex thus generating a too long distance between them for

FRET to occur. As the spatial organization of the FRET biosensors can be difficult to predict in some experimental settings, the method is challenging and cannot be applied in all protein interaction research (Vogel, Thaler & Koushik 2006). Also, stoichiometric donor:acceptor ratios over 1:10 and 10:1 (Chen *et al.* 2006), major difference in fluorescence brightness (Piston, Kremers 2007) or even autofluorescence of cellular components can affect the FRET outcome.

In FRET experiments a potentially false-positive result producing phenomenon, namely photoconversion, has been suggested to arise in acceptor photobleaching FRET microscopy (Valentin *et al.* 2005). According to some studies on photoconversion (Valentin *et al*. 2005, Kirber, Chen & Keaney 2007), subjecting YFP or other similar acceptor fluorochromes to high energy laser causes it to change its fluorescence properties, *i.e.* to shift its emission spectrum to similar of the donor or form a "CFP-like species". Therefore, there is a risk of the photoconverted YFP emission to be misinterpreted as increased donor fluorescence due to acceptor photobleaching (*i.e.* FRET signal) and subsequently a false positive result of the FRET experiment. However, there are also several papers stating that the photoconversion could not be achieved in the reported conditions or even after a longer exposure of the YFP (Thaler *et al*. 2006, Verrier, Soling 2006, Chial, Lenart & Chen 2010). The mechanism causing photoconversion is currently unidentified. In addition, recently Seitz and coworkers were able to quantify photoconversion of YFP and describe a correction method to deduce the true FRET signal (Seitz *et al.* 2012).

While photoconversion remains a controversial phenomenon and has not been undeniably accepted as an error source in FRET experiments based on acceptor photobleaching, we recognize the possibility that it may, in certain conditions, occur. However, it can be either avoided by using a FRET detection not utilizing photobleaching (*e.g.* FACS-FRET) or corrected from the data (Seitz *et al.* 2012).

2.5 Using rare genetic disorders in the research of basic biological mechanisms: the Finnish disease heritage

The Finnish disease heritage (FDH) was first used as a concept in 1973 by Norio, Nevanlinna and Perheentupa (Norio, Nevanlinna & Perheentupa 1973). Currently, FDH is considered to include approximately 40 rare, monogenic, mostly autosomal recessive disorders, including neurological, metabolic and growth disorders to mention a few (Kestilä, Ikonen & Lehesjoki 2010). The FDH diseases have an exceptionally high prevalence in Finland but most are described also in other populations. The main feature is the genetic uniformity of the Finnish patient cohort, in many cases characterized by a single or few founder mutations. On the other hand, mechanisms underlying the enrichment of the FDH mutations in the Finnish population contribute also to another feature of the genetic makeup in the population,

namely the absence or relative rarity of some of the most common inherited disorders in Caucasian population in general, such as phenylketonuria (PKU) and cystic fibrosis (Peltonen, Jalanko & Varilo 1999, Peltonen, Pekkarinen & Aaltonen 1995). The basis of the FDH is the genetic isolation of the Finnish population, further modified by small founder population(s), several population bottlenecks and genetic drift (Peltonen, Pekkarinen & Aaltonen 1995, Norio 2003a). However, the current Finnish population is not a single genetically uniform group but rather strikingly geographically divided into eastern and western subpopulations especially by Y chromosomal markers, depicting a more recent male-biased gene flow from Scandinavia to western Finland (Palo *et al.* 2009). Palo and coworkers also challenge the idea that the higher incidence of many of FDH illnesses in late settlement region is due to the bottleneck caused by the immigration in the 16th century by relatively small founder subpopulations. Instead, they state that the late settlement founder population represented a subset of the total population variation which was further modified by genetic drift in the small isolates causing the random enrichment of FDH alleles. The more varying gene pool in the early settlement became further influenced and mixed by the Scandinavian gene flow thus increasing the difference between western and eastern populations. Still, the genetic deviation in the eastern-northern vs. western Finland is seen in the distribution of some of the Finnish diseases; for example the birthplaces of LPI patients' grandparents are mostly located in the late settlement regions suggesting that the LPI founder mutation enriched among the population that came to inhabit the eastern and northern region (Lauteala *et al.* 1997b).

The molecular genetics underlying the FDH diseases was in the research focus from the 1990's. The genetic uniformity of Finnish patients has enhanced the mapping and characterization of the disease genes. Consistent with the genetic isolate, in most cases the Finnish patient cohort was found to be genetically homogenous carrying one or two founder mutations. In some cases such as LPI, prior to the characterizing of a single founder mutation, a common founder marker haplotype was observed at the mutation locus (Lauteala *et al.* 1997b, Lauteala *et al.* 1998) which was utilized in calculating the age of the mutation.

By now, the causative FHD genes have mostly been identified and the affected protein products characterized. The research aiming for identifying genetic defects causing Mendelian disorders in general has widened its scope to identify entire reaction pathways, signaling routes and complex interactions between proteins affected by the primary defect. The FHD research has resulted in novel findings in central biological processes such as ciliary function affected in Meckel syndrome (Kyttälä *et al*. 2006) and nephrin protein deficient in Finnish type congenital nephrosis having a function in cardiovascular development (Wagner *et al.* 2011). In LPI research the focus is now aimed at characterization of the relationship between the primary transport defect and the multiple clinical symptoms of the disease, particularly those that are more difficult to explain such as immunological and

hematological abnormalities often reported in patients (Tringham *et al.* 2012). Also, the detailed modeling of the y^+LAT1/4F2hc complex is not by any means completed despite the progress on understanding the 4F2hc ectodomain structure (Turnay *et al.* 2011). As y^+LAT1 belongs to the actively studied HAT transporters, the advancements in that area will most likely benefit the y^+LAT1 knowledge and further, add to the knowledge of molecular pathogenesis of LPI.

3. THE AIMS OF THE STUDY

The LPI gene *SLC7A7* encoding cationic amino acid transporter y^+LAT1 and several causative mutations including the Finnish founder mutation LPI_{Fin} were identified in a thesis by Juha Mykkänen (2003). The present study further elucidates the cellular defect caused by several LPI mutations and designed C-terminal deletion constructs utilizing green fluorescent protein fusions in confocal microscopy and flow cytometry FRET. Also, the expression level of mutant y^+LAT1 transporter was studied in transfected cells and the *SLC7A7* gene promoter was defined.

The specific aims of the present study were:

1. To analyze the localization of LPI mutant y^+LAT1s as GFP fusions in mammalian cells
2. To study the dimerization process between LPI mutant or C-terminally deleted y^+LAT1 and 4F2hc using flow cytometry FRET
3. To study the effects of mutant y^+LAT1 expression on cell viability
4. To define *SLC7A7* promoter region and characterize the promoter function

4. MATERIALS AND METHODS

4.1 *SLC7A7* cDNAs and fluorescent protein expression vectors

The GFP variant fluorescent proteins used in the current study are EGFP (enhanced green fluorescent protein), ECFP (cyan) and EYFP (yellow) as well as DsRed, whose excitation and emission maxima, as well as quantum yields are shown in Table 4.

The expression vectors pEGFP-C2, pECFP-C1 (cyan), pEYFP-C1 (yellow), DsRed-C1 and DsRed-N1 (Living Colors®, BD Biosciences Clontech, Palo Alto, CA) were used to create the fluorescent fusion protein constructs. The 4F2hc cDNA and y^+LAT1 wild type and mutant cDNAs (LPI_{Fin}, G54V, 1548delC and W242X) were originally cloned in pSPORT vector. The LPI mutation nomenclature is explained in Table 5 and their localization in the *SLC7A7* cDNA is shown in Figure 6.

Table 5. The nomenclature of LPI-causing mutations.

Nucleotide change[1]	Amino acid change	Alternative nomenclature	Comment	Reference
c.161G→T	p.G54V	447G→T, G54V[2]		(Mykkänen *et al*. 2000)
c.726G→A	p.W242X	1012G→A 967G→A, W242X[2]		(Mykkänen *et al*. 2000, Sperandeo *et al*. 2000)
c.1262delC	p.P421RfsX98	1548delC[2]		(Mykkänen *et al*. 2000)
c.895-2A→T	p.T299IfsX10	1181-2A→T, LPI_{Fin}[2] 1136-2A→T IVS5-2A→T	Acceptor splice site error	(Torrents *et al*. 1999, Borsani *et al*. 1999)

[1] Nucleotide numbering is based on the cDNA sequence NM_003982.3 and NT_026437; nucleotide +1 corresponds to the A in the ATG translation initiation codon in the SLC7A7 reference sequence (according to (den Dunnen, Antonarakis 2000)).[2] The mutation nomenclature used in this study

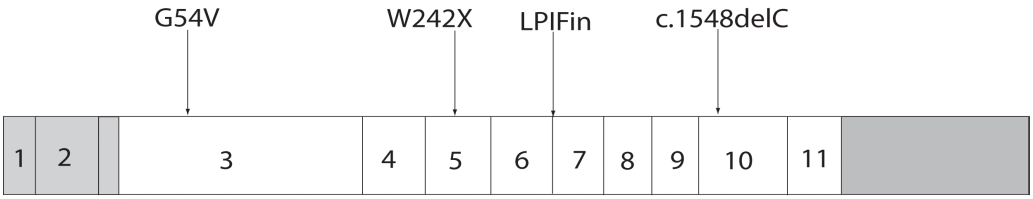

Figure 6. Mutations used in this study in the *SLC7A7* cDNA. Exons 1-11 are numbered; the coding region is in white.

4.2 Promoterless luciferase plasmid

To study the promoter activity of 5' non-coding region of *SLC7A7* gene, overlapping fragments of the putative upstream region were amplified by PCR and cloned into promoterless firefly luciferase-coding plasmid pBL-Luci6. Upstream sequence up to 1185 bp from 5' of exon 1 was considered the core promoter region and included in the study. Also, introns 1 and 2 were analyzed using the luciferase system.

4.3 Plasmid construction

4.3.1 Fluorescent protein fusion expression plasmids

The open reading frame (ORF) inserts of y^+LAT1 and 4F2hc to be cloned in frame with the fluorescent protein expressing vector backbones were first amplified utilizing cDNA templates and *Pfu* proof-reading DNA polymerase (Promega Corporation, Madison, WI) according to manufacturer's instructions. The primers are described in the "Material and Methods" section in I (y^+LAT1-EGFP and 4F2hc-DsRed) and section 2.1 in II (y^+LAT1-ECFP and 4F2hc-EYFP). All the used fluorescent protein vector backbones were purchased from BD Biosciences Clontech. The PCR products were run on 1% agarose gel, the DNA concentration was estimated and the product purified using the GFX DNA and Gel Band Purification Kit (Amersham Biosciences, UK). The purified products were A-tailed utilizing the *Taq*-DNA polymerase to enable the ligation into the linear pGEM-T subcloning plasmid (Promega). The PCR products were then ligated into pGEM-T according to the manufacturer's instructions using T4 DNA ligase (Promega, Madison, WI, USA), transformed into *E. coli* supercompetent cells (JM109 strain; Promega) and cultured overnight on LB agar plates containing 50 µg/ml ampicillin (Sigma Aldrich), 0.5mM IPTG and 40 µg/ml X-Gal (Promega). Positive colonies were picked by color selection and screened by colony PCR, grown overnight in LB medium followed by plasmid miniprep isolation using Strataprep™ Plasmid Miniprep Kit (Stratagene Cloning Systems La Jolla, CA, USA) following the manufacturer's instructions. Plasmids were double digested with appropriate restriction enzymes (New England Biolabs) to isolate the insert. The gel-purified inserts were then introduced to the identically digested final vectors to create y^+LAT1-EGFP-C2, 4F2hc-DsRed-C1, y^+LAT1-ECFP-C1 and 4F2hc-DsRed-N1- plasmids coding C or N terminally fluorescently tagged, wild type or mutant y^+LAT1 or 4F2hc. The ligation, transfection and miniprep preparation steps were repeated as above, except for the LB agar plate composition: antibiotic kanamycin (30 µg/ml) replaced ampicillin and no color selection reagents were added. Positive clones were sequenced using the BigDye sequencing kit and ABI Prism 377XL DNA Sequencer (Applied Biosystems). The final insert sequences were analyzed on Sequencher 4.0.5 software (Gene Codes Corporation). To produce an overexpressing 4F2hc plasmid construct without any fluorescent labels (4F2hc-stop-DsRed), a stop-codon was introduced by point mutation in the 3' end of the 4F2hc

in 4F2hc-DsRed-N1 plasmid. The point mutation was created using QuickChange™ Site-Directed mutagenesis Kit (Stratagene) (described in detail in I).

For the transfections, maxiprep scale plasmid isolation was performed for each construct (Qiagen Plasmid Maxi prep, Qiagen GmbH Hilden, Germany or NucleoBond® PC 500 EF Kit, Macherey-Nagel GmbH & Co. KG, Duren, Germany).

Cell populations were double transfected using the FRET vector combinations described in Table 7. Of the CFP- and YFP fusion proteins generated in the LPI project (Figure 7), the combination of N-terminally labeled y^+LAT1 and 4F2hc was chosen, as it gave the highest FRET signal in our previous studies (Kleemola *et al.* 2007). The C1 in the plasmid nomenclature originates from the expression vector name and refers to the multiple cloning site (MCS) in relation to the fluorescent protein gene in the vector backbone.

To construct the YFP tagged 4F2hc fusion proteins, the corresponding C- terminally labeled 4F2hc ORF was digested from the 4F2hc-DsRed-C1 plasmid. The C1 insert was isolated and the target vector was double digested by *SalI*/*BamHI*. The gel-purified 4F2hc ORF was then joined to the target vector by direct ligation. The resulting 4F2hc-EYFP-C1 plasmid was transformed, cultured, screened and sequenced as described above. Plasmids carrying sequence-confirmed inserts were grown into maxi preps and used in transfections.

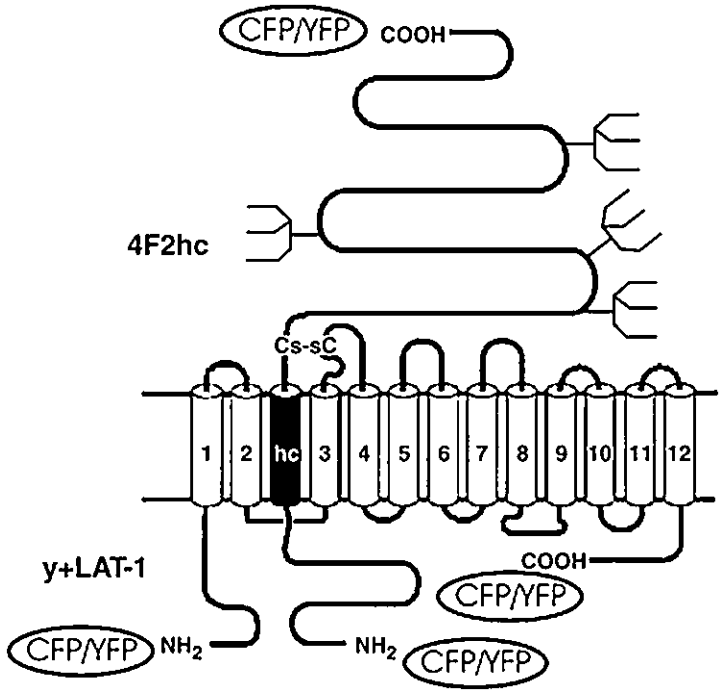

Figure 7. A schematic diagram of the y+LAT1/4F2hc dimer in its predicted conformation and CFP and YFP tag positions in the construct termini (Kleemola *et al.* 2007). The image is modified from (Verrey *et al.* 1999).

The transformed bacterial strains were pure cultured overnight in appropriate antibiotic supplemented LB and stored as glycerol stocks in -70°C.

4.3.2 Luciferase plasmids for promoter region analysis

To generate luciferase vectors for promoter activity analysis, the putative 5' promoter region and the first two introns of *SLC7A7* were amplified by PCR in fragments varying in size between 1185 bp and 153 bp. The names of the fragments refer to fragment (insert) size in bp and the coverage of the 5' region upstream of exon 1. The primer combinations are described in Table 6.

Table 6. Primers sequences used in cloning the promoter region segments into the pLuci vector

Construct	Primer	Sequence 5'-3'
Prom 153	73F	GTCGACCAGTCCCCGTTACCCTCTGC
	LPI107RBamHI	GCGGATCCCAGTGACCTTTGGGCAGGT
Prom 168	168F	GTCGACTCTGGGTCTGACCAGGCAGT
	LPI 5'RCBamHI	GTCGACTCTGGGTCTGACCAGGCAGT
Prom 192	113F	GTCGACGCCGGCCAGAGTCACACAT
	LPI107RBamHI	
Prom 232	232F	GTCGACGACTCAGGTATTTGGTGCCAGTG
	LPI 5'RCBamHI	
Prom 420	420F	GTCGACTGGGATCGTATCCCCTCTGC
	LPI 5'RCBamHI	
Prom 1185	1185F	GTCGACGATGCATTGTGAATGCTGCT
	LPI 5'RCBamHI	
Intron 1	LPI25FSalI	GTCGACCTTGGTTCTCCTAACTAGCAC
	LPI209RCBamHI	GCGGATCCAGTGTGAGCGGCAGTCAGGGAG
Intron 2	LPI190FSalI	GTCGACTCCCTGACTGCGGCTCACAC
	LPI411RCBamHI	GCGGATCCCCGTTAAGCAGTGAGATCTCC

The amplified fragments were first ligated into pGEM-T vectors as described in the fluorescent fusion protein plasmid protocol, grown over night following plasmid miniprep extraction. Isolated plasmids were digested using appropriate restriction enzymes following the purification and ligation of the detached inserts into promoter-less luciferase vectors. Final luciferase plasmids with putative promoter fragments were transformed into bacteria, grown into maxi preps and used in transfections.

4.3.3 Cell lines

Human epithelial cells of renal origin (HEK293 cells, human embryonic kidney 293, #CRL-1573; American Type Culture Collection, Manassas, VA) were used to express fluorescent fusion proteins both in the confocal microscopy and flow cytometry experiments (I, II)

as well as promoter analyses by the promoterless luciferase plasmid (III). Also, human adenocarcinoma cell line 2 was used in the confocal microscopy experiments in the experimental set-up (ATCC HTB-37; CaCO2). Cells were maintained in EMEM supplemented with 10% fetal calf serum (FCS). Fibroblasts derived from skin biopsies of control subjects and LPI patients were cultured in F-10 medium (80% Ham's F-10 and 20% FCS). Lymphoblasts were maintained in RPMI medium (90% RPMI and 10% FCS). All cell culture media were supplemented with 1 mM L-glutamine, 100U/ml penicillin-streptomycin and occasionally 30 µg/ml gentamycin or 0,25-0,5 µg/ml Fungizone® Antimycotic (Gibco® Invitrogen Cell Culture) and cultured in a humidified incubator in +37°C and 5% CO_2.

4.3.4 Transfection and sample preparation (I-III, unpublished)

To express the y^+LAT1 and 4F2hc fluorescent fusion proteins in cells, 2 µg of each plasmid DNA was used to transfect HEK293 cells. In localization studies (I, unpublished) as well as expression and mortality studies (II, unpublished) the y^+LAT1-EGFP-C2 plasmids were double transfected with an equal amount of 4F2hc expression vector. Transfection was performed by FuGENE6 Transfection Reagent (Roche Molecular Biochemicals, Mannheim, Germany) using 3:1 transfection reagent: total DNA mass (µg) ratio, and transfection complex incubation of 30 minutes. When multiple plasmids were used for transfection, the plasmid DNAs were mixed prior to adding of transfection reagent to ensure maximal amount of double transfected cells.

To prepare live cells for confocal microscopy (I), cell culture and transfection were carried through as described above. In the end of the culture period, the cover slips with attached cells were removed from the culture medium and mounted on object glasses using Hepes-buffered EMEM medium to maintain pH in normal $C0_2$ atmosphere. Confocal microscopy was carried out immediately.

In the protocol for FACS sample preparation, cells were cultured on six-well plates followed by transfection using the plasmid combinations described in Table 7 (for FACS-FRET) or y^+LAT1-EGFP-C2 constructs with equal amount of 4F2hc-stop-DsRed (for expression and viability analysis). Cells were detached from the plate by a brief trypsin treatment, washed with PBS and collected by centrifugation. Cell pellets were then re-suspended in either PBS or Hepes-buffered EMEM resulting in a total concentration of ~2 x 10^6 cells per ml, and the FACS run performed within 1-2 hours. To detect the dead cells in transfected cell populations, propidium iodide (PI) was added to final concentration of 0.3 µg/ml to the cell suspension just prior to the run. During the run, the PI positive, dead cell population was separated from the PI negative, live cell population.

Table 7. Control and FRET combinations used in II.

CFP	YFP	FRET combination
pECFP-C1 vector	pEYFP-C1 vector	negative FRET control
CFP-YFP tandem		forced FRET, positive control
y^+LAT1/ECFP-C1	-	expression/ negative FRET control
-	4F2hc/EYFP-C1	expression/ negative FRET control
y^+LAT1/ECFP-C1	4F2hc/EYFP-C1	FRET

4.3.4.1 GFP imaging (I, unpublished)

EGFP fusion proteins were visualized using Leica TCS SP1 MP spectral confocal microscope (Leica Microsystems, Mannheim, Germany) with 63x NA 1.4 plan apo chromat oil immersion objective. The argon laser line 488 nm was used to excitate the fluorochrome, and the fluorescence emission was collected through band pass filter of 500-550 nm. The ECFP-Golgi vector (I) was detected using the above mentioned confocal microscope connected with a Tsunami® Spectra-Physics Mode-Locked Ti-Sapphire laser (Millenia V, Spectra Physics), providing two-photon excitation at 790 nm. CFP emission was collected through 465-495 nm. The optical slice was set to <1.1µm and the detector gains were adjusted to an optimal signal level (I).

4.3.4.2 Fluorescence-activated cell sorting (FACS): cell viability analysis (II, unpublished)

FACS was utilized to study the expression and dimerization of EGFP, ECFP and EYFP amino acid transporter fusion proteins in cell population level and to gain information of their effects on the viability and proliferation of the cell populations. To study cell viability, the y^+LAT1-EGFP and 4F2hc-stop-DsRed expressing live cell samples, both the wild type and mutant variants of the light subunit, were run on BD FACSCalibur flow cytometer (BD Biosciences). The 488 nm laser excitation was used and GFP emission was detected at FL1 channel. The PI added prior to the FACS run penetrates dead cells only, and enables them to be detected on separate channel (FL2). The resulting dot plot diagrams were divided in four quarters: live and dead, GFP positive and negative, and the relative amount of live cells was calculated in GFP positive and negative groups separately. Runs were repeated three times to reduce the variation between samples caused by cell manipulation and sample preparation.

4.3.4.3 FRET analysis using FACS (II, unpublished)

The FRET assays were controlled by single transfections of each of the constructs, non-transfected and pseudo-transfected cell populations as well as CFP-YFP-tandem construct, representing forced FRET. The FACS-FRET analysis was performed using the LSRII flow cytometer (Becton Dickinson, San Jose, USA). The cell concentration during the FACS run was ~10^6 cells/ml. 30000 cells were analyzed from each sample. During the run, the donor

CFP was excited by 405 nm violet laser diode and the emitted CFP (donor) and YFP (acceptor) fluorescence signals were collected using detectors with 480/40BP filter and 520/50BP filter, respectively. The 405 nm laser is absorbed only by the donor resulting in a minimal background signal from YFP to the detector. Therefore, a positive FRET can be observed as an average increase of the acceptor (YFP) fluorescence intensity and the simultaneous decrease of the donor CFP fluorescence in double transfected cell populations.

The FACS-FRET results were displayed as CFP *vs.* YFP dot plots (Figure 1 in II). When FRET occurs, the fluorescence of the CFP-positive cells decreases and the population shifts to up left in the dot plot.

4.3.4.4 Statistical analysis (II)

The fusion protein expression rate and mortality rate data of wild type and LPI mutant transfected cell samples from the flow cytometer runs were statistically analyzed using the Poisson regression test. Results were expressed using relative risk (RL) with 95% confidence interval (CI). P-values lower than 0.05 were considered statistically significant. Statistical analysis was done with SAS System for Windows, release 9.1 SP 4 (SAS Institute Inc., Cary, NC, USA).

5. RESULTS AND DISCUSSION

5.1 Subcellular localization of wild type and mutant y⁺LAT1 in mammalian cells (I, unpublished)

At the start of the current study, the knowledge on the defect caused by LPI mutant y⁺LAT1 at cellular level was based on experiments on *X. laevis* oocytes injected with c-myc tagged y⁺LAT1 (Torrents *et al*. 1999, Mykkänen *et al*. 2000, Sperandeo *et al*. 2005a, Sperandeo *et al*. 2005b). These functional tests on various LPI causing mutations of y⁺LAT1 had revealed a differential cellular targeting of missense and frameshift mutants in the oocyte experiments. The analysis confirmed the dependence of plasma membrane trafficking of y⁺LAT1 on 4F2hc. So far attempts on direct visualizing of y⁺LAT1 in cell and tissues using polyclonal antibodies had failed, probably due to the low amount of y⁺LAT1 in cells. Also, the attempts to isolate and purify the protein have not succeeded, most likely due to the y⁺LAT1 structure that is highly hydrophobic and bound integrally in the plasma membrane. Curiously, the antibody against the mouse y⁺lat1 does not recognize the human homolog, limiting the possibility to use immunohistochemistry to study the cell biology of LPI. Thus, an alternative approach to visualize human y⁺LAT1 in intact cells had to be utilized. In this thesis, the target was to test whether the various types of mutant proteins behave differentially in mammalian cells. For this purpose GFP-y⁺LAT1 fusion protein expression vectors were designed. A wide number of proteins have been expressed as GFP or its spectral variant fusions, also a nonfunctional transporter of the SLC7 transporter family (Wolf *et al.* 2002).

The fusion protein expression plasmid was constructed on a commercial GFP expression vector backbone pECFP-C2 (Clontech), which produced an N-terminally tagged GFP fusion protein of y⁺LAT1. The plasmid was then transiently transfected into HEK293, CaCO2 and MDCK cell lines. The fusion protein was expressed in all the mentioned cell lines, but due to the best transfection efficiency HEK293 cell line was chosen for subsequent experiments. Wild type and mutations found in more than one LPI patient (point mutation G54V, frameshift mutations LPI_{Fin} and 1548delG and nonsense mutation W242X; the mutation nomenclature is explained in Table 5) were chosen for the targeting experiments in mammalian cells, since they have been functionally tested for transport activity and shown equally poor transport activity of cationic amino acids. Also, their localization had been previously tested in *X. laevis* oocytes using the c-myc tag (Mykkänen *et al*. 2000). Since y⁺LAT1 requires 4F2hc to reach the plasma membrane (Pfeiffer *et al.* 1999b), equal amount of unlabeled 4F2hc-expressing plasmid was co-transfected to the cells..

All the GFP fusion proteins were expressed in moderate to high levels in HEK293 cells enabling the visualization by confocal microscopy using live cell samples. However, other

than the missense mutant G54V, LPI mutant transfections constantly resulted in smaller amount of GFP positive cells (further discussed in the chapter focused on the expression level of mutant y$^+$LAT1). To visually differentiate between intracellular compartments relevant for protein translation and dimer formation (ER and Golgi), the GFP expressing cells were transfected with a Golgi visualization vector pECFP-Golgi (Clontech).

The imaging of the GFP-expressing cells revealed that the wild type and missense mutant fusion protein G54V localize in an identical pattern in the plasma membrane in the presence of the exogenous 4F2hc (Figure 2 in I). They were also detected partially in the intracellular membranes, but in significantly smaller amounts than following transfections of the y$^+$LAT1-EGFP alone, indicating a facilitating effect on membrane trafficking of overexpressed 4F2hc in the transfected cells. The fact that some of the wt y$^+$LAT1-EGFP is localized to the plasma membrane without exogenous 4F2hc is most likely due to the endogenous 4F2hc in the human kidney-originating HEK293 cell line. However, since the overexpressing system of the GFP vector containing the CMV promoter is highly active, it forces the cell to produce excessive amounts of y$^+$LAT1, and the protein synthesis machinery is not able to produce enough endogenous 4F2hc to form heterodimers with all the produced y$^+$LAT1-EGFP. In contrast, the truncating mutant fusion proteins localized in the ER and entered the trans-Golgi-network, where the 4F2hc glycosylation maturation takes place prior to the plasma membrane trafficking of the holotransporter, but were not carried to the plasma membrane. Therefore, mammalian cells transfected with fusion protein plasmids confirmed the observations from the oocyte experiments: as transfections of solely y$^+$LAT1-EGFP resulted in mostly intracellular localization of the fusion protein, y$^+$LAT1 requires 4F2hc to be trafficked to the plasma membrane.

The non-mammalian expression system on the LPI mutant proteins revealed an equally low transport activity of y$^+$LAT1 substrates of point mutants as well as frameshift or nonsense mutant proteins, even though the point mutant proteins are carried to the plasma membrane, while mutants resulting in larger fragments of aberrant amino acid sequence or truncation of polypeptide remain cytoplasmic (Mykkänen *et al*. 2000). The cytoplasm-localizing mutant proteins are detected as granular structures, which overlap with 4F2hc-fusion protein fluorescence in mammalian cells; however, the double transfection of y$^+$LAT1-EGFP and DsRed-4F2hc resulted only a few cells expressing both tagged proteins, suggesting that the cytotoxicity of DsRed affects the cell viability in this expression system (Strack *et al.* 2008). Therefore, to acquire viable cells with normal morphology, y$^+$LAT1-EGFP was co-transfected with 4F2hc without a fluorescent label (4F2hc-stop-DsRed).

All the studied mutant y$^+$LAT1 transporters are localized to both endoplasmic reticulum and Golgi (Figure 2 in I). Since the interaction between the transporter complex subunits takes place at ER (Kleemola *et al.* 2007), this localization pattern leaves open the possibility of

aberrant y⁺LAT1 polypeptides to dimerize with the heavy chain. The interaction between y⁺LAT1 and 4F2hc may thus be necessary for the recognition of falsely folded y⁺LAT1, or the correct and stabile dimerization may be the condition for the transporter complex to be carried to the plasma membrane. However, the basic confocal imaging of fluorescent protein tagged y⁺LAT1 and 4F2hc used in subproject I of the current thesis cannot resolve between colocalization and dimerization, leaving the possibility of defective dimerization as a cause of mistargeting open.

The plasma membrane localization of 4F2hc when expressed alone was discovered already in the *X. laevis* oocyte studies, and was observed repeatedly in every mutant y⁺LAT1/4F2hc imaging performed on mammalian cells (I, unpublished). Also, the 4F2hc homolog rBAT point mutations causing recessive type A of cystinuria cause trafficking failure or delay, indicating the crucial role of the heavy chain for the transporter complex to be correctly localized (Chillaron *et al.* 1997). In the current work, the trafficking defect was found to be restricted only to the truncating mutant y⁺LAT1s, while 4F2hc was at least partially normally carried to the plasma membrane when co-expressed with these y⁺LAT1 mutants. The simultaneous transfection of y⁺LAT1-EGFP and 4F2hc-DsRed was observed to result in increased cellular mortality; thus, to produce sufficient amount of live transfected cells for the imaging of y⁺LAT1 localization, the experiment was carried out using double transfections of y⁺LAT1-EGFP and non-tagged over-expressed 4F2hc (I). When transfected without additional 4F2hc, y⁺LAT1 was translocated to the plasma membrane but in lower amounts and in a slower rate than in double transfected samples.

According to Shaw and coworkers, 4F2hc has at least three endogenous LSHATs expressed in the HEK293 cells: y⁺LAT1, y⁺LAT2 and LAT1 (Shaw *et al.* 2002). Therefore, the observed correct localization of 4F2hc-DsRed independent of the y⁺LAT1-EGFP localization pattern is a result of dimer formation between 4F2hc and the endogenous LSHATs instead of 4F2hc and y⁺LAT1-EGFP since the ER-Golgi transition of the transporter heterodimer requires the light subunit (Sakamoto *et al*. 2009, Ganapathy 2009). Thus, not all of translated 4F2hc is interacting with the overexpressed y⁺LAT1-EGFP. Since the chosen method of transfection is not quantitative at the level of translated protein products we were not able to evaluate the amount of each subunit produced by a transfected cell population.

5.2 Subcellular localization of C-terminally truncated y⁺LAT1-EGFP (unpublished)

Interestingly, the basic concept of categorizing the LPI mutations in correctly targeted point mutant transporters and cytoplasm-remaining truncated transporters is not valid without exceptions. A series of GFP fusion proteins of truncated y⁺LAT1 was generated, specifically lacking amino acid residues of the C-terminal polypeptide in 10 amino acid intervals (Figure

8) in order to study the role of the C-terminal tail on the plasma membrane trafficking. Confocal imaging of transfected HEK293 cells expressing the truncated y⁺LAT1 revealed that only the transporters lacking all of the C-terminal polypeptide completely remain intracellular whereas the transporters truncated by equal or less than 10-30 terminal amino acid residues are partially carried to the plasma membrane (Figure 9). y⁺LAT1-EGFP fusions lacking 40-50 C-terminal residues are mostly intracellular, whereas all of the constructs completely lacking the C-terminal polypeptide *i.e.* from Δ60-y⁺LAT1-EGFP on are not trafficked to the plasma membrane.

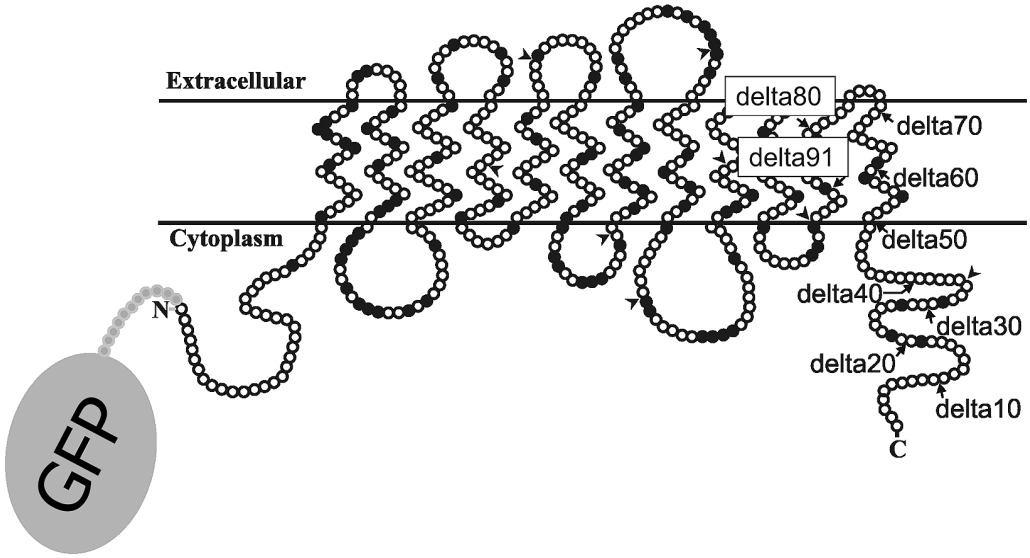

Figure 8. A schematic representation of C-terminal deletions on y⁺LAT1-EGFP fusions. y⁺LAT1 polypeptide structure in the membrane is predicted on the basis of hydrophobicity of amino acid sequence (Torrents *et al.* 1999). Deletion breakpoints are indicated by an arrow and named; open circle: amino acid residue; filled black circles: conserved amino acid residue; filled grey circles: linking peptide of the EGFP-C2 expression vector; arrowheads: exon-exon boundaries. (Modified from Torrents *et al.* 1999).

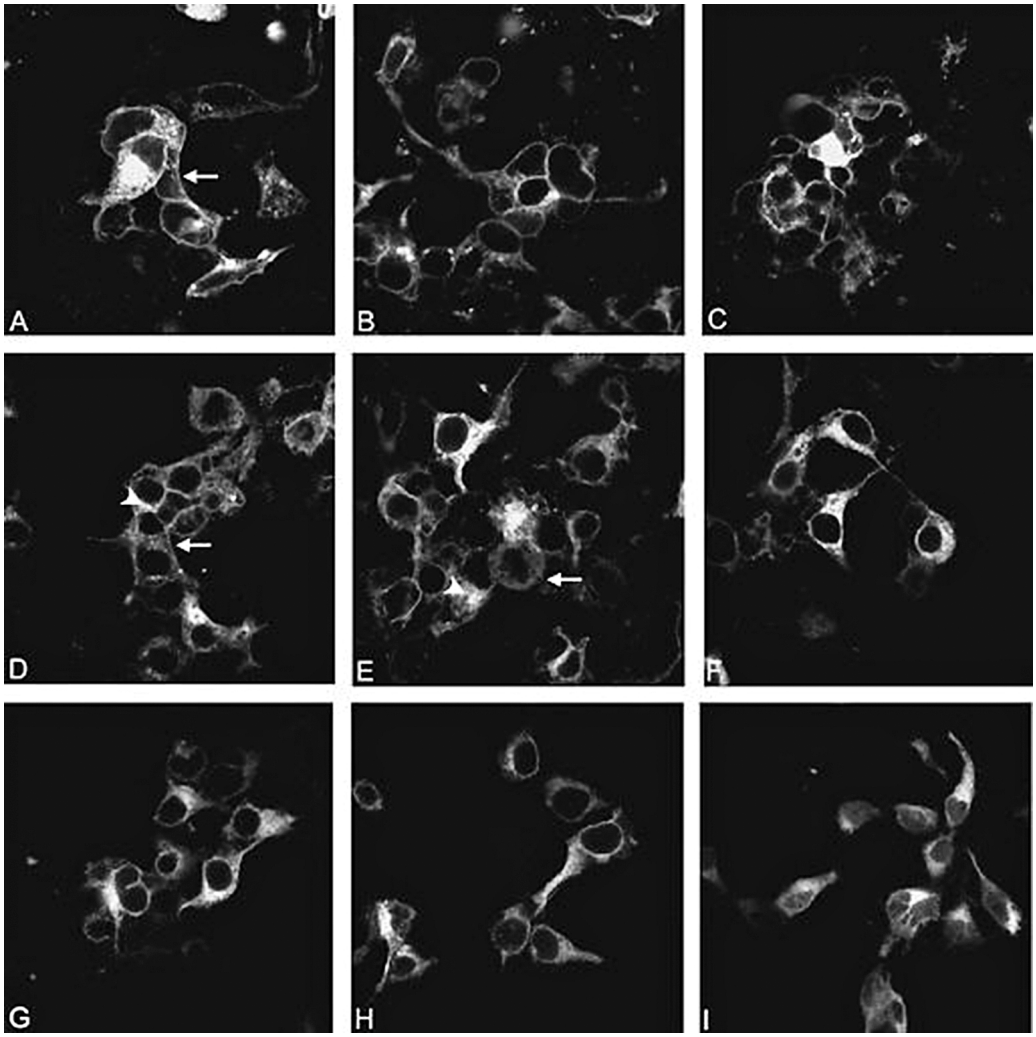

Figure 9. A: Δ10-y⁺LAT1-EGFP, B: Δ20- y⁺LAT1-EGFP, C: Δ30- y⁺LAT1-EGFP: truncated protein is partially localized to the plasma membrane D: Δ40- y⁺LAT1-EGFP, E: Δ50- y⁺LAT1-EGFP: most fusion protein intracellular. F: Δ60- y⁺LAT1-EGFP, G: Δ70- y⁺LAT1-EGFP, H: Δ80- y⁺LAT1-EGFP, I: Δ91- y⁺LAT1-EGFP: After the deletion of 60 amino acids, the fusion protein is not detected at the plasma membrane. Arrow: plasma membrane; arrowhead: Golgi.

Taken together, truncated y⁺LAT1 fusion proteins that have the cytoplasmic C terminus partially left are targeted to the plasma membrane, but the size of the deletion affects the trafficking dynamics: when the remaining portion of the C-terminal polypeptide grows smaller, more truncated y⁺LAT1 remain cytoplasmic. The gradual decrease in proportion of the membrane-localizing transporter may be due to the decreased rate of subunit assembly, slower trafficking rate or increasing instability of the transporter heterodimer, but since time-lapse imaging was not utilized, the explanation for the trafficking defect is currently elusive. However, the results indicate that the C terminus contains intrinsic elements or sequence signals important for the dimer stabilizing and/or plasma membrane sorting of

the resulting transporter complex which may be similar to those in the related transporter $b^{0,+}$AT (Sakamoto *et al*. 2009, Ganapathy 2009). In the article by Sakamoto and coworkers they report that the removal of the C-terminus does not affect the general membrane trafficking or complex formation with the corresponding heavy chain rBAT but abolishes the amino acid transporter activity. Also, a C terminal tripeptide signal responsible for the apical membrane targeting of $b^{0,+}$AT is described: The $V^{480}P^{481}P^{482}$ motif is partially conserved in Asc1, LAT1 and xCT, and is logically absent in the basolateral y^+LAT1 and y^+LAT2. Currently no basolateral sorting signal peptides either have been described in the y^+L transporters, leaving their targeting mechanism unresolved.

The sequential deletion constructs shown in Figure 8 have not been functionally tested, and thus the effect of these deletions on the transporter function is unknown. The defective subcellular sorting of LPI-associating, truncating mutant y^+LAT1 transporters causes the depletion of functional transporter heterodimers at the plasma membrane resulting in deficient amino acid transport in the cell, thus causing the disease. The two reported truncating LPI mutations that locate to the C-terminal polypeptide (p.Arg473Ter and c.1460delG) result in defective transporter *in vivo*, but their subcellular localization or *in vitro* function has not been tested. To speculate on the amino acid transporter activity of these C-terminally truncated y^+LAT1 proteins, it is feasible to think that those missing at least 60 amino acids most likely are defective as transporter subunits as they are not carried to the plasma membrane. Even though the transporters lacking less than 60 residues are partly membrane targeted, their transport activity may still be totally abolished, as is the case with point mutants causing LPI. In cystinuria, the associating $b^{0,+}$AT transporter subunit partially lacking the C-terminus was assembled to a transporter complex and carried to the plasma membrane, but showed no transporter activity (Sakamoto *et al.* 2009). Furthermore, $b^{0,+}$AT is required for the complete folding of rBAT via involvement in cysteine bond formation within the rBAT ectodomain (Rius and Chillaron 2012). The observed trafficking defect may be caused by improper recognition of the truncated y^+LAT1 by 4F2hc unlike the $b^{0,+}$AT/rBAT complex in the work by Sakamoto and coworkers (Sakamoto *et al.* 2009). When these y^+LAT1-EGFPs are over-expressed together with the 4F2hc, the truncated y^+LAT1 may be unable to fold in a conformation that is stably dimerized with 4F2hc, subsequently causing the trafficking defect of the transporter.

5.3 Dimerization of y^+LAT1/4F2hc (II, unpublished)

To expand the knowledge on y^+LAT1/4F2hc transporter complex formation and its role in the subunit assembly and targeting process, we generated fusion proteins of each of the subunits, y^+LAT1 and 4F2hc tagged with either EYFP or ECFP to perform FRET experiments in transfected HEK293 cells. By the means of FRET analysis of potentially interacting proteins one can distinguish between colocalization and dimer formation which is not achieved

by basic imaging techniques. Since the fluorescent fusion protein expression and imaging had proven successful in the previous study (I), the approach was adapted for the FRET experiments as well.

Several earlier studies have shown that y$^+$LAT1 and 4F2hc colocalize at the basolateral membrane of polarized epithelial cells of kidney and small intestine, and that y$^+$LAT1, among other LSHATs requires the corresponding HSHAT to be targeted correctly at the membrane (Pfeiffer *et al*. 1999b, Broer and Wagner 2002). Also, the *X. laevis* oocyte studies on the subcellular localization of LPI mutation-carrying y$^+$LAT1 proteins revealed, that mutations causing an amino acid substitution in the polypeptide did not affect the protein trafficking at cellular level, whereas the mutations changing the polypeptide structure more drastically, *i.e.* frameshift and nonsense mutations, resulted in an intracellular y$^+$LAT1 (Mykkänen *et al*. 2000). To study the subcellular localization of LPI mutant y$^+$LAT1 we expressed them as GFP fusion proteins in mammalian cells, an approach which proved the usefulness of the fusion protein technique in visualizing the y$^+$LAT1 protein (I). Also utilizing the GFP variant fusion proteins of y$^+$LAT1 and 4F2hc, acceptor photobleaching FRET microscopy was proven to be a reliable technique to study the dimerization of wt y$^+$LAT1 and 4F2hc (Kleemola *et al.* 2007). In the mentioned study, the interaction detected as positive FRET signal between the wild type transporter subunits was observed throughout the biosynthetic pathway: from ER to Golgi and at the plasma membrane. Thus, further studies utilizing FRET were indicated to clarify the importance of dimer formation step in the subcellular sorting of mutated y$^+$LAT1 proteins. To test the hypothesis according to which the earlier-detected trafficking defect of truncating mutant y$^+$LAT1 proteins (I) was caused by defective dimerization with 4F2hc, the FACS-FRET application was used. This technique was selected to overcome some potential hazards associated with the conventional acceptor photobleaching FRET microscopy, such as the controversial phenomenon of photoconversion (Valentin *et al*. 2005, Thaler *et al*. 2006, Verrier and Soling 2006, Kirber *et al.* 2007), which has been described in FRET assays involving photobleaching of YFP or a comparable acceptor (see chapter 2.4.2 FRET in practice). The FACS-FRET, in which FRET is measured in live transfected cell suspensions, does not involve photobleaching, but instead the donor is excited with specific wavelength following the emission detection from both the donor and the acceptor. When no FRET occurs, only donor emission is detected. In a positive FRET assay, acceptor fluorescence can be detected and the donor fluorescence is weakened. The results are obtained in average cell population shift in dot plots, where the CFP fluorescence is depicted in the X axis.

The LPI associating y$^+$LAT1 mutant transporters are known to be dysfunctional. In contrast to the truncated transporters, the point mutated and the E36del mutant (p.Glu36del) lacking one residue without a frameshift are carried to the plasma membrane (Mykkänen *et al*. 2000, Sperandeo *et al*. 2005b and subproject I). Interestingly, all the LPI mutated y$^+$LAT1s in this study (LPIFin, G54V, W242X, 1548delC) as well as the y$^+$LAT1 transporters carrying C-terminal deletions of 10-91 amino acids were detected to form dimers in equal

proportions of the CFP positive cell population when compared to the wild type y⁺LAT1 (Figure 1 and Table 2 in II, Figure 10, respectively). The average CFP emission of the analyzed cell population was quenched while the YFP emission was enhanced as the FRET transition of energy occurred in double transfected samples. In the FACS-FRET experiments the FRET induced fluorescence emission changes were visualized on a dot plot graph where the fluorescence emission intensity of CFP positive cells was compared to that of both CFP and YFP positive cell populations. All the studied y⁺LAT1 constructs gave similar results: FRET occurred between all of the mutated y⁺LAT1 proteins and 4F2hc.

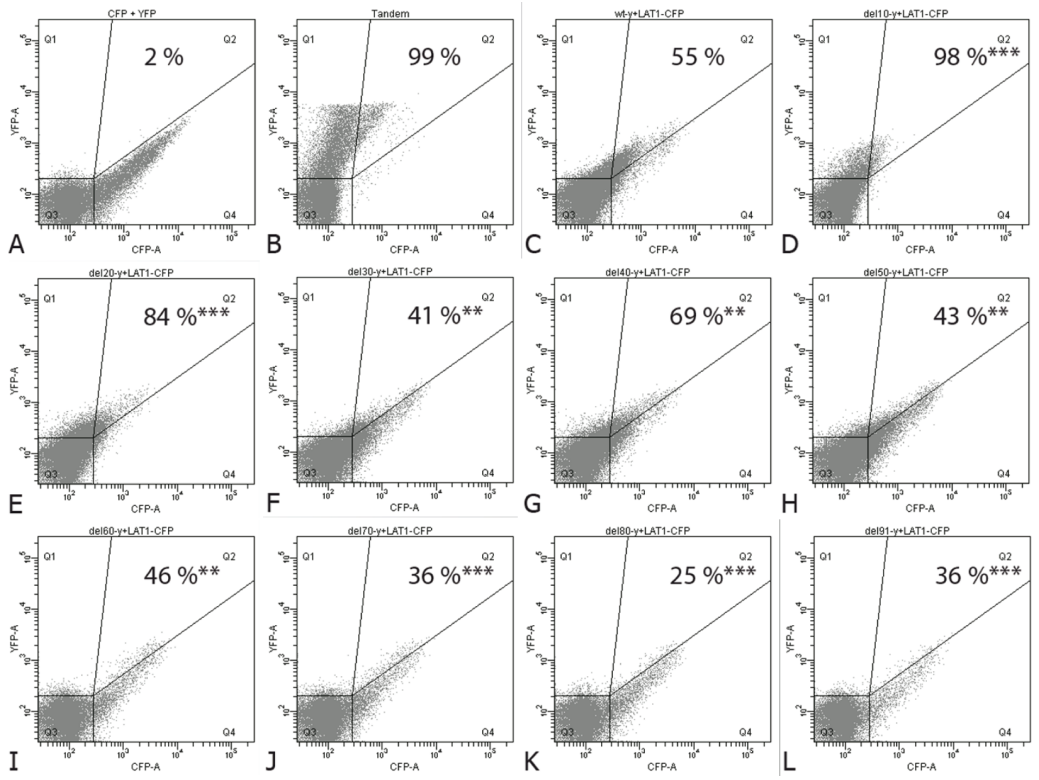

Figure 10. FACS-FRET analysis on sequentially deleted y⁺LAT1-ECFP-C1 double transfected with 4F2hc-EYFP-C1. The percentage in each panel represents the amount of CFP-positive cells (Q1+2+3) detected in the FRET quadrants Q1 and 2. The asterisks mark the statistical significance of the difference between each mutant y⁺LAT FRET and wild type y⁺LAT-induced FRET values. Panels: A: negative control with separate ECFP and EYFP expression plasmids; B: Forced FRET via tandem ECFP-EYFP construct; C-L: all ECFP-y⁺LAT1 constructs were transfected with equal amounts of EYFP-4F2hc constructs: C: wild type ECFP-y⁺LAT1; D: Δ10 ECFP-y⁺LAT1; E: Δ20 ECFP-y⁺LAT1; F: Δ30 ECFP-y⁺LAT1; G: Δ40 ECFP-y⁺LAT1; H: Δ50 ECFP-y⁺LAT1; I: Δ60 ECFP-y⁺LAT1; J: Δ70 ECFP-y⁺LAT1; K: Δ80 ECFP-y⁺LAT1 and L: Δ91 ECFP-y⁺LAT1.

According to these results, LPI mutation-carrying or C-terminally truncated y⁺LAT1 both interact with 4F2hc physically as efficiently as the wt y⁺LAT1 and 4F2hc. Therefore, the false cellular targeting of the truncated y⁺LAT1 observed in subproject I may not be caused by

the inability of the subunits to interact. However, the FACS-FRET analysis does not detect the localization of the interaction within a cell or give any information the stability of the resulting complex, something that could only can be achieved with microscopy based FRET approaches involving time-lapse. Therefore, one can only speculate whether the dimer is stable after assembly or disassembled after initial contact before the insertion to the plasma membrane. However, the results from the FACS-FRET analysis suggest that even the truncating mutations hindering the plasma membrane transport of y^+LAT1 do not prevent the dimerization process to initiate. The current paradigm on amino acid transporter biosynthesis suggested by Palacin and Kanai in 2004 and modified by Ganapathy in 2009 states that the light subunit is folded independently of the heavy subunit in the ER, where the subunits subsequently dimerize (Palacin and Kanai 2004, Ganapathy 2009). The heavy subunit goes through glycosylation first in ER and then in Golgi as a part of the transporter complex and only after completing the heavy subunit glycosylation, the transporter complex is translocated into the plasma membrane (Rius and Chillaron 2012).

The FRET analysis on cell populations using flow cytometry is faster and more straightforward when compared to acceptor photobleaching microscopy FRET assays. However, data on subcellular localization of the complex interaction is not achieved, making microscopy indispensable in most studies. In addition, some problems occurred in the FRET-FACS assays due to the constantly rather small amount of especially truncating mutation carrying y^+LAT1 proteins tagged with any fluorescent proteins. Since the relative lack of one counterpart of FRET, very large number of cells was required to reveal the donor quenching in the sample population. However, the FRET-induced change in both CFP (quenching) and YFP (dequenching) visualizing dot plots was clearly seen in all the studied mutant expressing cell populations (Figure 1 and Table 2 in II, Figure 10).

5.4 The effect of y^+LAT1-EGFP expression on cell proliferation (II, unpublished)

The expression level of y^+LAT1 fusion proteins was continuously observed to be lower in transfections producing truncating mutant fusion proteins. These transfections resulted in smaller amounts of fluorescent protein positive cells with the same experimental procedure and both in microscopy and flow cytometry, when compared to wild type or point mutant transfections (For flow cytometry results, see Figure 2 in II and Table 8). The low amount of mutant transfected cells was clearly a result from the mutant transporter expression and not from an unrelated effect of fluorescent protein expression as such. Since a potential explanation for the low amount of fusion protein expressing cells is increased mortality, we compared the cellular mortality rates after transfections in fusion protein positive and negative sub-populations.

To quantitate the y^+LAT1-EGFP expression level and to compare the proportion of living and dead cells between GFP-expressing and non-expressing cell subpopulations, the transfected live cell suspensions were treated with propidium iodide, which penetrates and dyes only dead cells. During the FACS run, the cell subpopulations were defined on the basis of GFP expression and PI intake. The y^+LAT1-EGFP positive population was clearly larger in wild type transfected samples when compared to any mutant transfected samples (Table 8), which supported the previous observations on both microscopy experiments (I) and FACS-FRET (II).

Table 8. y⁺LAT1-EGFP expression in wild type and LPI mutant transfected samples and the effect on cell mortality (Table 2 from original publication II).

Construct	GFP-positive cells, %	RL (95% CI)	Mortality GFP-, %	RL (95% CI)	Mortality GFP^+, %	p- value [c]	RL (95% CI) [c]	Mortality difference GFP-vs.GFP^+: p-values[d]	RL (95% CI)[d]
WT	20.7 [a]	1	4.2	1	3.6		1	<0.0001	1.17 (1.08-1.26)
LPI_{Fin}	5.2	0.25 (0.24-0.26)	2.0 [b]	0.48 (0.46-0.51)	3.2	ns	0.88 (0.75-1.04)	<0.0001	0.64 (0.55-0.75)
G54V	8	0.38 (0.38-0.39)	1.7 [b]	0.41 (0.38-0.43)	1.6	<0.0001	0.44 (0.36-0.53)	ns	1.09 (0.91-1.30)
1548delC	10.9	0.53 (0.51-0.54)	2.9 [b]	0.70 (0.66-0.73)	4.1	0.042	1.13 (1.00-1.26)	<0.0001	0.72 (0.66-0.80)

RL= Relative Risk; CI= Confidence interval; ns = not statistically significant

a The wild type expression level is higher than any mutant expression: p<0.0001

b The mortality rate of any mutant transfected, GFP-negative cell population differs significantly when compared to the wild type: p<0.0001

c The mortality rate of each mutant-GFP-positive cell population was compared to wild type-GFP-positive population mortality

d Mortality in GFP negative and GFP positive subpopulations of each construct transfected sample was compared.

The mortality rate was calculated in both GFP positive and negative subpopulations, and the results from samples resulting from wild type and mutant y⁺LAT1-EGFP transfections were compared. The mortality rate of fusion protein expressing cells was significantly higher in LPI_{Fin}, 1548delC and W242X transfected cells than in the wt y⁺LAT1-EGFP transfected cells. In contrast, wild type and G54V mutant positive cells showed decreased mortality and in the wild type samples the difference was also statistically significant (Table 8). Also, the difference in the mortality rates of each mutant positive cells correlates with the subcellular localization: The expression of y⁺LAT1 protein that is sorted to the plasma membrane (wt and G54V) reduces mortality in the GFP positive cell population, whereas production of cytoplasm-localizing y⁺LAT1 mutants increases it. However, the comparison of GFP negative groups of each transfected cell population gave the highest value of mortality in wt y⁺LAT1-EGFP transfected sample (4.2%); the percentage is significantly higher than caused by any LPI mutation transfections (1.7-2.9%). This observation is intriguing since it contradicts the apparent benefit that the cells acquire following the wt y⁺LAT1-EGFP transfection: when comparing the mortality of GFP positive and negative groups after transfection, the mortality significantly decreases in the positive group. When comparing GFP positive groups the difference in mortality is less clear, as the wt y⁺LAT1-EGFP associated mortality is second highest within the y⁺LAT1-EGFP positive cells. In the wt y⁺LAT1-EGFP transfected samples, the reduced viability within the GFP negative group following transfection most likely is caused by external stress factors, *i.e.* not the overexpression of fusion proteins as such. The wt y⁺LAT1-EGFP expressing cells divide faster than LPI mutation expressing cells resulting in relatively higher cell density in the culture dish at the end of the culture period, which may inhibit the cell division or even promote apoptosis. These effects, especially the latter, are subsequently detected as increased mortality in the GFP negative population. The latter, *i.e.* the slightly less prominent mortality rate in the wt y⁺LAT1-EGFP positive population, may be due the production of the highly over-expressing exogenous proteins, which consumes energy and causes cellular stress.

As all the LPI mutations in this study have been tested functionally, and none of them show any amino acid transport *in vitro* (Mykkänen *et al*. 2000), the observed difference in cell viability according to subcellular localization of the GFP fusion proteins cannot be explained by the amino acid transport activity or the lack of it. In contrast, the increased mortality can be derived from the deficiency of the y⁺LAT1/4F2hc complex, or more specifically, 4F2hc, at the plasma membrane. This deficiency in turn is caused by the dimerization of the transporter subunits in the ER, and the following scavenging of 4F2hc by the mutated y⁺LAT1 that is halted intracellularly and not carried to the cell surface. Therefore, the lower expression level of intracellular-localizing LPI mutant y⁺LAT1/4F2hc and the increased mortality can be explained by the relative lack of 4F2hc at the plasma membrane, leading to slower cell division rate and increased cellular mortality, as 4F2hc up-regulates cell division and promotes survival (Bulus *et al.* 2012). Thus, the truncated mutant y⁺LAT1 overexpression causes a selective disadvantage for an individual cell and prevents its division as efficiently

as a non-transfected cell in the same population. This causes the mutant positive cells to be rapidly outnumbered in the population.

To understand the effect of LPI mutant y⁺LAT1 expression on cell viability and proliferation fusion protein turn-over rate studies would provide more detailed insights into the protein trafficking process. This would help to explain the noticeable expression rate difference between wild type and mutant y⁺LAT1 proteins described in II. A major disadvantage of y⁺LAT1 protein studies is the lack of a functional antibody against the human y⁺LAT1, as this prevents quantitative analysis of wild type and mutant y⁺LAT1 in naturally expressing tissues. This would be crucial in understanding the cell and tissue level processing of y⁺LAT1 and the dimerization event with 4F2hc when the former is mutated. However, our more recent paper reports that the expression of LPI_{Fin} mutated *SLC7A7* is down-regulated to approximately 20% to that of healthy controls, possibly due to nonsense-mediated mRNA decay (Tringham *et al.* 2012), indicating that the level of LPI_{Fin} y⁺LAT1 in patient tissues may be close to nonexistent, therefore making attempts to detect it with antibodies challenging. Another approach to study the y⁺LAT1/4F2hc dimerization dynamics in cells would be live cell imaging applications such as FRAP, which would provide information on the effect of y⁺LAT1 mutations to the dimer stability and lifetime. Also, time-lapse microscopy could be utilized in studying the trafficking event of the complex. Furthermore, the three-dimensional modeling of y⁺LAT1 would provide interesting insights into the prediction of mutant polypeptide folding, dimer formation and subsequently the transport process of substrates of the y⁺LAT1/4F2hc complex.

5.5 Regulation of SLC7A7 transcription in epithelial cells (III and unpublished)

5.5.1 Detection SLC7A7 mRNA in lymphoblasts and fibroblasts

To determine the difference between the *SLC7A7* mRNA levels in control subject and LPI patient derived lymphoblasts and fibroblasts, the total RNA was isolated and Northern blot analysis was performed. The overall *SLC7A7* mRNA level was very low both in LPI patient and control derived cells and was detected only after long exposure. A single transcript was present in the cells (Figure 1A in III) in both Northern blot as well as rapid amplification of cDNA ends (RACE) –assay on adult kidney, in which only one active transcription initiation site was discovered. No significant variation was detected in the *SLC7A7* mRNA amounts between normal and LPI fibroblasts or lymphoblasts in the Northern blot autoradiographs. In contrast to these findings, Shoji and coworkers (Shoji *et al.* 2002) reported significantly lower *SLC7A7* mRNA levels in Japanese LPI patient derived lymphoblasts compared to normal cells. Also, Shoji and coworkers discovered that *SLC7A6* was up-regulated in the LPI lymphoblasts, *i.e.* cells with down-regulated *SLC7A7* expression, and suggested a compensatory or co-regulatory mechanism of y⁺LAT1 and y⁺LAT2 expression. However,

prior to that Dall'Asta and coworkers (Dall'Asta *et al.* 2000) had reported normal y^+ activity (arginine or arginine and lysine export) in LPI fibroblasts, thus suggesting a functional y^+L transporter other than y^+LAT1 in the cells.

After the initial experiments on *SLC7A7* expression by Northern blot (I, III), our group has performed an RNA microarray analysis as well as verified the results with an extensive set of quantitative PCR analyses on the blood samples of Finnish LPI patient cohort. In contrast to the Northern blot experiments using lymphoblasts and fibroblasts, it was discovered that the *SLC7A7* mRNA is indeed strongly down-regulated in LPI patient derived blood cell samples (Tringham *et al.* 2012). Therefore, the initial Northern blot results probably were not reliable but biased due to the following factors: 1) the use of patient derived, cultured cells as the starting material rather that non-cultured cells. The cells growing in a culture are provided with excess amount of nutrients as complete medium, whereas the cells that are derived directly from the extracted blood have adapted to the *in vivo* nutrient status in LPI patients; 2) the more sensitive approach using qRT-PCR in (Tringham *et al.* 2012) reveals more subtle variation in mRNA levels than Northern blotting. However, the compensatory mechanism using *SLC7A6* up-regulation when *SLC7A7* is down-regulated as suggested by Shoji and coworkers (2002) was not confirmed by the microarray/qPCR analysis in our studies. The *SLC7A6* mRNA levels were highly variable among the Finnish patient cohort, but the mean value did not differ from the control cohort. In addition, the LPI causing mutations in these two studies [LPI_{Fin} (Tringham *et al.* 2012) vs. p.Arg410Ter and/or p.Ser238Phe (Shoji *et al.* 2002)] might also have different effects on transcription regulation.

5.5.2 Characterization of the *SLC7A7* gene promoter region

To localize the promoter region of *SLC7A7* gene the sequence analysis of the 5' region of the non-coding exon 1 was performed by the TRANSFAC database search. The immediate upstream region covering approximately 1000 bp 5' to the transcription initiation site was screened to detect any transcription factor binding sites. No promoter elements typical for housekeeping genes such as TATA or CAAT boxes, Sp1 binding motifs or Inr elements were detected at functional positions of the scanned genomic region. However, several *cis*-acting promoter motifs were found, the most proximal among them being an E-box (Enhancer box binding protein) motif at 64 to 69 bp and an AP-2 (activating protein 2) binding site located 95 to 102 bp upstream the transcription initiation site. Both the E-box and the AP-2 motifs bind several transcription factors that can either enhance or repress the transcription initiation. Generally, the sequence analysis suggests a highly controlled and/or tissue specific transcription initiation control of *SLC7A7* rather than a ubiquitous expression represented by *SLC3A2* (4F2hc) or *SLC7A5* (LAT1).

To screen the 5' region of *SLC7A7* gene for transcription regulatory sequence elements we utilized a promoter-less luciferase plasmid backbone where fragments of the 5' region

sequence were cloned into. In the luciferase system the putative promoter elements in the cloned fragment induce transcription of the firefly luciferase gene. The subsequent promoter activity is measured as bioluminescence, produced by the enzymatic oxidation reaction and the following release of a photon of the luciferase substrate, luciferin. The amount of emitted bioluminescence from transfected cells is then used to quantitate the promoter activity by comparing the reading to the baseline acquired by using an empty luciferase vector.

Using the luciferase assay covering the 5' region up to nucleotide position -1155, relatively low luciferase activity was detected, except for the -141 fragment containing an enhancer element (E-box motif) as well as an AP-2 binding site resulting in about 10-fold activity compared to the empty reporter vector (Figure 3 in III). Curiously, when the E-box motif sequence was mutated, it initiated twice as high promoter activity than the wild type motif did. In contrast to the moderate promoter activity of the 5' region, the activity induced by the minigene construct (introns 1 and 2 together with the 5' non-coding region) yielded a 35-fold luciferase activity compared to the baseline (Figure 3 in III). Also, the promoter activity was orientation dependent, *i.e.* only the minigene cloned in *cis*-orientation increased promoter activity, whereas the trans-orientation clone resulted in activity comparable to the baseline. The result indicates a strong intronic enhancer element in the *SLC7A7* gene region, similar to what has been reported for the *SLC3A2* gene coding for 4F2hc (Karpinski *et al.* 1989).

More recently, the *SLC7A7* regulation has been explained in further detail in the work by Puomila and coworkers (Puomila *et al.* 2007), suggesting a model of two alternative, tissue specific promoters. According to their results a conserved TATA box-containing downstream promoter located 5' to exon 2, is active in the tissues where the primary cellular LPI defect is seen and the highest *SLC7A7* expression is detected, namely small intestine and kidney. The promoter is not associated with a CpG repeat sequence, which is typical for TATA box promoters. Interestingly, also another TATA-less promoter, referred as the upstream promoter was identified at 25 nucleotides 5' from the TATA box-containing core promoter.

Thus, the presence of both the core promoter and luciferase activity inducing elements in the 5' and 3' ends of intron 1 explains the high luciferase activity produced by the minigene construct (III). Instead, another promoter situated upstream exon 1 is active in the tissues expressing *SLC7A7* only in small amounts such as brain (Puomila *et al.* 2007). The exact significance of the alternative promoter usage in *SLC7A7* transcription and further, on LPI pathophysiology, is currently unclear.

6. SUMMARY AND CONCLUSIONS

The research of primary inherited aminoacidurias, among them lysinuric protein intolerance, has expanded rapidly towards the fields of medical genetics and cell biology in the last 15 years. The research, which started as disease characterization, and later proceeded into biochemistry and defining transporter systems and molecules, raised questions on the underlying factors causing distinct phenotypes. These questions have been answered first by the means of molecular genetics in the late 1990's and the early 2000's, when genes for several diseases such as cystinuria type B and LPI genes and their mutations were identified. More recently, the research has proceeded on the protein and cell biology level as well as gene expression regulation studies and knockout mice models of the diseases.

Heteromeric amino acid transporters function in a complex network of interactions. Only the core of these interactions has been characterized to date: transporters and their amino acid substrates as well as transporter-associating proteins such as 4F2hc. To understand the relationship between structural and functional properties of an individual transporter subunit it must be linked to a wider context of other subunits in the complex in question and furthermore, to the other transporters connected either functionally via shared substrates or by subcellular localization. To begin with, this requires the transporter heterodimer characterization at the level of subcellular localization and interaction and the analysis of possible disturbances in the process caused by mutations of the coding gene associated with the primary defect.

In this study, the aim was to utilize research methods, which would first allow the direct visualization of y^+LAT1 in intact mammalian cells and further to analyze the nature of mutations in y^+LAT1 when present in cells. During the study the lack of a specific, applicable antibody against the human y^+LAT1 expressed in low levels in normal tissue prevented the use of naturally y^+LAT1 expressing tissues in the localization studies of the transporter. This necessitated the use of an alternative method in imaging the protein.

This was obtained by the formation of fluorescent fusion protein of y^+LAT1, first with GFP and later the spectral variants CFP and YFP, which were all successfully expressed in cultured human cells. This gave an opportunity to visualize the transporter subunits at subcellular level. Subsequently, specific defects caused by mutant expression were identified: the frameshift and nonsense mutant *i.e.* truncated y^+LAT1 proteins remain cytoplasmic in mammalian cells whereas the point mutated proteins are carried to the plasma membrane. Furthermore, the utilization of GFP fusion proteins made it possible to manipulate y^+LAT1 sequence and thus to study features of the transporter that would otherwise be missed. This was obtained by designing series of truncating deletions on the C-terminal tail of y^+LAT1, which were aimed at studying the significance of the C-terminal part of the protein on the

cellular trafficking. Interestingly, the localization of the truncated constructs harboring the C-terminal polypeptide revealed an exception on the localization rule dictated earlier by the LPI mutants: they were sorted correctly to the plasma membrane. This primary observation indicates that the lack of small parts of C-terminus does not prevent the protein trafficking machinery to function completely, but detailed studies are required to further analyze the cellular sorting pattern and transport function of these truncated proteins.

The most notable achievement of the current study is the use of FRET flow cytometry in the dimerization process analysis. The obtained results on the dimerization indicate that 4F2hc is able to form a heterodimeric complex even with the frameshift and nonsense mutant y^+LAT1 proteins that are not transported to the plasma membrane, and thus the aberrant subcellular localization is not the result of unsuccessful dimerization process. This indicates that 4F2hc is not controlling the y^+LAT1 folding or function prior to transporter complex formation, but instead the heterodimeric complex is quality controlled via another mechanism prior to trafficking to cell surface.

The successful utilization of the FACS-FRET method introduced significant new insights into the transporter complex assembly and localization in intact cells. This method enabled fast screening of dimerization on cell population level added with data on fusion protein expression level and even cellular mortality, which could not have been obtained using FRET based on observation of fixed cell by microscopic methods. Also, the effects of long term laser exposure possibly affecting the FRET microscopy results *i.e.* photoconversion were avoided by using the flow cytometry approach, making the obtained results more reliable.

In addition to the localization data obtained by using GFP fusion protein, the overexpressed y^+LAT1-EGFP mutants lead to an observation on the effect of y^+LAT1 variants on cell viability. The expression rates of the truncated (LPI mutation carrying) proteins included in the study were significantly lower than wild type y^+LAT1 expression level, a finding that was explained by the increased mortality in fusion protein positive cell population. However, even though the point mutant G54V expression did not increase the mortality rate it resulted in equally poor expression level as the other studied mutants. This observation indicates a relationship between the severity of the defect in protein structure and the disturbances it causes in cells: truncated proteins may have properties that disturb cell division or cause cells to die, whereas the point mutant associating defect may be limited to a low proliferation rate, while the mortality rate of positive cells is not increased.

Importantly, the LPI mutation defect on the transporter is also reflected in the subcellular localization pattern of the transporter dimer. As the y^+LAT1-4F2hc complex formation takes place directly after translation, also 4F2hc surface expression is affected along with the actual transporter channel targeting. Therefore, as 4F2hc is an important cell survival

and proliferation factor, the observed effects on cell viability could be partially or even completely due to the relative deficiency of 4F2hc at the plasma membrane.

Although the human y^+LAT1 protein has not yet been structurally modelled and the details of pathogenic effects of mutant transporters in human target tissue still remain unknown, the current study has succeeded in providing new insights and useful tools for further studies on the research of molecular pathogenesis in LPI.

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