Ornithine decarboxylase is the receptor of regulatory protein RS1 (RSC1A1) mediating RS1 dependent short-term regulation of glucose transporter SGLT1

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1. Introduction

1.1 Glucose transport and SGLT proteins

The facilitated glucose transporters of the sodium-coupled glucose cotransporters, the SGLT or SLC5 gene family (Hediger and Rhoads, 1994; Wright and Turk, 2004) and GLUT or SLC2 gene family (Uldry and Thores, 2004), are involved in the absorption of glucose in the small intestine, the reabsorption of glucose in the kidney, uptake across the blood-brain barrier, and the uptake and release of glucose from all cells in the body. GLUT proteins allow the transport of glucose down the concentration gradient (Brosius et al., 2005), while SGLT proteins transport glucose against its concentration gradient (Wright et al., 2007). The transport of glucose into epithelial cells is mediated by a secondary active cotransport system, or SGLT, driven by a sodium gradient generated by the Na^+/K^+ ATPase. GLUT proteins transport the glucose accumulated in the epithelial cell further into the blood across the plasma membrane by diffusion. The different types of membrane-associated glucose transporters include three members of SGLT and fourteen members of GLUT transporters (Scheepers et al., 2004). The SGLT family comprises of high-affinity, low-capacity sodium-glucose symporter SGLT1, low-affinity, high-capacity Na^+-dependent glucose co-transporter SGLT2, the glucose sensor SGLT3, the widely distributed inositol (Berry et al., 1995) and multivitamin transporters SGLT4 and SGLT6 (Balamurugan et al., 2003) and the thyroid iodide transporter SGLT5 (Smanik et al., 1996). The SGLT1 (gene name *SLC5A1*) (Hediger et al., 1989) has a sodium to glucose coupling ratio of 2:1 (Wright et al., 2001) and is expressed mainly in intestine, heart, and kidney (Zhou et al., 2003). The SGLT2 (gene name *SLC5A2*) (Wells et al., 1993) is in contrast to SGLT1 a low-affinity, high-capacity sodium-glucose symporter with a sodium-to-glucose coupling ratio of 1:1. The protein shows an ubiquitous expression pattern with highest expression levels in kidney (Zhou et al., 2003) where it mediates the reabsorption of most of the filtered glucose in the proximal convoluted tubule (Wright et al., 2001). SGLT3 expressed in cholinergic neurons of the small intestine and in skeletal muscle at the neuromuscular junctions acts mainly as a glucose sensor (Diez-Sampedro et al., 2003).

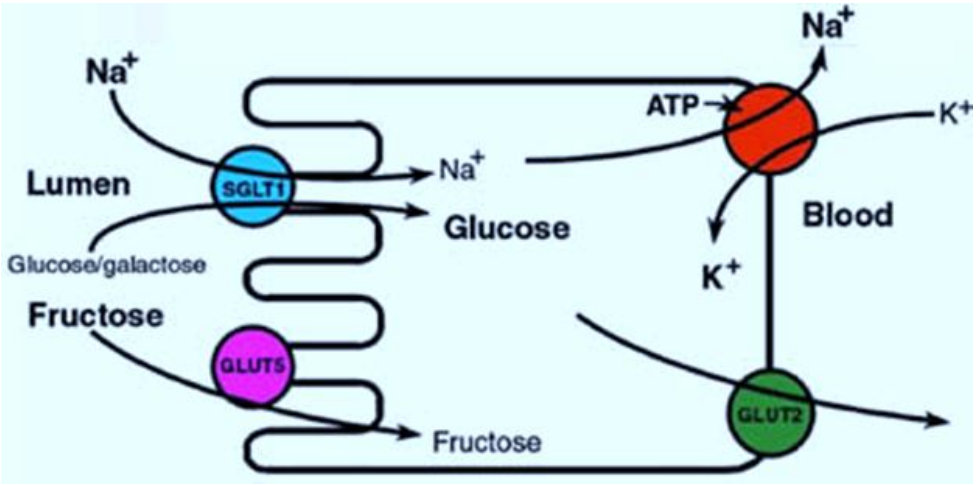

Fig 1: Classical model of intestinal sugar transport. SGLT1 is the sodium dependent glucose/galactose transporter on the brush border membrane (BBM). The Na+K+-ATPase on the basolateral membrane (BLM) maintains the gradient necessary for the functioning of SGLT1. GLUT5 is a facilitative transporter on the BBM which transports fructose into the cell. GLUT2 on the BLM transports glucose, galactose and fructose out of the cell (modified from Wright, 1998).

1.2 Regulation of glucose absorption in small intestine

The absorption of glucose in small intestine changes dramatically during development. Glucose absorption in small intestine is regulated in response to diet and food intake via changes of expression, location, and activity of SGLT1 and/or GLUT2 (Kellett, G. L., 2001, Shirazi-Beechey et al., 1991, Ferraris et al., 1989, Miyamoto et al., 1993). Polyamines play a prominent role in the maturation of the glucose transporters (SGLT1 and Glut2) in the small intestine during development (Wild et al., 2007). Inhibition of the rate limiting enzyme of the polyamine biosynthesis ornithine decarboxylase (ODC) by an inhibitor α-difluoromethylornithine (DFMO) resulted in decreased glucose absorption in rabbit brush border membrane vesicles (Johnson et al., 1995). Few factors that are involved in the regulation of expression of SGLT1 are beta-adrenergic innervation (Ishikawa et al., 1997), glucagon- like peptide 2 (Cheeseman, C. I., 1997), and cholecystokinin (Hirsh et al., 1998). Different protein kinases like, protein kinase A, protein kinase C (PKC) (O'Donovan et al., 2004) and phosphoinositol 3-kinase and Cyclic AMP are also involved in the regulatory pathways (Ishikawa et al., 1997, Cheeseman, C. I., 1997, Hirsch et al., 1996). Regulation of SGLT1 can either be by changes in transcription (Miyamoto et al., 1993, Martin et al., 2000, Vayro et al., 2001), mRNA stability (Loflin et al., 2001), intracellular trafficking (Cheeseman

C. I., 1997, Hirsch et al., 1996, Khoursandi et al., 2004, Veyhl et al., 2006), or transporter activity (Vayro et al., 1999). However, the individual regulatory pathways, their cross-talk, and their physiological importance are not understood. Since the regulation of glucose disposal through SGLT proteins has been considered as an important biological target that can be used for the development of innovative therapies to effectively control blood glucose levels, different regulatory pathways of SGLT1 regulation and the molecules involved in these pathways are being investigated extensively.

1.3 Polyamines and Ornithine decarboxylase (ODC)

Polyamines (putrescine, spermidine and spermine) are polyorganic cations that are critically involved in the regulation of many biological functions such as embryonic development (Kusunoki et al., 1978), cell cycle (Seidenfeld et al., 1981, Alm et al., 2000), cancer (Pegg et al., 1988, Seiler et al., 1998). Stimulation of growth of the cells by exogenous polyamines in polyamine depleted medium emphasizes the importance of polyamines in cell growth (McCann et al., 1987, Thomas et al., 1987, Porter et al., 1983). Cells deprived of polyamines cease to grow and proliferate, but usually do not die (Balasundaram et al., 1991; Balasundaram et al., 1993). Polyamines have specific roles in neurochemistry (Seiler N., 2000), as well as pulmonary (Hoet et al., 2000) and immune system functions (Seiler et al., 1994, Thomas et al., 1992). Polyamines in the cell are regulated tightly at different levels such as synthesis, degradation, as well as cellular uptake and release (Tabor et al., 1984, Casero et al., 1993, Morgan D. M. L., 1999, Seiler et al., 1996). They bind to the negatively charged moieties of the cell like DNA, RNA, proteins. Polyamines modulate different cell processes either positively or negatively depending on the cell type and the regulatory mechanism (Feuerstein et al., 1990, Kusama-Eguchi et al., 1991, Yoshida et al., 1999, Hölttä & Hovi 1985). Polyamine biosynthesis is regulated by two key enzymes, ornithine decarboxylase and S-adenosylmethionine decarboxylase. Putrescine is synthesized by the decarboxylation of ornithine by the enzyme ornithine decarboxylase (ODC) (Tabor et al., 1984, Casero et al., 1993, Morgan D. M. L., 1999). Polyamines themselves act as negative feedback inhibitors for these two enzymes emphasise the significance of maintaining polyamine concentration in appropriate levels within the cell. The concentration of these two enzymes in the cells is generally low in quiescent state but increases drastically after growth inducing stimuli. The precursor putrescine concentration in the cell is generally low when compared to the millimolar range of spermidine and spermine (Morgan 1990; Watanabe et

al., 1991). The free and potentially reactive concentrations of polyamines are much lower than the total concentrations as polyamines are generally present in a bound state to the negative macromolecules. The concentration of polyamines in the cell is controlled by the protein antizyme (AZ), the synthesis of which is induced by polyamines (Fong et al., 1976, Heller et al., 1976). Antizyme has the ability to inhibit the activity of ODC apart from inhibiting the uptake of polyamines by the cell (Mitchell et al., 1994). Antizyme binds to ODC and targets it to proteasomal dependent degradation via a unique ubiquitin independent pathway (Murakami et al., 1992a).

Ornithine decarboxylase (ODC, EC 4.1.1.17) is a 51 KDa protein comprising of 461 amino acids. Hamster ODC with 455 amino acids is an exception (Grens et al., 1989; Yao et al., 1995). Active mammalian ODC is a homodimer and catalyses the conversion of L-ornithine to putrescine. All known ODC´s require pyridoxal 5`-phosphate (PLP) as a cofactor. ODC is a very labile protein with half-life values between 10-20 min (Seely et al., 1982, Isoma et al., 1983) and 1-2 h (Hayashi et al., 1996). ODC induction is always accompanied by a similar increase in the amount of ODC protein. The main demonstrated post-translational modification of mammalian ODC is phosphorylation (Rosenberg-Hasson et al., 1991, Worth et al., 1994).

ODC is degraded via an exceptional pathway. Inhibitor protein antizyme, induced by polyamines binds to the ODC monomer, inhibits its enzyme activity and targets the protein for degradation by 26S proteasome (Murakami et al., 1992a). 26S proteasome was thought to degrade only proteins tagged with poly-ubiquitin chain and ODC was the first exception to this. Very few proteins were shown to be degraded via this exceptional pathway of ubiquitin independent-26S proteasome dependent pathway [Cyclin-dependent kinase inhibitor $p21^{cip1}$ (Sheaff et al., 2000) and the NS2 protein parvovirus minute virus (Miller and Pintel 2001)]. The protein antizyme is recycled and is seldom degraded (Tokunaga et al., 1994).

Three isoforms of antizyme (Antizyme 1, 2 and 3), were described to date (Ivanov et al., 2000, Ivanov et al., 2007) in mammalian species. The most highly investigated form is antizyme-1 which is ubiquitously expressed, was originally identified with an ODC inhibitory activity whose synthesis is stimulated by increased polyamine concentration (Fong et al., 1976, Heller et al., 1976). The high affinity of antizyme to ODC monomers and the weak association between the two ODC subunits sets the basis for the ability of antizyme to neutralize ODC activity (Coleman et al., 1994, Rosenberg-Hasson et al., 1991). Although ODC inactivation is a known function of antizyme, it is actually an intermediate step in a

process of targeting ODC subunits to ubiquitin-independent degradation by the 26S proteasome (Murakami et al., 1992). Antizyme also regulates polyamine transport across the plasma membrane through a yet undefined mechanism (Mitchell et al., 1994, Coffino et al., 2001, Mangold et al., 2005).

Antizyme-2 has tissue distribution similar to that of antizyme-1, but it is expressed at significantly lower levels (Ivanov et al., 1998). Antizyme-2 also inhibits ODC activity and polyamine uptake as efficiently as antizyme-1, but does not promote ODC degradation. The third member of the antizyme family, antizyme-3 is a testis-specific antizyme observed only in haploid germinal cells (Ivanov et al., 2000, Tosaka et al., 2000). Mutagenesis and structural studies have revealed the amino acids of ODC that are responsible for interaction with antizyme and for ODC degradation. The antizyme binding site required for antizyme binding and antizyme dependent degradation is present in between amino acids 117 and 140 (Li & Coffino 1992). Interaction with antizyme induces a conformational change in ODC, resulting in the exposure of a C-terminal ODC segment that serves as the proteasome recognition signal (Ghoda et al., 1989, Ghoda et al., 1990, Li et al., 1993, Rosenberg-Hasson et al., 1991). The C-terminal half of the antizyme is required for its interaction with ODC. However, while this interaction is sufficient to inactivate ODC, the ability to promote ODC degradation depends on the integrity of a small N-terminal segment (Mamroud-Kidron et al., 1994, Li et al., 1994, Almrud et al., 2000). Although the specific role of the N-terminal segment is still unknown, it was suggested that it aids the ability of the C-terminal segment of ODC to mediate proteasomal recognition (Coffino et al., 2001, Li et al., 1996).

Recent studies have demonstrated that antizyme is subjected to regulation by an ODC-related protein termed antizyme inhibitor. Antizyme inhibitor is a distinct protein which has antizyme inhibitory function (Fujita et al., 1982) and lacks ornithine decarboxylating activity (Koguchi et al., 1997). Antizyme inhibitor inhibits all members of the antizyme family (Mangold et al., 2005).

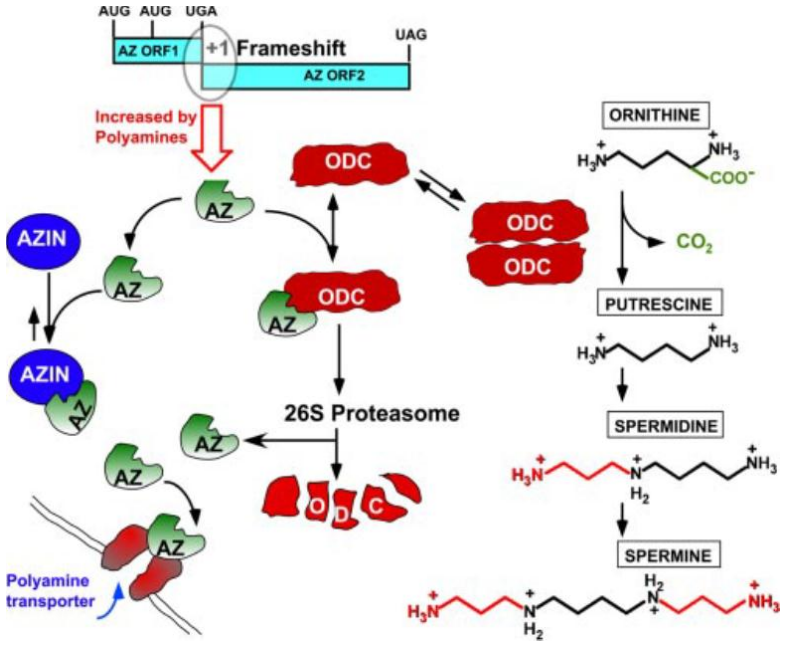

Fig 2: Role of antizyme and ODC in polyamine metabolism. The ODC dimer catalyzes the production of putrescine, which is then converted into the higher polyamines. Antizyme is synthesized via a+1 frameshift in translation of the mRNA fusing ORF1 and ORF2 in a manner stimulated by polyamines. Antizyme (*AZ*) can bind to ODC bringing about degradation by the 26 S proteasome or to antizyme inhibitor (*AZIN*) or block the polyamine uptake (adapted from Antony E Pegg., 2006)

1.4 Regulation of ODC in small intestine

ODC is a highly regulated enzyme at the protein level and the activity of the enzyme is regulated by many cell processes and it either increases or decreases drastically to various cell stimuli like growth factors or external stimuli. The activity of ODC is crucial in various cell processes. Although the activity of ODC in the small intestinal mucosa is high compared with most other tissues (Ball et al., 1976) it still increases dramatically in response to feeding. More and Swendseid reported 40 fold increase in enzyme activity 1hr after fasted rats received an amino acid mixture by gastric intubation (Moore et al., 1985). Fasting decreased the activity of ODC to approximately 20% of that found in fed animals (Tabata.K et al., 1986). In addition to food intake or increased exposure of gut mucosa to nutrients ODC activity in rat small intestinal mucosal is increased by various events which stimulate mucosal growth such as partial resection of small intestine (Luk et al., 1988), luminal obstruction (Seidel et al., 1984) and lactation (Yang et al., 1984). The inhibition of ODC activity by the specific inhibitor difluoromethylornithine (DFMO) prevented the growth response to refeeding and those associated with lactation, maturation and injury. Higher

levels of ODC activity were observed in differentiated enterocytes at the tips of the villi compared to proliferating enterocytes in the crypts (Baylin et al., 1978, Porter et al., 1980, Sepulveda et al., 1982). Measuring ODC activity by trapping $^{14}CO_2$ released from decarboxylation of l-^{14}C ornithine, two hours after refeeding fasted rats a 10 fold increase in ODC activity was observed in cells from villus tip and a 20 fold increase in cells from the mid villus region whereas no significant increase was observed in crypt cells (L R Johnson et al., 1988). Epidermal growth factor EGF increased enzyme activity significantly in cells from all three regions. Gastrin on the other hand did not increase the activity significantly in any of the fraction (Fitzpatric et al., 1987) suggesting that activity of ODC is not necessarily correlated with growth of gastro intestinal mucosa and that ODC activity may be regulated by different stimuli in villus and crypt cells (Fitzpatric et al., 1987, Seidel et al., 1985).

1.5 RS1 protein

RS1 is a 67-68 KDa protein (gene *RSC1A1*) which is encoded by an intron less single copy gene that is present only in mammals. RS1 has been cloned from human (Lambotte *et al.*, 1996), pig (Veyhl *et al.*, 1993), rabbit (Reinhardt *et al.*, 1999), and mouse (Osswald *et al.*, 2005). It exhibits 70% identity at the amino acid level. It has a wide tissue distribution of liver, kidney, Intestine and also in neurons. RS1 is also expressed in low levels in lung and spleen but not in skeletal muscle cells. In porcine kidney RS1 is found in the brush border membrane fraction (Valentin et al., 2000). In the porcine kidney derived epithelial cell line LLC-PK_1, RS1 localizes to the intracellular side of the plasma membrane, at the *trans*-Golgi network (TGN), and also to the nucleus in a confluence dependent manner. In sub-confluent LLC-PK_1, RS1 localizes to the nuclei and cytoplasm, whereas in confluent cells RS1 localizes to the cytoplasm (Kroiss et al., 2006). The confluence dependent localization of the protein has been attributed to the differential regulatory role (transcriptional and post-transcriptional) of the protein. Up on over expression in Xenopus laevis oocytes, RS1 localized mostly to the cytosol. However small fractions were also found associated with the plasma membrane.

1.6 Functions of RS1

Co-expression studies done in Xenopus laevis oocytes revealed that RS1 regulates the activity of various transporters such as Na^+-D-glucose co-transporter SGLT1, Na^+ myo-inositol co transporter SMIT, organic cat ion transporters OCT1 and OCT2 and the organic

anion transporter OAT1 and the Na^+-co-transporter for serotonin SERT. On the other had RS1 had no effect on the activity of H^+-peptide co-transporter PEPT1 and the sodium independent glucose transporter GLUT1 (Lambotte et al., 1996; Reinhardt et al., 1999; Vehyl et al., 2003). Although RS1 regulates several transporters belonging to different families, Na^+-D-Glucose co-transporter (SGLT1) has been studied extensively because of its physiological importance. $RS^{-/-}$ knockout mice were generated and the regulation of SGLT1 in small intestine was studied (Osswald et al., 2005). It turned out that the expression of SGLT1 in small intestine was increased after removal of RS1 and that the change of SGLT1 expression was accompanied by an increased velocity of glucose absorption. The obese phenotype of the RS1-/- mice described by Oswald et al. (2005) was not consistently observed in later generations.

The RS1 dependent down regulation of SGLT1 was tissue specific. RS1 protein is identified with a N-terminal functional domain of 100 amino acids responsible for posttranscriptional down regulation comprising of two regulatory tripeptide domains QSP and an octapeptide SDSDRIEP sequences and also consensus sequences for phosphorylation by a variety of protein kinases. It also hosts a nuclear localization sequence which steers the cell cycle dependent nuclear localization of the RS1 protein and a transcriptional activating domain controlling the transcriptional regulation of the SGLT1. The UBA domain at the C-terminal end has to be characterized.

The posttranscriptional regulation of RS1 was studied by co-expression of the cRNA of hSGLT1 along with hRS1 (Vehyl et al., 1993) or by injection of the purified RS1 protein into hSGLT1 expressing Xenopus laevis oocytes. RS1 decreased significantly the Phlorizin inhibitable AMG uptake by SGLT1. This short term posttranscriptional downregulation of AMG uptake by SGLT1 was due to the inhibition of dynamin dependent release of SGLT1 vesicles at the TGN and this inhibition is increased up on PKC stimulation (Vehyl et al., 2006). The posttranscriptional regulation of SGLT1 by RS1 is effected by intracellular AMG concentration, which is in contrast to the regulation of the organic cation transporter OCT1 mediated tetraethylammonium uptake. These data suggest that RS1 regulates different transporters in different ways and that the inhibition is regulated by an intracellular glucose binding protein (Vehyl et al., 2006).

In LLC-PK_1 cells the state of confluence governs the dynamic localization whether nuclear or cytoplasmic of the protein RS1 (Korn et al., 2001, Kroiss et al., 2006). RS1 protein is localized profoundly to the nucleus in subconfluent LLC-PK_1 cells in contrast to the

cytoplasmic localization in the confluent state (Kroiss et al., 2006). This data supports the functional relevance of the transcriptional downregulation of the transporter SGLT1 in the subconfluent LLC-PK_1 cells by RS1 (Korn et al., 2001). The confluence dependent nuclear localisation of the protein RS1 has been attributed to the cell cycle. The nuclear shuttling sequence of the protein RS1 (RNS) localized between the amioacids 349-369 steers a cell cycle dependent nuclear localization of the protein where it participates in the transcriptional downregulation of the transporter SGLT1 (Filatova et al., 2009, Korn et al., 2001). The RNS contains a novel non-conventional nuclear localization signal that binds importin β1, a nuclear export signal mediating export via CRM1 (Chromosome maintenance region 1/Exportin 1/Xpo1p/ Kap124p) and a Ca^{2+}-dependent calmodulin binding site along with a PKC phosphorylation site at its C-terminus. During confluence, phosphorylation at the PKC site in RNS mediates rapid nuclear export of the protein RS1 (Filatova et al., 2009).

In the frame of characterization of the functional domain of the RS1, Vernaleken et al., found that two tripeptides GlnCysPro (QCP) and GlySerPro (QSP) derived from the RS1 sequence were able to inhibit SGLT1 mediated AMG uptake with high affinity. They were able to block the release of vesicles containing SGLT1 at the TGN to the same extent as that of the pure protein and that this blockage is regulated by the intra cellular sugar concentrations. This data indicates that these tripeptides bind to a high affinity binding site on a protein which in turn is modulated by intracellualar monosaccharide concentrations at the TGN (Vernaleken et al., 2007). The N-terminal 100 amino acids of RS1 contain a domain (RS1-Reg) which is responsible for the posttranscriptional regulation of transporters at the trans-Golgi network (M. Veyhl-Wichmann and H. Koepsell, unpublished data). RS1-Reg contains two times the tripeptide motif QSP and the octapeptide SDSDRIEP, which are able to down regulate SGLT1 on their own. RS1-Reg contains 20 serine residues out of which 12 are located in consensus sequences for phosphorylation by various protein kinases.

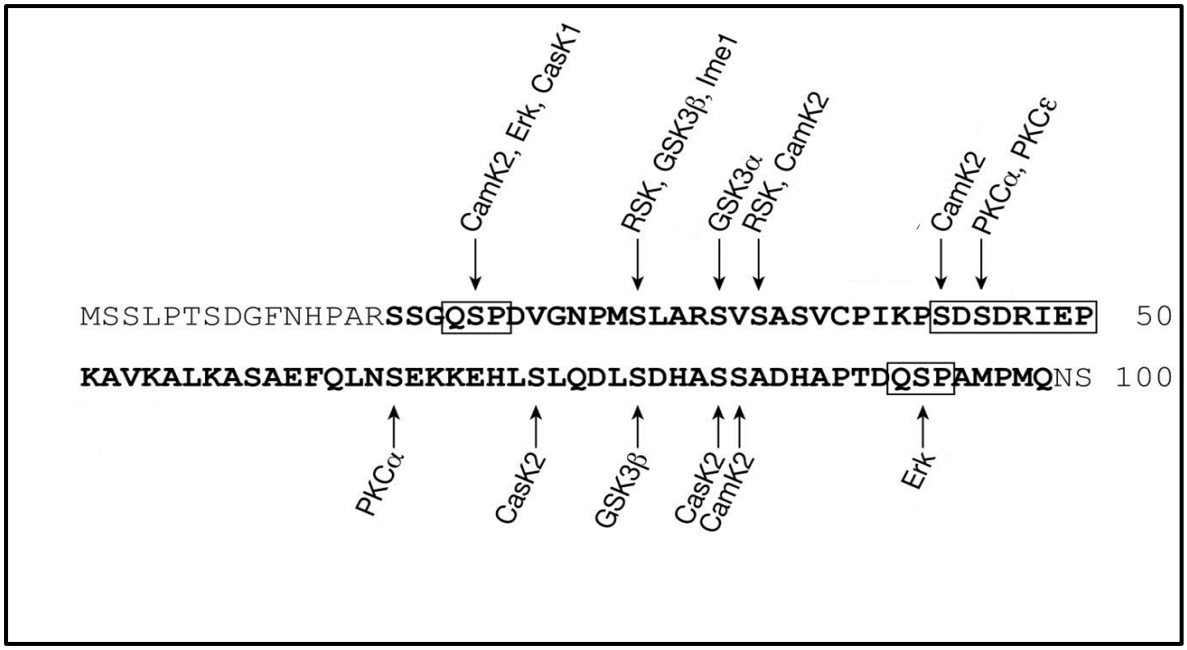

Fig 3: Sequence of 100 N-terminal amino acids of human RS1 containing the regulatory domain (hRS1-Reg). RS1-Reg is indicated in bold. Functional active motifs are boxed**.** Predicted phosphorylation sites of various protein kinases are indicated by arrows. (CamK2-Calcium/Calmodulin dependent protein kinase 2, ERK- Extracellular signal-regulated kinase, CasK- Casein kinase, RSK-Ribosomal protein S6 kinase, GSK-Glycogen synthase kinase, PKC- Protein kinase C)

Recent data showing that the down regulation of the Na^+- nucleoside cotransporter CNT1 by RS1-Reg and SDSDRIEP was not dependent on intracellular glucose concentration and that mimicking or preventing phosphorylation in one or more positions altered the affinity of RS1-Reg for downregulation of hSGLT1 differently from hCNT1 indicates that hRS1-Reg can address different transporters (M. Veyhl-Wichmann and H. Koepsell, unpublished data). The hypothesis was raised that differentially phosphorylated forms of RS1-Reg bind to different receptors for RS1 which are involved in the release of vesicles with different transporters from the TGN. Since it was shown that QSP downregulates hSGLT1 in a glucose dependent manner similar to total hRS1 protein and hRS1-Reg it was concluded that the glucose binding site responsible for glucose dependence is located on the hRS1-Reg receptor for SGLT1 regulation or on a protein which is involved in the regulation downstream of this receptor (Vernaleken et al., 2007). RS1 was shown to interact with a 28 KDa protein called ischemia/reperfusion inducible protein IRIP, which is upregulated in kidney after ischemia/reperfusion (Jiang et al., 2005). IRIP also has a broad tissue distribution, and is expressed highly in testis, ovary, bronchial epithelia, thyroid, kidney, colon and brain and is found in low levels in spleen, heart and small intestine. Interestingly IRIP also regulates the activity of plasma membrane transporters belonging to different families including SGLT1,

the organic cation transporters OCT2 and OCT3, the anion transporter OAT1, the Na+-co-transporter for serotonin SERT, the dopamine transporter DAT and the norepinephrine transporter NET (Jiang et al., 2005) which of the most are shown to be regulated by RS1 (Lambotte et al., 1996; Reinhardt et al., 1999; Vehyl et al., 2003). RS1 when coexpressed with IRIP in oocytes expressing OCT2 did not show any additive effect on the regulation of the activity, in contrary the effect of RS1 was lost when a dominant negative mutant of IRIP was co-expressed. This experimental data suggest that both the proteins may be involved in the same regulatory pathway in regulating the plasma membrane transporters (Jiang et al., 2005). The investigation of binding partners involved in the transcriptional and posttranscriptional regulatory mechanism of RS1 may bring new insights in understanding the regulatory pathways of many plasma membrane transporters involved.

2. Aim of the study

The aim of this study was to identify proteins which bind to the N-terminal domain of hRS1 and may be involved in the posttranscriptional regulation of transporters by hRS1. Since we were not sure whether the functional relevant interactions of proteins have a high affinity to allow co-precipitation, we performed two hybrid screening trying to detect the functionally relevant interaction partners. When the study was initiated the regulatory domain hRS1-Reg had not been described in detail. Thus we performed two-hybrid screening with a 312 amino acid long N-terminal fragment of hRS1. Being aware that false positive interacting partners may be identified on one hand and that hRS1-Reg may interact with many different proteins due its complex role in the regulation of release of vesicles with various transporters, we wanted to focus on an interacting partner which may be relevant for the regulation of SGLT1. Selection of an appropriate partner and characterization of its functional relevance proved to be the main challenge of this study.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1 Chemicals

All laboratory chemicals were of p.a. grade and purchased from Sigma-Aldrich (Taufkirchen, Germany), Merck (Darmstadt, Germany), Carl Roth GmbH (Karlsruhe, Germany), Serva (Heidelberg, Germany), Biozym Diagnostik (Hameln, Germany) or AppliChem (Darmstadt, Germany).

3.1.2 Antibodies used in this work:

Table 1: Primary antibodies used in this work. *IB*, immunoblot

Antigen	Species, specification	Application, Dilution	Company
GFP	Mouse monoclonal (MMS-118P)	IB, 1:5000	Covance, Freiburg, Germany
TurboGFP	Mouse monoclonal (TA50041)	IB, 1:2000	Origene (amsbio), UK
myc	Mouse monoclonal (OP10)	IB, 1:1000	Calbiochem, Darmstadt, Germany
KLF-5	Rabbit polyclonal (ab24331)	IB, 1:1000	Abcam, Cambridge, UK
RACK1	Mouse polyclonal (610177)	IB, 1:5000	BD biosciences, Heidelberg, Germany
ODC	Mouse monoclonal (O1136)	IB, 1:1000	Sigma Aldrich Taufkirchen, Germany
FLAG	Mouse monoclonal (F1804)	IB, 1:5000	Sigma Aldrich Taufkirchen, Germany
GAPDH	Rabbit polyclonal (SC25778)	IB, 1:1000	Santa Cruz, Heidelberg, Germany

Table 2: Secondary antibodies

Antibody	Application, Dilution	Company
Anti-mouse IgG HRP-conjugated, (115-035-062)	IB, 1:5000	Jackson Immuno research, Suffolk, UK
Anti-rabbit IgG HRP-conjugated, (A0545)	IB, 1:5000	Sigma Aldrich, Taufkirchen, Germany

3.1.3 Markers, Enzymes and Inhibitors

DNA markers 1kb Ladder and PageRuler Prestained Protein Ladder (MBI Fermentas, St. Leon-Rot, Germany) were used.

Restriction endonucleases (XhoI, PstI, Acc65I, BamHI, and BsrGI), Pfu DNA polymerase, and T4 DNA ligase were obtained from MBI Fermentas.

The following inhibitor mix was used in this work: protease inhibitor cocktail set III (Calbiochem, Darmstadt, Germany) (the final concentrations of inhibitors were: 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 0.8 μM aprotinin, 50 μM bestatin, 15 μM N-(trans-epoxysuccinyl)-L-leucine-4-guanidineobutylamide, 20 μM leupeptin, 10 μM pepstatin A).

3.1.4 Reaction kits and affinity matrices

The indicated reaction kits were used according to the manufacturer's instructions: Plasmid Purification Kit (Qiagen, Hilden, Germany); ECL PlusTM Detection Kit (GE Healthcare, Munich, Germany); Pierce ECL Western Blotting Substrate (Pierce, Bonn, Germany), LR Clonase II Enzyme mix (Invitrogen). For immunoprecipitation the following agarose beads were used: S protein agarose (Merck, Darmstadt, Germany), Anti-c-Myc agarose (Sigma-Aldrich, Taufkirchen, Germany)

3.1.5 Plasmids and constructs

Majority of vectors used in this work were generated by Dr. V. Gorboulev, GFP-TEV-S tag control vector was given by A. Filatova, vectors used in split ubiquitin system and cDNA library were kindly provided by Prof. Dr. Guliano Ciarimboli, University Klinik, Münster.

Plasmid	Description	Source
GFP-S tag	Mammalian expression vector constructed on the basis of pEGFP-C1(Clontech, Heidelberg, Germany) expressing fusion protein GFP and S tag	Filatova et al., 2009
GFP-S tag-RS1-(1-98)	GFP-S tag vector expressing N-terminal 98 amino acids of protein hRS1	delivered by V.Gorboulev
GFP-S tag-RS1-(1-312)	GFP-S tag vector expressing N-terminal 312 amino acids of protein hRS1	delivered by V.Gorboulev
ODC-myc	Mammalian expression vector pcDNA3 encoding full length Ornithine decarboxylase with a myc tag at the C-terminus	obtained from Shu-ichi Matsuzawa (Matsuzawa et al., 2005)
AZ-myc	Mammalian expression vector pcDNA3 encoding antizyme with a myc tag at the C-terminus	obtained from Shu-ichi Matsuzawa (Matsuzawa et al., 2005)
RS1-reg-FLAG	Mammalian expression vector pcDNA3.1 expressing the N-terminal 98 amino acids of human protein RS1 with a FLAG tag at the C-terminus	V.Gorboulev
RS1-(1-312)-FLAG	Mammalian expression vector pcDNA3.1 expressing the N-terminal 312 amino acids of human protein RS1 with a FLAG tag at the C-terminus	delivered by V.Gorboulev
pMetYCgate	pMetYCgate is a low-copy plasmid with the selection marker *LEU2*, comprised of the Met-repressible *MET25* promoter, B1-KanMX-B2 cassette, and CubPLV. pMetYCgate is suited for Y-CubPLV fusions of bait peptides Y.	obtained from Dr. Guliano Ciarimboli (Obrdlik et al., 2004)
hRS1-RSSP vector	The Human RS1 cloned into pRSSP vector which contains non-translating regions of the *Xenopus* β-globin gene providing high expression in oocytes.	(Busch, 1996) delivered by V.Gorboulev

3.1.6 Bacteria, yeast strain and cell line

The bacterial *E.coli* strain DH10B (Grant *et al.*, 1990) was used for selection and amplification of plasmids.

THY.AP4 yeast strain (Obrdlik *et al.*, 2004) was used as host strain for Split ubiquitin system (provided by Dr. Guliano Ciarimboli, University Klinik, Münster)

HEK 293 is a human embryonic kidney cell line (Graham *et al.*, 1977).

3.1.7 Radioactive compounds

Name	Specific activity	Manufacturer
[^{14}C]-methyl-α-D-Glucopyranoside ([^{14}C]AMG)	300 mCi/mmol	American Radiolabeled Chemicals, St Lois, USA
D,L-[1-^{14}C] ornithine	56 mCi/mmol	Hartmann Analytic, Braunschweig, Germany
[^{3}H]- Uridine	20 Ci/mmol	American Radiolabeled Chemicals, St Lois, USA

3.1.8 Materials used for ODC enzyme activity assay

5ml Vaccutainer [BD biosciences, Heidelberg, Germany. Ref No.367614]

Combi stopper [B. Braun, Melsungen, Germany, Cat.No: 4495101]

Whatmann filter paper Grade 1 [GE Healthcare Europe GmbH, Freiburg, Germany. Cat.No 1001030]

21G needle [A. Hartenstein, Wuerzburg, Germany]

3.1.9 Software

Search of the putative phosphorylation sites and calmodulin binding motifs was performed employing Minimotif Miner (Balla *et al.*, 2006). Densitometric analysis was performed using program Image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2005; Abramoff M.D., 2004).

3.2 Methods

3.2.1 Molecular biology

3.2.1.1 Polymerase chain reaction (PCR)

PCR reaction was performed in a volume of 50 µl in reaction buffer using 10 ng of template DNA (hRS1/RSSP), 5 pmol of forward and reverse oligonucleotide primers and 12.5 nmol of dNTPs. Before the reaction initiation, the reaction mix was covered with paraffin oil. After

heating to 94^0C, 1.25 units of Pfu DNA polymerase were added to initiate the PCR reaction. Following an initial denaturation step for 1 min at 94^0C, 25 cycles with the following parameters were performed: denaturation for 30 s at 94^0C, annealing for 30 s at 55^0C, and elongation for 30 s at 72^0C with the final elongation time for 5 min at 72^0C. Elongation time in amplifying cycles was 1 minute per kb to be amplified. The reaction efficiency was controlled by analytical agarose gel electrophoresis. Prior to restriction digestion, paraffin oil, and polymerase were removed with chloroform extraction.

3.2.1.2 DNA isolation by chloroform extraction

To precipitate DNA after PCR and remove paraffin oil and proteins, the chloroform extraction was performed. 50 µl of chloroform-isoamyl alcohol (24:1 (v/v)) was added to PCR mix, mixed gently, and two phases were separated by centrifugation at 14 000 g for 5 min. The upper (aqueous) phase containing DNA was used for precipitation of DNA by addition of 0.1 volume of 3M sodium acetate, pH 5.0 and 2.5 volumes of absolute ethanol for 2 hr at -20^0 C followed by centrifugation at 14000g for 10 min. The pellet was washed with 70% (v/v) ethanol to remove excess of the salt, air-dried and dissolved in water.

3.2.1.3 Digestion of DNA and analytical agarose gel electrophoresis of DNA

TAE buffer: 40 mM Tris-acetate, pH 8.0, 1 mM EDTA

Gel-loading buffer (final concentration): 7.5% (v/v) glycerin, 0.06% (w/v) bromphenol blue

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1µg of the plasmid DNA was mixed with 0.25 µl (10 units) of the respective enzyme in 10 µl of the appropriate buffer and was incubated at 37^0C for 2 hours. Samples containing plasmid DNA were mixed with gel-loading buffer and subjected on horizontal 1% (w/v) agarose gel containing 0.3 µg/ml ethidium bromide. The electrophoresis was performed for 1 h in TAE buffer at a voltage of 5 V/cm. The DNA molecular weight marker, 1kb (MBI Fermentas), was used as a reference for size determination of the DNA fragments. The DNA bands were visualized by illumination in UV light (254 nm) using a Dual Intensity Ultraviolet Transilluminator and photo-documented.

3.2.1.4 Transformation of bacteria and clone selection

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SOC medium: 10g/l yeast extract, 20g/l bacto-tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM D-glucose

LB medium: 2 % (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 170 mM NaCl

LB-agar: LB-media, 1.5 % (w/v) agar

Plasmid DNA was introduced into bacteria by the electroporation method (Dower *et al.*, 1988). 20 µl of electrocompetent *E.coli* cells were carefully thawed on ice, mixed with 10 ng plasmid DNA and transferred into the prechilled electroporation cuvette. After electrical pulse (1.6 kV, 5 ms) bacteria were suspended with 1 ml of SOC medium and incubated for 1 h at 37^0C to express the antibiotic resistance conveyed by the plasmid. 250-500 µl of the bacterial suspension were plated on an agar plate containing the corresponding antibiotic and incubated overnight at 37^0C. After 16 h the single colonies of transformed bacteria were observed, and 10 colonies were selected for further analysis. The cells were transferred to a new agar plate and into tubes with 3 ml LB medium and incubated for 16 h at 37^0C

3.2.1.5 Isolation of plasmid DNA from E. coli

Plasmids were isolated from overnight cultures inoculated with a single colony. Cultures were grown at 37^0C in LB medium containing the corresponding antibiotic.

Mini preparation of plasmid DNA

P1 buffer 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A

P2 buffer 200 mM NaOH, 1% (w/v) SDS

P3 buffer 10 mM Tris-HCl, pH 8.0, 1 mM EDTA

Cells from 3 ml of the overnight culture were transferred to a snap vial (Eppendorf). After centrifugation for 3 min at 7000 g, the pellet was resuspended in 300 µl of P1 buffer and incubated for 5 min at RT. After addition of 300 µl of P2 buffer tubes were gently mixed and incubated for 5 min. 300 µl of P3 buffer was added and the samples were incubated for 15 min on ice. The cell debris and chromosomal DNA were spun down by the centrifugation for 15 min at 13000 g at RT, and plasmid DNA was precipitated from 800 µl of supernatant with 640 µl of isopropanol and centrifuged for 15 min at 13 000 g at RT. The pellet was washed with 70% (v/v) ethanol, air-dried and resuspended the plasmid DNA pellet in 20 µl of water. 1 µl of DNA solution was used for an analytical restriction digest. The positive samples were subjected to sequencing. DNA samples were stored at -20^0 C.

Large scale isolation of plasmid DNA

For large scale purification of plasmid DNA, 100 ml of an overnight *E.coli* culture transformed with the plasmid of interest were used. The purification was performed according to the manufacturer's instructions (HiSpeed Midi Kit, Qiagen, Hilden, Germany). The DNA concentration was determined by spectrophotometry, adjusted to 1 µg/µl concentration, and DNA was stored at -20^{0}C.

3.2.1.6 Determination of DNA concentration by spectrophotometry

The DNA concentration was determined by measuring the optical density of a sample at a wavelength of 260 nm. For estimation of the concentration of the DNA the following formula is used. $OD_{260} \times 50 \times 60$ gives the amount of DNA in mg/ml. Where 50 is the specific value for plasmid DNA and 60 is the dilution factor. Purity of DNA was estimated from the ratio of absorbance at 260 nm and 280 nm. The sample was considered to be free of protein contamination if the ratio was 1.8 - 2.0.

3.2.2. SPLIT UBIQUITIN SYSTEM:

The split ubiquitin system is based on the reconstitution of ubiquitin, a small and highly conserved protein which tags other proteins for degradation. During the process of intracellular protein degradation by the 26S proteasome, a series of enzymatic reactions covalently attach a chain of ubiquitin molecules to the protein destined for degradation. The ubiquitin-tagged protein is then transported to the 26S proteasome and is degraded. To recycle the ubiquitin, the cell has evolved a mechanism to remove the polyubiquitin chain from the target protein. This process is mediated by the ubiquitin specific proteases (UBPs). The UBPs specifically recognize intact folded ubiquitin and cleave the polypeptide chain after the last residue of ubiquitin (a Gly-Gly motif). Free monomeric ubiquitin is thus released back into the cytosol, whereas the target protein is degraded. When yeast ubiquitin is split into two halves (N-terminal ubiquitin (Nub) and C-terminal ubiquitin (Cub)) both parts are folded incompletely and not recognized by UBPs when they are expressed separately. When both parts are co-expressed in the same cell, they assemble forming so called split ubiquitin and are recognized and cleaved by UBPs. The affinity between Nub and Cub is largely reduced when isoleucine at position 3 of the Nub is mutated to glycine (NubG). When NubG and Cub are coexpressed they do not assemble. In the employed split ubiquitin protein complementation assay a protein of interest (protein Y) is fused to Cub which is also coupled to the artificial transcription factor LexA-VP16. Proteins (X proteins) which are investigated for interaction with protein Y are coupled to NubG. An interaction between proteins X and Y bring NubG and Cub into close proximity. This leads to assembly of the two halves forming

split ubiquitin. The split ubiquitin is recognized by UBPs which cleave the polypeptide chain between Cub and LexA-VP16. As a result the transcriptional factor translocates into the nucleus and binds to the LexA DNA binding domain of the LexA promoter which is located upstream of a reporter gene such as β-galactosidase or auxotrophic growth markers. The two auxotrophic growth markers used in the employed system are HIS3 and ADE2. Activation of HIS3 or ADE2 enables the yeast THY AP4 strain to grow on selective media lacking histidine or adenine, respectively. Thus in the split ubiquitin system the interaction between two proteins is translated into a transcriptional read out, resulting in the growth of yeast on selective media lacking amino acids histidine and adenine or color development in a β-galactosidase assay.

In contrast to the conventional yeast two hybrid system (Fields and song 1989) where the interacting proteins are required to be located in the nucleus, the split ubiquitin system developed by Johnsson and Varshavsky (Johnsson and Varshavsky 1994) allows (1) to assay full length proteins, (2) to detect interactions at the plasma membrane, (3) to detect interactions involving integral membrane proteins, membrane associated proteins, soluble proteins, and proteins that are modified posttranscriptionally.

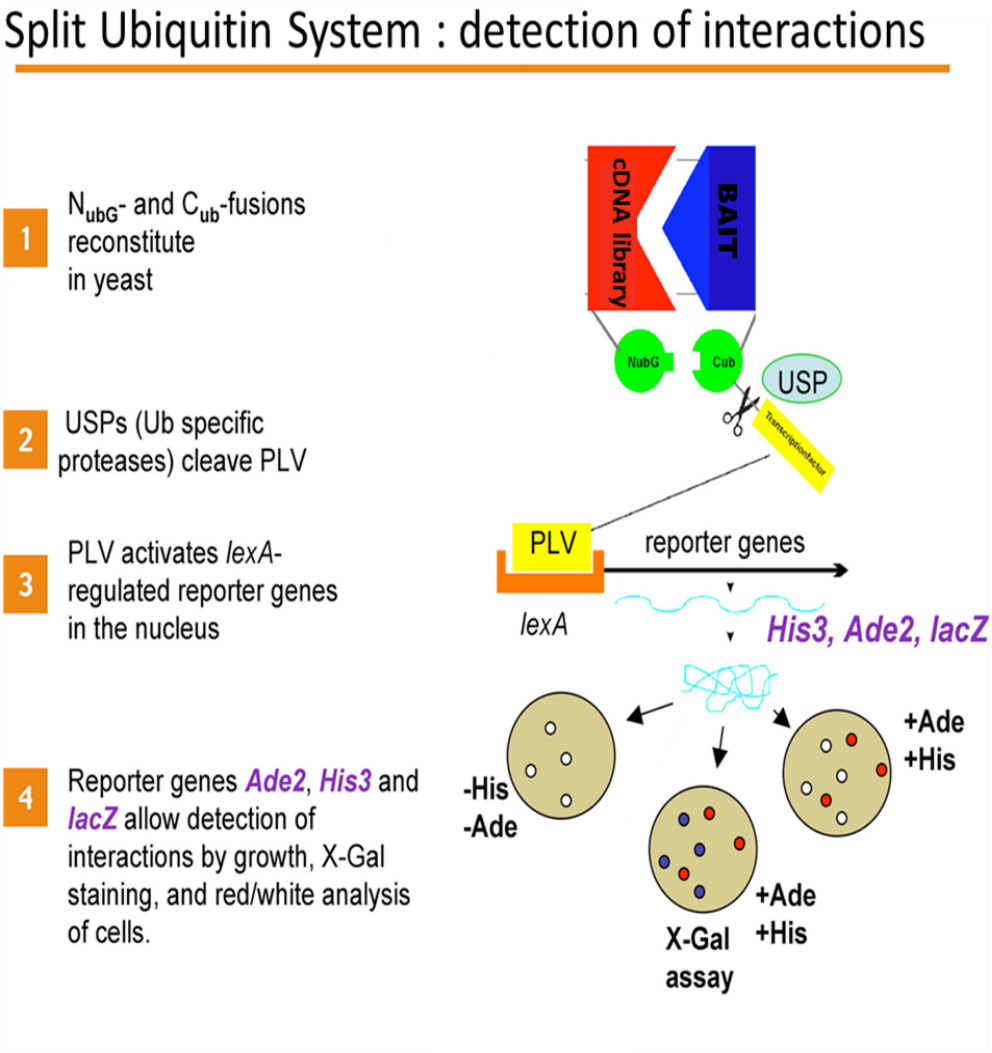

Fig 1: Schematic representation of the split ubiquitin system.

Source: Dualsystems membrane kit 3 user manual

3.2.2.1 Growth medium for yeast

All aqueous solutions were prepared with deionised water and generally autoclaved at 120^0 C for 20 min unless specified for filter sterilisation

YPAD Medium and Plates

Rich medium for the routine growth of yeast

For 1 litre of the medium 10 g of bacto-yeast extract, 20 g of bacto-peptone, 20 g of dextrose, and 100 mg of adenine sulphate were added and the pH was adjusted to 6.0 with HCl and

autoclaved. For YPAD plates 20 g of bacteriological-grade agar was mixed to the same composition as liquid medium. After autoclaving the medium was cooled to 55^0C, dispensed into sterile petri dishes, cooled for solidification and stored at $+4^0$ C until use.

Synthetic Complete Medium (**SC Medium) and Plates**

Synthetic Complete medium consists of a nitrogen base, a carbon source, and a "dropout" solution containing essential amino acids, nucleic acids, trace elements and vitamins. For selection purposes, certain amino acids are omitted or "dropped out" (e.g., leucine, tryptophan, histidine, adenine) from the dropout solution. For liquid medium, the agar is omitted. Amino acid powder mix was prepared by mixing equal amounts of all the amino acids omitting the auxotrophic selected amino acids that are used for preparing the drop out solutions (for example 2-3 g for each compound) of the following: alanine, arginine, aspartic acid, asparagine, cysteine, glutamic acid, glutamine, glycine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine

Composition of ingredients per litre of medium:

2% w/v Glucose

0.17% w/v yeast nitrogen base without ammonium sulfate and amino acids

0.5% ammonium sulfate

1.5 gms of aminoacid powder mix

Chemicals for auxotrophy selection

All dissolved in distilled water and sterilized by filtering

Chemical	Stock conc. (g/100ml)	Vol (ml) stock storage for 1L medium	Storage temperature
Adenine sulfate	0.2	10	RT
Uracil	0.2	10	RT
L-tryptophan	1	2	4°C
L-leucine	1	10	4°C
L-histidine HCl	1	2	4°C
L-methionine	1	2	4°C

Depending on the auxotrophies (e.g., for SC-Leu, all except Leucine) the respective amounts as given in the above table are mixed and autoclaved. After autoclaving the medium was

cooled to 55^0 C, dispensed into sterile petri dishes, cooled for solidification and stored at $+4^0$ C until use.

3.2.2.2 Preparation of competent THY.AP4 yeast

Single colony of the THY.AP4 yeast strain was inoculated in 5ml of YPAD medium and incubated overnight at 30^0C. The overnight culture was inoculated into 100ml YPAD to an OD_{600} 0.08-0.1. It was incubated at 30^0C on a shaker till OD_{600} reaches 0.5-0.6 (2-3 duplications). The culture was placed in to two sterile 50 ml falcon tubes and centrifuged at 2500 g for 5min. The medium was removed and the pellets were suspended separately in 5ml sterile ddH_2O and recentrifuged as described above. Water was removed and the pellets were suspended in 2.5ml LiAC/TE, pooled, and recentrifuged as described above. After centrifugation and removal of the supernatant, the pellet was suspended in 0.5-0.8 ml LiAc/TE buffer and incubated at room temperature for 30 min to make the competent cells ready for use.

3.2.2.3 Creation of the bait plasmid for split ubiquitin system

For creation of the bait plasmid in the split ubiquitin system an *in vivo* cloning method is used. For *in viv*o cloning into the split ubiquitin system vectors, the open reading frame (ORF) has to be flanked by B1 and B2 linkers via PCR. B1 and B2 linkers contain attB1 and attB2 sites of the GATEWAY cloning system (Invitrogen) respectively. B1 primer contains at its 5` end the sequence corresponding to attB1 site of the GATEWAY cloning system followed by the 5`end sequence of the gene of interest (RS1-[1-312]). B2 primer contains at its 5` end the sequence corresponding to attB2 site of the GATEWAY cloning system followed by the 3`end sequence of the gene of interest. The GATEWAY technology is a universal cloning method based on the site specific recombination properties of bacteriophase lamba (landy, 1989). The GATEWAY technology provides a rapid and highly efficient way to move DNA sequences into multiple vector systems for functional analysis and protein expression (Hartley et al., 2000). Homologous recombination between B1 and B2 sequences of the B1-RS1-(1-312)-B2 and of the linear vector produces circular vector harboring the RS1 (1-312) fragment. The bait plasmid pMetYC is restricted with the restriction enzymes Pst I and Hind III (Obrdlik *et al*., 2004). The B1-RS1-(1-312)-B2 PCR product and the linearized vector are co transformed into THY.AP4 yeast strain. Transformants are selected on SC-Leu auxotrophic selective medium plates. The following primers have been used to generate the RS1-(1-312) ORF that is used in *in vivo* transformation to generate the bait plasmid pMetYC.

Forward primer with B1 linker underlined

5´- ACA AGT TTG TAC AAA AAA GCA GGC TCT CCA ACC ACC ATG TCA TCA TTA CCA ACT TCA GAT GGG-3´

Reverse primer with B2 linker underlined

5´- TCC GCC ACC ACC CAC TTT GTA CAA GAA AGC TGG GTA GGG CTG TAA ATC CTG AGT GGA AAT GG-3´

3.2.2.4 Transformation of yeast strain THY.AP4 (Obrdlik *et al*., 2004)

Working solutions:

LiAc/TE: (1 ml 10 x TE, 1 ml 1M LiAc, 8 ml ddH2O)

PEG/LiAc mix: (0.5 ml 10 x TE, 0.5 ml 1M LiAc, 4.0 ml 50% PEG)

Stock solutions:

1M LiAc (Filter sterlised)

10 x TE: (100 mM Tris HCl, 10 mM EDTA, pH 7,5; adjusted with NaOH)

50% PEG 4000 (Fluka) sterilized by autoclaving

5mg/ml salmon sperm DNA (SSDNA), in 1xTE

Before the transformation was started salmon sperm DNA was boiled for 3 min and chilled on ice.

For transformation the following reagents are added

20µl carrier salmon sperm DNA (5-10 mg/ml)
20µl DNA mix consisting of DNA (linear pMetYC 100 ng and insert [B1-RS1 (1-312)-B2] > 100 ng) with a molecular ratio of linearized bait vector and insert in 1:4 ratio in 1X TE bufer
4.5µl 1M LiAc
50µl competent cells
300µl PEG/LiAc mix

The components were mixed well after each addition and the reaction mix was incubated for 20 min at 30^0C on a thermo mixer. After 20 min the cells were subjected to heat shock for 20 min in a water bath at 42^0C. Thereafter the cells were centrifuged at 5000 g for 1 min and the supernatant was removed. The pellet was suspended in 100 µl sterile water or 1x TE buffer

and was spread on selective media plates lacking leucine. The plates were incubated for 2-4 days at 30^0C for the growth of yeast which has been transformed with bait plasmid. For cDNA library transformation the yeast THY.AP4 strain containing the bait plasmid was cultured and transformed with the cDNA library according to the manufacturer's instructions (Dualsystems membrane kit 3, Dualsystems Biotech, Zurich, Switzerland).

3.2.3 Protein analysis methods

3.2.3.1 Determination of protein concentration

The protein content of the samples was determined according to Bradford test using bovine serum albumin as a standard (Bradford, 1976). 1 µl of protein solution was diluted in 99 µl of water and 900 µl of Bradford reagent (Bio-Rad) and incubated for 5 min at RT. The extinction of the samples was measured at 595 nm and correlated with the extinction of the solvent and control bovine serum albumin samples (2, 4, 6, 8, and 10 µg).

3.2.3.2 Co-precipitation of the GFP/myc tagged proteins and associated proteins

All the buffers used are pre chilled and the experiments were done on ice. Transiently transfected HEK 293 cells were washed three times with pre chilled PBS buffer. The cells were scrapped with a rubber policeman into 1 ml of the lysis buffer (10 mM HEPES pH 7.2, 142 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 0.5% NP-40 containing 0.1 µM PMSF, and 10 µl/ml of the protease inhibitor cocktail from calbiochem). The cells were lysed by sonication (3 min of total sonication time with repeating on and off steps for 15 s and 45 s, respectively). The cell debri was cleared by centrifugation at 100000 g for 1 hr at 4^0C. Protein concentration was determined as described in section 3.2.3.1 and the concentration was adjusted to 1 mg/ml. The samples were freezed in liquid nitrogen and were stored in -20^0 C. For pull down assay 500 µg of the protein sample was used. 30 µl of the affinity matrix (agarose beads) was added to the protein sample for the precipitation of the respective recombinant proteins and their associated protein complexes. After 1 hr rotation at $+4^0$ C, the agarose beads were collected by centrifuging the sample at 6000 g. the beads were washed thrice using the lysis buffer. The protein complex bound to the beads was eluted by boiling the beads for 5 minutes at 95^0 C in SDS sample buffer (0.001 % (w/v) bromphenol blue, 10 % (v/v) glycerol, 0.25 M β-mercaptoethanol, 1 % (w/v) SDS, 15 mM Tris-HCl, (pH 6.8)). The eluted proteins were analyzed by western blotting.

3.2.3.3 SDS-polyacrylamide gel electrophoresis

Proteins or cell extracts were separated by the discontinuous SDS-polyacylamide gel electrophoresis according to Laemmli (Laemmli, 1970). The gels were composed of two layers, the separating gel containing the corresponding amount of acrylamide/bisacrylamide, 375 mM Tris-HCl, pH 8.8, and 0.1% SDS and a stacking gel (5% acrylamide/bisacrylamide, 0.1% SDS, 125 mM Tris-HCl, pH 6.8). Depending on the required separation range the acrylamide concentration was adjusted to 10%, 12.5%, 15%, or 17.5% in separating gel buffer using the Rotiphorese gel, acrylamide/bisacrylamide 37.5:1 mixture (Carl Roth GmbH). Shortly before casting, polymerization of the stacking or separating gels was initiated by addition of ammonium persulfate (APS) and N,N,N',N'-Tetramethylethylendiamine (TEMED) to a final concentration of 0.01% (v/v) of each component. Protein samples were prepared by heating for 5 min at 95^0C in SDS sample buffer (0.001 % (w/v) bromphenol blue, 10 % (v/v) glycerol, 0.25 M β-mercaptoethanol, 1 % (w/v) SDS, 15 mM Tris-HCl, (pH 6.8). The samples were loaded onto polyacrylamide gel. The electrophoresis was performed in SDS running buffer (24.8 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% (w/v) SDS) at 25 V/cm using Mini Protean-3 electrophoresis chambers (Biorad) and the electrophoresis power supply EPS601 (GE Healthcare). The Page Ruler Prestained Protein Ladder (MBI Fermentas) was used as a size reference.

3.2.3.4 Western blot and Immunodetection

Western blot

TBST buffer: 137 mM NaCl, 50 mM Tris-HCl, pH 8.0, 2.7 mM KCl, 0.05 % (v/v) Tween 20
Blotting buffer: 25 mM Tris, pH 8.3, 192 mM glycine, 10 % (w/v) methanol

For immunodetection of proteins, the samples were subjected to immunoblot analysis. Proteins were separated by SDS-PAGE, transferred onto a Polyvinylidene Difluoride (PVDF) membrane using a semi-dry system (Gershoni and Palade, 1983). For semi-dry transfer of proteins the horizontal semi-dry transferblot (chamber type SD 18) with two graphite plates was used. Before transfer, the PVDF membrane was pre-soaked in methanol for 5 min. Thereafter, Whatman filter papers, the gel and the membrane were soaked in the blotting buffer. Subsequently, the sheets of Whatman paper, the PVDF membrane, and the gel were assembled on a graphite plate of the transfer blot in the following order: (cathode), 3 x (3 mm) Whatman filterpaper, SDS-PAGE gel, PVDF membrane, 3 x (3 mm) Whatman filter paper, and (anode). Blotting was performed in the blotting buffer at 1.5-2 mA/cm^2 for 2 hr.

Immunodetection of the proteins.

First, all non-specific binding sites on the membrane were blocked by incubation with blocking buffer (5 % (w/v) milk powder in TBST buffer) for 1 h at room temperature. Followed by three 5 minute washes, the membrane was incubated with the primary antibodies diluted in TBST containing 1% (w/v) milk for 1 h at RT or overnight at $+4^0$C. After three 5 min washing steps with TBST containing 1% (w/v) milk, the membranes were incubated with the horse radish peroxidase (HRP)- coupled secondary antibodies diluted in TBST containing 1% (w/v) milk for 1 h at RT. Unbound antibodies were removed with next three 5 min washing steps with TBST. During all incubation steps the membrane was kept for shaking on the rotor. The bound label was visualized by enhanced chemiluminescence using Pierce ECL Western Blotting Substrate (Pierce) according to the manufacturer's instructions. The obtained pictures were scanned and densitometric analysis was performed using program Image J (http://rsbweb.nih.gov/ij/index.html).

3.2.3.5 Membrane stripping:

Bound antibodies were removed from the Western Blot membranes by incubation in the stripping buffer (0.1M glycine, pH 2.9) for 1 h at RT. Afterwards membranes were washed three times in TBST and blocked with the blocking buffer for 1 h. After this procedure Western blots could be used for the re-probing with other antibodies.

3.2.4 Cell Culture

3.2.4.1 Cultivation of mammalian cells

Native and transiently transfected human embryonic kidney HEK 293 cells were grown at 37^0C in a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) containing 10% (v/v) foetal calf serum (FCS, Sigma-Aldrich), 1% L-glutamine (PAA, Pasching, Austria), and 1% penicillin/streptomycin (PAA).

3.2.4.2 Transfection of HEK 293 cells

Transfection mixture was made ready by mixing the following components and was allowed to stand for 30 mins. For 15 cm culture plate, 37 µg plasmid DNA, 80µl Polyethylenimine (PEI) (1mg/ml) (Sigma-Aldrich) and 2.5 ml DMEM without FCS and L-glutamine and antibiotics were mixed. Subconfluent HEK 293 cells were transiently transfected with the vectors encoding different proteins using the transfection mixture. Medium was changed following 5 hour incubation and cells were observed for vector expression after 18-24 hours.

3.2.5. Oocyte measurements

3.2.5.1 Preparation of *Xenopus laevis* oocytes and injection of cRNA

Solutions	
ORi buffer (oocytes Ringer solution):	5 mM HEPES pH 7.6, 100 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$
Ca^{2+}- free ORi buffer:	5 mM HEPES pH 7.6, 100 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$
K-ORi buffer:	5 mM HEPES pH 7.6, 100 mM KCl, 3 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$

Xenopus laevis toads were obtained from H. Kähler (Hamburg, Germany). Animals were housed and handled in compliance with institutional guidelines and German laws. Oocytes at the stages V and VI were obtained by partial ovariectomy of mature female *Xenopus laevis* anesthetized by immersion in anesthetizing solution (1 mg/ml of Tricaine supplemented with 1 mg/ml of $NaHCO_3$).The oocytes were treated overnight with collagenase I (10 mg/ml in ORi). The treated oocytes were then washed twice with Ca^{2+}-free ORi and kept at 16^0C Incubator in ORi buffer. Selected oocytes were injected with 50.6 nl of water containing cRNA-solutions (see table 3) using a nanoliter injector with boro-silicate glass capillaries. For protein expression, injected oocytes were kept for 2 days at 16^0C incubator. Non-injected oocytes served as a control.

cRNA Injected	**Amount (ng/oocyte)**
hSGLT1	10
hODC	5
hCNT1	10

Table 3: Amounts of cRNA injected into Xenopus oocytes

3.2.5.2 Injection of RS1-reg protein and biochemicals into oocytes

Different concentrations (10 nM, 100 nM, 200 nM, 500 nM, 1 µM, and 3,5 µM) of RS1-Reg protein, 3 mM difluoromethylornithine (DFMO) Inhibitor of ODC, 3 nM brefeldin A (BFA; inhibitor of vesicle release from *trans*-Golgi network), 250 µM AMG [(methyl-α-D-glucopyranoside) a non- metabolized derivative of glucose and specific substrate of SGLT1], 1 µM putrescine dissolved in K^+ Ori buffer were injected into oocytes. After injection of the protein and/or biochemical, the oocytes were incubated in ORi buffer for one hour before the tracer-flux measurements. To investigate the influence of intracellular sugar on DFMO-dependent down-regulation of hSGLT1, different concentrations of AMG were injected into the hSGLT1 expressing oocytes with or without DFMO

3.2.5.3 Tracer-flux experiments

For tracer-flux measurements, oocytes expressing transporters, oocytes injected with protein or biochemicals or non-injected control oocytes were divided into groups containing 10 oocytes per each and were transferred into 2 ml eppendorf tubes with 200 µl ORi uptake medium containing the radiolabelled substrate (Veyhl et al., 1993, Veyhl et al., 2003). 25 µM methyl-α-D-[^{14}C]-glucopyranoside ([^{14}C]-AMG) and 5µM ^{3}H Uridine were used as substrates for oocytes expressing hSGLT1 and hCNT1 respectively. The oocytes were incubated for 20 mins at room temperature on a shaker. After incubation the tracer uptake was terminated by aspiration of the ORi buffer followed by 3 washes in ice cold buffer. Individual oocytes were transferred into 5 ml scintillation vials and were dissolved in 200 µl of 5% SDS. After solubilizing, 1ml of Lumasafe scintillation cocktail (Lumac LSC (Groningen, Netherlands) was added and the radioactivity was analyzed by the scintillation counting.

3.2.5.4 Statistical analysis

The test for significance of differences between mean values was performed using one-way ANOVA test with a post hoc tukey comparison for at least three experiments performed with different batches of oocytes.

3.2.6 ODC Activity assay

Solutions

1. 50 mM Tris/HCl
2. 0.5 M DTT in Tris/HCl

2b. Tris/DTT- 1 ml Tris/HCl + 5 µl DTT (2)

3. 0.01 N NaOH in water
3b. NaOH (1:100) dilution in water
4. 0.7 mM Pyridoxal-5-Phospate (P-5-P) in Tris/HCl (heat to 50^0C to dissolve)
4b. 1:100 dilution of solution of P-5-P
5. 10mM EGTA

Homogenising buffer: 1 ml Tris/HCl (1) + 10 µl DTT (2) + 10 µl EGTA (5)
Incubation buffer : 2.5 µl NaOH (3b) + 11 µl Tris/DTT (2b) + 2.5 µl radioactive [^{14}C] ornithine

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The activity of the enzyme ODC was assayed with a radiometric technique in which the amount of $^{14}CO_2$ liberated from D, L-[1-^{14}C] ornithine was estimated, as described earlier (Milovic et al., 2001). HEK 293 cells were grown to subconfluent stage. At 60% confluence the cell were transiently transfected with respective plasmids (section 3.2.3.3). After 24 hours the cells were checked for plasmid expression and they were washed thrice with ice cold PBS. The washed cells were collected by scrapping using a rubber policeman in homogenizing buffer. They were lysed by sonication. The cell lysates were centrifuged at 100,000 g for 1 hr to clear the insoluble material and protein concentration of the supernatant was determined using Bradford protein assay (section 3.2.3.1). 10 µl of solution 4b was added to 100 µl of the supernatant (test) for which 100 µl of Tris/HCl served as control (For the activity experiment using pure ODC protein, 80 ng in 100 µl Tris/HCl was used). Before the start of the reaction the filterpapers pierced on to the needle of the vaccutainer were soaked in 20 µl of 1M benzethonium hydroxide solution. To start the reaction 16 µl of the incubation buffer was added and the vaccutainer was closed and incubated for 1hr at 37^0C in a water bath. To stop the reaction, 200 µl of 0.6 N perchloric acid was added through the needle and incubated for 30 min at 37^0C in the water bath. The filter paper was collected after 30 minutes and was transferred into the 5 ml scintillation vials containing the scintillation mixture prepared by adding 75 µl 10% acetic acid to 3 ml of lumasafe scintillation cocktail and were kept for scintillation counting.

3.2.6.1 Preparation of the setup for ODC activity assay:

Close the top of a 21G needle with a red colored combi stopper. Plunge the closed 21G needle into the vaccutainer through the rubber cap. Pierce carefully a 0.6x0.6 cm whatmann filter paper through the needle and close the vaccutainer.

4. Results

4.1 Identification of proteins that interact with hRS1 by two-hybrid screening

To understand how protein RS1 regulates the expression of transporters, interacting proteins must be identified. For this purpose a two-hybrid screening using the split ubiquitin system (Obrdlik et al., 2004) has been employed. Human embryonic kidney cDNA library has been screened using the N-terminal fragment of hRS1 (amino acids 1-312) as bait in the split ubiquitin screening. This fragment contained a domain (RS1-Reg) which mediates the posttranscriptional regulation of different transporters including the Na^+-D-glucose co-transporter SGLT1 (Veyhl and Koepsell, unpublished data). The yeast transformed with bait and the prey library plasmids was grown on selective media (SC media) lacking amino acids leucine, tryptophan and adenine (SC-leu-trp-ade). After 2-3 days of incubation the yeast colonies grown on these plates were replica plated on to auxotropic selection media lacking amino acid histidine (SC-leu-trp-ade-his). The yeast colonies grown on these selection plates were supposed to contain the interacting partners and hence were processed for further analysis by ß-galactosidase assay. The interaction was identified by the change of yeast colonies expressing the interacting partner to blue color. The colonies that turned blue were selected to be harboring true interactions. Prey plasmid from these colonies was isolated and transformed into DH10B E.coli strain by electroporation (section 3.2.1.5) and selected by antibiotic resistance of ampicillin. The prey plasmid isolated from E.coli was sequenced, checked for the open reading frame (ORF). Clones which are in ORF are selected and the protein encoded by the cDNA library plasmid was identified by BLAST analysis.

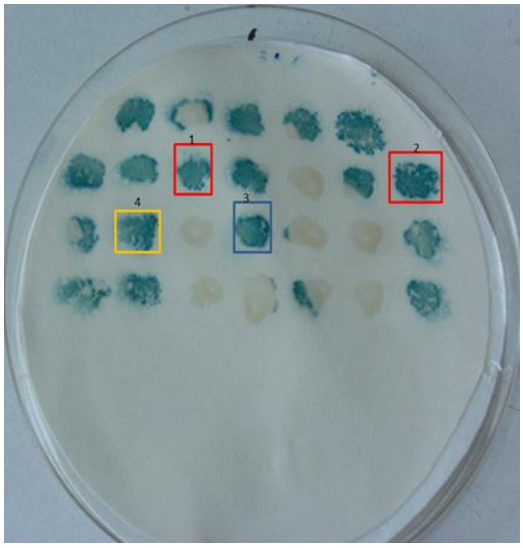

Fig 1: Plate showing the ß-galactosidase assay of the selection procedure in split ubiquitin system

Positive interactions were identified by the blue coloration of the yeast colonies. The yeast colony marked in the red colored box was identified to contain osteopontin (Twice) as the interacting partner. The blue colored box was ornithine decarboxylase and the yellow colored box was ß-actin. The rest of the clones were not in the open reading frame, hence regarded as false positives.

The following proteins were identified as interacting partners

Serial Number	Name of the clone	Genebank Accession number
1	Secreted phosphoprotein I (Osteopintin)	AAA59974.1
2	Ornithine decarboxylase I	NP_002530.1
3	Iron sulphur domain-2	
4	Ferritin Light peptide	AAA52439.1
5	ß-Actin	AAH08633.1
6	Mitochondrial carrier Adenine nucleotide translocator	AAH56160.1
7	superoxide dismutase-2 (Mitochondrial)	AAH12423.1
8	Phospho fructokinase (liver)	
9	Nudix (Nucleoside diphosphate linked moiety-x)	
10	Uromodulin	AAA36799.1
11	Procollagenase-c-endopeptidase enhancer 2	
12	Annexin-2	AAH68065.1
13	HSP 60 KD protein 1	NP_955472.1
14	Small nuclear RNA activating complex (SNAPC1)	NP_003073
15	Lamin beta 1	NP_005564.1
16	Annexin A2	AAH68065.1
17	Guanine Nucleotide binding protein (G protein) ß polypeptide like protein 2 like 1 (GNB2L1)	NP_006089.1
18	Ferritin	

Table 1: Interacting partners of RS1- (1-312) from the screening of human embryonic kidney cDNA library.

From the several proteins that were identified as positive interacting partners in the screening, the above mentioned proteins (table 1) were in the open reading frame. Ornithine

decarboxylase, the rate limiting enzyme involved in the formation of the diamine putrescine, a precursor molecule for polyamines spermidine and spermine (Morris DR, Pardee AB. 1966) was selected for further study because it had been reported that inhibition of ODC reduced glucose absorption in small intestine (Johnson L.R. 1995) and that oral polyamine administration increased expression of glucose transporters SGLT1 and GLUT2 in small intestine (Wild et al., 2007).

4.2 Bio-physical confirmation of the interaction of ODC with hRS1

To verify the interaction of ODC and RS1 (1-312) by a biochemical method, a pull-down assay has been performed. Subconfluent HEK 293 cells were transiently transfected with plasmids expressing GFP-S-tag-RS1 (1-312) and ODC-myc or with the control vector (GFP-S-tag) and ODC-myc. After overnight incubation the cells were lysed by sonication in the presence of lysis buffer (10 mM HEPES pH 7.2, 142 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 0.5% NP-40) containing 0.1 µM PMSF, and 10 µl/ml of the protease inhibitor cocktail mix from calbiochem . The lysates were cleared from cell debri by centrifugation at 100000 g. The protein content of the supernatant was measured and the protein concentration was adjusted to 1 mg/ml. 500 µg was taken, 30µl of the S-protein- agarose was added and the suspension was rotated for 1 h at 4^0 C. The agarose beads were collected by centrifugation and washed three times with the lysis buffer. Thereafter proteins bound to the beads were removed by centrifugation after boiling the beads in the presence in SDS sample buffer (section 3.2.3.2). The supernatants were separated by SDS-PAGE and analyzed by western blotting and immunoreaction with antibody against myc tag (Fig. 1A). Where a polypeptide band with apparent molecular mass of 51 kDa was obtained after expression of GFP-S-tag-RS1 (1-312) no immunreaction was detected in supernatants obtained after GFP-S tag (control vector) and ODC (Fig. 2 A). A pull down experiments after expression of GFP-S-tag-RS1 (1-312)/GFP-S-tag plus ODC-myc were also performed using myc agarose for precipitation ODC plus associated proteins. In this setting co-precipitated GFP-S-tag-RS1 (1-312) was identified using an antibody against GFP (Fig: 2 B). To demonstrate the specificity of the interaction of RS1 (1-312) with ODC we tested whether RS1 (1-312) is also co-precipitated with antizyme an inhibitory protein to ODC that is generated in response to high polyamine levels in the cell (Fong et al., 1976, Heller et al., 1976), which binds to ODC and targets it for degradation (Murakami et al., 1992a) (Fig. 2 C). Here antizyme-myc (AZ-myc)

was coexpressed with GFP-S-tag-RS1 (1-312) or GFP-S-tag. AZ-myc was precipitated with anti-myc-agarose, and associated GFP fusion proteins were identified using anti-GFP antibody. Fig. 1C indicates that antizyme does not interact with RS1 (1-312). The data demonstrate physical and specific interaction of ODC with the N- terminal RS1 (1-312) protein fragment.

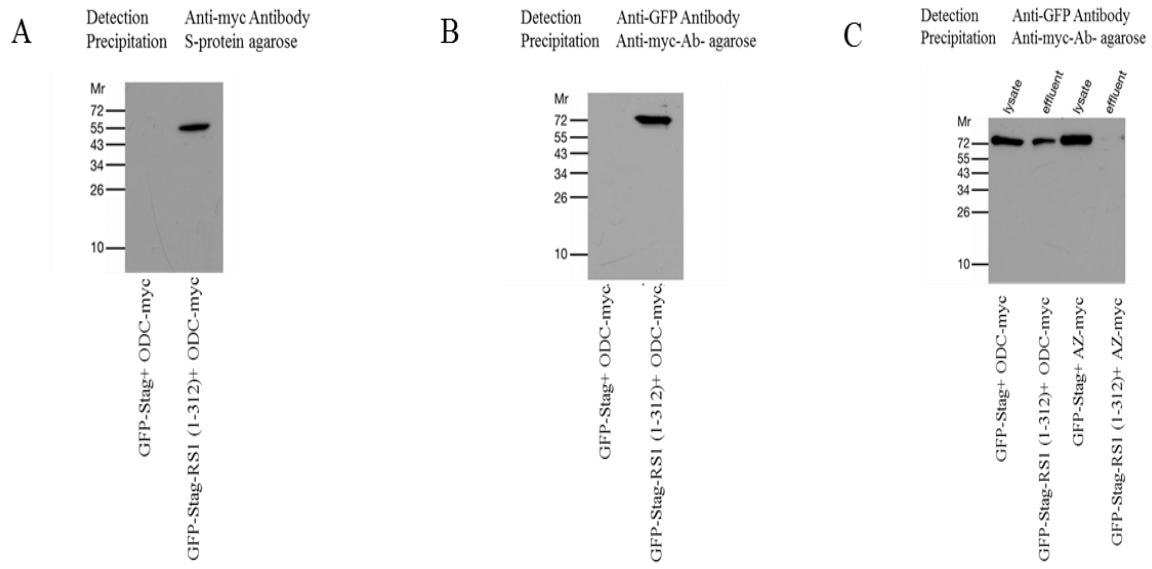

Fig 2: Interaction of the N-terminal 312 amino acid fragment of RS1 with ODC

Pull down assay using S protein agarose from the HEK 293 cell lysates expressing GFP-S-tag-RS1 (1-312) and ODC-myc or the control vector (GFP-S-tag) and ODC-myc confirmed the interaction of ODC with RS1 (1-312) fragment but not the control vector by anti myc antibody (A). Co-precipitation experiment using the above mentioned lysates by anti-myc agarose yielded GFP-S-tag-RS1 (1-312) but not the control vector (GFP-S-tag) in the associated protein complex which is detected by anti GFP antibody (B). Pull down experiment using myc-agarose with lysates expressing GFP-S-tag-RS1 (1-312) together with ODC and /or antizyme (AZ-myc) confirmed the specific interaction of RS1 (1-312) with ODC but not with antizyme using anti- GFP antibody (C).

4.3 Demonstration that the regulatory domain of RS1 (RS1-Reg) binds to ODC.

It was observed in our group that hRS1 down-regulates the expression of SGLT1 post-transcriptionally by blocking the release of SGLT1 containing vesicles from the TGN (Vernaleken et al. 2007) and that this effect is mediated by the NH2 terminal regulatory domain of hRS1 comprising amino acids 16-98 called hRS1-reg (M. Veyhl-Wichmann and

H. Koepsell, unpublished data). RS1-Reg contains two different peptide motifs (QSP and SDSDRIEP) which are able to down-regulate SGLT1 by their own but are not additive. RS1-Reg also contains many consensus sequences for phosphorylation by various kinases suggesting that the function of hRS1-Reg is modulated by phosphorylation. To determine whether ODC binds to hRS1-Reg we expressed either GFP-S-tag or GFP-S-tag-RS1-Reg together with ODC-myc, precipitated ODC-myc with agarose coupled to anti-myc antibody and investigated whether GFP-S-tag and/or GFP-S-tag-hRS1-Reg was co-precipitated using anti GFP antibody. Expression of proteins, cell lysis, precipitation, Western blotting and immune detection using anti-GFP antibody was performed as described in section 3.2.3.2. In contrast to GFP-S-tag, GFP-S-tag-hRS1-Reg was co-precipitated with ODC indicating that ODC binds to hRS1-Reg.

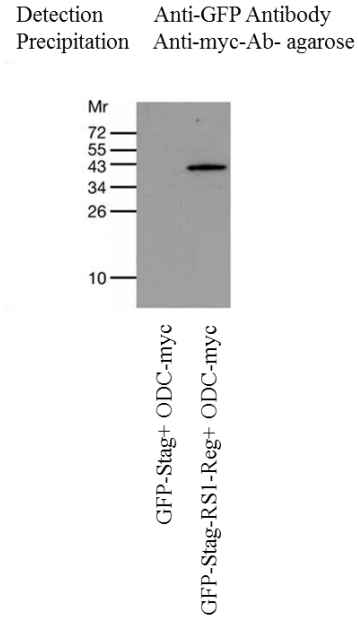

Fig 3: Regulatory domain of RS1 (RS1-Reg) interacts with ornithine decarboxylase.

We wanted to check whether the regulatory fragment of RS1 is involved in the binding of ODC. To this end co-precipitation experiments have been performed. Co-precipitation experiments from the HEK 293 cell lysates expressing GFP-S-tag-RS1-Reg and ODC-myc or the control vector (GFP-Stag) and ODC-myc by anti-myc agarose demonstrated that RS1-Reg binds with ODC. The control vector (GFP-Stag) did not show any interaction with ODC when blotted with anti GFP antibody confirming the specificity of interaction.

4.4 ODC stimulates the expressed AMG uptake mediated by SGLT1

The activity of the rate limiting enzyme of the polyamine biosynthesis, ODC is regulated by factors such as cell volume, cell-cell contact, amino acids, growth factors either transcriptionally or post-transcriptionally. One of such interesting factors that regulate ODC activity is glucose via SGLT1. Glucose along with sodium transported via SGLT1 increases the activity of ODC. Phlorizin a specific inhibitor of SGLT1 could block this increase of activity of ODC (Lundgren et al., 1990). An increase of SGLT1 mRNA and protein was identified in the intestine of post natal rats fed 3 days with polyamine with a wash out effect of 3 days (Wild et al., 2007). Since hRS1-Reg regulates the release of SGLT1 containing vesicles from the TGN in a glucose dependent manner it was investigated whether ODC is involved in this pathway. It was investigated whether co-expression of ODC with hSGLT1 in *X. laevis* oocytes alters expressed transport activity of hSGLT1. We injected hSGLT1 cRNA alone or together with ODC cRNA into oocytes, incubated the oocytes for two days for expression, and measured hSGLT1 expressed uptake of 25 µM [^{14}C] AMG. Co-expression of ODC led to a 50% increase of expressed AMG uptake suggesting that ODC stimulates hSGLT1 expression posttranscriptionally.

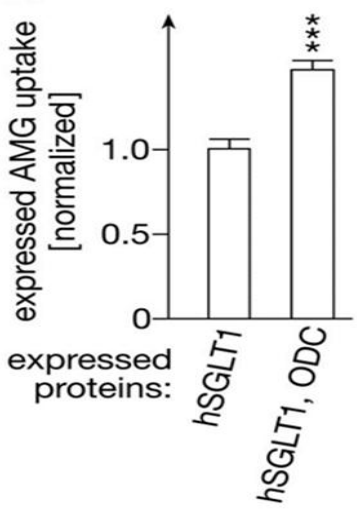

Fig 4: Coexpression of ODC with hSGLT1 in oocytes leads to an increase in SGLT1 mediated AMG uptake The tracer AMG uptake was measured in oocytes expressing SGLT1 alone or SGLT1 along with ODC. AMG uptake when measured for 20 min, oocytes expressing SGLT1 alone showed less uptake than the oocytes expressing SGLT1 and ODC. ODC increases the SGLT1 mediated AMG uptake when co expressed. Uptake measurements were normalized to parallel performed measurements in the control (SGLT1). Mean values ± SE

of 3 individual experiments are presented. ***, P<0.001 for difference to control, determined according to the unpaired T test.

4.5 Polyamine dependent down regulation of SGLT1 activity by α-difluoromethylornithine (DFMO)

DFMO is an irreversible inhibitor of ODC that blocks the activity of ODC. To check whether the AMG tracer uptake by SGLT1 is decreased by blocking the activity of ODC, the irreversible ODC blocker DFMO has been used. Ornithine decarboxylase is endogenously expressed in oocytes and is involved in the maturation of oocytes (Osborne et al,. 1989). Henceforth the activity of SGLT1 was analyzed by blocking the endogenous ODC present in the oocytes by injecting DFMO. The effect of the DFMO in the presence and absence of putrescine the product formed from the decarboxylation of ornithine involving ODC was also examined. For this, oocytes expressing SGLT1 (oocytes injected with SGLT1 cRNA and incubated at 16^0 C for two days for expression) were injected with 3 mM DFMO alone or DFMO along with 1µM putrescine or with 1µM putrescine alone and incubated for 1 hr at room temperature. After incubation the effect of DFMO on SGLT1 was evaluated by performing an uptake measurement using radiolabelled AMG, the substrate of SGLT1. DFMO was able to decrease the uptake of radiolabelled AMG by SGLT1. Importantly putrescine blunted the inhibition of SGLT1 mediated AMG uptake by DFMO whereas putrescine exhibited no effect on SGLT1 expressed AMG uptake. The data suggest that the effect of DFMO on SGLT1 is mediated by depletion of polyamine putrescine due to a decrease of ODC activity.

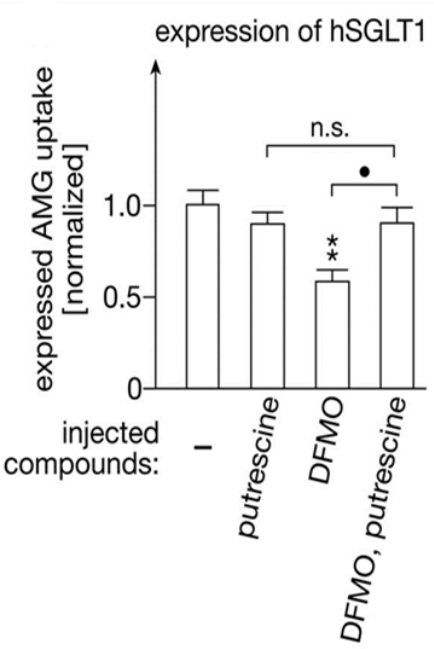

Fig 5: DFMO inhibition of SGLT1 is putrescine dependent

Oocytes expressing SGLT1 were injected with either 3mM DFMO, an irreversible inhibitor of ODC or DFMO along with 1µM putrescine or 1µM putrescine alone. After 1 hr incubation at RT in ori buffer, the SGLT1 mediated AMG tracer uptake was measured. DFMO was able to decrease the SGLT1 mediated AMG uptake to a significant level of 40% and this decrease was not seen when putrescine was injected together with DFMO. Uptake measurements were normalized to parallel performed measurements in the control (SGLT1). Mean values ± SE of 3 independent experiments are indicated. *, $P<0.05$, **, $P<0.01$ for difference to control, determined according to the ANOVA with post hoc Tukey's test.

4.6 Inhibition of ODC by DFMO is sugar dependent and is biphasic

Lundgren et al., showed that in LLC-PK1 (porcine kidney epithelial cells) cells Na^+-dependent co transported glucose analogues 1-O-methyl- α -D-glucopyranoside (α-MDG) and 1-O-methyl-ß-D-glucopyranoside (ß-MDG) via SGLT1 increases the activity of ODC. α-MDG which is a Na^+-dependent substrate for SGLT1 showed an increase in the activity of ODC which could be blocked by phlorizin (Lundgren et al., 1990). Vernaleken et al., showed that intracellular sugar concentrations had a detrimental effect on the regulation of RS1 derived tripeptides on SGLT1. The involvement of a sugar binding protein in the regulatory mechanism of RS1 has been hypothesized (Vernaleken et al., 2007). Henceforth we wanted to study the effect of sugar on the inhibition of SGLT1 activity by DFMO. For this, oocytes expressing SGLT1 were injected either with 3 mM DFMO alone or DFMO along with

different concentrations of the non-metabolized glucose analogue AMG. Sugar abolishes the inhibitory effect of DFMO showing that the effect of DFMO is sugar dependent (Fig.A). The effect of AMG on the inhibitory effect of DFMO has been checked with pure ODC protein. AMG had a biphasic effect on the action of DFMO with low concentration (1mM) inhibiting the effect of DFMO and with higher concentration (10mM) showing no effect.

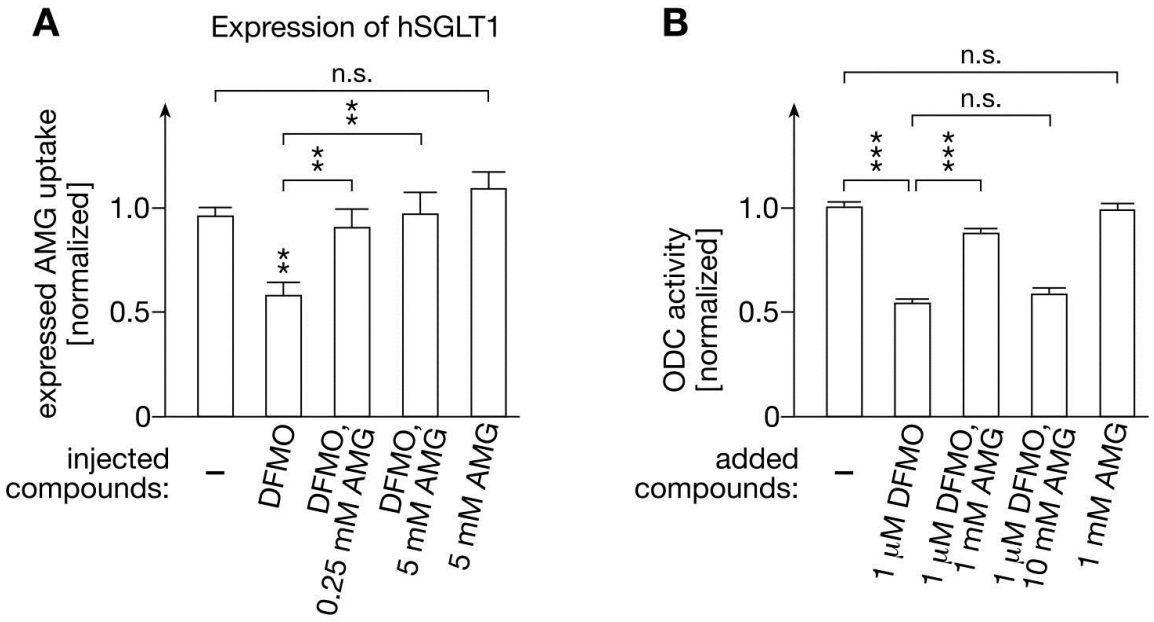

Fig 6: Effect of AMG on the inhibitory function of DFMO is biphasic

250 µM or 5 mM of AMG when injected along with DFMO (3 mM) into oocytes expressing SGLT1 had an inhibitory effect on the function of DFMO (A). In the activity assay performed using pure ODC protein, AMG had a biphasic effect. 1 mM concentration of AMG prevented the inhibition of ODC activity by DFMO whereas 10 mM had no effect (B). Uptake measurements were normalized to parallel performed measurements in the control (SGLT1). Mean values ± SE of 3 independent experiments are indicated. ***, P<0.001, **, P<0.01 for difference to control.

4.7 ODC acts on the exocytotic pathway of release of vesicles at the *trans*-Golgi network

RS1 is an intracellular 67 KDa protein that is present in the cytoplasm and also migrates to nucleus. Immuno hisochemical studies by Kroiss et al., showed that RS1 also localized to *trans*- Golgi network. Veyhl et al., and Vernaleken et al., showed that RS1 and its tri peptide derivatives QSP and QEP (phosphorylated form of QSP) regulate the activity of SGLT1 by blocking the exocytotic release of vesicles containing SGLT1 at the *trans*-Golgi network (Kroiss et al., 2006, Veyhl et al., 2006 Vernaleken et al., 2007). We have shown that RS1-

Reg interacts with ODC (Fig. 3). In the process of functional characterization of this interaction, we wanted to investigate the mechanism of downregulation of SGLT1 by the ODC inhibitor DFMO and wanted to check whether the decrease of activity of ODC leads to the blockage of exocytotic release of vesicles at the *trans*-Golgi network. To check this, exocytosis blocker brefeldin A (BFA) was used. Brefeldin A (BFA) inhibits the guanosine nucleotide exchange factors that activate ADP-ribosylation factors, which regulate the assembly of coat complexes at the TGN and endosomes involved in protein sorting and release of vesicles. Oocytes expressing SGLT1 were injected either with BFA (3 nM) or BFA along with DFMO (3 mM) an irreversible inhibitor of ODC. After incubation for 2 hours at room temperature, the oocytes were checked for SGLT1 mediated AMG tracer uptake. BFA was able to decrease the SGLT1 mediated AMG uptake by blocking the release of vesicles from the *trans*-Golgi network. The same effect was shown if DFMO was used. BFA along with DFMO did not show any additive effect. This shows that both BFA and DFMO act via the same pathway of blocking the release of vesicles from *trans*-Golgi i.e exocytosis.

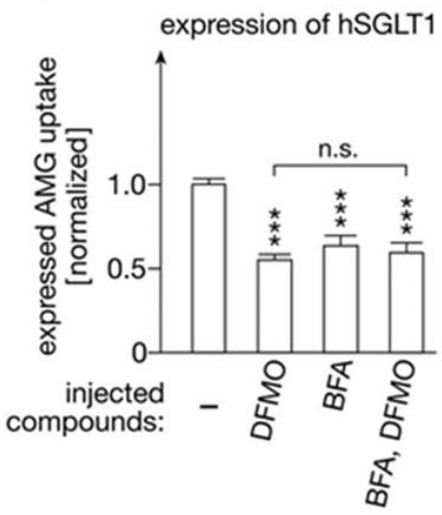

Fig 7: DFMO decreases the activity of SGLT1 by blocking the release of vesicles from TGN

To identify the regulatory mechanism involved in the blockage of SGLT1 activity by DFMO, oocytes expressing SGLT1 were injected either with ODC blocker DFMO (3mM) alone or the exocytosis blocker BFA (3nM) alone or BFA together with DFMO. BFA was able to inhibit the SGLT1 mediated AMG uptake and BFA together with DFMO had no additive effect. This data suggest that both BFA and DFMO regulate SGLT1 via the same pathway of blocking exocytosis at *trans*-Golgi network. Uptake measurements were normalized to parallel performed measurements in the control (SGLT1). Mean values ± SE are indicated. ***, $P<0.001$, for difference to control.

4.8 RS1-Reg decreases the activity of ODC.

To verify whether the binding of RS1-Reg regulate the activity of ODC, an activity assay (as described in methods section 3.2.6) has been performed using transiently transfected HEK 293 cell lysate. The activity is assayed by measuring the amount of CO_2 released during the process of conversion of radiolabelled L-ornithine to putrescine (Milovic et al., 2001). For the activity assay the lysate of HEK 293 cells transfected with plasmids expressing either ODC or ODC along with RS1-Reg-FLAG was used. ODC expressed along with the control vector (pcDNA 3.1) served as control. RS1-Reg when co-transfected with ODC showed a significant effect on the activity of ODC, whereas the control vector did not show any effect on the activity of ODC. The data suggests that RS1-Reg decreases the activity of ODC.

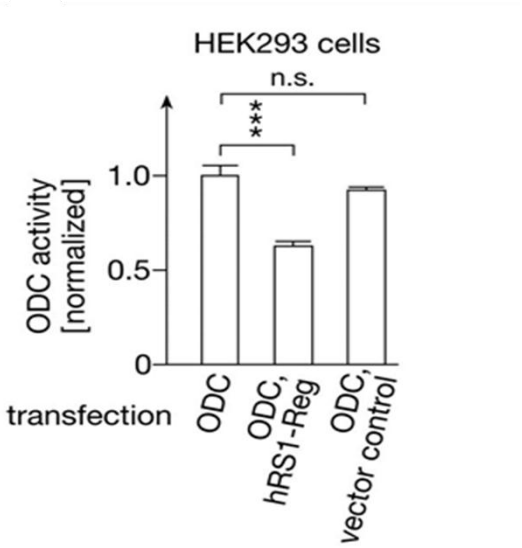

Fig 8: RS1-Reg when co-transfected with ODC in HEK 293 cells decreases the activity of ODC

Subconfluent HEK 293 cells were transfected with vectors expressing either ODC or ODC together with RS1-reg or ODC with the control vector (pcDNA 3.1). After overnight expression the cells were lysed in the lysis buffer by sonication and centrifuged at 100000 g for 1hr at 4^0C. The cleared lysate (supernatant) was used to estimate the activity of ODC as explained in methods section (3.2.6). ODC activity was reduced significantly when RS1-Reg was co-transfected but not control vector. The data shows that RS1-Reg decreases the activity of ODC. Activity measurements were normalized to parallel performed measurements in the control (ODC). Mean values ± SE of 3 independent experiments are indicated. ***, P<0.001, for difference to control, ns-not significant.

4.9 RS1-reg and DFMO decrease the SGLT1 mediated AMG uptake by decreasing the activity of ODC

RS1 has an inhibitory effect on the AMG uptake mediated by the sodium dependent glucose transporter SGLT1. The N-terminal regulatory domain RS1-Reg also decreases the SGLT1 mediated AMG uptake with an IC_{50} value of 130 nM (done in this work). It has been shown that the regulatory domain of RS1 (RS1-Reg) decreases the activity of ODC in HEK 293 cell lysate (Fig.8). We wanted to investigate whether RS1-reg mediated SGLT1 regulation is by decreasing the activity of ODC or both the proteins act in two different pathways. To this end the ODC blocker DFMO (3mM) was injected in the oocytes either with purified RS1-Reg (3.5µM) or alone and the SGLT1 mediated AMG tracer uptake was measured after incubation at room temperature for 1 hr. The ODC blocker DFMO was able to decrease the SGLT1 mediated AMG uptake to the same extent as RS1-Reg and both together did not show any additive effect on the AMG (25 µM) uptake in the oocytes. This shows that both RS1-Reg and ODC act in the same regulatory pathway.

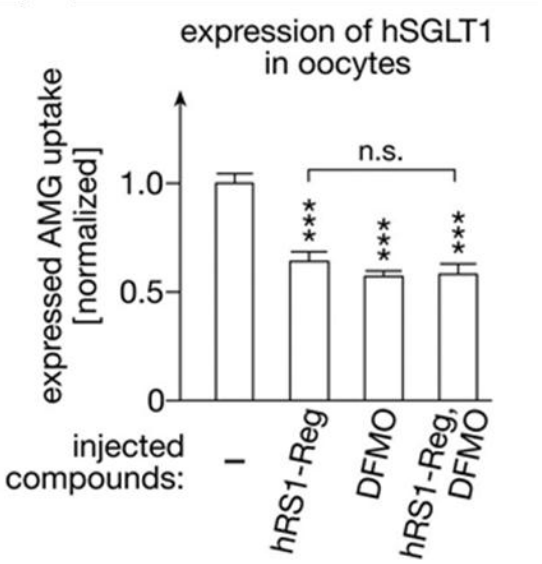

Fig 9: Inhibition of ODC by DFMO results in decreased AMG uptake by SGLT1 and RS1-Reg has no additive effect

Oocytes expressing SGLT1 were injected either with protein RS1-Reg (3.5µM) and DFMO (3mM) or together. After 1hr incubation at RT in ori buffer, SGLT1 mediated AMG uptake was checked for 20 minutes in a final concentration of 25µM radioactive AMG. RS1-Reg inhibits the AMG uptake to the same extent as DFMO and both had no additive effect on AMG uptake by SGLT1. Uptake measurements were normalized to parallel performed measurements in the control (SGLT1). Mean values ± SE of 5 independent experiments are

indicated. ***, P<0.001, for difference to control, determined according to the ANOVA with post hoc Tukey's test.

4.10 Polyamine dependent regulatory mechanism of RS1-Reg

We wanted to check whether the effect of RS1-Reg on SGLT1 is polyamine dependent. To this end, oocytes expressing SGLT1 were injected with either purified protein RS1-Reg or RS1-Reg along with putrescine and the effect on AMG tracer uptake by SGLT1 was measured. Purified RS1-Reg protein when injected alone was able to decrease the AMG tracer uptake by SGLT1, and this effect was blocked in presence of putrescine. This shows that RS1-Reg regulates the activity of SGLT1 by regulating the activity of ODC.

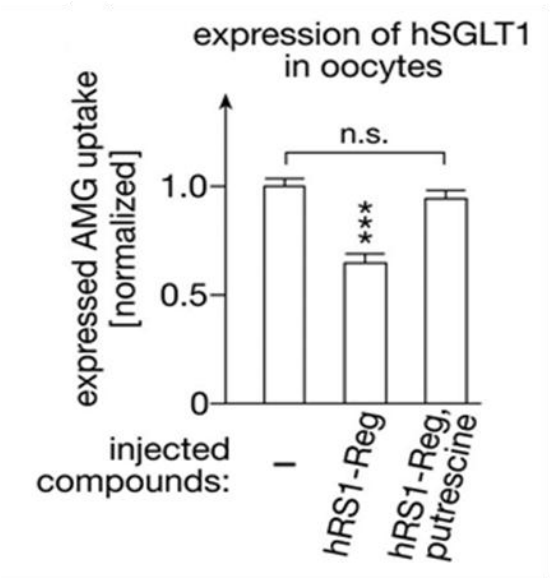

Fig 10: Inhibition of RS1-Reg on SGLT1 is abolished in presence of putrescine

Oocytes expressing SGLT1 were injected with 3.5 µM RS1-Reg protein or RS1-Reg protein along with 1µM putrescine. After 1hr incubation and AMG tracer uptake, oocytes injected with RS1-reg alone had a significant decrease in the AMG uptake compared with oocytes injected with putrescine along with RS1-Reg protein. The inhibitory function of RS1-Reg is lost in presence of the polyamine precursor putrescine showing that RS1-Reg inhibits the SGLT1 mediated AMG uptake by decreasing the activity of ODC. Uptake measurements were normalized to parallel performed measurements in the control (SGLT1). The number of independent experiments was 3 in number. ***, P<0.001, for difference to SGLT1expressing oocytes.

4.11 Putrescine restores the inhibitory effect of tripeptide QEP on SGLT1 activity

Vernaleken et., al showed that the tri peptide QEP (phosphorylation mimicked form of tripeptide QSP derived from RS1-Reg) was able to down regulate the SGLT1 mediated AMG uptake with high affinity. QEP blocks the release of vesicles containing SGLT1 from the *trans*-Golgi network (Vernaleken et al., 2007). To check whether the polyamine dependent effects that were found with RS1-Reg (Fig:10) could be mediated by the tripeptide QEP, oocytes expressing SGLT1 were injected either with the tri peptide QEP alone or QEP along with putrescine. Putrescine when injected together with QEP was able to rescue the inhibition of SGLT1 mediated AMG uptake that was seen with QEP. This supports the hypothesis that the effect of QEP is polyamine dependent.

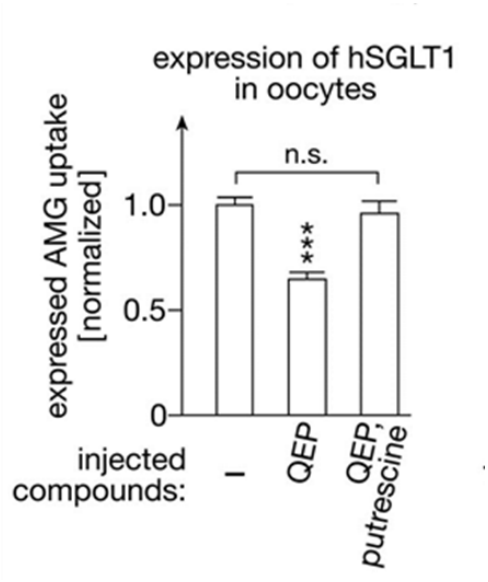

Fig 11: Polyamine dependent regulatory mechanism of the tripeptide QEP

Oocytes expressing SGLT1 were injected either with the tripeptide QEP (1µM) alone or QEP with 1µM putrescine and incubated for 1hr at room temperature. After incubation they were checked for the AMG tracer flux measurement. Oocytes incubated with 1µM tripeptide QEP showed significantly less tracer uptake than with oocytes expressing only SGLT1 (buffer injected). Putrescine was able to block the inhibition seen with QEP suggesting that the inhibition of SGLT1 seen in presence of QEP is by blocking the activity of ODC. Mean values ± SE of 3 independent experiments are indicated. ***, P<0.001, for difference to control (SGLT1), determined according to the ANOVA with post hoc Tukey's test.

4.12 RS1 derived tripeptide QEP decreases the activity of ODC

N-terminus of RS1 protein consists of individual regulatory tripeptides that can block the release of vesicles containing SGLT1 from the *trans*-Golgi network. Tripeptide QSP and its phosphorylation mimicked form at the amino acid serine, QEP along with octapeptide SDSDRIEP were shown to be effective in down regulating the SGLT1 mediated AMG uptake (Vernaleken et al., 2007; Veyhl-Wichmann and H.Koepsell unpublished data). We wanted to check whether the tripeptide QEP binds to ODC and decreases the activity of ODC. For this, pure ODC protein (abnova) with a GST tag at the N-terminus was used. The activity of ODC was measured in presence and absence of 100µM QEP as described in the methods section. QEP decreased the activity of ODC to nearly 50%. The reverse tripeptide PEQ used as a control of the same concentration did not show any effect on the activity of ODC. This shows that tripeptide QEP binds to ODC and decreases its activity.

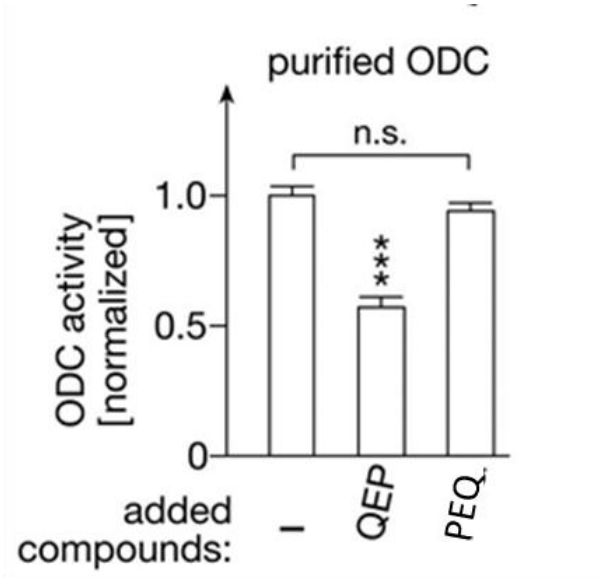

Fig 12: Tripeptide QEP dependent down regulation of the activity of ODC

Pure ODC protein (80 ng) when incubated with 100µM of the tripeptide QEP for 1hr at 37^0C showed significantly less activity when compared to the non treated form. The amount of radiolabelled CO_2 released from the decarboxylation of ^{14}C- ornithine by ODC in presence of QEP (100µM) was approximately 50% less than the control. The reverse tripeptide PEQ did not show any effect on the activity of ODC. ODC activity measurements were normalized to parallel performed measurements in the control (ODC). Mean values ± SE of 3 independent experiments are indicated. ***, P<0.001, for difference to control, determined according to the ANOVA with post hoc Tukey's test.

4.13 RS1 mediates ODC dependent specific regulatory mechanism of SGLT1

RS1-Reg regulates the transport activities of several plasma membrane transporters belonging to different families. It regulates the activity of various transporters such as Na^+-D-glucose co transporter SGLT1, Na^+ myo-inositol co transporter SMIT, organic cat ion transporters OCT1 and OCT2 and the organic anion transporter OAT1 and the Na+-co-transporter for serotonin SERT (Lambotte et al., 1996; Reinhardt et al., 1999; Vehyl et al., 2003). RS1 participates in the short term posttranscriptional regulation of SGLT1 by blocking the release of vesicles containing SGLT1 at the *trans*-Golgi network. The detailed mechanisms of regulation of other transporters have not been clearly understood. Murrugaren et al., showed that RS1 also blocks the exocytotis pathway of release of vesicles containing the nucleoside transporters CNT1, CNT2 and CNT3 belonging to the family of SLC28 (Murrugaren et al., 2012). We have shown that RS1-Reg regulates the activity of SGLT1 by decreasing the activity of ODC (Fig. 9). The effects of polyamines vary depending on the transport system examined. Polyamines appear to be involved in the upregulation of glucose transport. Conversely polyamines appear to down regulate proline transport (Johnson et al., 1995). We wanted to check whether polyamines are also involved in RS1 mediated regulatory mechanism of CNT transporters. Henceforth we injected the ODC specific blocker DFMO into the Xenopus laevis oocytes expressing hCNT1, incubated for 1hr at room temperature and checked the hCNT1 specific substrate H^3-uridine uptake. Surprisingly DFMO did not inhibit the hCNT1 mediated uridine uptake in contrast with the inhibition of SGLT1 mediated AMG uptake. RS1-Reg served as positive control blocking the hCNT1 mediated uridine uptake. The data suggest that RS1-Reg regulates different transporters via different regulatory mechanisms. It could be hypothesized that ODC serves as a receptor specific for RS1 mediated SGLT1 regulation.

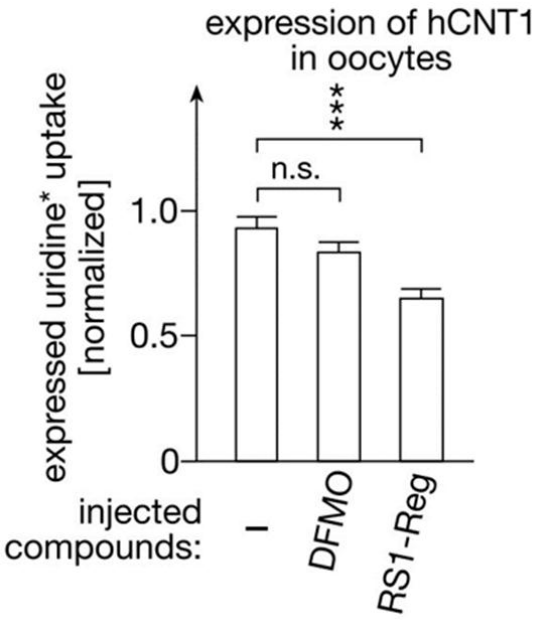

Fig 13: ODC inhibitor DFMO does not regulate the activity of hCNT1

Oocytes expressing the human concentrated nucleoside transporter 1 (hCNT1) were injected with either 3mM concentration of DFMO or 3.5 µM RS1-Reg protein. After 1hr incubation in ori buffer at room temperature the CNT1 specific radiolabelled substrate uridine (5 µM) uptake was measured. The ODC inhibitor DFMO had no effect on the uptake mediated by CNT whereas RS1-Reg was able to inhibit the same significantly. This shows that ODC differentially regulates various transporters. Uptake measurements were normalized to parallel performed measurements in the control (hCNT1). Mean values ± SE of 3 independent experiments are indicated. ***, P<0.001, for difference to control, determined according to the ANOVA with post hoc Tukey's test.

4.14 ODC protein levels are higher in the intestinal mucosa of RS1 knockout mice

RS1 regulates SGLT1 transcriptionally and post-transcriptionally. To investigate the role of RS1 in vivo the RS1-KO mice were generated (Osswald *et al.*, 2005). The regulation of sodium dependent glucose transporter SGLT1 by RS1 has been studied widely because the RS1 knockout mice developed obese phenotype with high levels of SGLT1 expressed in the intestine with high glucose uptake from the intestine. As RS1-reg decreases the activity of ODC (Fig. 5), we wanted to check the level of ODC protein expressed in the intestine of RS1 knockout mice compared to wild type. For this the intestinal mucosal cell scrapping has been used. The everted intestines of RS1 wildtype and knockout mice were scrapped gently and the mucosa has been collected into the homogenizing buffer (10 mM HEPES pH-7.2, 142 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 0.5% NP-40, 0.1 µM PMSF with (10 µl/ml) protease

inhibitor cocktail mix). The collected mucosal lining was homogenized using a glass homogenizer on ice. The homogenate was centrifuged at 200000 g for 1 hr at 4^0C. The supernatant was collected and the protein concentration was analyzed by bradford analysis. 30µg of the lysate was loaded on to the gel for electrophoresis. The proteins from the electrophoresed gel were transferred on to a nitro cellulose membrane by semi dry blotting (section 3.2.3.4) and the amount of ODC on the membrane was identified using the antibody against ODC. The densitometric intensities of the bands on the blot were quantified using the program Image J. ODC protein expressed in the intestinal mucosa of RS1 knockout mice was significantly higher than that expressed in wild type mice. This confirms the finding that RS1 negatively affects the ODC protein level.

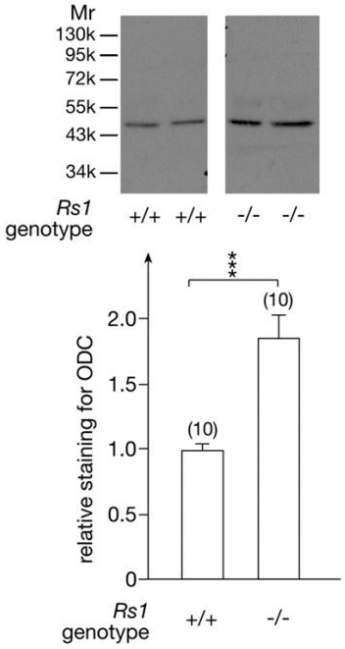

Fig 14: Western blot showing ODC protein levels in the mucosal scrapping of RS1$^{+/+}$ and RS1$^{-/-}$ mice intestine.

RS1$^{+/+}$ and RS1$^{-/-}$ mice were fasted for 18 hrs prior to sacrifice. The intestine was washed, everted and scrapped gently to collect the mucosa. The mucosa was homogenized in the homogenizing buffer and centrifuged at 200000g. The supernatant was collected. 30 µg of the protein was loaded on to the gel for electrophoresis. The proteins separated on the gel were analyzed by western blotting using antibody against ODC. The intensity of the bands was measured densitometrically using the program Image J.

5. Discussion

In an attempt to understand the mechanism involved in the regulation of several transporter proteins by the protein RS1 and to find the interacting partners involved in the pathway of regulation by the protein, a yeast two hybrid screening has been performed using split ubiquitin system. To localise the interaction on the protein RS1 which is a 67 KD protein, the N-terminal 312 amino acid fragment has been used for the screening of human embryonic kidney cDNA library. From the many number of proteins that were seen as positive in the screening procedure ODC was selected for further confirming and validating the interaction because the enzyme ODC is the rate limiting enzyme of the polyamine biosynthesis and polyamines are the polyorganic cation molecules that are necessary for the vital functioning of the cells. They participate in cell growth, proliferation, division, protein synthesis ((Jänne et al., 1978, Pegg and McCann 1982, Tabor and Tabor 1984, Williams-Ashman and Canellakis 1979) and are shown to be involved in the regulation of several ion channels. Polyamines are shown to activate, inhibit or block different receptor channels belonging to glutamate activated receptor channels (Ransom & Stec 1988, Williams *et al*. 1989, Kamboj *et al*. 1995). Other cation channels blocked by polyamines are voltage-gated Ca^{2+} (Scott *et al*. 1993) and Na^{+} (Huang & Moczydlowski 2001) channels, inwardly rectifying K^{+} channels (Fakler *et al*. 1994, Ficker *et al*. 1994, Lopatin *et al*. 1994), cyclic nucleotide-gated channels (Lu & Ding 1999). They are involved in the regulatory mechanism of several transport proteins transcriptionally and posttranscriptionally. Post natal rats when fed spermidine daily for 3 days from day 7 to 9 showed a precocious increase in the abundance to mRNA for sucrose isomaltase (SI), SGLT1, and Glut-2 and NA^{+}-K^{+}- ATPase and α1 and β1 isoform gene expression of Na^{+}-K^{+}-ATPase on day 10 compared to controls. ODC activity, mRNA and protein were also increased on day 10. The increased expression of these genes was not found after a washout period of 3 days suggesting that these effects were transient and 3 days of oral polyamine administration induces the maturation of glucose transporters in the postnatal small intestine which may be mediated by alterations in ODC expression (Wild et al., 2007). Henceforth ODC was selected as a prime candidate for further study to clarify whether ODC and polyamines play a role in RS1 mediated regulation of SGLT1 and the sodium-nucleoside transporter CNT1. The interaction of RS1 (1-312) with ODC has been verified by co-precipitation experiments and the N terminal regulatory domain of RS1 (RS1-

Reg) has been shown to be responsible for the binding with ODC. Shu-ichi Matsuzawa et al. showed that expressing whole proteins or protein domains fused to N-terminus of ODC promotes proteasomal dependent degradation of the chimeric protein and their interacting cellular target proteins (Shu-ichi Matsuzawa et al., 2005). The generation of polyamines by ODC results in the synthesis of a protein antizyme (AZ) a 228 amino acid protein that inhibits the activity of ODC by a feedback mechanism (Fong et al., 1976, Heller et al., 1976). Antizyme binds to ODC monomers and blocks its activity by preventing the formation of active homodimers and targets the protein for degradation by 26S proteasome in an ubiquitin independent pathway (Murakami et al., 1992a). Mutational studies confirmed the antizyme binding site on ODC between amino acids 117-140 (Li et al., 1992, Almrud et al., 2000). Liu et al., showed that amino acids asparagine at position 125 and methionine at position 140 were crucial for the binding of antizyme and when amino acids at these positions were mutated it resulted in stable monomeric form of ODC (Liu et al., 2011). Ju-yi Heish et al. demonstrated that fragments of antizyme were able to restrict the activity of ODC to the same level of whole antizyme protein. A truncated version of antizyme [AZ (95-176)] was able to reduce the activity of ODC as strong as the wild type (Hsieh J-Y et al., 2011). To check whether the interaction of RS1-Reg with ODC has an effect on the activity of the enzyme, an activity assay has been performed. For this the endogenous ODC activity is checked in non-transfected HEK 293 cell lysates. As this is too low, transiently transfected cell lysates either with ODC or ODC and RS1-Reg were used. RS1-Reg decreased the activity of the ODC, but not the control vector .This effect could also be non-specific with the involvement of various other factors in the cell, which need to be verified.

The polyamines spermidine and spermine and their precursor putrescine are polyorganic cations found in virtually all cells of higher eukaryotes. Although the exact mode of action of these compounds at the molecular level is still not clear polyamines are intimately involved in and required for cell growth and their intracellular concentrations are closely regulated. In the mucosa of the small intestine, as in other tissues an increase in the activity of ODC is one of the earliest events after a proliferative stimulus. ODC in small intestinal mucosa increases dramatically in response to refeeding. After 2 hours of refeeding the fasted rats, ODC activity from mid villus and villus tip regions had increased 10 and 20 fold respectively (Johnson L.R etal., 1989) Thus the majority of the intestinal ODC resides in the cells that are mature and do not divide. Though crypt cell ODC is increased by tropic hormones such as gastrin and epidermal growth factor, villus cell ODC is increased in response to luminal nutrients

indicating that ODC of the differentiated villus cells is regulated differently from that of proliferative cells (Johnson L.R etal., 1989).

With the possible question of what is the function of ODC in non-proliferative mature enterocytes Johnson et al, clarified the role of polyamines in the primary functioning of enterocytes i.e. absorption of nutrients, fluid and electrolytes. Glucose transport was measured in brush border membrane vesicles (BBMV) from control rabbits, rabbits treated with DFMO and control and DFMO treated rabbits given polyamines exogenously. BBMVs from rabbits treated with 5% DFMO in the drinking water 24 hours before they were killed transported significantly less glucose than vesicles from the control animals. Orogastric administration of spermidine, spermine or putrescine to DFMO treated animals 24 hours before they were killed prevented the decrease in the overall glucose uptake in the intestine (Johnson L.R. 1995).

As polyamines were shown to be involved in the regulation of glucose transporters in the intestine (Johnson L.R. 1995) and RS1 protein was shown to regulate the activity of the plasma membrane transporters belonging to various families such as Na^+-D-glucose co-transporter SGLT1, Na^+ myo-inositol co transporter SMIT, organic cat ion transporters OCT1 and OCT2 and the organic anion transporter OAT1 and the Na+-co-transporter for serotonin SERT (Lambotte et al., 1996; Reinhardt et al., 1999; Veyhl et al., 2003), we wanted to know whether RS1 regulates the activity of SGLT1 by decreasing the activity of ODC. For these experiments a system with no endogenous RS1 would be an ideal condition. As RS1 is a widely expressed protein in mammals, Xenopus laevis oocytes were selected to be the best system to perform these experiments as they do not have endogenous RS1 protein. Xenopus laevis oocytes were shown to express ODC which is essential in the process to development of the oocytes (Osborne et al,. 1989). For the investigation of the mechanism of regulation of SGLT1 by ODC oocytes were injected with the cRNA of either SGLT1 alone or SGLT1 along with cRNA of ODC. Oocytes expressing both SGLT1 and ODC showed an increase in the AMG uptake compared to SGLT1 alone showing that ODC when over expressed enhances the expression of SGLT1. Oocytes expressing SGLT1 when injected with protein RS1-Reg showed a significant decrease in the SGLT1 mediated AMG uptake. As ODC is endogenously expressed in oocytes (Osborne et al,. 1989), a hypothesis has been raised that RS1-Reg protein may decrease the SGLT1 mediated AMG uptake by decreasing the activity of ODC. To confirm this hypothesis an irreversible blocker of the ODC activity difluoromethylornithine (DFMO) was used. As ODC is involved in the maturation of

oocytes (Osborne et al,. 1989), to block the endogenous ODC available in the oocytes, 3mM DFMO was injected into the oocytes expressing SGLT1 either alone or along with the protein RS1-Reg. DFMO decreased the activity of SGLT1 to the same level as RS1-Reg protein and both RS1-Reg and DFMO together did not show any additive effect. As it had been shown that RS1-Reg decreases the activity of ODC in HEK cell lysates (Fig: 8) this data supports the hypothesis that RS1-Reg decreases the SGLT1 mediated AMG uptake by decreasing the activity of ODC. The polyamine putrescine inhibited the regulatory function of RS1-Reg and also DFMO. This shows that polyamines are involved in a short term posttranscriptional regulatory mechanism of the transporter SGLT1, and that RS1-Reg decreases the SGLT1 mediated AMG uptake by inhibiting the polyamine synthesis by blocking the activity of the rate limiting enzyme of the polyamine biosynthesis ODC.

RS1 blocks the exocytotic release of vesicles containing SGLT1 from the TGN. Brefeldin A (BFA) which inhibits the guanosine nucleotide exchange factors that activate ADP-ribosylation factors, which regulate the assembly of coat complexes at the TGN and endosomes involved in protein sorting and release of vesicles was able to inhibit the SGLT1 mediated AMG uptake when injected in oocytes to the same extent as RS1-Reg protein. Both upon co injection did not show any change in the extent of inhibition suggesting that RS1 may be involved in the blocking of exocytotic release of vesicles at the TGN (Vehyl et al., 2006). In an attempt to check whether by inhibiting the activity of ODC also leads to the inhibition of the exocytotic release of vesicles containing SGLT1, the ODC blocker DFMO was injected into the oocytes either alone or together with brefelding A. Both BFA and DFMO were able to block the AMG uptake by SGLT1 to the same extent and co injection did not enhance the inhibitory effect hinting that both act via the same mechanism. This supports the hypothesis that by blocking the activity of ODC, exocytotic release of vesicles containing SGLT1 can be inhibited which is the speculated mechanism of action of RS1-Reg protein. Kanerva et al., showed that antizyme inhibitor 2 an activator of ODC which is mainly localized to the vesicles at TGN regulates the transport of secretory vesicles by locally activating ODC and polyamine biosynthesis (Kanerva et al., 2010). They reported that in MCF-7 cells (breast cancer cell line) polyamine depletion by DFMO for 4 days induced fragmentation of the TGN, the same effect seen as with the siRNA of antizyme inhibitor 2 resulted in the blockage of release of the secretory vesicles of the exocytotic pathway at TGN. They report that after treatment with DFMO the distribution of β-cop, a marker for intra and post golgi transport vesicles also dispersed in to the cytoplasm and formed

aggregates, in contrast to no change observed in the distribution of GM130 a marker of cis - Golgi indicating the intact morphology of the cis-Golgi. The morphological changes of TGN observed by DFMO were reversed after addition of putrescine for 3 hours. Taking into consideration the short treatment times described in this work, the effect of blockage of exocytosis by DFMO could be due to the regulation in ODC activity supporting the theory of kanerva et al. that activation of ODC locally at the vesicles generating polyamines enhances the trafficking at the TGN. With the short treatment times used in this work and from the result that DFMO treatment did not influence the activity of the human uridine transporter CNT1 it's unlikely that the golgi apparatus is disturbed by DFMO, inhibiting the release of vesicles and disrupting the trafficking mechanism as described by kanerva et al, where the treatment time is as long as 4 days (Kanerva et al., 2010).

Glucose plays an important role in the regulatory mechanism of the RS1 protein in the selection of the transporter to be regulated. Vehyl et al., showed that intracellular concentration of AMG had an inhibitory effect on function of RS1 protein in regulating the SGLT1 mediated AMG uptake, Vernaleken et al., showed that intracellular sugar concentrations had a detrimental effect on the regulation of RS1 derived tripeptides on SGLT1, however the absence of AMG had the same effect on the regulation of RS1 on hOCT2 mediated TEA uptake. This selectivity in the regulation of transporters by intracellular AMG concentration by RS1 hints glucose dependent sensing mechanism which could be mediated by a glucose binding protein that aids as a receptor at the TGN. Hence the involvement of a sugar binding protein in the regulatory mechanism of RS1 has been hypothesized (Vernaleken et al., 2007). We checked the effect of sugar on the inhibition of SGLT1 that is exerted by DFMO. Intracellular 250 µM AMG was able to abolish the inhibition of DFMO on SGLT1 showing that the inhibition of SGLT1 mediated AMG uptake by DFMO was sugar dependent. When tested for the same effect with pure ODC protein the effect of AMG on the inhibition of ODC activity was biphasic. 1 mM concentration of AMG abolished the inhibitory effect of DFMO whereas 10 mM of AMG had no effect on the inhibitory effect of DFMO. Sodium dependent co transported analogues of glucose were shown to activate the specific activity of ODC. Glucose in the growth medium was also shown to increase the activity of ODC in vero cells ((Lundgren et al., 1990, Benis et al., 1993 Lundgren et al., 1988). This biphasic effect suggests that ODC might be the sugar binding protein involved in the regulatory mechanism of RS1 and it might have multiple binding sites for the sugar which results in the conformational change in the active site on ODC. It has

been shown by mutational studies that conformational changes in the active site of ODC might lead to displacement of the inhibitory adduct formed at the active site by decarboxylation of DFMO (Metcalf et al., 1978, Pegg et al., 1987, Coleman et al., 1993). In the process of elucidating the selectivity of the regulatory mechanism of RS1 protein on different transporters, it was shown that the ODC inhibitor DFMO which blocked the SGLT1 mediated AMG uptake had no effect on the hCNT1 mediated uridine uptake which is also inhibited by RS1-Reg. This data suggest that ODC acts as a receptor specific for RS1 mediated SGLT1 regulation.

Posttranscriptional modifications play a major role in the activity of any protein and phosphorylation is one of such major posttranscriptional modification that regulates the protein function. The only posttranscriptional modification seen with ODC is the phosphorylation (Rosenberg-Hasson et al., 1991, Worth et al., 1994). Protein kinase C (PKC) phosphorylation regulates the function of many proteins. It also regulates the nuclear transportation of the protein RS1 (Filatova et al., 2009. The N-terminal regulatory fragment of RS1 protein also has several consensus sequences for phosphorylation by different kinases. QEP is the phosphorylation mimicked form of the RS1 derived tripeptide QSP at the position serine. Vernaleken et al., showed that the RS1 derived tripeptide QEP was able to decrease the SGLT1 mediated AMG uptake significantly (Vernaleken et al., 2007). QEP is the tripeptide that was shown to be the active form which was able to down regulate SGLT1 mediated AMG uptake in a sugar independent mechanism (Veyhl-Wichmann, H. Koepsell unpublished data).

In the course of understanding the posttranscriptional regulatory mechanism of RS1-Reg fragment, we asked ourselves if this down regulation of SGLT1 mediated AMG uptake by QEP is due to the decrease of the activity of ODC and thus ultimately by the decrease of polyamines. To answer this question, oocytes expressing SGLT1 transporter are injected with either QEP alone or QEP along with the polyamine precursor putrescine. After one hour incubation and tracer flux measurement of AMG, oocytes injected with QEP alone showed less uptake as described earlier and in oocytes injected with QEP along with putrescine, this inhibition is lost. This supports the hypothesis that the regulatory mechanism involving either RS1-Reg or the RS1 derived phosphorylation mimicked tri peptide QEP is by regulating the activity of the enzyme ODC and is polyamine dependent. In order to verify this hypothesis and to check whether the tripeptide QEP down regulates the activity of the enzyme ODC and demonstrate that QEP directly blocks the activity of ODC, ODC activity using pure ODC

protein was measured in presence and absence of 100µM QEP. As expected the tripeptide QEP was able to inhibit the activity of the pure ODC enzyme to nearly 50%. The control reverse tripeptide PEQ did not show any effect on the activity of the enzyme.

RS1 exhibits a confluence dependent localization pattern in the cell which is attributed to its transcriptional and post-transcriptional regulatory functions. Sub-confluent cells exhibits more of the protein in the cytoplasm compared to the confluent cells which have less (Kroiss et al., 2006). The sub-confluent state which could be compared to the dividing cells of the crypt also hosts ODC in smaller amounts compared to the matured and differentiated villus cells (Johnson L.R etal., 1989) resembling the state of confluent cells which has the contrasting feature with high levels of ODC and less RS1 protein. In correlation to the level of ODC and RS1 protein, the amount of SGLT1 protein in the membrane and activity is also low in proliferative differentiating cells where RS1 is high and ODC is low than in differentiated and mature cells where SGLT1 protein in the plasma membrane is high and activity is also high where RS1 is low and ODC is high. Basing on the results obtained in this study that RS1 decreases the activity of ODC and these interesting regulatory mechanisms of the SGLT1 protein depending on the state of confluence tempts to the hypothesis that RS1 when high may decrease the activity of ODC thereby decreasing the amount of SGLT1 by influencing the trafficking of vesicles containing SGLT1 at TGN and vice versa. The mucosal scrapping of the intestines of the $RS1^{-/-}$ compared to $RS1^{+/+}$ mice show higher amounts of ODC protein (Fig: 14) and SGLT1 (Osswald et al., 2005) supporting the hypothesis that RS1 might regulate SGLT1 via ODC.

ODC in the intestinal epithelium behaves differently from the other tissues with higher activity in matured and differentiated epithelial cells in the villus than rapidly dividing cells in the crypt. The increase in the activity could be because of the not much known signaling mechanism generated from the stimulus due to presence of nutrients in the lumen or it could be from the entry of glucose via SGLT1 along with sodium which in turn could trigger ODC and activated ODC may further enhance the glucose uptake by the release of vesicles containing SGLT1 as speculated from the results of this work. The effects of polyamines vary depending on the transport system examined. Polyamines appear to be involved in the upregulation of glucose transport, conversely polyamines appear to down regulate proline transport. Johnson et al. showed that in contrast to glucose, in vivo spermine decreased the J_{max} for proline uptake, whereas DFMO treatment significantly increased both J_{max} and K_m (Johnson et al., 1995). Previous studies indicate that the Na^+ -dependent glucose co

transporter is regulated independently from and opposite to amino acid transporters. EGF has been shown to increase J_{max} of glutamine uptake into fibroblasts by 60% while causing a significant decrease in glucose uptake in the same system (Dudrick et al., 1993). Similarly a two fold increase in J_{max} has been reported for glutamine and alanine uptake in rat jejunal BBMV after systemic treatment with EGF, whereas glucose transport was decreased by 50% (salloum et al., 1993). BBMV´s from rabbit intestine with lowered polyamine levels transported less glucose. The decrease was due to a decrease in J_{max} that could be best attributed to fewer carriers in the membrane. Treatment of rabbits with oral polyamines prevented the effect of DFMO. Polyamine depletion altered proline transport in a manner opposite to its effects on glucose indicating specificity (Johnson et al., 1995). Thus it has been shown that polyamines have the property of regulating the glucose transport independent to that of other transporters which is in best correlation with the results obtained in this study. Treatment of oocytes with DFMO decreased the SGLT1 mediated AMG uptake but not the CNT1 dependent uridine uptake. The novel finding that ODC acts as a specific receptor for RS1-Reg and RS1 derived tripeptide QEP in regulating SGLT1 could be of great pharmacological importance since sodium-dependent glucose transport (SGLT) proteins represent an excellent target for the development of innovative therapies to better manage diabetes by controlling hyperglycemia, as it is of utmost importance in the treatment of diabetes and its complications. In recent years, novel approaches for the treatment of diabetes suggest that affecting glucose absorption in the intestine and/or glucose reabsorption in the kidney might be a possible strategy to control blood glucose levels. As a result, inhibitors of glucose absorption have been developed to inhibit digestion of sucrose and lactose by disaccharidases in the intestinal lumen. Few examples of inhibitors that are available in the market include acarbose (Precose R® or Glucobay R®), voglibose (Basen R®), and miglitol (Glyset R®) (Asano N., 2003). These agents block alpha-glucosidase in the brush border of the small intestine. Inhibition of these enzymes reduces the rate of digestion of complex carbohydrates and, consequently, glucose absorption in the small intestine. However, these beneficial effects of glycemic control are accompanied by gastrointestinal side effects such as abdominal discomfort, flatulence, and diarrhea. For these reasons, the clinical application of these agents is limited. As the understanding of glucose transport at the molecular level has progressed, inhibitors of the transport molecule itself have been synthesized, some of which are currently undergoing preclinical and clinical trials. One such attempt is the study conducted in this work. An attempt has been made to understand the detailed regulatory

mechanism of the protein RS1 involved in the down regulation of SGLT1. ODC has been identified as a receptor specific for SGLT1 that is involved in this regulatory mechanism of RS1. The tripeptide QEP (phosphorylation mimicked form) derived from RS1 which was shown to down-regulate SGLT1 in a monosaccharide independent manner (Veyhl-wichmann, H. Koepsell unpublished data) was shown to act via down regulating of the activity of ODC. This opens a new horizon for the possibility of developing this tripeptide QEP as a new drug for targeting the SGLT1 specifically which could be a used to reduce the glucose uptake in the small intestine and also reduce the reabsorption of glucose in the proximal tubule of kidney, thus aiding for the control of hyperglycemia and to manage diabetes. With the involvement of ODC as a target specific for the regulation of SGLT1 by RS1 or RS1 derived tripeptides, this innovative treatment by RS1 derived tripeptides would pose a minimal threat for side effects, which need to be validated with extensive research. This work identifies ODC as a receptor for protein RS1 in the regulatory mechanism involving specifically SGLT1 by the protein RS1 which regulates many transporters at the TGN.

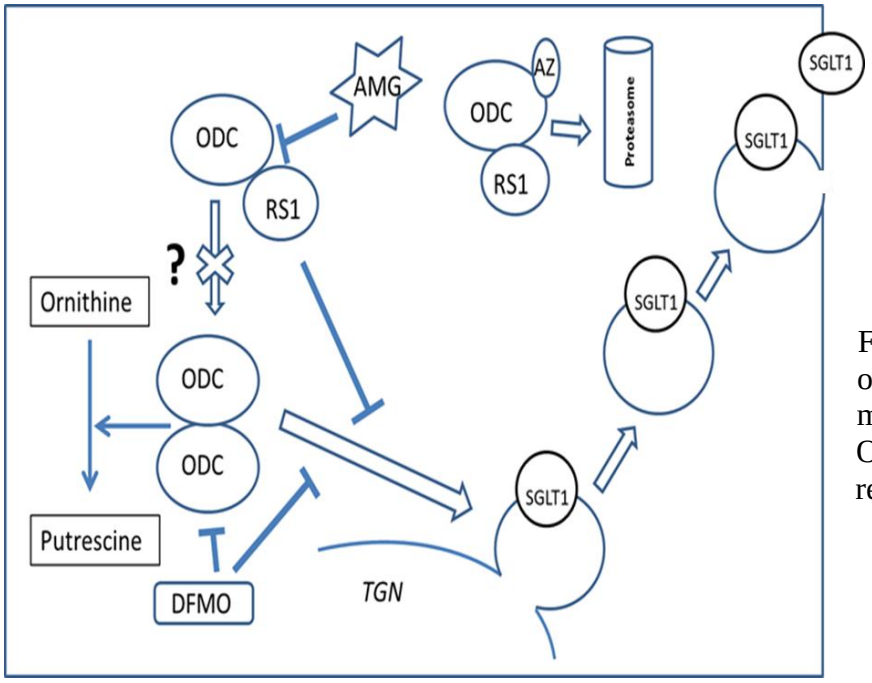

Fig 15: Schematic view of the proposed mechanism of receptor ODC in RS1 dependent regulation of SGLT1

6. SUMMARY

The mammalian specific protein RS1 is a 67 KDa protein that is encoded by an intron less single copy gene. It is involved in the regulation of different plasma membrane transporters. Na+-D-glucose transporter SGLT1 is the best studied target that is regulated transcriptionally and post-transcriptionally. RS1 protein localized at the *trans*-Golgi Network (TGN) blocks the release of vesicles containing different transporters. It is identified with an N terminal 83 amino acid post-transcriptional regulatory fragment termed as RS1-Reg. Presence of 20 serine residues in consensus sequence for phosphorylation by different protein kinases, two times the tripeptide QSP and an octapeptide SDSDRIEP which regulate individually different transporters determines the complexity of regulation by this RS1-Reg fragment. In the present study the protein ornithine decarboxylase (ODC) was identified as a receptor for RS1 in the specific regulation of the release of SGLT1 containing vesicles at the TGN. ODC was identified as an interacting partner for N-terminal 312 amino acid fragment of RS1 using yeast two hybrid split ubiquitin system. The regulatory fragment (RS1-Reg) was demonstrated to be mediating this interaction. ODC was shown to enhance the SGLT1 activity when co-expressed in Xenopus oocytes. ODC was shown to be involved in the regulation of the release of vesicles containing SGLT1 from the TGN, as the ODC blocker difluoromethylornithine (DFMO) had no additive effect over the exocytosis inhibitor brefeldin A. Using pure ODC protein it was shown that the downregulation of ODC activity by DFMO was monosaccharide dependent. RS1-Reg was involved in the post-transcriptional down regulation of SGLT1. This regulation of SGLT1 was observed due to the inhibition of the activity of ODC by RS1-Reg, as the polyamine putrescine was successful in blocking the RS1-Reg effect. RS1 derived tripeptide QEP (phosphorylation mimicked form of tripeptide QSP) which was known to regulate the exocytosis of SGLT1 in a monosaccharide dependent manner was found to inhibit the activity of ODC in oocytes as polyamine putrescine was successful in blocking the inhibitory effect seen in presence of QEP. The interpretation that QEP blocks the activity of ODC was proved in an enzyme activity assay using pure ODC protein. By blocking the endogenous ODC in Xenopus laevis oocytes by an irreversible inhibitor DFMO, it was shown that the regulation by ODC was specific for SGLT1 and not human nucleotide transporter CNT1. The present work identifies ODC as a receptor for RS1 in the regulation of release of vesicles containing specifically SGLT1 from the trans-Golgi network. The finding that RS1-Reg and RS1 derived tripeptide QEP decreases the activity of

SGLT1 by specifically targeting ODC can further be used in the development of new pharmacological therapies with a better understanding about the regulatory mechanism involving RS1 aiming at a specific target SGLT1 to control glucose absorption/and or reabsorption and better manage diabetes.

7. Zusammenfassung

Das Protein RS1 welches spezifisch in Säugetieren vorkommt hat ein Molekulargewicht von 67 kDa und wird von einem intronfreien „ single copy gene“ kodiert. Es ist an der Regulation verschiedener in der Plasmamembran enthaltenen Transportern beteilig. Der am besten untersuchte Zieltransporter für RS1 ist der Na^+-Glukose-Kotransporter SGLT1, welcher sowohl auf transkripioneller, wie auch auf posttranskripioneller Ebene reguliert wird. RS1 ist am Transgolgi-Netzwerk (TGN) lokalisiert und blockiert hier die Abschnürung von Transporter-enthaltenen Vesikeln. Es enthält N-Terminal ein 83 Aminosäure langes Fragment (RS1-Reg) welches für die glukoseabhängige posttranskripionelle Regulation von SGLT1 verantwortlich ist. Die Komplexität dieser Regulation wird dadurch ersichtlich, dass RS1-Reg 20 Serine in Konsensussequenzen für verschiedenste Kinasen sowie zweimal das Tripeptid QSP und einmal das Oktapeptid SDSDRIEP enthält. Diese Peptide können SGLT1 wie RS1-reg glukoseabhängig regulieren. In der vorliegenden Studie wurde das Protein Ornithin-Decarboxylase (ODC) als Rezeptor für die von RS1 vermittelte spezifische Regulation von SGLT1 identifiziert. Die Identifizierung erfolgte zunächst durch „yeast two hybrid screening“ und dann durch Immunkopräzipitation. Zunächst wurde die Interaktion von ODC mit den ersten 312 Aminosäuren von RS1 gefunden. In weiterführenden Experimenten zeigte sich, dass bereits RS1-Reg für die Interaktion ausreicht. Nach Koexpression von ODC mit SGLT1 in Xenopus laevis-Oocyten konnte eine erhörte Aktivität des SGLT1 gezeigt werden. Durch Hemmung der endogenen ODC Aktivität in Oozyten durch den spezifischen ODC-Blocker Difluoromethylornithin (DFMO) wurde die durch SGLT1 vermittelte Aufnahme von Glukose gehemmt. Da DFMO keinen additiven Effekt gegenüber dem Exocytoseblocker Brefeldin A zeigte, scheint diese Aktivierung auf einer Regulation der Abschnürung Transporter-enthaltender Vesikel zu beruhen. Es wurde außerdem gezeigt, dass Putrescin, ein Produkt von ODC, in der Lage ist die Regulation von SGLT1 durch RS1-Reg zu blockieren. Dies legt den Schluss nahe, dass die Regulation von SGLT1 durch RS1-Reg auf einer Inhibition von ODC durch RS1-Reg beruht. In gleicher Weise konnte der inhibitorische Effekt des Tripeptids QEP durch Putrescin blockiert werden. QEP ähnelt der phosphorylierten Form QSP in RS1-reg. Die Tatsache, dass QEP die enzymatische Aktivität von ODC hemmte war ein Nachweis für die Interaktion eines aktivierten Peptidmotifs aus RS1-Reg. Die erzielten Ergebnisse sind

für die Entwicklung neuer pharmakologischer Therapien, bei denen die Regulation der Glukoseabsorption durch RS1 beeinflußt wird, von Bedeutung.

Appendix-I: Degradation studies of RS1 (1-312) and RS1-Reg

RS1 (1-312) and ODC interaction leads to antizyme dependent degradation of RS1 (1-312)

The- enzyme ornithine decarboxylase ODC (E.C 4.1.1.17) is a 51 KDa protein. It is one of the known short lived mammalian proteins with a half-life of 20-30 mins. Transcriptionally and post transcriptionally it is a tightly regulated protein. The activity of this rate limiting enzyme of the polyamine biosynthesis is controlled by the synthesized product putrescine. Putrescine stimulates the production of an inhibitory protein called antizyme. Antizyme binds to ODC and leads to its degradation in a unique ubiquitin independent proteasomal dependent degradative pathway (Murakami et al., 1992). Antizyme the inhibitory protein that is expressed in response to high polyamine levels is synthesized as a highly expressed 24.5 KDa protein and also as a less expressed 29 KDa protein. Antizyme binds to ODC with high affinity forming a hetero dimer thus preventing the formation of an active homodimer of ODC thus decreasing the activity of ODC and ultimately leading to its degradation via a unique ubiquitin independent proteasomal pathway. A degradative assay has been performed to check whether the interaction of RS1 (1-312) with ODC leads to the antizyme dependent degradation of RS1 (1-312). For this sub confluent HEK 293 cells have been transiently transfected with vectors expressing either GFP-S tag-RS1 (1-312) alone or GFP-S tag-RS1 (1-312) with ODC or GFP-S tag-RS1 (1-312) together with ODC and AZ. After 24 hours of transfection the cell lysates were checked for the amount of GFP-S tag-RS1 (1-312) in a western blot using anti GFP antibody. The degradation of RS1 (1-312) increased drastically when antizyme was co transfected along with ODC confirming that this degradation was antizyme dependent. To check whether this degradation is proteasome dependent, a proteasomal inhibitor MG-132 has been used. MG-132 restored significantly the amount of RS1 (1-312) protein (Fig: 14A). Control vector expressing GFP-S tag alone did not show any effect when co transfected with ODC and AZ (Fig: 14C). This shows that the interaction of RS1 (1-312) protein with ODC leads to its degradation in an antizyme dependent pathway via the proteasome.

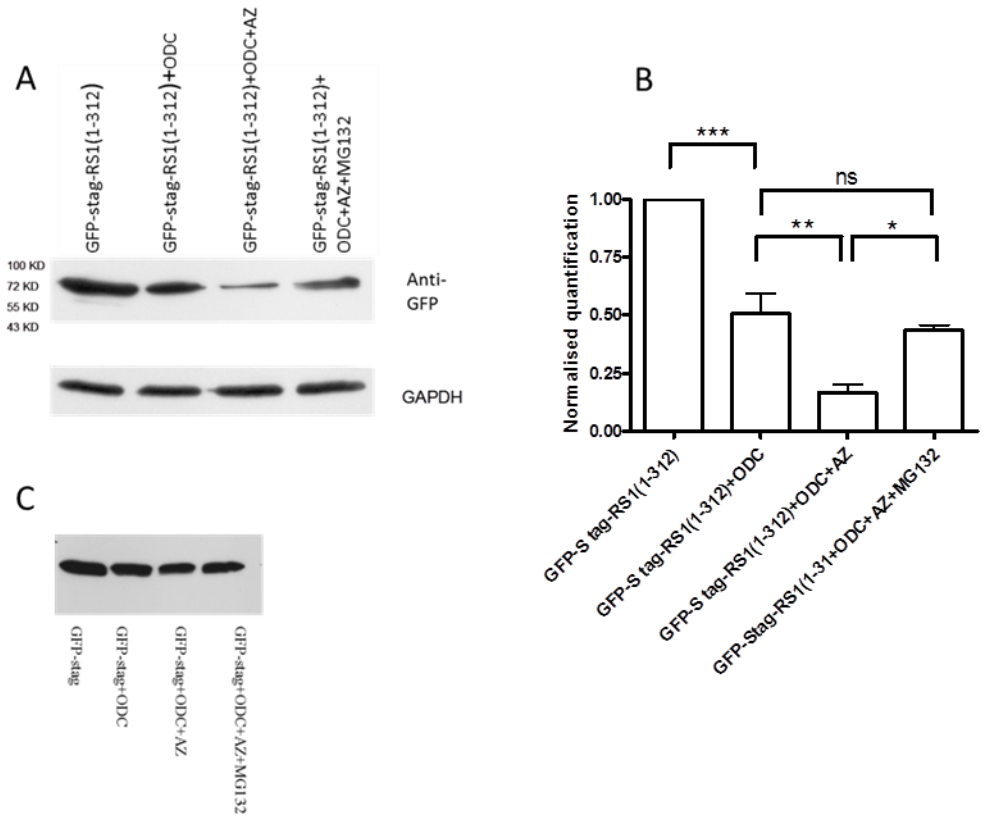

Fig 16: Antizyme dependent degradation of RS1 (1-312) and ODC complex.

Subconfluent HEK 293 cells were transfected with GFP- Stag-RS1 (1-312) or GFP- Stag-RS1 (1-312) /ODC or GFP-Stag-RS1 (1-312)/ODC/Antizyme. GFP- Stag-RS1 (1-312) with ODC and Antizyme transfected cells incubated with proteasomal inhibitor MG132 were used for the blockage of proteasome dependent degradation. After overnight expression, the cells were, cleared off cell debri by high speed centrifugation and the protein content was measured using Bradford method. 30 µg of the protein was taken for SDS-PAGE analysis (A). The bands were measured densitometrically using program Image J (B). GFP-Stag along with ODC and Antizyme transfected cells served as control (C). The results demonstrate an antizyme dependent degradation of the RS1 (1-312)/ODC complex.

RS1-Reg is not degraded by ODC/AZ complex

Ornithine decarboxylase the rate limiting enzyme involved in the production of polyamines is regulated in a feedback mechanism by putrescine via the production of an inhibitory protein

antizyme. It has been shown in fig: 3 that RS1-reg interacts with ODC. To investigate whether this interaction leads to the degradation of RS1-reg, and if degraded, it is antizyme dependent or not, a degradation assay had been performed by transiently transfecting subconfluent HEK 293 cells with RS1-Reg-FLAG tag alone or together with ODC or with ODC and AZ. The amount of RS1-Reg-FLAG was checked in the lysates by western blotting using anti FLAG antibody. ODC or ODC together with AZ did not show any effect on the degradation of RS1-reg protein.

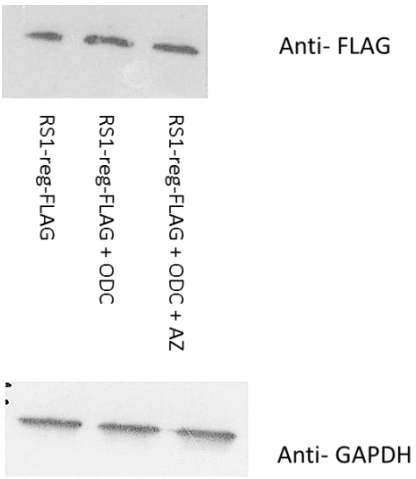

Fig 17: RS1-reg is not degraded by ODC in presence of Antizyme

Subconfluent HEK 293 cells were transfected with RS1-Reg-FLAG or RS1-Reg-FLAG along with ODC or RS1-reg-FLAG with ODC and Antizyme. After overnight expression the cells were lysed by sonication and the protein content was measured using Bradford method. 30 µg of the protein was taken for SDS-PAGE analysis. The proteins on the gel were analyzed by western blotting using anti-FLAG antibody. The amount of RS1-Reg in all the three conditions was the same with no significant difference, in contrast to RS1 (1-312) when co transfected with ODC and AZ. The data suggests that RS1-Reg is not degraded in an antizyme dependent manner when bound to ODC.

From the degradation studies done in HEK-293, LLC-PK_1 and CaCo2 cells (Koepsell et al., unpublished data) it is known that RS1 is a rapidly degraded protein. We want to check if this degradation is due to the interaction of RS1 (1-312) with ODC. Co-transfection studies done in HEK 293 cells with RS1 (1-312) and ODC with or with-out antizyme demonstrated the specific degradation of RS1 (1-312)/ODC complex in presence of antizyme (Fig: 15). The same degradation experiments using RS1-reg protein confirmed that the degradation of RS1-

reg was not influenced by ODC/AZ complex (Fig: 16). To check whether the interaction of RS1 (1-312) with ODC has an effect on the activity of the enzyme ODC, an activity assay using the HEK293 cell lysate expressing ODC and RS1 (1-312) showed no influence of RS1 (1-312) on the activity of ODC.

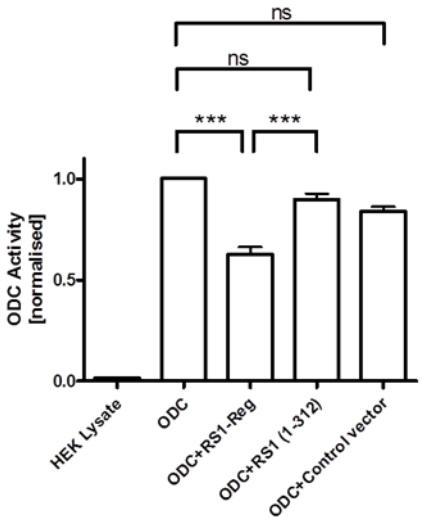

Fig 18: RS1 (1-312) fragment of RS1 is not involved in the downregulation of ODC activity

Subconfluent HEK 293 cells were transfected with vectors expressing either ODC alone or ODC/RS1-reg or ODC/RS1 (1-312) and ODC/control vector (pcDNA 3.1). After overnight expression the cells were lysed in the lysis buffer by sonication and centrifuged at 100000 g for 1hr at 4^0C. The cleared lysate (supernatant) was used to estimate the activity of ODC as explained in methods section (3.2.6). Non-transfected cell lysate was also used to check the level of ODC activity. ODC activity was reduced significantly when RS1-reg was co-transfected but not RS1 (1-312) and empty vector. The data shows that RS1-reg decreases the activity of ODC but not RS1 (1-312). Activity measurements were normalized to parallel performed measurements in the control (ODC). Mean values ± SE are indicated. ***, P<0.001, for difference to control, determined according to the ANOVA with post hoc Tukey's test.

This shows that RS1-reg was able to decrease the activity of the ODC enzyme but not RS1 (1-312). The inhibitory protein antizyme is a protein of 228 amino acids. Mutational studies confirmed the antizyme binding site on ODC between amino acids 117-140 (Li et al., 1992, Almrud et al., 2000). Ju-yi Heish et al. demonstrated that fragments of antizyme were able to restrict the activity of ODC to the same level of whole antizyme protein. A truncated version of antizyme [AZ (95-176)] was able to reduce the activity of ODC as strong as the wild type (Hsieh J-Y et al., 2011). RS1-reg protein when co-transfected with ODC or ODC along with antizyme was not degraded in an antizyme dependent manner as compared to RS1 (1-312)

protein. It was speculated that RS1-reg may bind to the same position as that of antizyme and henceforth it's also decreasing the activity of the enzyme ODC same as done by the inhibitory protein antizyme. Antizyme decreases the activity of ODC by blocking the formation of active homo dimer by binding to the monomers of ODC and targets it to degradation by 26S proteasome. According to the speculation if RS1-reg binds to the antizyme binding site on ODC and decrease the activity of ODC, then upon antizyme transfection into the HEK 293 cells expressing ODC and RS1-reg, the degradation of ODC by proteasome should be rescued when compared with the lysates containing only ODC and antizyme. But this was not observed. ODC was more readily degraded when co transfected with either antizyme alone or antizyme along with RS1-reg. This suggests that 1) either antizyme has higher affinity to the antizyme binding site on ODC when compared to RS1-reg protein or 2) RS1-reg may also act in the same way as antizyme, by targeting the ODC to proteasome and getting turnover without being degraded or 3) the binding of RS1-reg on ODC makes a conformational change that it is not able to form a homo-dimer thus leading to the decrease of activity. To clarify this, further more detailed study of the binding site of RS1-reg on ODC and its mode of action in decreasing the activity of the enzyme ODC has to be investigated.

Appendix-II: Bio-physical confirmation of interacting partners of RS1

The following clones have been demonstrated as positive interacting partners using the yeast two hybrid (Invitrogen) screening of the CaCo2 cDNA library as described in my diploma thesis. Calmodulin has been demonstrated to interact with the nuclear shuttling sequence of RS1 and the calmodulin binding sequence has been identified and characterized (Filatova et al., 2009). In an attempt to confirm the interaction by bio-physical method, co-precipitation experiments have been done with the proteins that were identified as positive in yeast two hybrid screening.

Results of the yeast two hybrid system

Serial number	Name of the clone	Gene bank accession number
1	Glutathione S transferase Pi	X06547
2	Serpin peptidase clade A1	NM_000295
3	Apolipoprotein H	NM_000042
4	Kruppel like factor 5	AF287272
5	Calcium\calmodulin dependent protein kinase 2 Gamma	NM_172170
6	Receptor for activated protein kinase 1 (RACK1)	NP_006089

Table 2: List of positive interacting partners obtained from screening CaCo2 cDNA library by yeast two hybrid system.

Demonstration that RS1 (1-312) interacts with Kruppel like factor-5 (KLF-5)

Transcription factors regulate diverse cellular processes, including proliferation, cell cycle, apoptosis, migration, and differentiation, by controlling gene expression. The KLF family consists of 20 members in humans, and is structurally characterized by three tandem zinc-finger domains at the C-terminus. Several members of the KLF family, such as KLF2 (Wu et al., 2004), KLF4 (Pandya et al., 2004, Rowland et al., 2005), KLF5 (McConnell et al., 2007), KLF6 (Narla et al., 2001, Chen et al., 2003), and KLF8 (Wang et al., 2007), have been

demonstrated to play vital roles in the development of various human cancers. KLF5 is widely expressed at varying levels in different tissues. Based on Northern blot analysis, high levels of KLF5 mRNA are present in the human and mouse digestive tract including intestine, colon, and stomach, and pancreas, placenta, testis, prostate, skeleton muscle, and lung (Sogawa et al., 1993, Conkright et al., 1999, Shi et al., 1999). KLF5 mRNA was also detected in human and rabbit bladder and uterus (Watanabe et al., 1999, Ohnishi et al., 2003). Although the expression of KLF5 appears mostly to be epithelial, KLF5 is also expressed in cardiovascular SMCs (Watanabe et al., 1999), cornea (Chiambaretta et al., 2004), lymphoid cells (Yang et al., 2003), and neuronal cells (Yanagi et al., 2008). Accumulated evidence suggests that KLF5 is more highly expressed in proliferating cells than in differentiated cells (Conkright et al., 1999). KLF5 mRNA is shown to express in high levels in the basal layer of the epidermis and in the base of the intestinal crypts (Ohnishi et al., 2000). Consistently, the KLF5 protein is also exclusively expressed in proliferating epithelial cells at the base of the crypts of the intestine but not in the terminally differentiated epithelial cells in the villi (McConnell et al., 2007). The KLF5 protein is primarily expressed in the nucleus (Shi et al., 1999). KLF5 also contains a nuclear export signal (NES) and sumoylation facilitates KLF5 nuclear localization by inactivating NES (Du et al., 2008). KLF5 has been demonstrated to regulate many genes involved in cell proliferation, cell cycle, survival, migration, angiogenesis, stemness, and differentiation (Chenet al., 2006, Guo et al., 2009, Usui et al., 2006, Chanchevalap et al., 2006, Jiang et al., 2008, Shinoda et al., 2008, Nagai et al., 2000) in different contexts. Most KLF5 target gene proximal promoters contain one or more GC rich sites (Sogawa et al., 1993, Kojima et al., 1997, Zhang et al., 2003). Although KLF5 has been shown to bind to Sp1 sites, GC boxes, and CACCC boxes, there are no strictly conserved consensus core sequences (Sogawa et al., 1993, Kojima et al., 1997, Zhang et al., 2003). To confirm the results of the yeast two hybrid screening by bio-physical method, co-precipitation experiments have been performed using HEK 293 cells lysates expressing either KLF5 and control vector (GFP-Stag) or KLF5 together with N-terminal 312 amino acid fragment of RS1 [GFP-Stag- RS1 (1-312)]. S protein agarose beads were used to precipitate the RS1 fragment and its associated protein complex from the lysate and blotted with anti KLF antibody to verify the interaction in western blotting.

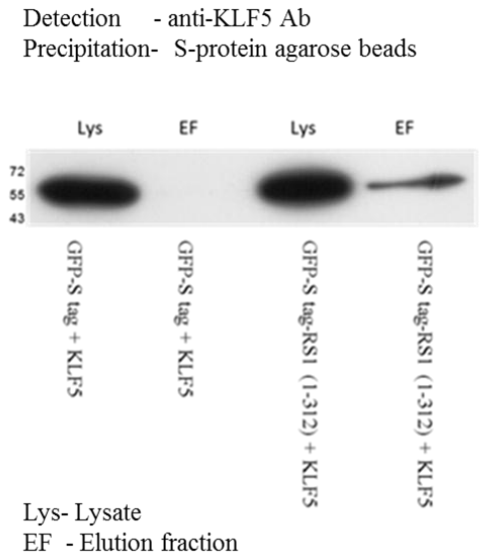

Fig 19: KLF 5 interacts with N-terminal (1-312) aa fragment of RS1. HEK 293 cells lysates expressing either KLF5 and control vector (GFP-Stag) or KLF5 together with GFP-Stag- RS1 (1-312) fragment of RS1 were used for co-precipitation experiments (section 3.2.3.2). S protein conjugated agarose beads were used for the pull down assay and the protein complex precipitated with the beads was probed with anti-KLF5 antibody in the western blot to detect the KLF5 in the complex. KLF5 showed interaction with RS1 (1-312) but not the control vector confirming the interaction.

RS1 is involved in the transcriptional and post-transcriptional regulation of SGLT1 (Korn et al., 2001, Veyhl et al., 1993). Korn et al, showed that RS1 is ivolved in the regulation of SGLT1 transcriptionally by over expressing porcine RS1 in confluent LLC-PK1 cells (Korn et al., 2001). Martin et al, showed that SGLT1 gene expression is regulated by SP1 transcriptional factor belonging to SP/Kruppel like factor transcriptional factor family (Martin et al., 2000) by binding to the GC box sequences in the promoter region, which are also the potential sites of KLF5 binding (Sogawa et al., 1993, Kojima et al., 1997, Zhang et al., 2003). The rabbit SGLT1 promoter was also observed to have two CACCC boxes at nucleotide positions -266 and -476 apart from the two GC boxes which are also the binding sites of KLF5. It would be interesting to investigate the role of the transcription factor KLF5, which is an intestinal specific isoform in the regulation of SGLT1 and evaluate the role of RS1 in this mechanism of regulation.

Demonstration of interation of Ca^{2+}/Calmodulin depedent protein kinase 2 Gamma (CaMKII G) with RS1-Reg and RS1 (1-312)

Ca^{2+}/Calmodulin (CaM)-dependent kinases belong to the family of serine/threonine protein kinases that are activated by Ca^{2+}/CaM. CaM dependent kinases are critically important for proper cellular function and are involved in several signal transduction pathways and regulatory processes such as gene transcription, cell survival/cell death (apoptosis), cytoskeletal reorganization, and learning and memory (Matthews et al., 1994, Sun et al., 1994). The subcellular localisation of the CaMK2 gamma is nuclear and cytoplasmic. One striking difference between CaMK2 and the other multifunctional CaM kinases is that it is the only one that does not exist as a monomer in cells. Upon elevation of intracellular Ca^{2+} concentration, Ca^{2+}-saturated CaM can bind to and fully activate each subunit of the CaMK2 holoenzyme. Once Ca^{2+}/CaM is bound, one subunit can be autophosphorylated at Thr286 by a neighbouring activated CaMK2 subunit, generating Ca^{2+}-independent activity so that even when Ca^{2+} levels are reduced, the kinase is fully active until CaM dissociates (Miller et al., 1986, Sun et al., 1994). This could serve as a mechanism to increase sensitivity of CaMK2 to changes in intracellular Ca^{2+} concentration (Meyer et al., 1992, Hanson et al., 1994). Calmodulin along with CaMK2 and also individually has been shown to play a regulatory role in the trafficking of many membrane proteins and receptors (Hidaka et al., 1985, de Figueiredo et al., 1995, Clint et al., 2007). As RS1 is localized to *trans*-Golgi network (Kroiss et al., 2006), and shown to regulate the process of release of vesicles containing different transporters at TGN (Veyhl et al., 2006), it would be interesting to investigate the role of calmodulin and CaMK2 in RS1 mediated trafficking of various transporter-containing vesicles. Interestingly, as it has been shown that CaMK2 gamma binds to RS1-Reg and also N-terminal 312 amino acid residues of RS1 in a calcium dependent as well as calcium independent manner. The two consensus sequences for phosphorylation by CaMK2 (RSV**S** at serine 31 and **S**AD at serine 83) in the RS1-Reg domain of RS1 would be of high interest to investigate. Thus, it may be speculated that this fragment of RS1 in association with calmodulin and CaMK2 might be phosphorylated by CaMK2 and thus may modulate the post-transcriptional regulatory mechanism of different transporters.

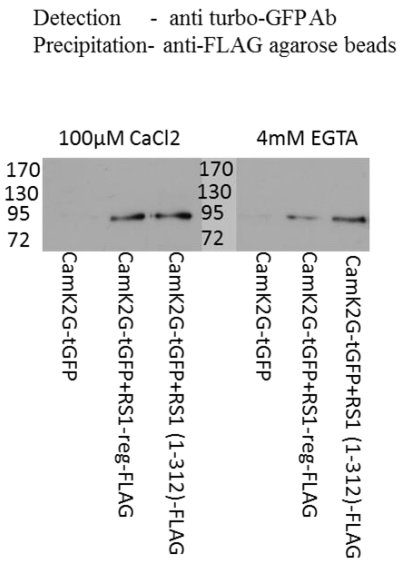

Fig 20: CaMK2 gamma isoform interacts with RS1-reg and RS1 (1-312) in presence and absence of calcium HEK 293 cells lysates expressing either CamK2G alone or together with RS1-reg or RS1 (1-312) fragments of RS1 were used for co-precipitation experiments (section 3.2.3.2) in the presence and absence of calcium. Anti- FLAG antibody conjugated agarose beads were used for the pull down assay and the protein complex precipitated with the beads was probed with anti-turbo-GFP antibody to detect the CaMK2 in the complex. CaMK2 interacted with both the fragments of RS1.

8. ABBREVIATIONS

DMEM	Dulbecco`s modified Eagle`s medium
DMSO	Dimethyl sulphoxide
DMF	N,N-Dimethyl formamide
dNTP	deoxynucleotide triphosphate
hr	Hour
GFP	Green fluorescent protein
LB-Amp	Luria bertani medium containing ampicillin
LiAc	Lithium Acetate
NSD	Nuclear shuttling domain
OAT	Organic anion transporter
OCT	Organic cation transporter
ODC	Ornithine decarboxylase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEI	Polyethylenimine
PVDF	Polyvinylidene difluoride
RT	Room temperature
SGLT	Na^+-D-glucose co-transporter
SC	Synthetic complete media
SC/-Leu	Synthetic complete media lacking leucine
SC/-Leu/-Trp	Synthetic complete media lacking leucine and tryptophan
SC/-Leu/-Trp /-His/-Ura	Synthetic complete media lacking leucine, tryptophan, histidine and uracil
TEMED	N,N,N',N'-Tetramethylethylendiamine
TEV	Tobacco etch virus

TGN	*trans*-Golgi network
X-Gal	5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

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Affidavit

I hereby declare that my thesis entitled “Ornithine decarboxylase is the receptor of regulatory protein RS1 (RSC1A1) mediating RS1 dependent short-term regulation of glucose transporter SGLT1” is the result of my own work. I did not receive any help or support from third parties, i.e. commercial consultants and others. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I verify that this thesis, either in identical or in similar form, has not been submitted as part of another examination process.

I confirm that the information is true and complete.

Chakravarthi Chintalapati.

Würzburg, 2013.