RS1 protein dependent and independent short and long term regulation of sodium dependent glucose transporter -1

RS1 Protein abhängige und unabhängige Kurz- und Langzeitregulation des Natrium-abhängigen Glukosetransporter -1

Doctoral thesis

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1. Introduction:

Carbohydrate mismanagement leads to chronic disorders such as obesity and type 2 diabetes. Sedentary lifestyles and high caloric diet intake have been noted as causes for increase in carbohydrate mismanagement disorders. Therapeutic drug development against these disorders relies on understanding the mechanism of carbohydrate metabolism. Carbohydrates forms one of major nutrients in diet and are a major source of energy for mammals. They are hydrolysed during digestion to monosaccharides by enzymes in the gastrointestinal tract. Monosaccharides cannot infuse the selectively permeable plasma membrane surface of intestinal epithelial cells (enterocytes). This entry is mediated by transporters present on the apical surface of the enterocytes. Later the monosaccharides exit into the blood stream via the transporters located in the enterocyte baso-lateral surface (Hediger, Coady et al. 1987, Wood and Trayhurn 2003). The sodium dependent glucose co-transporter (SGLT) of solute carrier family 5A (SLC5A) (Wright, Loo et al. 1994, Wright, Loo et al. 2004, Krimi, Letteron et al. 2009, Wong, Debnam et al. 2009) and the facilitative glucose transporter (GLUT) of the solute carrier family 2 (SLC2) family (Uldry and Thorens 2004) are involved in the absorption of glucose and some structurally related substances in the small intestine. These transporters are also involved in reabsorption of glucose from the glomerular filtrate, uptake of glucose across the blood-brain barrier, and in uptake and release of glucose from all cells in the body.

1.1 Sodium Dependent Glucose Co-transporter 1 (SGLT1):

SGLT1 is identified as a high affinity low capacity transporter (Hediger, Coady et al. 1987, Corpe and Burant 1996, Tavakkolizadeh, Berger et al. 2001, Wright, Martin et al. 2003, Houghton, Zarroug et al. 2006). Various organs (intestine, trachea, kidneys, heart, brain, testis and prostate glands) in humans and rats contain SGLT1 at distinct locations (Balen, Ljubojevic et al. 2008, Wright, Loo et al. 2011). The importance of SGLT1 in intestinal glucose transport was recognised when it was observed that the absence of functional SGLT1 in human intestine led to glucose-galactose mal-absorption (Shirazi-Beechey, Gribble et al. 1994, Wright 1998). SGLT1 is most strongly expressed in the apical membrane of enterocytes where it mediates the first step in small intestinal glucose absorption (Hediger, Coady et al. 1987, Kellett and Brot-Laroche 2005, Gorboulev, Schurmann et al. 2012). SGLT1 mediates glucose transport by symport of sodium ions and glucose with a stoichiometric ratio of 2:1. This symport occurs via a secondary active transport where the

sodium gradient is generated by the Na^+/K^+ ATPase (Figure 1) (Wright, Loo et al. 1994, Wright, Martin et al. 2003, Wright, Hirayama et al. 2007).

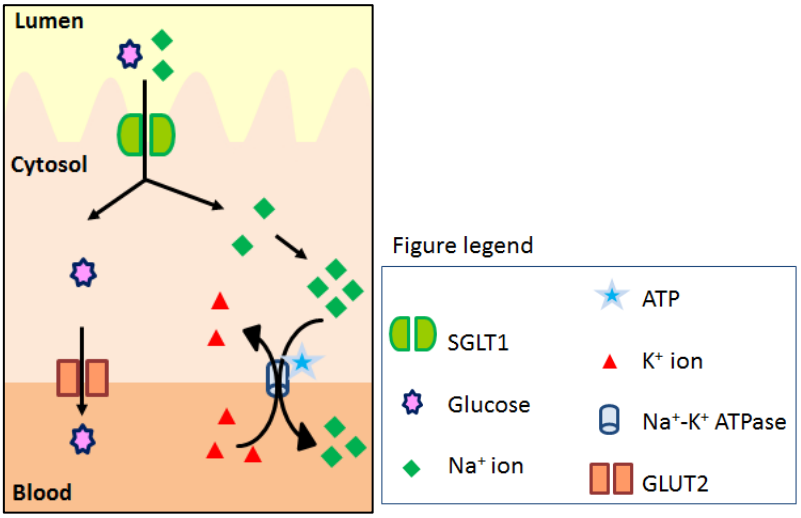

Figure 1: Model for intestinal secondary active glucose (and galactose) transport across the small intestine: The sketch depicts the mechanism of glucose transport occurring within a mature enterocyte at the upper villus of the small intestine. Glucose (or galactose) is co-transported with Na^+ across the brush border membrane by SGLT1. Glucose then exists across the basolateral membrane by GLUT2 or by exocytosis. Na^+ is actively transported across the basolateral membrane by Na^+/K^+ ATPase. The energy provided by Na^+ gradient is used for the transport of glucose. This figure is based on a scheme provided by Wright et al., 2004.

1.1.1 Regulation of SGLT1:

The functional capacity of SGLT1 is regulated to meet up with physiological demands of the species (Ferraris 2001).

Depending on the mechanism, the regulation of SGLT1 can be classified into post-transcriptional short term regulation and transcriptional and post-transcriptional long term regulation (Karasov and Debnam 1987, Wright, Hirsch et al. 1997).

Post-transcriptional short term regulation occurs in the brush border membrane of enterocytes layered with an intact mucosa layer (Sharp, Debnam et al. 1996). This regulation enables adaptation to rapid changes of luminal glucose concentration (Wright, Hirsch et al. 1997, Kellett and Brot-Laroche 2005). They include modifications of SGLT1 that may alter either the affinity, turn over number, vesicular trafficking or degradation bringing change in the number of transporter molecules within the plasma membrane (Hirsch, Loo et al. 1996, Vayro and Silverman 1999).

Several factors mediate the short term post-transcriptional regulation of SGLT1. Protein RS1 identifies SGLT1 as a physiological target for regulation (Osswald, Baumgarten et al. 2005). It mediates post-transcriptional short term regulation of SGLT1 by modulating the recruitment of vesicles containing SGLT1 from the intracellular pool at Trans Golgi network (TGN) to enterocyte apical surface (Veyhl, Keller et al. 2006). This regulation is dependent on intracellular glucose concentration and Protein kinase C (PKC) activation (Veyhl, Wagner et al. 2003). Peptides such as Proadrenomedullin N-terminal 20 peptide (PAMP) and adrenomedullin (AM) can also post-transcriptionally modulate the exocytosis of intracellular vesicles containing SGLT1 for short term regulation. This regulation is suggested to depend on cyclic adenosine monophosphate (cAMP) - Protein kinase A (PKA) pathway (Fernandez de Arcaya, Lostao et al. 2005). Hormones which can mediate short term regulation of SGLT1 include glucagon like peptide-2 (GLP-2) (Shirazi-Beechey, Moran et al. 2011), epinephrine (Ishikawa, Eguchi et al. 1997), portal insulin (Stumpel, Kucera et al. 1996), glucagon-37 (Stumpel, Scholtka et al. 1997), leptin (Ducroc, Guilmeau et al. 2005), cholecystokinin (CCK) (Hirsh and Cheeseman 1998) and prostaglandin E (PGE2) (Scholtka, Stumpel et al. 1999). These hormones and peptides may involve activation of secondary messengers such as G-protein (α-gustiducin) (Margolskee, Dyer et al. 2007) and cAMP (Ishikawa, Eguchi et al. 1997, Luz Sdos, de Campos et al. 1997, Stumpel, Scholtka et al. 1997, Lee, Loflin et al. 2000, Fernandez de Arcaya, Lostao et al. 2005, Shirazi-Beechey, Moran et al. 2011). Protein kinases such as PKC (Hirsch, Loo et al. 1996, Wright, Hirsch et al. 1997, Vayro and Silverman 1999, Veyhl, Wagner et al. 2003, Barrenetxe, Sainz et al. 2004, Kim, Lee et al. 2004, Castaneda-Sceppa, Subramanian et al. 2010), PKA (Ishikawa, Eguchi et al. 1997, Fernandez de Arcaya, Lostao et al. 2005, Subramanian, Glitz et al. 2009), Serum and glucocorticoid inducible kinase-1 (SGK-1) (Shojaiefard, Strutz-Seebohm et al. 2007) and glycogen synthase kinase 3 beta (GSK-3β) (Rexhepaj, Dermaku-Sopjani et al. 2010) can be involved in post-transcriptional regulation of SGLT1.

Transcriptional long term regulation of SGLT1 occurs in the cells present in lower villus and crypts which later migrate to upper villus and manifest the regulated SGLT1 function (Ferraris, Villenas et al. 1992). Although long term regulation of SGLT1 mainly involves transcriptional mechanism, it can also be regulated by post-transcriptional mechanism. Long term regulation enables adaptation to different diet conditions (Shirazi-Beechey, Hirayama et al. 1991), ontogenetic development (Shirazi-Beechey, Smith et al. 1991) differentiation of enterocyte for maturation (Freeman, Heavens et al. 1992) and also involves changes during

chronic metabolic disorders like diabetes (Fedorak, Gershon et al. 1989, Dyer, Garner et al. 1997, Dyer, Wood et al. 2002), hypo- or hyperinulinaemia (Philpott, Butzner et al. 1992), carcinoma (Alesutan, Sopjani et al. 2012, Hanabata, Nakajima et al. 2012, Lai, Xiao et al. 2012), obesity (Krimi, Letteron et al. 2009, Sopjani, Bhavsar et al. 2010) and inflammatory bowel diseases (Thomson and Wild 1997, Thomson and Wild 1997). These regulations of SGLT1 also control its diurnal rhythmic expression depending on the feeding pattern of the species (Furuya and Yugari 1974, Fisher and Gardner 1976, Corpe and Burant 1996, Rhoads, Rosenbaum et al. 1998, Tavakkolizadeh, Berger et al. 2001, Pan, Terada et al. 2004, Houghton, Zarroug et al. 2006). The long term SGLT1 regulation involves modulations in mRNA level or change in membrane integrity. Modulation in mRNA level could include changes in promoter activity (Dyer, Vayro et al. 2003), mRNA expression (Miyamoto, Hase et al. 1993) or mRNA stability (Loflin and Lever 2001, Pedder, Ford et al. 2008). Modulations at membrane integrity could include changes in height of microvilli (Smith, Peacock et al. 1991), membrane physio-chemical composition (Schwarz, Hostetler et al. 1985, Schwarz, Bostwick et al. 1989, Vazquez, Rovira et al. 1997), membrane actin polymerization (Chung, Wong et al. 1999) or change in the fraction of transporting to non-transporting cells (Fedorak, Gershon et al. 1989). These modulations at mRNA level and membrane integrity may lead to changes in density of transporter molecules (Ferraris and Diamond 1992, Ferraris and Diamond 1993), change in surface area for absorption (Smith, Peacock et al. 1991), change in rate of transport (Diamond, Karasov et al. 1984), change in the cells intended for transporter activity (Fedorak, Gershon et al. 1989) or increase translocation of other transporters to apical membrane surface (Krimi, Letteron et al. 2009, Sopjani, Bhavsar et al. 2010).

Prevalence of diet dependent SGLT1 regulation has been observed with changes in sodium (Donowitz, De La Horra et al. 1998), fibre (Reimer, Field et al. 1997) and carbohydrate content. High consumption of carbohydrate in diet leads to increase in rate of intestinal glucose transport by changing SGLT1 mRNA and protein abundance (Shirazi-Beechey, Gribble et al. 1994, Dyer, Hosie et al. 1997, Ferraris 2001). Carbohydrate based SGLT1 diet regulation is reported among vertebrates like mammals (rats, mice, cows, sheep and humans), amphibians (tadpoles) and fishes (tilapia and catfish) but not in birds (Ferraris 2001). Several factors are involved in the diet dependent regulation of SGLT1. Protein RS1 regulates SGLT1 transcriptionally for adaptation to diet having reduced glucose-galactose content (Filatova, Leyerer et al. 2009). High nutrient diet shows transcriptional upregulation of

SGLT1 activity by elevation of cAMP levels. Elevated levels of cAMP lead to PKA dependent increase in SGLT1 promoter activity (Dyer, Vayro et al. 2003).

Regulation of SGLT1 with regards to animal development depends on the gestation period, age and feeding (Ferraris 2001). These regulations include maintenance of high intestinal SGLT1 activity in foetal and neonatal stage (Buddington and Diamond 1989, Vazquez, Rovira et al. 1997, Buddington, Malo et al. 2000), gradual decrease in SGLT1 mRNA levels from duodenum to ileum of adults (Kojima, Nishimura et al. 1999, Yoshikawa, Inoue et al. 2011), accumulation of SGLT1 mRNA and insertion of SGLT1 transporters at the apical membrane during enterocyte maturation (Delezay, Baghdiguian et al. 1995), maintenance of SGLT1 diurnal rhythm (Rhoads, Rosenbaum et al. 1998, Martin, Wang et al. 2000, Vayro, Wood et al. 2001, Balakrishnan, Stearns et al. 2008, Kekuda, Saha et al. 2008) and abnormal SGLT1 activity during chronic occurrence of various metabolic disorders (Fedorak, Gershon et al. 1989). Several factors are involved for regulation of SGLT1 for animal development and cellular differentiation. Protein RS1 mediates transcriptional regulation of SGLT1 in LLCPK1 cells (porcine kidney epithelial cell line) dependent on cell confluence (Korn, Kuhlkamp et al. 2001, Filatova, Leyerer et al. 2009). Other proteins like a 38kDa nucleo-cytoplasmic protein (Lee, Loflin et al. 2000) and a RNA binding protein (HuR) (Loflin and Lever 2001) are involved in SGLT1 regulation during cell differentiation. Some factors which are involved in regulation of SGLT1 during chronic disorders or diseases such as carcinoma, diabetes, obesity, short bowel syndrome and intestinal inflammation include protein complexes like Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (Lee, Heo et al. 2007), hormones like angiotensin II (Wong, Debnam et al. 2009) and resistin like molecule β (RELMβ) (Krimi, Letteron et al. 2009, Sopjani, Bhavsar et al. 2010), growth factors like epidermal growth factor (EGF) (Chung, Wong et al. 1999) protein kinases like 5' Adenosine Mono-phosphate activated protein kinase (AMPK) (Krimi, Letteron et al. 2009, Sopjani, Bhavsar et al. 2010) and Tau tubulin kinase 2 (TTBK2) (Alesutan, Sopjani et al. 2012) and transcriptional factors like hepatocyte nuclear factors 1 (HNF1) and specificity protein 1 (Sp1) (Rhoads, Rosenbaum et al. 1998, Martin, Wang et al. 2000, Vayro, Wood et al. 2001, Balakrishnan, Stearns et al. 2008, Kekuda, Saha et al. 2008). Vagus nerve contributes in diurnal rhythm regulation of SGLT1 (Stearns, Balakrishnan et al. 2008, Stearns, Balakrishnan et al. 2012). Few of the different secondary messengers and protein kinases involved in the long term regulation of SGLT1 include cAMP (Peng and Lever 1995, Loflin and Lever 2001), PKA (Dyer, Vayro et al. 2003) and PKC (Castaneda-Sceppa,

Subramanian et al. 2010). PKC is involved in transcriptional regulation of SGLT1 by involving several intracellular signalling pathways such as p38/MAPK (Mitogen activated protein kinase), Extracellular signal regulated kinase (ERK)/MAPK, c-Jun N-terminal kinase (JNK)/MAPK and Phosphatidylinositide 3-kinases (PI3K)/Protein kinase B (Akt)/ mammalian target of rapamycin (mTOR) (Castaneda-Sceppa, Subramanian et al. 2010).

1.1.1.1 The RS1 protein and its regulation on SGLT1:

The Mammalian specific, intronless single copy gene *RSC1A1* encodes a 67- 68 KDa protein named RS1. Depending on the mammalian species, *RSC1A1* gene expression shows a wide tissue distribution. RS1 is expressed in kidneys, small intestine, liver, spleen, brain and lungs (Veyhl, Spangenberg et al. 1993, Lambotte, Veyhl et al. 1996, Poppe, Karbach et al. 1997, Reinhardt, Veyhl et al. 1999, Osswald, Baumgarten et al. 2005). Within the cell RS1 protein is distributed below the apical plasma membrane, around the Trans Golgi network (TGN) and within the cell nucleus (Valentin, Kuhlkamp et al. 2000, Osswald, Baumgarten et al. 2005, Kroiss, Leyerer et al. 2006).

RS1 regulates the activity of different transporters from different families such as SGLT1 (Veyhl, Spangenberg et al. 1993), SGLT1 homologous Na^+ myo-inositol co-transporter (SMIT) (Lambotte, Veyhl et al. 1996), organic cation transporters (OCT1 and 2) and concentrative nucleoside transporters (CNT1, 2 and 3) (Reinhardt, Veyhl et al. 1999, Veyhl, Wagner et al. 2003, Errasti-Murugarren, Fernandez-Calotti et al. 2012). Regulation of SGLT1 has been studied extensively due to its physiological importance. The deletion of *RSC1A1* gene in mice (RS1-/-) lead to increase in SGLT1 activity. Hence SGLT1 is identified as a physiologically important target for RS1 (Osswald, Baumgarten et al. 2005).

RS1 is involved the transcriptional and post-transcriptional regulation of SGLT1 (Korn, Kuhlkamp et al. 2001, Veyhl, Keller et al. 2006). The transcriptional regulation of SGLT1 by RS1 was studied in the porcine kidney epithelial cell line - LLCPK1. In LLCPK1 a confluence dependent inverse relationship occurs between the nuclear distribution of RS1 and the expression of SGLT1. When RS1 expression is down-regulated by antisense strategy or overexpressed artificially it resulted in opposite effects on SGLT1 mRNA expression and activity. RS1 thereby reveals a transcriptional regulation dependent on cell confluence (Korn, Kuhlkamp et al. 2001). In mice fed with glucose-galactose reduced diet (GGRD) RS1 transcriptionally regulates SGLT1 to mediate higher SGLT1 mRNA and protein expression in wild type mice as compared to RS1-/- mice (Filatova, Leyerer et al. 2009). The post-

transcriptional regulation by RS1 was studied in *Xenopus laevis* oocytes system where co-expression of human RS1 (hRS1) and human SGLT1 (hSGLT1) cRNA decreased SGLT1 activity (Veyhl, Spangenberg et al. 1993, Lambotte, Veyhl et al. 1996, Veyhl, Wagner et al. 2003). Down regulation of SGLT1 activity also occurred on injecting hRS1 protein in hSGLT1 expressing *Xenopus laevis* oocytes (Veyhl, Keller et al. 2006). This indicated RS1 as a post-transcriptional regulator of SGLT1. This regulation occurs by inhibiting dynamin dependent exocytotic release of hSGLT1 containing vesicles from TGN (Veyhl, Wagner et al. 2003, Kroiss, Leyerer et al. 2006, Veyhl, Keller et al. 2006).The regulation is enhanced by PKC activation and is dependent on intracellular glucose concentration (Veyhl, Wagner et al. 2003, Veyhl, Keller et al. 2006).

Functional characterization of RS1 protein identified an N-terminal domain (TGN-Reg) consisting of about 90 residues from 16-98 and 15-92 in human and mouse RS1 (hRS1 and mRS1) respectively responsible for post-transcriptional regulation of SGLT1. Two peptide sequences - Gln-Ser-Pro (QSP) (Vernaleken, Veyhl et al. 2007) and Ser-Asp-Ser-Asp-Arg-Ile-Glu-Pro (SDSDRIEP) (M. Vehyl-Wichmann, et al data unpublished) in the TGN-Reg are identified capable to mediate this regulation of SGLT1. This domain also comprises of a 21 residue long nuclear shuttling domain (RNS) to mediate the cell confluence dependent nuclear localization of RS1 protein and another domain for controlling the transcriptional regulation of SGLT1 (Filatova, Leyerer et al. 2009). The C-terminal sequence of RS1 contains a tripeptide Gln-Cys-Pro (QCP) which can also mediate post-transcriptional regulation of hSGLT1 (Vernaleken, Veyhl et al. 2007) and an ubiquitin associated domain (UBA) which has not been characterized (Valentin, Kuhlkamp et al. 2000). QCP and QSP are also substrates of H-peptide co-transporter PepT1 and are effectively absorbed when applied in the intestine lumen (Vernaleken, Veyhl et al. 2007). The TGN-Reg domain also consists of several consensus sequences for phosphorylation by a variety of protein kinases. The putative phosphorylation sites in this domain of hRS1 and mRS1 were screened and their comparative alignment is depicted in Figure 2.

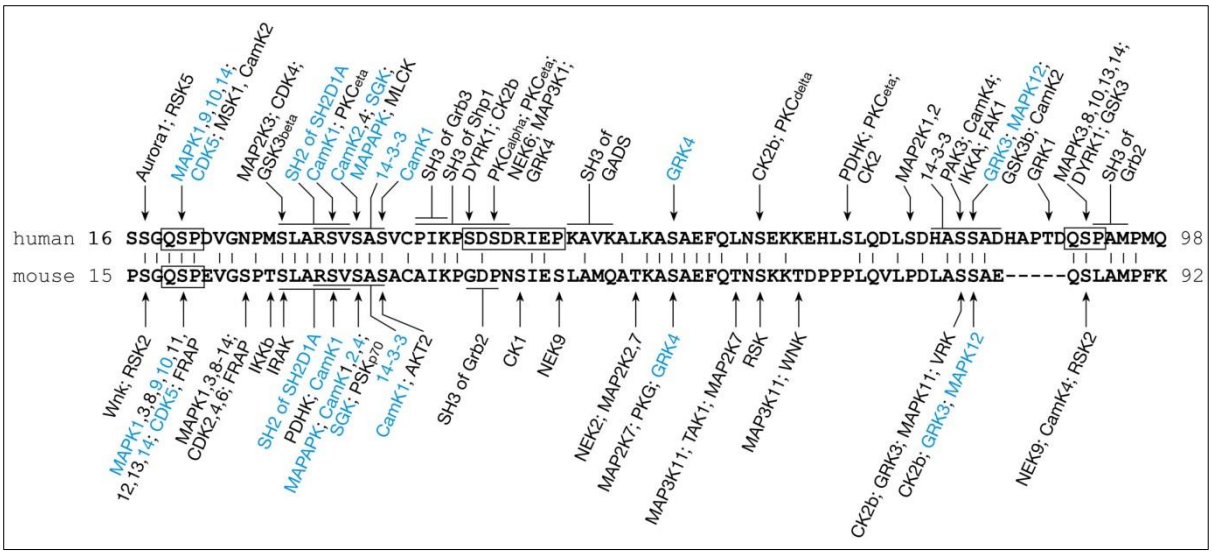

Figure 2: Comparative alignment between TGN-Reg Domains of hRS1 with mRS1: Both hRS1 and mRS1 have several serine and threonine putative phosphorylation sites. The sites common in both have been marked in blue. Two QSP motifs are present in hRS1 whereas only one QSP motif is present in mRS1. SDSDRIEP motif is specifically present only in hRS1 sequence. SDSDRIEP is a putative site for PKC α phosphorylation while QSP is a putative site for CamK2 phosphorylation.

1.1.1.2 PKC dependent regulation of SGLT1:

Several factors mediate the regulation of SGLT1 by involving activation of secondary messengers and protein kinases. Involvement of PKC in the regulation of SGLT1 is shown during sepsis caused by bacterial lipopolysaccharide (LPS) (Amador, Garcia-Herrera et al. 2007), during hormonal stimulus like epinephrine (Kim, Lee et al. 2004), Angiotensin II (Han, Park et al. 2004), leptin (Ducroc, Guilmeau et al. 2005) and in RS1 protein dependent post-transcriptional regulation of SGLT1 (Veyhl, Wagner et al. 2003, Veyhl, Keller et al. 2006). PKC regulates SGLT1 by involving modulation in level of cAMP (Hirsch, Loo et al. 1996). PKC regulates SGLT1 both transcriptionally and post-transcriptionally. This regulation might modulate SGLT1 protein level in the apical surface or affect the rate of SGLT1 dependent transport. Post transcriptional regulations that can modulate SGLT1 number in apical surface include changes in trafficking of SGLT1 containing vesicles, SGLT1 degradation rate or SGLT1 mRNA stability. The post-transcriptional regulations that can alter SGLT1 affinity or turn over number can modulate the rate of SGLT1 mediated transport. PKC dependent diverse post-transcriptional modulations have been observed in different systems. PKC decreases rabbit SGLT1 turn over number in monkey kidney derived fibroblast like cell line (COS-7) (Vayro and Silverman 1999). In Chinese hamster ovary cells expressing rabbit SGLT1 (CHO-G6D3), PKC activation lead to increase in transcription of SGLT1 mRNA (Castaneda-Sceppa, Subramanian et al. 2010). In LLCPK1 cells, PKC

demonstrates a decrease in SGLT1 mRNA due to mRNA degradation (Shioda, Ohta et al. 1994). In SGLT1 expressing *Xenopus laevis* oocytes (Hirsch, Loo et al. 1996, Wright, Hirsch et al. 1997, Veyhl, Keller et al. 2006), in rat intestinal brush border membrane vesicles (Ishikawa, Eguchi et al. 1997), in differentiating human cell clone HT-29-D4 (Delezay, Baghdiguian et al. 1995), CHO-G6D3 cell line (Castaneda-Sceppa, Subramanian et al. 2010) and LLCPK1 cells (Shioda, Ohta et al. 1994, Peng and Lever 1995) PKC mediates SGLT1 regulation by modulating exo – and endocytosis of SGLT1 carrying vesicles. The different systems not only show different forms of post-transcriptional modulations but also show diversity in respect to the effect (increase or decrease) on SGLT1 activity, protein or mRNA caused due to PKC activation.

The different effects caused by PKC on SGLT1 regulation are dependent not only on the expression system but also on the species homolog of SGLT1 sequence. In *Xenopus laevis* oocytes expressing rabbit and rat SGLT1, PKC activation mediates a negative regulation decreasing the rate of substrate transport whereas when human SGLT1 is expressed it mediates a positive regulation increasing substrate transport. The SGLT1 sequence in rat and human has 5 consensus PKC sites while rabbit contains 4 sites (Wright, Hirsch et al. 1997). The amino acid residues between positions 550-636 of SGLT1 cytoplasmic loop sequence are considered to be the most likely domain involved in PKC dependent sequence specific regulation of SGLT1. (Hirsch, Loo et al. 1996). Serine 418 residue in hSGLT1 sequence is speculated for SGLT1 regulation by phosphorylation/ de-phosphorylation (Kumar, Tyagi et al. 2007). These complementary mechanisms for PKC dependent SGLT1 regulation may also depend on the choice of path followed. The activation of MAPK and PI3K/Akt/mTOR signalling pathway by PKC lead to increase in SGLT1 dependent uptake. The activation of p38/MAPK signalling pathway by PKC exerts the inhibitory action on SGLT1 uptake (Castaneda-Sceppa, Subramanian et al. 2010).

The further investigation of PKC in the transcriptional and posttranscriptional regulatory mechanism of RS1 may bring new insights in understanding SGLT1 regulation and thereby might impart knowledge for the better management of carbohydrate metabolism.

2. Aim of the study:

The aim of this study was to further elucidate the role of the regulatory protein RS1 in the short term post-translational and long term transcriptional regulation of SGLT1by employing tools which were available in the laboratory of Professor H. Koepsell. These tools were mice in which RS1 was removed genetically (RS1-/- mice) and tripeptides derived from the regulatory domain of RS1 which mediates post-translational regulation of SGLT1. Since it is known that post-translational regulation of SGLT1 is modulated by PKC (Hirsch, Loo et al. 1996, Wright, Hirsch et al. 1997) and that PKC also influences the post-translational regulation mediated by RS1 (Veyhl, Wagner et al. 2003) the effect of PKC in wild type mice versus RS1-/- mice was compared. Concerning investigation of the role of RS1 in transcriptional regulation the present study was focussed on the investigation of effects of diets on the expression of SGLT1 in small intestine. Due to restriction of time and technical difficulties arising during the study the investigation was focussed on functional analyses.

3. Materials & Methods

3.1 Materials

3.1.1 Chemicals:

All laboratory chemicals used were of pro analysi (p.a.) grade and were purchased form one of the firms namely Sigma-Aldrich (Taufkirchen, Germany), Merck (Darmstadt, Germany), Perkin Elmer (Darmstadt, Germany) or AppliChem (Darmstadt, Germany).

3.1.2 Animals:

Institutional guidelines and German laws were met in order to handle mice as the model system. Wild type mice and RS1-/- mice of 129/OLA/C57BL/6 or C57BL/6 background were used for comparative studies on SGLT1 activity. Animals of age 2 – 3 months were chosen for study. All animals were housed in a temperature – controlled environment with a 12h – light/12h – dark cycle with free access to specific diet and tap water.

3.1.3 Diets:

For the study of diet effects on SGLT1 regulation, weaning mice of 10-14 days old were segregated to different groups. Each group were fed with free access to specific diet and tap water for 2 months. Standard control or normal diet (ND) (Ssniff V1534-000R/M-H, 10mm), High fat high glucose diet (HFHGD) (Ssniff® EF R/M by D12492 mod.) and Glucose-galactose reduced diet (GGRD) (Ssniff® EF R/M Glucose-arm) were purchased from Spezialdiäten GmbH, (Soest, Germany). The specific composition of each diet is mentioned in (table 1) and their total metabolizable energy is mentioned in (table 2)

Table – 1: Diet composition

Diet Components	**Standard control diet (%)**	**High fat high glucose diet (%)**	**Glucose-galactose reduced diet (%)**
Starch	36.4	2.2	5.8
Protein	19	24.1	29.7
Fibre	4.9	6	28.8
Mono and Di saccharides	4.7	22.4	0.5
Fat, Minerals, Vitamins	3.3	34	20.5

Table -2: Total metabolizable energy

	Standard control diet (MJ/kg)	High fat high glucose diet (MJ/kg)	Glucose-galactose reduced diet (MJ/kg)
Total metabolizable energy	12.8	25.2	22.9

3.1.4 Inhibitors and Activators:

Protease inhibitors used for the study were obtained from Roche Diagnostics Deutschland GmbH (Mannheim, Germany). The working concentrations of the same are specified in the following table.

Table -3: Working stock concentration of protease inhibitor:

Protease Inhibitor	Working concentration (µM)
Aprotinin	0.8
Benzamidin	1000
Leupeptin	20
Phenylmethylsulfonyl fluoride (PMSF)	100
Pefabloc SC (AEBSF)	2000

Phorbol 12 - myristate 13 - acetate (PMA) which is an activator of PKC was obtained from the firm Sigma-Aldrich (Taufkirchen, Germany). The working concentration of the PMA used was 5 µM.

3.1.5 Synthetic tripeptides:

Two synthetic tripeptides QSP (Gln-Ser-Pro) and QEP (Gln-Glu-Pro) were obtained from the lab of Dr. Rüdiger Pipkorn, German Cancer Research Center (Heidelberg, Germany). The working concentration of the peptides used was 15 mM.

3.1.6 Radioactive substrate:

ARC0131 C^{14} radiolabelled Methyl–α-D-Glucopyranoside (AMG), [glucose-^{14}C (U)]; (11.1 Gbq/mMol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Radioactive [^{14}C] AMG was added in tracer amounts to a required concentration of AMG for performing SGLT1 uptake and affinity studies. The final AMG working

concentration for uptake studies in everted segment method was 10 µM while for affinity measurements in brush border membrane vesicles (BBMV), a concentration range from 0.02 – 5 mM was used.

3.1.7 Buffers and solutions:

Deionised water was used to prepare all aqueous buffer solutions. Compositions of the different buffer solutions are mentioned in the respective method sections.

3.1.8 Software:

Putative phosphorylation motifs in TGN-Reg domain of human and mice RS1 sequence were searched using MnM 3.0 (Minimotif Miner, version 3.0)(http://minimotifminer.org) and GPS 2.1 (Group-based Prediction System, version 2.1) (http://gps.biocuckoo.org) software. Statistical analyses were performed using PRISM (GraphPad software, Inc., San Diego, Calif.) software.

3.2 Methods

3.2.1 Small intestinal brush border membrane vesicle (BBMV) preparation:

Isolation of BBMVs was performed using a modified magnesium precipitation - centrifugation procedure (Koepsell, Fritzsch et al. 1990).

Each set of BBMV preparation required 3 mice. The mice were starved for 24 hours prior to use for BBMV preparation. Small intestine from these mice were isolated, everted and washed either directly with 20 ml ice cold homogenization buffer or after incubation for 1 hour at 37°C in a shaking water bath under different study conditions. After washing, the small intestines were mechanically homogenized using the Power Gen 125 homogenizer (Fischer Scientific, Waltham, Massachusetts, USA). The homogenization was performed in 35 ml ice cold homogenization buffer with protease inhibitors. The homogenate obtained was precipitated after adding magnesium chloride ($MgCl_2$) to a final concentration of 10 mM. For precipitation the homogenate was incubated with $MgCl_2$ for 20 min in ice with intermittent shaking at every 5 min. The precipitated homogenate was centrifuged at 3000 relative centrifugal force (rcf), 4°C for 15 min in a super speed centrifuge (Sorvall® Evolution RC centrifuge, Thermo Fisher Scientific, Langenselbold, Germany) using rotor SS-34. Pellet containing mitochondria and nuclei was discarded and the remaining supernatant was centrifuged for 30 min at 27000 rcf, 4°C. 35 ml of ice cold vesicle buffer containing protease inhibitor was added to the resulting pellet and homogenized using glass Potter Elvehjem homogenizer (Sartorius, Gottingen, Germany). The homogenate was centrifuged for 30 min at 27000 rcf, 4°C and the pellet was collected. To this pellet 300 µl of ice cold vesicle buffer with protease inhibitors was added and it was aspirated using syringe with needle having pore size 26G x 3/8". The BBMVs of each preparation were divided in 3-4 portions and stored in liquid nitrogen until use.

Homogenization buffer	(100 mM mannitol, 2 mM Hepes) pH 7.1 adjusted using 1 M Tris
Vesicle buffer	(100 mM mannitol, 20 mM Hepes) pH 7.4 adjusted using 1 M Tris

3.2.2 AMG affinity uptake in BBMVs:

Substrate uptake in BBMVs was performed using rapid filtration technique (Hopfer, Nelson et al. 1973).

Aliquots of 2-3 different BBMV preparations for the same condition were thawed at 37°C in a water bath and pooled to overcome differences between preparations. Hence each preparation used for uptake measurement was freeze-thawed only once. AMG, an SGLT specific non-metabolizing substrate, was used at concentrations 0.02 – 5 mM, to measure the substrate dependence of SGLT1 mediated glucose transport. Different AMG concentrations were prepared with tracer amount of [^{14}C] AMG in uptake buffer containing either 100 mM sodium thiocyanate or 100 mM potassium thiocyanate. Sodium dependent uptake measurements were performed by incubating 20 µl BBMV for 2 seconds in 50 µl of sodium or potassium containing uptake buffer with different concentrations of AMG. Uptake in the presence of sodium was blocked with 1 ml ice cold stop buffer containing 100 mM sodium chloride plus 0.2 mM phlorizin. Uptake measured in the absence of sodium was stopped using 1 ml ice cold stop buffer containing 100 mM potassium chloride. The vesicles were washed on nitrocellulose membrane filters using respective ice cold blocking buffer. The filters were transferred into 5 ml scintillation vials. 1ml of scintillation fluid - Lumasafe scintillation cocktail (Lumac LSC (Groningen, Netherlands) was added and the vials were placed in a shaker for 1 hour for mixing. The radioactivity on the filters was measured using Beckman Coulter TM LS 6500 Multi-purpose scintillation counter (Beckman Coulter, Inc., Fullerton, CA, USA).

Na^+ containing uptake buffer	100 mM mannitol, 20 mM Hepes, 100 mM sodium thiocynate pH 7.4 adjusted with 1 M Tris
K^+ containing uptake buffer	100 mM mannitol, 20 mM Hepes, 100 mM potassium thiocynate pH 7.4 adjusted with 1 M Tris
Na^+ containing stop buffer	100 mM mannitol, 20 mM Hepes, 100 mM sodium hydroxide pH 7.4 adjusted with 5 M sodium hydroxide
K^+ containing stop buffer	100 mM mannitol, 20 mM Hepes, 100 mM potassium hydroxide pH 7.4 adjusted with 5 M potassium hydroxide

3.2.3 Protein estimation in BBMVs:

To measure the amount of protein in BBMV preparations, Lowry's method for protein estimation was used (Lowry, Rosebrough et al. 1951).

Bovine serum albumin (BSA) at concentrations 5 µg, 10 µg, 20 µg and 40 µg were used as controls. BBMV samples to be analysed were diluted 17.5 times in 0.5 % sodium dodecyl sulphate (SDS) and 100 µl was taken for analysis. 1 ml Lowry solution was added to both samples and controls and incubated for 10 min. Later 100 µl of 1 X Folic and Ciocalteu's phenol reagent was added and vortexed. The controls and samples were incubated for 30 minutes at room temperature and measured for optical density at absorbance of 760 nm wavelength using tungsten light in Pharmacia LKB Ultraspec III UV/visible scanning spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). A graph was plotted using the values of optical density measured against control concentrations and the slope, $y = m(x) + c$, (where y = optical density values, x = unknown concentration and m and c are linear equation constants) was obtained. Using this slope, sample concentration was determined in µg/µl after correcting to dilution factor and sample volume.

Lowry solution	Lowry's reagent (2 % sodium carbonate, 0.4 % sodium hydroxide) + 0.02 % potassium sodium tartarate + 0.01 % copper sulphate

3.2.4 AMG uptake in small intestinal everted segments:

Mice were starved for 24 hours and AMG uptake measurements were performed in small intestinal everted segments at 10 am.

To determine the distribution of SGLT1 activity along the small intestine segments from duodenum (2-6 cm distal to pylorus), jejunum (8-12 cm distal to pylorus) and ileum (4-8 cm proximal to ileocecal valve) were used. Short term regulation of SGLT1 mediated transport was studied using jejunal segments derived from 6 cm – 14 cm distal to pylorus. The small intestine isolated from the mice was first washed with Krebs ringer solution (20 ml) kept at room temperature. The small intestine from mice was then everted using a steel rod and 1 cm long segments of the above defined regions were dissected. The Krebs ringer buffer used for AMG uptake was bubbled for few seconds with 95% O_2 5% CO_2 gas. AMG uptake into the mucosa of the segments was either measured directly or after the segments had been pre-incubated under different study conditions for 1 hour at 37°C in a shaking water bath.

Phlorizin inhibitable uptake of AMG was measured after 2 min incubation at 37°C in shaking water bath in Krebs ringer buffer containing 10 µM [^{14}C] AMG without or with 1 mM phlorizin Uptake was stopped by incubating the segments for 5 min in ice cold Krebs ringer buffer containing 1 mM phlorizin. The segments were washed on a nitrocellulose filter with ice-cold Krebs ringer buffer. The accurate length of the individual segments was determined under a light microscope using a 1/10 mm scale. The segments were dissolved in 500 µl of tissue solubilizer - Soluene® 350 (PerkinElmer, Massachusetts, USA) by heating them in a water bath at 60°C for 2 hours. 200 µl from each dissolved sample was taken in duplicates into 5 ml scintillation vials. 1 ml of scintillation fluid - Lumasafe scintillation cocktail (Lumac LSC, Groningen, Netherlands) was added and the vials were placed in a shaker for 1 hour for mixing. The radioactivity of the dissolved samples was measured was measured using Beckman Coulter TM LS 6500 Multi-purpose scintillation counter (Beckman Coulter, Inc., Fullerton, CA, USA).

Krebs Ringer solution	(10 mM Hepes, 103 mM sodium chloride, 4.8 mM potassium chloride, 1.2 mM magnesium sulphate, 1.2 mM potassium di-hydrogen phosphate, 1.2 mM calcium chloride) pH 7.35 adjusted with 5 M sodium hydroxide
Krebs Ringer solution for pre-incubation	(10 mM Hepes, 103 mM sodium chloride, 4.8 mM potassium chloride, 1.2 mM magnesium sulphate, 1.2 mM potassium di-hydrogen phosphate, 1.2 mM calcium chloride, 5 mM MES) pH 6.5 adjusted with 5 M sodium hydroxide

3.2.5 Calculation and Statistics

Uptake measurements in everted segments of small intestine were performed at least in 3 mice for every condition. The radioactive measurements were calculated in Picomoles/cm/2 min. The uptake measurements were normalized twice in order to overcome two types of variations. One variation was the technical difference in controls measured on different days. The other variation was the segmental difference within a particular region of small intestine (Appendix III). The measurement obtained for the control segment in the different mice used

per day was averaged. This average value was used to normalize the other conditions measured in the different segments of these mice. These values were then re-normalized according to the regional segmental difference. The mean value ± standard error obtained for the twice normalized values were compared between wild type or RS1-/- control to understand the effect of a particular study condition.

Aliquots from different BBMV preparations of the same conditions were thawed and pooled for affinity measurements. This was done to avoid the variation between individual BBMV preparations. At least two different BBMV preparations were pooled for every affinity uptake. Sodium dependent SGLT1 affinity uptake was assayed at least 3 times. The radioactive counts measured for every affinity assay were determined in Picomoles/mg/2 seconds. These values of each assay were fitted in non-linear regression to obtain K_m value using the Michaelis-Menten enzyme kinetic equation (Y=Vmax*X/(K_m+X); where X symbolizes the different AMG concentrations and Y is the relative transport velocity obtained). Sodium-dependent uptake rates of individual experiments were normalized to the respective Vmax values. The mean uptake rates ± standard error values obtained from independent experiments was represented in non-linear Michaelis-Menten curve and the K_m. value was obtained. To find the change in SGLT1 affinity, the K_m values obtained from independent experiments of different conditions were compared.

Significance of difference between standard error of mean (SEM) of three or more groups were tested using one way ANOVA with post hoc Tukey comparison, marked as asterisk (*). Significance of difference between SEM values of two groups was calculated using one sided student's unpaired or paired t-test. One sided student's t test was used for comparing the effect of difference in respect to only control condition. Unpaired t-test was used when different mice sample SEM values were compared and significant differences were marked with exclamation (!). Paired t-test was done to compare differences between segment SEM values of the same mice subjected to different conditions and significant differences were marked as section sign (§).

3.2.6 Experimental design setup:

The mechanism underlying RS1 dependent and independent short term and long term regulation of SGLT1 in mouse system was investigated in the present study.

SGLT1 activity and affinity for transport were assessed in wild type and RS1-/- mice. For estimating SGLT1 activity, phlorizin inhibited uptake of 10 µM AMG, labelled with tracer

amount of [^{14}C] AMG was performed in everted segments of mice small intestine. Affinity of SGLT1 was determined by measuring Na^+ specific uptake at different concentrations of AMG, labelled with tracer amount of [^{14}C] AMG, in brush border membrane vesicles of mice small intestine.

Short term regulation of SGLT1 was studied after 1 hour pre-incubation of mice small intestine in presence and absence of glucose. For understanding diet adaptations of SGLT1 direct measurements were performed in mice subjected to diets varied in their glucose content. RS1 dependent regulation for these changes was studied by measuring the difference in SGLT1 mediated AMG uptake and kinetics in small intestine of wild type and RS1-/- mice. Further study was done to characterize RS1 in the role of short term SGLT1 regulation by using its functional tripeptide QSP (Vernaleken, Veyhl et al. 2007) and its phosphorylation mimicking mutant QEP. Since RS1 has many putative phosphorylation sites (Figure 2) and its regulation of SGLT1 is known to be PKC dependent (Veyhl, Wagner et al. 2003), the role of PKC in RS1 dependent SGLT1 short term regulation was also investigated. QSP has a CamKII phosphorylation putative site (Figure 2) hence the addition of QSP phosphorylation with PKC activation was also studied to infer whether they follow a common pathway for mediating SGLT1 short term regulation. Since PKC is also known for its role as a post-transcriptional regulator of SGLT1 (Wright, Hirsch et al. 1997) the effect of PKC on SGLT1 affinity for transport was checked to understand its mechanism of regulation.

4. Results

In this study attempt was made to improve the understanding of glucose in short term and long term regulation of SGLT1. In particular, effort was made to understand better, the dependence of the regulator protein RS1 in these regulations. For this, functional studies were performed in wild type and RS1-/- mice in combination with application of the RS1 derived regulatory tripeptide QSP. In addition, experiments were also made to elucidate the role of PKC in RS1 dependent short term regulation of SGLT1.

4.1 Effect of glucose and RS1 on SGLT1 activity:

SGLT1 plays a pivotal role in small intestine for regulation of glucose absorption. After application of D-glucose in mice by gavage *in vivo* and analyses of glucose transport in isolated brush-border membrane vesicles (BBMV) it has been shown that glucose bolus increases the amount and transport activity of SGLT1 in wild type mice (Gorboulev, Schurmann et al. 2012). On the other hand mice lacking *RSC1A1* gene which shows enhanced glucose absorption in small intestine in brush border membrane (BBM) and increased amount of SGLT1 protein in plasma membrane enriched (PME) fractions (Osswald, Baumgarten et al. 2005), did not show any further increase in SGLT1 amount and activity in the BBM after gavage with glucose. The upregulation of SGLT1 in wild type mice after a glucose bolus equals SGLT1 activity of RS1-/- mice (H. Kipp and H. Koepsell, unpublished data). RS1 is involved in the post-transcriptional short term regulation of SGLT1 in a glucose dependent manner (Veyhl, Keller et al. 2006).

In my study the effects of RS1 removal or incubation with D-Glucose on SGLT1 mediated transport activity was investigated by performing uptake measurement in everted small intestinal segments. These measurements allowed more direct control of the experimental conditions such as luminal glucose concentrations compared to *in vivo* gavage with D-glucose. However this experimental setup had the drawback that pre-incubation of the everted small intestinal segments led to decrease in SGLT1 activity (Appendix I). In figure 3 the phlorizin inhibited uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured in everted jejunal segments of wild type and RS1-/- mice after pre-incubating them in the presence or absence of 5 mM D-glucose. In the absence of glucose, AMG uptake was slightly higher in RS1-/- mice, compared to wild type mice. In the presence of glucose, AMG uptake was increased in wild type and RS1-/- mice. This increase reached the same level of transport activity in both cases. The data are consistent with the uptake measurements

in isolated BBMV obtained after glucose gavage, with the exception that the activity of SGLT1 in the absence of glucose in RS1-/- mice was smaller, compared to activity in the presence of glucose observed in wild type mice.

Figure 3

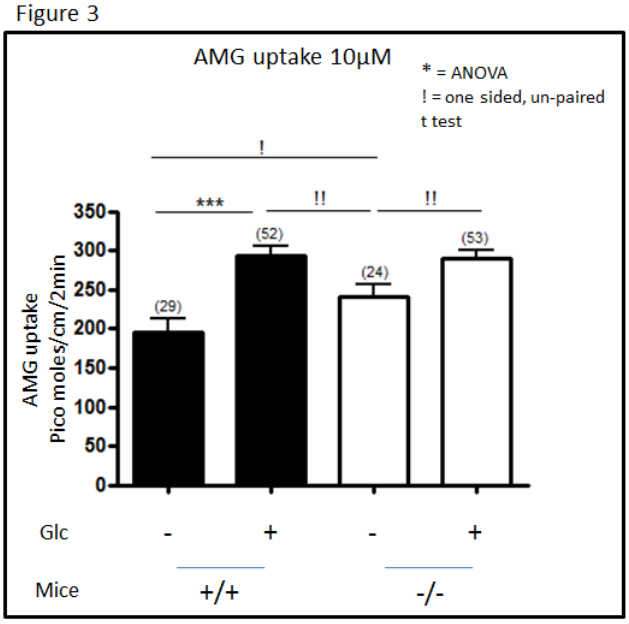

Figure 3: Glucose alters SGLT1 activity: Wild type (▮) and RS1-/- mice (▯) were starved for 24 hours prior to measuring SGLT1 dependent uptake. Phlorizin inhibited uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured for 2 minutes in everted segments of mice jejunum after pre-incubating the segment in buffer or 5 mM glucose for 1 hour. Radioactivity in the segments was measured and was calculated in terms of picomoles/cm/2 min. RS1-/- mice showed elevated AMG uptake in comparison to wild type mice (P=0.0294) by 1 sided, un-paired t test, marked by exclamation (!). Glucose effect in wild type mice was highly significant (p<0.001) by ANOVA test, indicated by asterisk (*). Glucose effect in RS1-/- mice was slightly significant (P = 0.0116). The SGLT1 activity observed in RS1-/- mice in absence of glucose was smaller compared to activity in wild type mice in presence of glucose (P = 0.0047).

4.2 Effect of glucose and RS1 on SGLT1 affinity:

To determine whether the up-regulation of SGLT1 activity observed after removal of RS1 and/or after pre-incubation with glucose include changes of SGLT1 affinity, the substrate dependence of SGLT1 mediated AMG uptake in isolated BBMVs was measured. Pre-incubation of everted small intestine segments for 1 hour led to decreased SGLT1 activity but did not change the Michaelis Menten (K_m) values for AMG uptake into BBMV (Appendix I and II). Hence K_m values for AMG uptake were measured using BBMVs prepared from small intestines of mice which had been either pre-incubated for 1 hour with 5 mM D-glucose or were not pre-incubated. AMG uptake measurements for 2 seconds were performed at AMG concentrations (0.02 – 7.14 mM) containing tracer amount of [^{14}C] AMG in the presence and

absence of 100 mM sodium. Based on the obtained sodium dependent uptake rates, the K_m values were calculated. The data presented in Figure 4A and 4B indicate similar K_m values for AMG uptake in RS1-/- mice compared to wild type mice. They showed that the K_m values in RS1-/- and wild type mice were significantly increased when the small intestines had been pre-incubated with D-glucose. This indicated that the glucose dependent up-regulation of SGLT1 in BBM is correlated with a decrease in affinity which appears to be independent of RS1. Thus my data suggested a RS1 independent strong glucose induced increase of SGLT1 in the BBM.

Figure 4 A

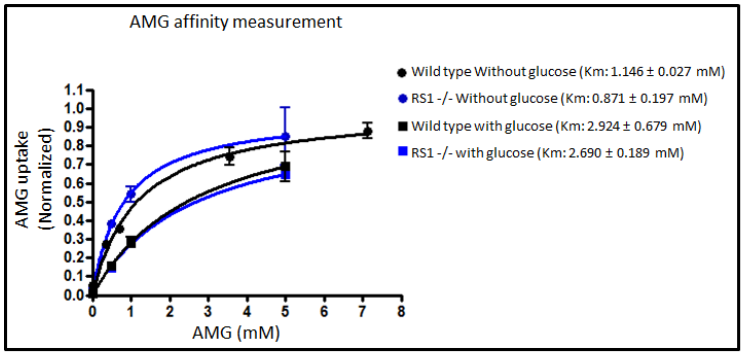

Figure 4 B

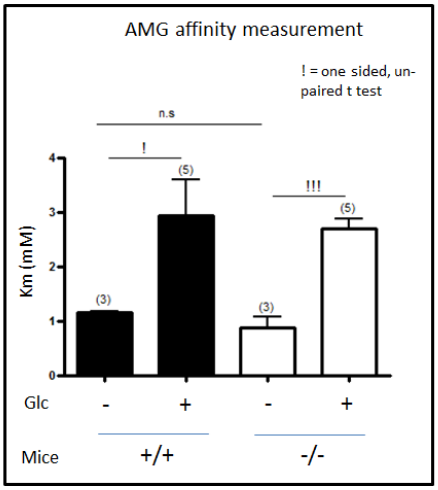

Figure 4: Glucose alters SGLT1 affinity independent of RS1: Wild type and RS1-/- mice were starved for 24 hours prior to BBMV preparation. Sodium dependent uptake measurements were performed for 2 sec in BBMVs incubated in Na^+ containing buffer and K^+ containing buffer at different radioactive AMG concentrations (0.02 – 7.14 mM). Radioactivity measured for uptake was calculated in picomoles/mg/2 seconds. The values obtained were analysed using Michael-Menten equation to determine the K_m value. Figure 4A represents the sodium-dependent uptake rates of individual experiments that were normalized to the respective Vmax values. Mean ± standard error values from independent experiments are shown. Figure 4B represents the comparison between K_m values for sodium-dependent AMG uptake into BBMVs. Significant difference was observed between K_m values after pre-incubation with glucose and no pre-incubation in wild type (P=0.0485) and RS1-/- mice (P=0.0004) by 1 sided, un-paired t test (!). The SGLT1 affinity in wild type and RS1-/- mice did not differ (P=0.1202) by 1 sided, un-paired t test.

4.3 Effect of regulatory tripeptides (QSP and QEP) on glucose dependent short term regulation of SGLT1:

In small intestine, RS1 down-regulates the amount of SGLT1 in the BBM if the glucose concentration in small intestine lumen is low. In the presence of high glucose concentration in the intestine lumen, the RS1 dependent down-regulation is reduced, resulting in a higher

expression of SGLT1 (H.Kipp and H. Koepsell, unpublished data) . The TGN-Reg domain of human RS1 which mediates post-transcriptional down-regulation contains two copies of the active motif - QSP (figure 2). The data of Prashanth R Reddy and Alexandra Friedrich suggest that QSP and its phosphorylation mimicking variant QEP do not down-regulate SGLT1 in small intestine of wild type mice when the glucose concentration in the lumen is low, because the endogenous RS1 is effective under this circumstance (Prashanth Reddy, Alexandra Friedrich, Hermann Koepsell, unpublished data). Experiments of Maike Veyhl-Wichmann indicate that the affinity of QEP to down-regulate human SGLT1 expressed in oocytes of *Xenopus laevis* is higher, compared to QSP (M. Vehyl-Wichmann, Prashanth R. Reddy, A. Friedrich and H. Koepsell, unpublished data). In figure 5 I investigated whether the SGLT1 mediated glucose transport in small intestine of wild type and RS1-/- mice was down-regulated after pre-incubation without and with 5 mM D-glucose without or with 15 mM QSP. Phlorizin inhibited uptake of 10 µM AMG was measured in everted segments of mice small intestine. The measurements were normalized to the uptake after pre-incubation of small intestine everted segments of wild type mice or RS1-/- mice in buffer (Figure 5A and 5B) or D-glucose (Figure 5C and 5D). The mean ± standard error values in picomoles/cm/2 min used for normalization were: wild type mice pre-incubation without glucose 291.9 ± 15.67, RS1-/- mice pre-incubation without glucose 296.2 ± 13.47, wild type mice pre-incubation with glucose 324.5 ± 12.02, RS1-/- mice pre-incubation with glucose 354.2 ± 36.69. The results indicated a border line significance for glucose dependent up-regulation in wild type ($P = 0.1843$) and RS1-/- mice ($P = 0.0728$) (data not shown) which suggested the glucose dependent up-regulation of SGLT1 to be independent of RS1. The data represented in Figure 5 indicated significant down-regulation of SGLT1 dependent AMG uptake in wild type and RS1-/- mice after pre-incubation with 15 mM QSP in the absence as well as in the presence of 5 mM D-glucose. The effect observed in wild type mice in the absence of glucose was unexpected (see discussion).

Figure 5 A

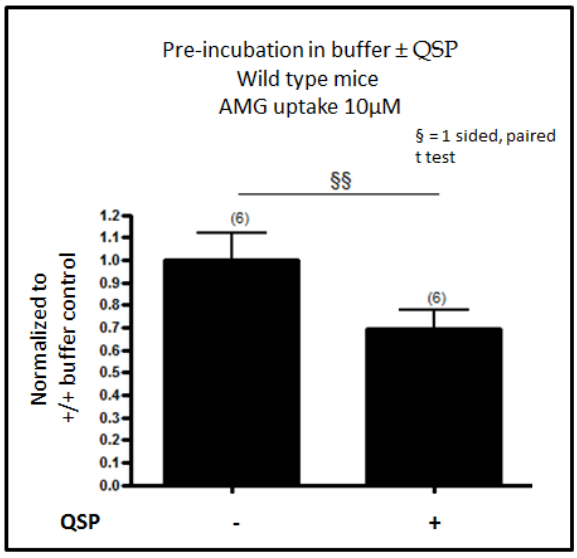

Figure 5 B

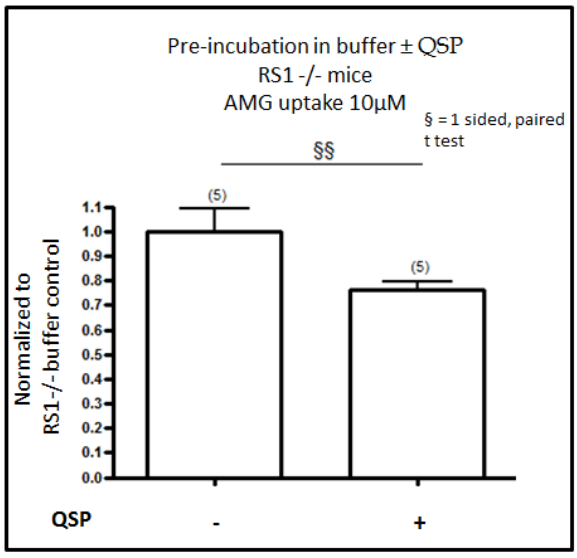

Figure 5 C

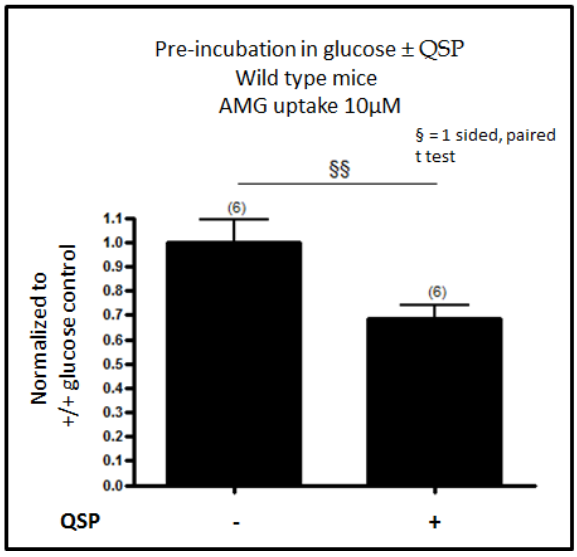

Figure 5 D

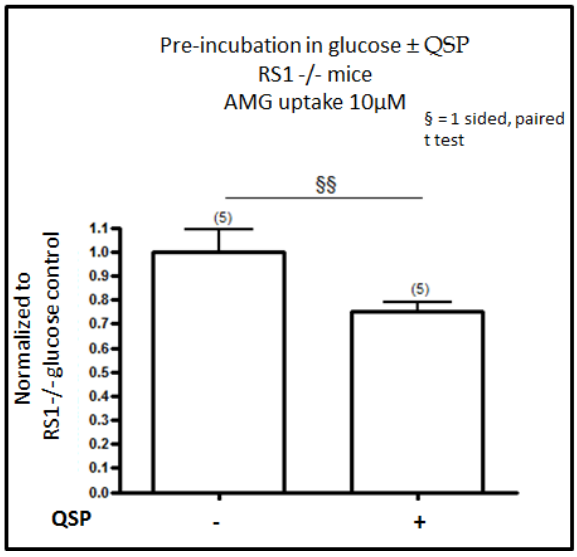

Figure 5: QSP at 15 mM concentration mediated down regulation of SGLT1 activity independent of glucose: Wild type and RS1-/- mice were starved for 24 hours prior to measuring SGLT1 dependent uptake. Phlorizin inhibited uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured for 2 minutes in everted jejunum segments of wild type mice (Figure5 A and C) and RS1-/- mice (Figure5 B and D). The segments were pre-incubated in buffer (Figure5 A and B) or in glucose (Figure5 C and D) with or without 15 mM QSP for 1 hour. Radioactivity was calculated in terms of Picomoles/cm/2 min and normalized to wild type or RS1-/- mice in buffer or glucose control respectively. QSP significantly down-regulated AMG uptake in wild type and RS1-/- mice independent of glucose ($P < 0.01$ by 1 sided paired t test, marked by section sign (§)).

Since QEP has a higher affinity than QSP to post-transcriptionally down-regulate human SGLT1 expressed in oocytes of *Xenopus laevis*, I investigated whether the down-regulation of SGLT1 in small intestine of wild type mice after pre-incubation in 5 mM D-glucose with 15 mM QEP was more pronounced compared to 15 mM QSP (Figure 6). Only wild type mice

were considered for this measurement as QSP down regulated SGLT1 in wild type and RS1-/- mice in the presence of 5 mM D-glucose (Figure 5 C and 5 D). The phlorizin inhibited 10 µM AMG uptake measurements were normalized to pre-incubation in glucose condition of wild type mice in the absence of tripeptides (Figure 6). The mean ± standard error values in picomoles/cm/2 min used for normalization of wild type mice segments pre-incubated with glucose was 293.9 ± 11.36. The results showed that 15 mM QSP and QEP significantly down-regulated SGLT1 dependent AMG uptake in wild type mice. No significant difference was observed between QSP and QEP mediated down regulation in wild type mice (P =0.3426, 1 sided un-paired t test). This indicated that QSP and QEP were equally effective at the employed concentration and experimental conditions.

Figure 6

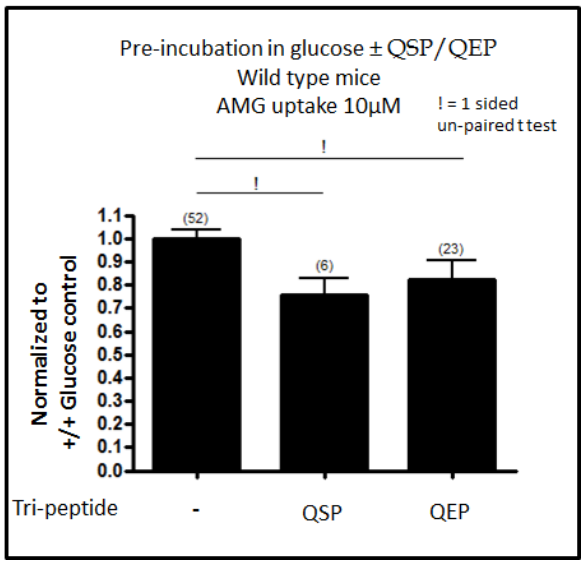

Figure 6: QSP and QEP at 15 mM concentration are equipotent in mediating down regulation of SGLT1: Wild type mice were starved for 24 hours prior to performing SGLT1 dependent AMG uptake. Phlorizin inhibited uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured for 2 minutes in everted jejunum segments of wild type mice after pre-incubating the segments in glucose with or without 15 mM concentration of tripeptide for 1 hour. Radioactivity was calculated in terms of Picomoles/cm/2 min and normalized to mice glucose control. QSP and QEP down regulate AMG uptake with a significance of (P = 0.0212) and (P = 0.0155) respectively by 1 sided un-paired t test marked by exclamation mark (!).

4.4 The role of PKC in RS1 dependent short term regulation of SGLT1:

PKC activation is involved in the post-transcriptional short term regulation of SGLT1 (Wright, Hirsch et al. 1997, Vayro and Silverman 1999, Castaneda-Sceppa, Subramanian et al. 2010). Previously, data were obtained showing that the activation of PKC influences the regulation of total RS1 protein on the human SGLT1 expressed in *Xenopus laevis* oocytes (Veyhl, Wagner et al. 2003, Veyhl, Keller et al. 2006). Since the TGN-Reg domain of mouse RS1 contains many putative phosphorylation sites for protein kinases including PKC (Figure 2), the previously observed effect of PKC activation could be due to activation of the TGN-Reg by phosphorylation. To investigate whether the short term down-regulation by RS1 on SGLT1 is PKC dependent in mouse small intestine, the effect of PKC activation on SGLT1 dependent AMG uptake was performed in wild type and RS1-/- mice. Everted segments of wild type and RS1-/- mice small intestine were pre-incubated for 1 hour in buffer or 5 mM D-glucose with or without 5 µM Phorbol 12-myristate 13-acetate (PMA), an activator of PKC (Castagna, Takai et al. 1982)). The phlorizin inhibited AMG uptake was measured in these everted segments. The measurements were normalized to pre-incubation in buffer (Figure 7A and B) or glucose (Figure 7C and D) condition of wild type (Figure 7A and C) and RS1-/- (Figure 7B and D) mice respectively. The mean ± standard error values in picomoles/cm/2 min used for normalization were: wild type mice pre-incubation without glucose 206.1 ± 26.52, RS1-/- mice pre-incubation without glucose 276.7 ± 27.39, wild type mice pre-incubation with glucose 295.0 ± 12.29, RS1-/- mice pre-incubation with glucose 296.6 ± 12.75. The data (Figure 7) showed a 20-30% down-regulation of SGLT1 dependent AMG uptake in wild type and RS1-/- mice after pre-incubation with 5 µM PMA which was independent of pre-incubation with 5 mM D-glucose. This indicated a PKC dependent short term down-regulation of SGLT1 which appeared to be independent of RS1 as well as glucose.

Figure 7 A

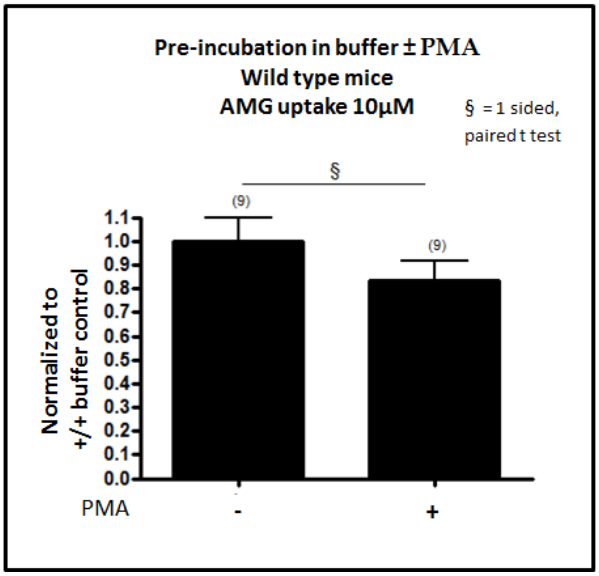

Figure 7 B

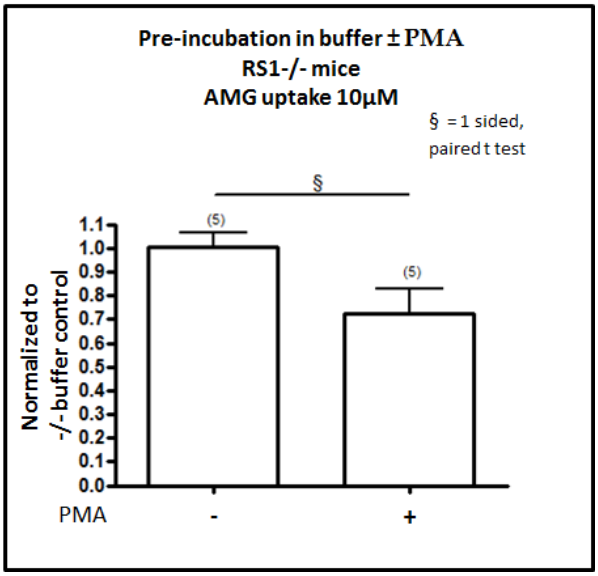

Figure 7 C

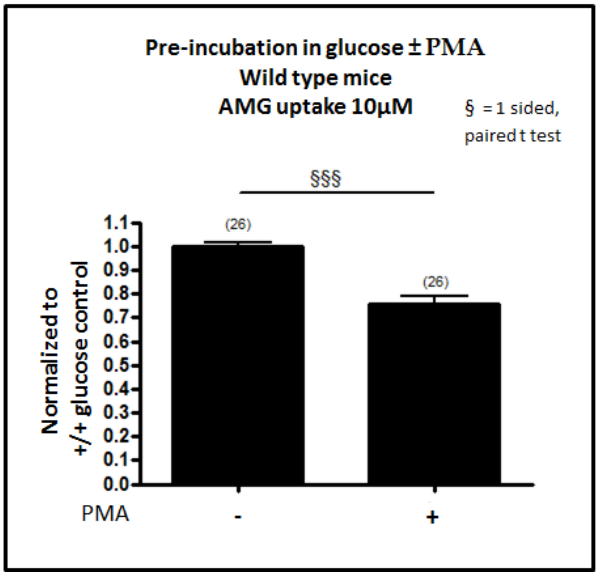

Figure 7 D

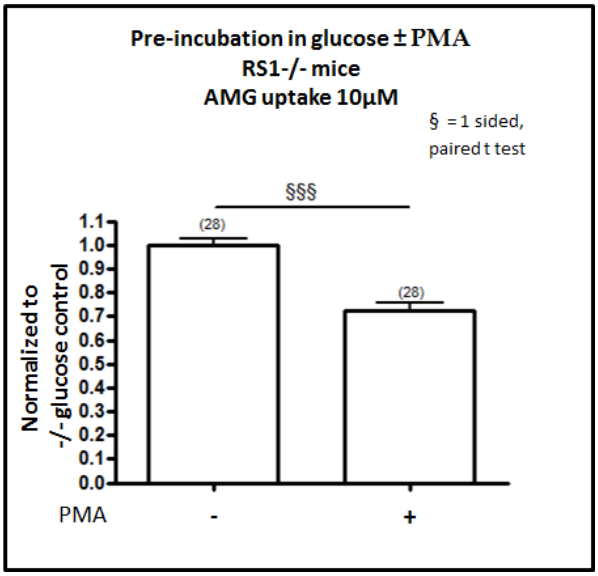

Figure 7: PKC down-regulated SGLT1 activity independent of glucose and RS1: Wild type and RS1-/- mice were starved for 24 hours prior to performing SGLT1 dependent AMG uptake. Phlorizin inhibited uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured for 2 minutes in everted jejunum segments of wild type mice (Figure 7A and 7C) and RS1-/- mice (Figure 7B and 7D) after pre-incubating the segment in buffer (Figure 7A and 7B) or glucose (Figure 7C and 7D) with or without 5 µM PMA for 1 hour. Radioactivity was calculated in terms of Picomoles/cm/2 min and normalized to wild type or RS1-/- mice in buffer or glucose control respectively. PKC when incubated with or without glucose was able to significantly down- regulate SGLT1 activity in wild type and RS1 -/- mice (§).

If short term down-regulation via RS1 and PKC are mediated by independent mechanisms, then the down-regulation of SGLT1 by both together should be additive. To test this I studied additive effects of QEP and PKC activation in everted segments of small intestine of wild

type and RS1-/- mice. Employing only the TGN-Reg modified motif QEP, the effect of PKC on the entire TGN-Reg domain was excluded. The measurements were performed in the presence of 5 mM D-glucose, as the endogenous RS1 in wild type mice is expected to be less active under this condition. Everted segments of mice jejunum were pre-incubated in 5 mM D-glucose either in the presence of 15 mM QEP or 5 µM PMA alone or QEP with PMA for 1 hour. The experiments were performed in wild type mice (Figure 8 A) and RS1-/- mice (Figure 8 B). Phlorizin inhibited uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured. The data were normalized to control AMG uptake after pre-incubation with D-glucose without QEP and PMA in wild type and RS1-/- mice respectively. The mean ± standard error values in picomoles/cm/2 min used for normalization were: wild type mice pre-incubation with glucose 293.9 ± 11.36 and RS1-/- mice pre-incubation with glucose 289.5 ± 10.45. The data showed no additive inhibition of SGLT1 activity mediated by PMA with QEP over QEP or PMA alone in wild type and RS1-/- mice. Since PMA inhibition was independent of RS1 (figure 7), the short term down-regulation of SGLT1 by RS1 and PKC suggested independent phenomenon for the same pathway of inhibition.

Figure 8 A

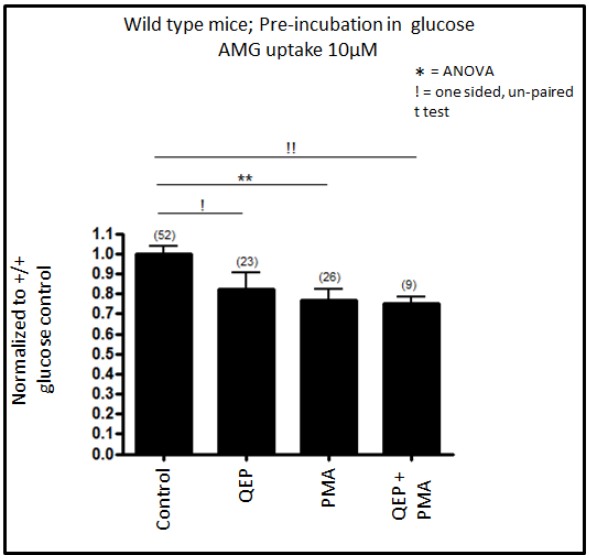

Figure 8 B

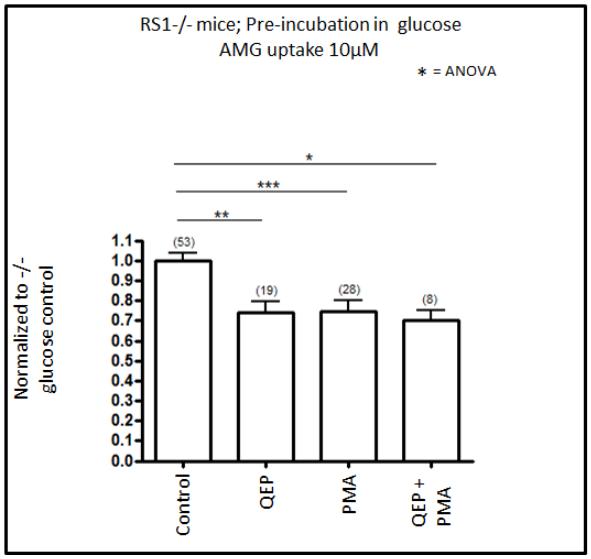

Figure 8: PKC has no additive effect on QEP dependent short term regulation of SGLT1: Wild type and RS1-/- mice were starved for 24 hours prior to performing SGLT1 dependent AMG uptake. Phlorizin inhibited uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured for 2 minutes in everted jejunum segments of wild type mice (Figure 8 A) and RS1-/- mice (Figure 8 B) after pre-incubating the segment in glucose with or without QEP, PMA and QEP + PMA for 1 hour. Radioactivity was calculated in terms of Picomoles/cm/2 min and normalized to wild type or RS1-/- mice in glucose control respectively. QEP with PMA showed no additive inhibition to QEP or PMA alone in wild type and RS1-/- mice.

4.5 Effect of PKC on SGLT1 affinity:

Trying to elucidate how PKC activation down-regulates SGLT1 mediated AMG uptake in small intestinal brush border membranes (BBM), we wondered whether the Michaelis Menten (K_m) value for SGLT1 dependent AMG uptake was altered. The *Km* value of sodium dependent AMG uptake was measured in brush border membrane vesicles (BBMVs) which were isolated from small intestine of RS1-/- mice pre-incubated without or with 5 mM D-glucose. BBMVs isolated from small intestine of RS1-/- mice were used because PKC effect appeared to be independent of RS1 (Figure 7). In these experimental series the pre-incubation of small intestine in the absence of glucose was performed in the presence of 9 mM sodium pyruvate for trying to avoid intracellular ATP depletion. Control experiments had suggested that sodium pyruvate exhibited some protection against SGLT1 inactivation during the pre-incubation period (Appendix I) without altering the K_m value for SGLT1 dependent AMG uptake (Appendix II). Small intestines were pre-incubated for 1 hour with 9 mM sodium pyruvate or 5 mM D-glucose with or without 5 µM PMA and BBMVs were isolated. Sodium dependent AMG uptake measurements were performed at AMG concentrations (0.02 – 5 mM) containing tracer amount of [^{14}C] AMG using an incubation time of 2 seconds. Uptake rates per mg protein were calculated (picomoles/mg/2 seconds).

K_m values were determined by fitting the Michael-Menten equation to the uptake rates obtained (Figure 9). The data indicated that the K_m value for SGLT1 mediated AMG uptake was decreased when PKC was active. In my experiment this effect was significant in the presence of glucose. Noteworthy the decrease of K_m after stimulation of PKC resulted in decrease of 10 µM AMG uptake mediated by SGLT1. Thus the data suggested that PKC stimulation might affect either the turnover number or amount of functional active SGLT1 at the BBM.

Figure 9 A

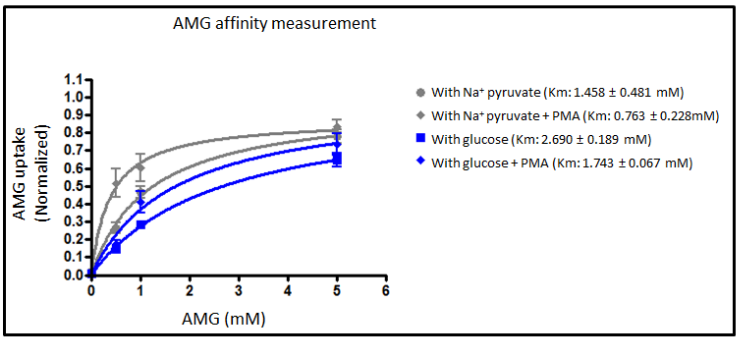

Figure 9 B

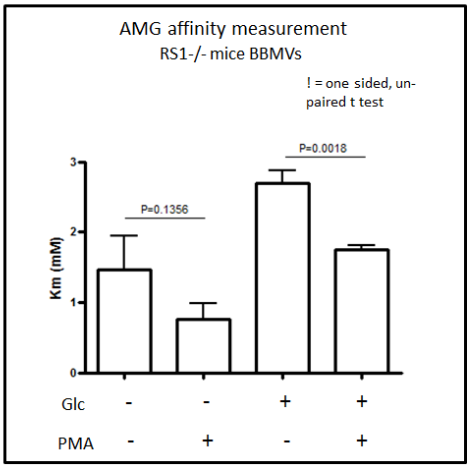

Figure 9: PKC altered SGLT1 affinity: Wild type and RS1-/- mice were starved for 24 hours prior to BBMV preparation. BBMVs were prepared after pre-incubation in 5 mM glucose or 9 mM sodium pyruvate with or without 5µM PMA for 1 hour. Sodium dependent uptake measurements were performed for 2 sec in BBMVs incubated in Na^+ containing buffer and K^+ containing buffer at different radioactive AMG concentrations (0.02 – 5 mM). Figure 9A represents the sodium-dependent uptake rates of individual experiments that were normalized to the respective Vmax values. Mean ± standard error values from independent experiments are shown. Figure 9B represents the comparison between K_m values for sodium-dependent AMG uptake into BBMVs. Border line significance was observed for the difference between K_m values of BBMVs prepared with pre-incubation with sodium pyruvate with or without PMA, P = 0.1356, 1 sided un-paired t test. Significant difference was observed between K_m values of BBMVs prepared with pre-incubation with glucose in presence or absence of PMA, P = 0.0019, 1 sided un-paired t test (!).

4.6 Effect of RS1 on SGLT1 activity during diet adaptation:

Diet dependent long term up-regulation of SGLT1 in small intestine increases the capacity for glucose absorption during high carbohydrate content in food (Shirazi-Beechey, Hirayama et al. 1991, Moran, Al-Rammahi et al. 2010). This glucose dependent regulation is influenced by types of ingested carbohydrate and is influenced by extrinsic factors (Luz Sdos, de Campos et al. 1997). Because RS1 is involved in the transcriptional regulation of SGLT1 and preliminary data suggest RS1 to influence SGLT1 regulation in mouse small intestine in response to hyper-caloric, fat rich but glucose-galactose reduced diet (GGRD, see Table 1) (Filatova, Leyerer et al. 2009), we compared the transport activity in different small intestinal segments of wild type and RS1-/- mice that were kept for 2 months on normal diet (ND), GGRD and high fat glucose rich diet (HFHGD). ND is normo-caloric and possesses high polysaccharide and low fat content while HFHGD is hyper-caloric and contains high monosaccharide and high fat content. The compositions and calorie contents of the three diets are shown in Table 1 and 2. After diet application, the mice were starved for 24 hours and the

phlorizin inhibited uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured in everted small intestinal segments taken from different parts of small intestine. These measurements were performed without pre-incubation of the everted small intestinal segments. The data were normalized to duodenal SGLT1 activity for AMG uptake in wild type and RS1-/- mice respectively. The mean ± standard error values in picomoles/cm/2 min used for normalization were: duodenum uptake in wild type mice fed with ND 370.7 ± 18.25 and duodenum uptake in RS1-/- mice fed with ND 320 ± 19.36.

The results are shown in Figure 10. Independent of diet in wild type and RS1-/- mice significant low activity was observed in ileum compared to duodenum and jejunum. In jejunum of wild type and RS1-/- mice, SGLT1 mediated uptake of 10 µM AMG in animals kept on hyper-caloric, fat rich diet (HFHGD and GGRD) were significantly reduced compared to animals kept on normo-caloric, low fat diet (ND). Since this effect was observed independent of the glucose or galactose content in diet, the effect might depend on the fat or total calorie content in diet. This fat dependent down-regulation was apparently also independent of RS1 as it was also observed in RS1-/- mice. In duodenum of wild type mice significant lower transport of SGLT1 was observed when mice were fed glucose-galactose reduced, fat rich diet (GGRD) compared to ND. This effect was absent in RS1-/- mice with different diet. Thus a glucose dependent long term regulation in duodenum was seen which was dependent on RS1.

Figure 10 A

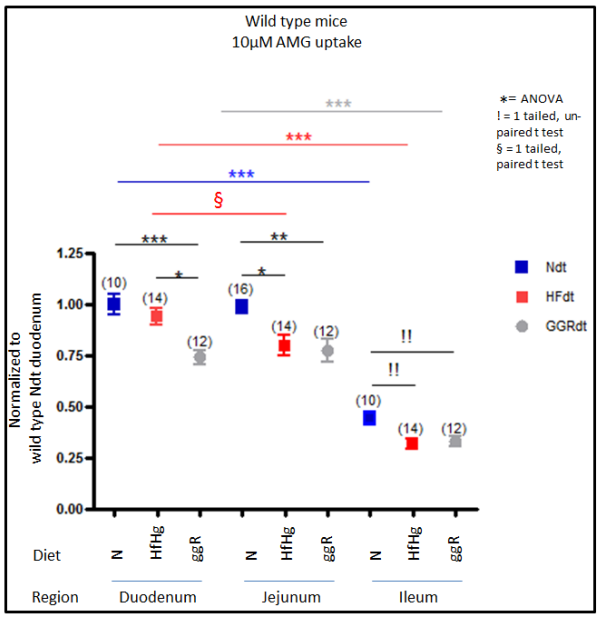

Figure 10 B

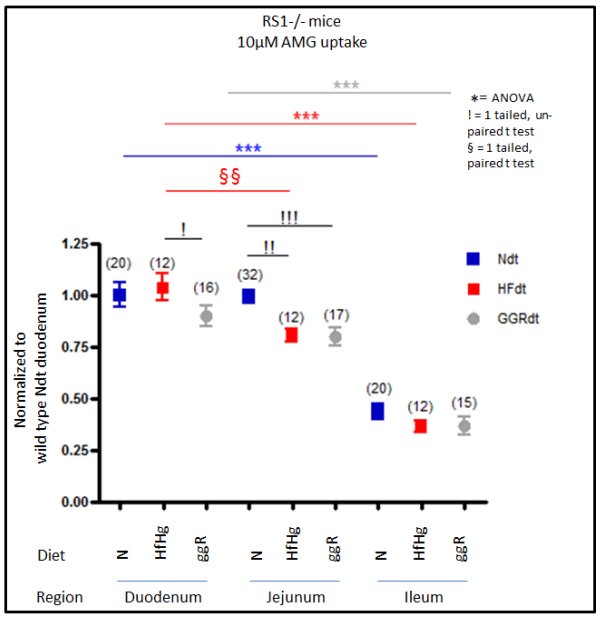

Figure 10: Long term regulation of SGLT1 in small intestine to dietary glucose and fat content shows region specific RS1 dependence: Wild type and RS1-/- mice were fed with different diet for 2 months. They were starved for 24 hours prior to performing SGLT1 dependent AMG uptake. Phlorizin inhibited uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured for 2 minutes in everted segments of different small intestine regions in wild type (Fig 10 A) and RS1-/- (Fig 10 B) mice. Radioactivity of AMG uptake was calculated in terms of Picomoles/cm/2 min and normalized to respective mice normal diet (ND) duodenum. Ileum in wild type and RS1-/- mice significantly showed less SGLT1 activity in all diets (P<0.001) by ANOVA (*). Wild type and RS1-/- mice fed with high fat glucose rich diet (HFHGD) showed regional difference between jejunum and duodenum (P = 0.0169 & 0.0024 respectively) by 1 tailed paired t test (§). Jejunum region of ND in wild type and RS1-/- mice showed significant difference to mice fed with HFHGD with (P<0.05) by ANOVA, (*) and (P = 0.0009) by un-paired t test (!). Jejunum region of ND in wild type and RS1 -/- mice showed significant difference to mice fed with GGRD with (P<0.01) by ANOVA (*) and (P = 0.0004) by un-paired t test (!). RS1-/- mice did not show significant difference in SGLT1 activity between ND and diet with low glucose and galactose concentration (GGRD) in duodenum (P=0.1046) and ileum (P=0.1289) by un-paired t test.

We also investigated whether the K_m values of AMG uptake measured in BBMVs prepared from mice kept on ND, HFHGD and GGRD. The results indicated a borderline significant higher K_m value for AMG in small intestine of RS1-/- mice that were kept on HFHGD which was not detected in wild type mice (Appendix VII). Since this observation may not reflect a relevant difference and was difficult to interpret it was not investigated further.

5. Discussion

In this study, data was presented for a PKC dependent short term down-regulation of SGLT1 function in the small intestine of mice which is independent of RS1. It is suggested that this effect may be due to PKC dependent phosphorylation of SGLT1. A second finding of this study is the observation that SGLT1 transport activity in jejunum is down-regulated independently of RS1 for long term adaptation to high fat containing diet. Thirdly it is observed that substrate transport by SGLT1 in duodenum is down-regulated in an RS1 dependent manner after reduction in glucose and galactose content from the diet.

I also obtained some data concerning the role of RS1 in SGLT1 regulation where absence of RS1 led to enhanced activity of SGLT1 (Figure 3). However this data showed relatively less enhanced effect in comparison to results previously demonstrated by other colleagues. Furthermore I also obtained some data concerning the role of RS1 in glucose dependent short term regulation of SGLT1 which contradicted the results obtained by colleagues in the laboratory, who used a different experimental approach. Dr. Helmut Kipp measured phlorizin inhibited AMG uptake in brush border membrane vesicles isolated from wild type mice small intestine after application of a 2.2 M glucose gavage *in vivo* or after incubation for 2 minutes of the small intestine with 20 mM AMG. He observed increased SGLT1 protein in the BBM and 2-4 times increase in SGLT1 mediated AMG uptake. In his experiments the RS1-/- mice was shown to already have an enhanced SGLT1 expression and no further increase in SGLT1 activity or protein was observed with increased glucose luminal concentration. Performing AMG uptake measurements in everted small intestinal segments of small intestine after pre-incubation in the absence or presence of 5 mM D-glucose I observed a relative small glucose dependent increase of AMG uptake in wild type mice as well as in RS1-/- suggesting an RS1 independent short term up-regulation by glucose. These differences in results may be explained on the basis of the different methods employed for SGLT1 dependent uptake measurements. Since Dr. Kipp detected that glucose dependent up-regulation in small intestine occurs very rapidly and could be observed after 2 minutes incubation period, it is probable that the up-regulation by 5 mM D-glucose in my experiments were reversed during the uptake measurements in the everted segments which were performed using an incubation period of 2 minutes. A second difficultly was that the transport activity of SGLT1 in small intestine was decreased during 1 hour pre-incubation period, which was employed to study the short term effects on SGLT1 regulation (Appendix I). Thus these different degrees of

inactivation during preincubation with and without glucose may have been determined in my experiments rather than the glucose dependent regulation. The inactivation by pre-incubation could have also altered the enhancement in SGLT1 activity of RS1-/- mice leading to the resultant small enhancement observed (Figure 3). Another reason for the differences in results could also be the difference in concentration of glucose applied for understanding its effect in short term regulation of SGLT1. Dr. Kipp employed a concentration of 20 mM and about 2 M to study the glucose dependent regulation of SGLT1. In my experiments I performed the study with only 5 mM D-glucose. Hence the decreased effect in the level of rise of SGLT1 activity for glucose dependent short term regulation may be dependent on the concentration of glucose along with the effect of inactivation during pre-incubation. Due to these limitations the further study to elucidate the effect of RS1 in short term regulation of SGLT1 were performed by employing the active motif QSP and its phosphorylation mimicking mutant QEP. QSP belongs to the N-terminal TGN-Reg domain of RS1 and mediate post-transcriptional down-regulation of SGLT1 (Vernaleken, Veyhl et al. 2007). Although QSP did mediate short term down-regulation of SGLT1, this down-regulation was independent of glucose and endogenous RS1 (Figure 5). This glucose independent down-regulation of SGLT1 by QSP could be explained in terms of concentration. 15 mM concentration of QSP might be sufficient to inhibit the up-regulation of SGLT1 by 5 mM glucose. However the down-regulation of QSP to be effective in absence of glucose, which is a condition when endogenous RS1 is active, can be explained by different reasons. It could be stated that the SGLT1 activity is not completely down-regulated by endogenous RS1 and additive 15 mM QSP aids in further inhibition. Another possible explanation could be the experimental conditions. Mice were starved for 24 hours prior to uptake measurements. Starvation has been shown to lower affinity of SGLT1 and increase its uptake activity (Marciani, Lindi et al. 1987, Gupta and Waheed 1992, Gal-Garber, Mabjeesh et al. 2000). Hence the starvation of mice might have elevated SGLT1 activity that could have allowed QSP to mediate down-regulation on AMG transport even under a condition when endogenous RS1 is expected to be in an active state.

The glucose dependent short term regulation of SGLT1 also involves increasing K_m value for SGLT1 dependent AMG uptake (Figure 4). However RS1 did not affect the affinity for SGLT1 dependent AMG uptake (Figure 4). Hence it can be said that the glucose effect on SGLT1 affinity is independent of RS1. The SGLT1 affinity was also altered on PKC activation (Figure 9), where the K_m value for SGLT1 mediated AMG transport is decreased

about 2 fold. In addition the PKC dependent short term regulation of SGLT1 occurred in an RS1 independent manner (Figure 7). But the observation of no additive down-regulation of SGLT1 when QEP was used with PKC activation (Figure 8) indicated that PKC and RS1 might act independently within the same pathway to down-regulate SGLT1. RS1 is known to mediate short term regulation of SGLT1 post-transcriptionally by inhibiting the trafficking of vesicles containing SGLT1 to BBM (Veyhl, Keller et al. 2006). PKA and PKC are involved in regulating SGLT1 by altering exo-endocytosis of SGLT1 carrying vesicles to BBM (Wright, Hirsch et al. 1997). Protein kinases like PKA has also been reported to mediate short term regulation of SGLT1 by causing SGLT1 phosphorylation to change its affinity for substrate transport (Subramanian, Glitz et al. 2009). The SGLT1 sequence also has several putative phosphorylation sites for PKC (Hirsch, Loo et al. 1996). Hence the possible mechanism for PKC to mediate an increase in SGLT1 affinity and down-regulate SGLT1 activity for glucose transport might involve the transporter phosphorylation that would affect post-transcriptionally SGLT1 turn over number and/or amount in the BBM.

Based on the preliminary data which stated that RS1 is involved in the transcriptional regulation of SGLT1 for adaptation to glucose-galactose reduced diet (Filatova, Leyerer et al. 2009) further data was obtained in this study to understand the dependence of RS1 in long term regulation of SGLT1 in small intestine for adaptation to diets different in glucose and fat content. The data obtained (Figure 10) showed the glucose dependent down-regulation of SGLT1 transport in duodenum of wild type mice for adaptation to glucose-galactose reduced diet (GGRD). The possible explanation for SGLT1 down regulation with low carbohydrate diet could be explained in terms of biosynthetic cost. In GGRD the cost of producing transporter might exceed the benefits of its activity. While on the other hand with high carbohydrate diet, energy synthesis would increase with increased absorption hence allowing increase in SGLT1 transporter activity (Ferraris and Diamond 1997). In support to this theory of biosynthetic cost, it was observed that the SGLT1 activity in wild type mice duodenum did not differ between polysaccharide rich (ND) or monosaccharide rich (HFHGD) diets. However this glucose dependent down-regulation of SGLT1 transport in duodenum was absent in RS1-/- mice. Hence the RS1 dependent long term down-regulation of SGLT1 for diet glucose adaptation was shown to be localized to duodenum region of small intestine.

It was also observed that in the jejunum the SGLT1 activity was decreased in wild type and RS1-/- mice fed with fat rich diets (GGRD and HFHGD) when compared to the jejunal SGLT1 activity in mice fed with normal diet (ND) (Figure 10). This indicated a fat dependent

but RS1 independent long term down-regulation of SGLT1. Studies have reported increased SGLT1 gene expression in jejunum of mice fed with high carbohydrate/fat ratio when compared to low carbohydrate/fat diet (Honma, Mochizuki et al. 2009, Inoue, Mochizuki et al. 2011). Likewise this fat dependent down-regulation of SGLT1 activity could also be dependent on carbohydrate concentration. The HFHGD is a diet rich in simple sugars like monosaccharides and disaccharides. As complexity in carbohydrate availability is known to shift SGLT1 expression from jejunum to ileum due to slow digestion (Shimada, Mochizuki et al. 2009) these simple monosaccharides could have been easily absorbed in duodenum thereby leading to decreased SGLT1 function at jejunum. This effect would also account for the RS1 independent regional difference of SGLT1 activity between duodenum and jejunum region in mice fed with HFHGD. Likewise the decrease in jejunual SGLT1 activity for mice with GGRD could be due to the reduced amount of glucose and galactose in diet. Hence the RS1 independent down-regulation of SGLT1 in mice fed with HFHGD and GGRD could be a fat dependent and a glucose dependent effect.

An RS1 independent and diet independent down-regulation of SGLT1 activity was observed in ileum. This could suggest that the age related long term regulation for prevalence of gradient distribution in SGLT1 transport from proximal to distal region in the small intestine (Toloza and Diamond 1990) is in-differed by dietary composition and RS1.

In conclusion it can be stated that the RS1 dependence for SGLT1 regulation differs with respect to time scale of glucose exposure and is also specific to region in the small intestine. PKC activation was involved in the short term down-regulation of SGLT1 independent of RS1, but may follow the same path of RS1 for causing SGLT1 down-regulation. RS1 mediated the long term down-regulation of SGLT1 specifically in duodenum region of small intestine in a manner dependent on glucose-galactose diet content. RS1 and diet independent long-term regulation of SGLT1 was observed to maintain posterior to distal gradient in SGLT1 function. An RS1 independent but dietary fat and/or glucose content dependent down-regulation of SGLT1 is present in mice jejunum region.

6. Appendix

Appendix I: Tissue pre-incubation alters SGLT1 dependent substrate transport:

In my study SGLT1 regulation was investigated by performing uptake measurement in everted small intestinal segments. These measurements allowed more direct control of the experimental conditions such as luminal glucose concentrations compared to *in vivo* gavage with D-glucose. The study of short term regulation of SGLT1 required pre-incubating the everted segments under required study condition for 1 hour. Pre-incubation of tissue might deprive tissue of energy source leading to decrease in SGLT1 efficiency for transport. In order to understand the effect of pre-incubation on SGLT1 activity phlorizin inhibited uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured in everted segments of wild type mice small intestine were compared without or with pre-incubation under 3 conditions – buffer (no energy resource), sodium pyruvate (energy resource) and glucose (substrate for SGLT1 and an energy resource). The result (Figure 11) showed no significant difference between buffer and sodium pyruvate pre-incubation. Pre-incubation with buffer and sodium pyruvate significantly decreased SGLT1 activity. However glucose showed significantly higher activity than pre-incubation with buffer or sodium pyruvate.

Figure 11

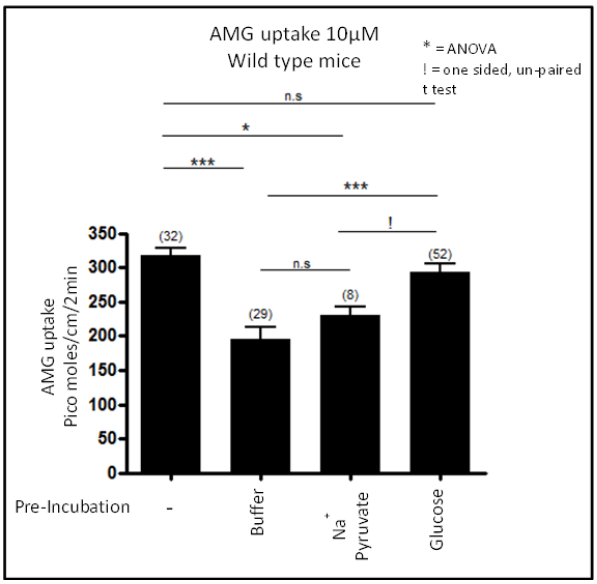

Figure 11: Pre-incubation alters SGLT1 activity: Wild type mice were starved for 24hrs prior to perform SGLT1 dependent AMG uptake. Phlorizin inhibited uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured for 2 minutes in everted jejunum segments of wild type mice either without pre-incubation or after pre-incubating the segment in buffer, sodium pyruvate or glucose for 1hr. Radioactivity of AMG uptake was calculated in terms of Picomoles/cm/2min. Pre-incubation decreases SGLT1 activity in buffer ($P<0.001$) and sodium pyruvate ($P<0.05$), by ANOVA (*).

It can be discussed that though pre-incubation itself alters SGLT1 activity, the significant difference observed between pre-incubation condition of sodium pyruvate and glucose indicated substrate specific upregulation of SGLT1. Hence this method of pre-incubation was considered for the study of glucose dependent SGLT1 regulation despite the limitation in alteration of SGLT1 activity by pre-incubation. Furthermore the presence of the energy source sodium pyruvate failed to completely remove the pre-incubation effect on SGLT1 activity. Hence pre-incubation in sodium pyruvate or buffer either could be used as control condition for glucose effect.

Appendix II: Tissue pre-incubation does not alter SGLT1 affinity:

Pre-incubation was shown to alter SGLT1 activity (Figure 11). Hence it was of interest whether pre-incubation altered SGLT1 affinity for transport. For this the K_m values for AMG uptake were measured using BBMVs prepared from small intestines of RS1-/- mice without and with pre-incubation in sodium pyruvate for 1hr. Radioactive uptake measurements of 2 seconds were made for different AMG concentrations (0.02 – 5 mM) in the presence and absence of 100 mM sodium and the K_m values for sodium dependent uptake rates were calculated. The data represented in Figure 12 showed that pre-incubation does not alter SGLT1 affinity (P = 0.2036).

Figure 12

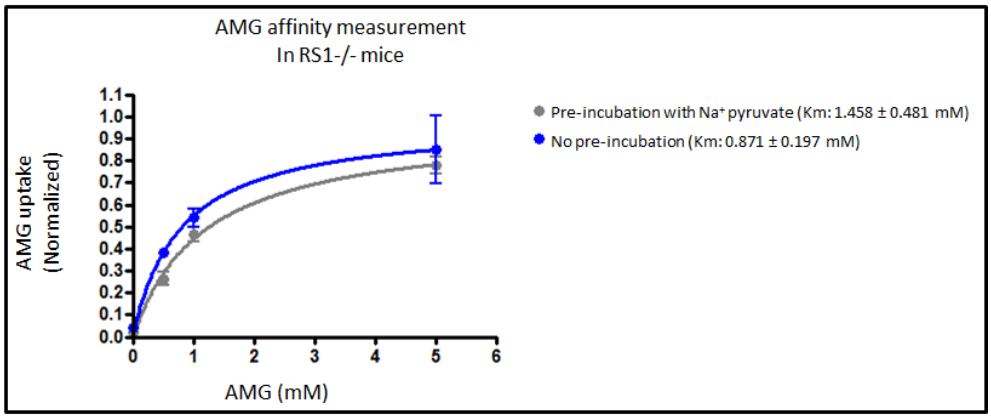

Figure 12: Pre-incubation did not alter SGLT1 affinity: RS1-/- mice were starved for 24 hours prior to BBMV preparation. BBMVs were prepared either with or without pre-incubation in 9 mM sodium pyruvate for 1 hour. Sodium dependent affinity uptake measurements were performed for 2 sec in BBMVs incubated in Na^+ containing buffer and K^+ containing buffer at different radioactive AMG concentrations (0.02 mM – 5 mM). Figure 12 represents the sodium-dependent uptake rates of individual experiments that were normalized to the respective Vmax values. Mean ± standard error values from independent experiments are shown. No significant difference was observed between K_m values of BBMVs prepared without or with pre-incubation in sodium pyruvate, P = 0.2036, 1 sided un-paired t test.

Appendix III: Segmental difference occurs within jejunum region of mice small intestine:

Different segments within the jejunum region of the same mice were considered to perform paired comparisons under different study conditions. However for these comparisons it was necessary to understand the difference within these segments to calculate the actual effect of the desired study condition. Hence phlorizin inhibited uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured within different sections of the mice jejunum region. To overcome the difference within day to day analysis the average of segment 1 within the jejunum region was used to normalize the measurements. The mean ± standard error values in picomoles/cm/2min used for normalization were: wild type mice pre-incubation without glucose 163.5 ± 19.84, RS1-/- mice pre-incubation without glucose 178.3 ± 17.88, wild type mice pre-incubation with glucose 337.9 ± 29.77, RS1-/- mice pre-incubation with glucose 315.2 ± 8.718. The data indicated a border line significant glucose dependent up-regulation in wild type (P = 0.1843) and RS1-/- mice (P = 0.0728). The result (Figure 13) showed the segmental difference observed in the jejunum region of wild type and RS1-/- mice under conditions of pre-incubation in buffer or glucose.

Figure 13 A

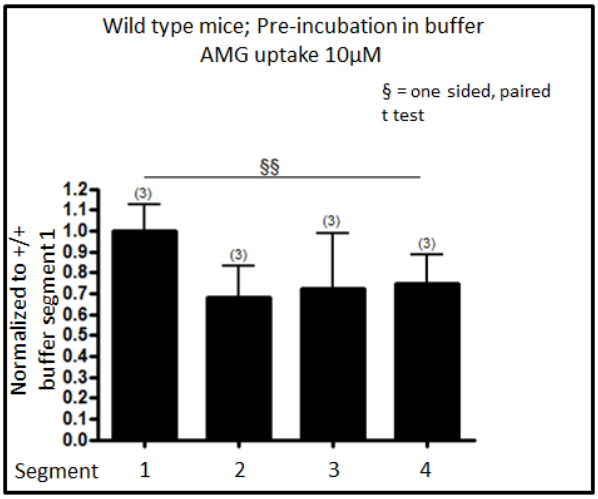

Figure 13 B

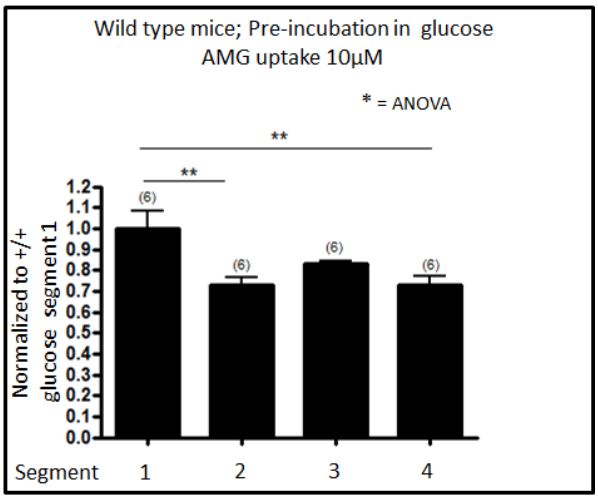

Figure 13 C

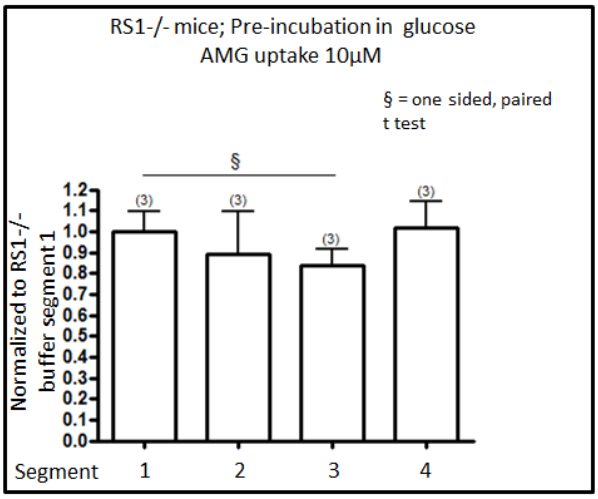

Figure 13 D

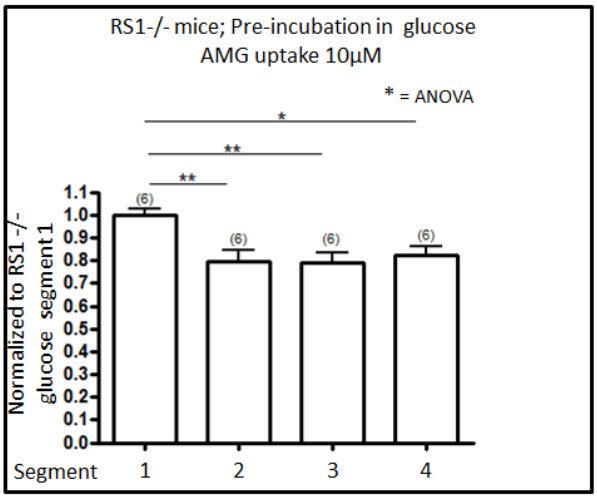

Figure 13: Jejunal region of small intestine shows segmental difference in AMG uptake activity: Wild type and RS1-/- mice were starved for 24hrs prior to perform SGLT1 dependent AMG uptake. Phlorizin inhibited uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured for 2 minutes in everted jejunum segments of wild type (Figure 13 A and B) and RS1-/- (Figure 13 C and D) mice after pre-incubation in buffer (Figure 13 A and C) or glucose (Figure 13 B and D). Radioactivity of AMG uptake was calculated in terms of Picomoles/cm/2min. The values were normalized to the first segment of wild type or RS1-/- mice jejunum in buffer or glucose condition respectively. Differences were analysed by ANOVA (*) or 1 sided paired t test (§) between 4 different segments of jejunum region within the mice small intestine. Significant segmental difference was observed in the region of jejunum in mice small intestine.

The values obtained in the above results for the different segments of jejunum region were used to normalize the respective segment in any study performed.

Appendix IV: Brefeldin A activity is dependent on glucose and RS1:

Brefeldin A is an exocytotic inhibitor. Since post-transcriptional short term regulation of SGLT1 can be mediated by vesicular trafficking of SGLT1 carrying vesicles, the role of Brefeldin A was investigated for short term regulation of SGLT1. For this, phlorizin inhibited uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured in wild type and RS1-/- mice small intestine everted segments after pre-incubation in buffer or glucose with or without Brefeldin A (12µM). The measurements were normalized to uptake after pre-incubation in buffer (Figure 14A and 14B) or glucose (Figure 14C and 14D) condition of wild type (Figure 14A and 14C) and RS1-/- (Figure 14B and 14D) mice respectively. The measurements were normalized to uptake after pre-incubation of small intestine everted segments of wild type mice or RS1-/- mice in buffer (Figure 5A and 5B) or D-glucose (Figure 5C and 5D). The mean ± standard error values in picomoles/cm/2min used for normalization were: wild type mice pre-incubation without glucose 120.6 ± 11.17, RS1-/- mice pre-incubation without glucose 144.3 ± 21.42, wild type mice pre-incubation with glucose 258.7 ± 17.32, RS1-/- mice pre-incubation with glucose 227.2 ± 13.89. The results showed a borderline significant downregulation of SGLT1 dependent AMG uptake in wild type and RS1-/- mice on pre-incubation with Brefeldin A in buffer. However in glucose, pre-incubation with Brefeldin A mediated only a borderline significant down regulation in wild type mice.

Figure 14 A

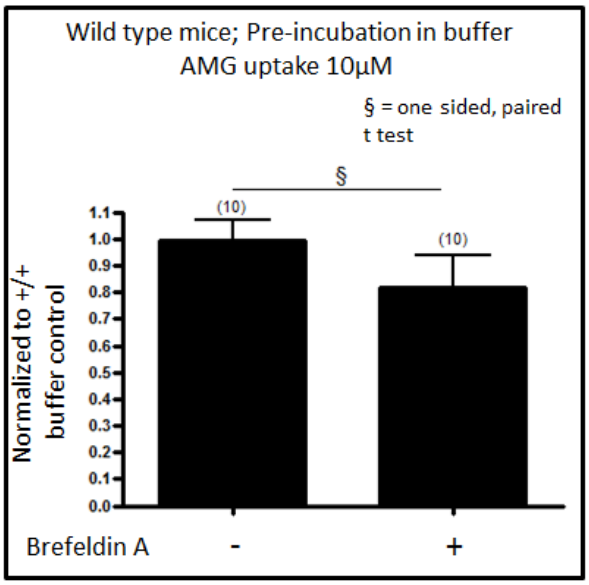

Figure 14 B

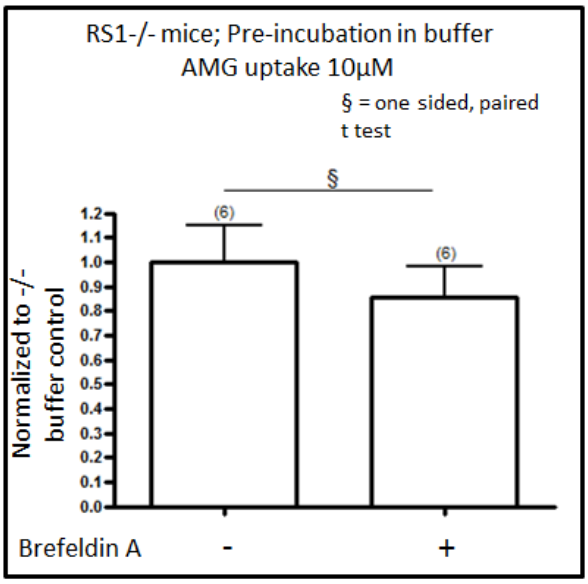

Figure 14 C

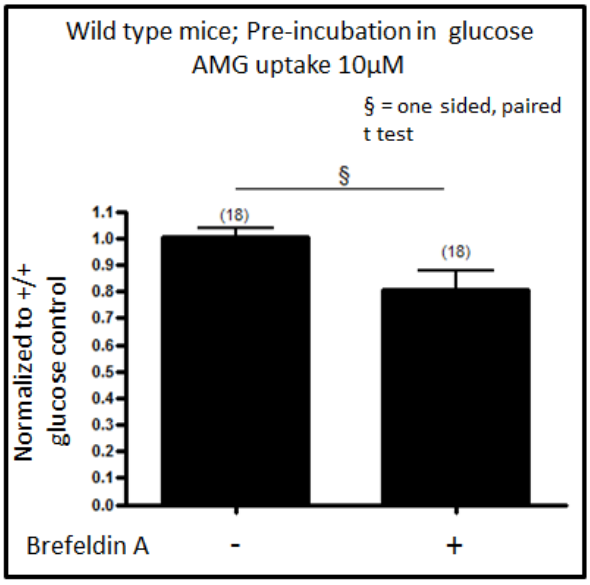

Figure 14 D

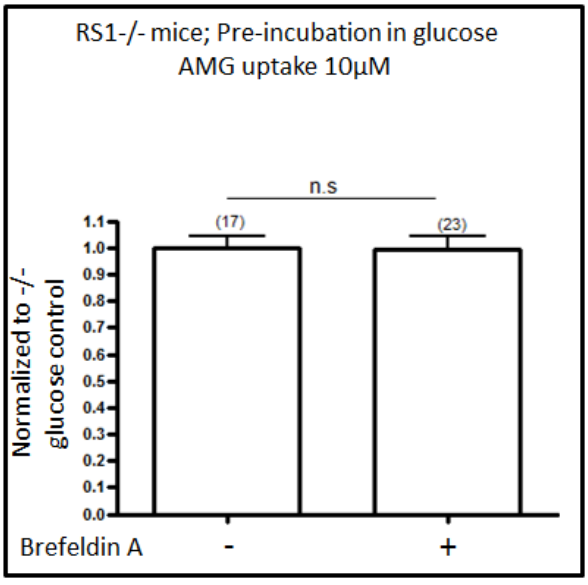

Figure 14: Brefeldin A inhibits exocytotic inhibition of SGLT1 vesicular trafficking in a complex manner: Wild type and RS1-/- mice were starved for 24hrs prior to perform SGLT1 dependent AMG uptake. Phlorizin inhibited uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured for 2 minutes in everted jejunum segments of wild type mice (Fig 14A and C) and RS1-/- mice (Fig 14B and D) after pre-incubating the segment in buffer (Figure 14A and B) or glucose (Figure 14C and D) with or without 7-12µM Brefeldin A for 1hr. Radioactivity of AMG uptake was calculated in terms of Picomoles/cm/2min and normalized to respective mice buffer or glucose control. Buffer pre-incubated with Brefeldin A inhibited SGLT1activity in wild type mice (P = 0.0472) and in RS1 -/- mice (P = 0.0440) tested by 1sided paired test (§). However Brefeldin A inhibited SGLT1 activity only in wild type mice when incubated in glucose (P = 0.0440) (§).

The difference in the inhibition by Brefeldin A between wild type and RS1-/- under glucose condition indicated a complex mechanism. SGLT1 regulation mediated by Brefeldin A might be dependent on intracellular glucose concentration and endogenous RS1.

Appendix V: QEP down regulates SGLT1 activity by exocytotic inhibition of SGLT carrying vesicles:

QCP a functional tripeptide within the RS1 sequence down-regulates SGLT1 without adding to the exocytotic inhibition of SGLT1 vesicular trafficking by Brefeldin A in the *Xenopus laevis* oocyte system (Vernaleken, Veyhl et al. 2007). QSP is another functional tripeptide of RS1 sequence. For understanding the mechanism of SGLT1 regulation by QSP, its phosphorylation mimicking mutant QEP effect on SGLT1 activity was compared to inhibitory effect of Brefeldin A. As Brefeldin A did not mediate inhibition in RS1-/- mice when glucose was present (Figure 15 D), phlorizin inhibited uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured in everted segments of wild type mice alone after pre-incubation in glucose with Brefeldin A (12 µM), QEP (15 mM) or Brefeldin A with QEP. The uptake measurements were normalized to the mean ± standard error value 293.9 ± 11.36 in picomoles/cm/2 min obtained for the everted segments of wild type mice small intestine after pre-incubation with glucose. The data in figure 15 showed no additive down-regulation of SGLT1 activity by QEP to the exocytotic inhibition of SGLT1 vesicles by Brefeldin A. This possibly indicated that QEP mediated down-regulation of SGLT1 by inhibiting the trafficking of SGLT1 carrying vesicles to the apical surface.

Figure 15

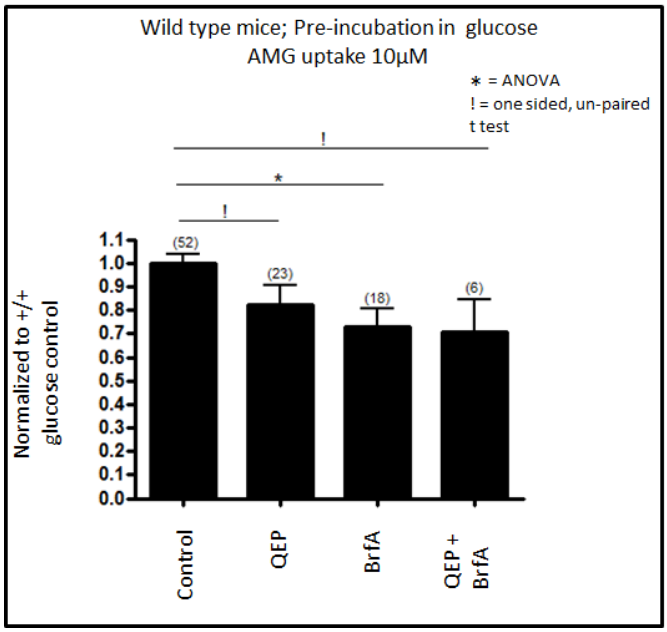

Figure 15: Brefeldin A and QEP inhibit exocytosis of SGLT1 carrying vesicles: Wild type mice were starved for 24 hours prior to perform SGLT1 dependent AMG uptake. Phlorizin inhibited uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured for 2 minutes in everted jejunum segments of wild type mice after pre-incubating the segment in glucose with or without QEP, Brefeldin A and QEP with Brefeldin A for 1hr. Radioactivity of AMG uptake was calculated in terms of Picomoles/cm/2min and normalized to wild type mice glucose control. No additive down-regulation of SGLT1 function was observed by QEP with Brefeldin A over the inhibition by QEP or Brefeldin A alone.

Appendix VI: PKC down regulates SGLT1 activity by aiding to exocytotic inhibition of SGLT1 vesicles:

QEP and Brefeldin A do not additively down-regulate SGLT1 activity (Figure 15). RS1 regulates SGLT1 in a PKC dependent manner (Veyhl, Wagner et al. 2003). PKC down-regulated SGLT1 activity independent of RS1 (Figure 7) however the PKC dependent down-regulation of SGLT1 was suggested to act by the same pathway of RS1 mediated down-regulation of SGLT1 (Figure 8). Since RS1 mediated down-regulation of SGLT1 by inhibiting its exocytotic trafficking to plasma membrane (Appendix V) the involvement of PKC activation was investigated for the exocytotic inhibition of SGLT1 vesicles. For this, phlorizin inhibited uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured in wild type mice jejunum everted segments after pre-incubation in 5 mM D-glucose with PMA (5 µM), Brefeldin A (12 µM) or PMA with Brefeldin A. The uptake measurements were normalized to the mean ± standard error value 293.9 ± 11.36 in picomoles/cm/2 min obtained for the everted segments of wild type mice small intestine after pre-incubation with glucose. Results (Figure 16) showed an additive PKC mediated down

regulation on SGLT1 activity to Brefeldin A inhibition. This data suggested that PKC and Brefeldin A alone might mediate only partial exocytotic inhibition of SGLT1 carrying vesicles and when together complete the down-regulation of SGLT1 or PKC and Brefeldin A inhibited SGLT1 by different pathways.

Figure 16

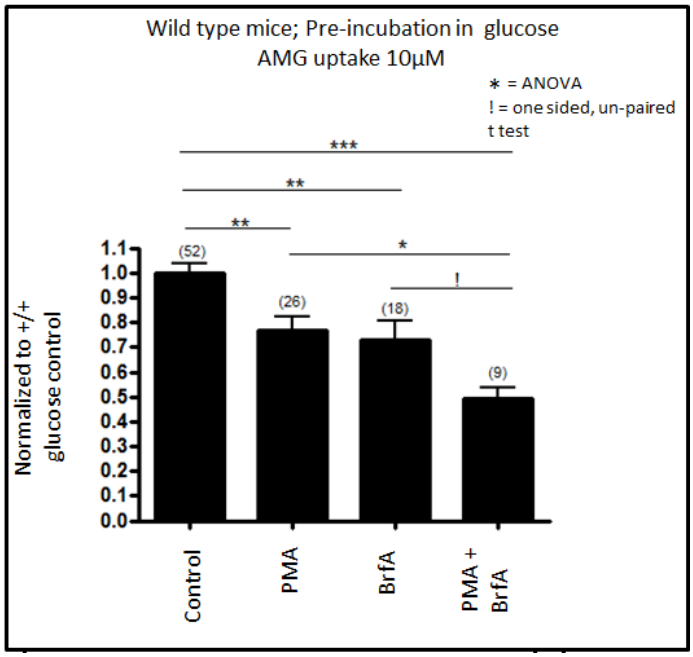

Figure 16: Brefeldin A and PKC inhibit SGLT1 additively: Wild type mice were starved for 24hrs prior to perform SGLT1 dependent AMG uptake. Phlorizin inhibited uptake of 10 µM AMG, labelled with tracer amount of [^{14}C] AMG was measured for 2 minutes in everted jejunum segments of wild type mice after pre-incubating the segment in glucose with or without PMA, Brefeldin A and PMA with Brefeldin A for 1hr. Radioactivity of AMG uptake was calculated in terms of Picomoles/cm/2min and normalized to wild type mice glucose control. PMA with Brefeldin A inhibition were additive to PMA ($P<0.05$) and Brefeldin A ($P=0.015$) inhibition alone by one sided un-paired, t test (!).

Appendix VII: SGLT1 affinity for substrate transport during diet adaptation might be RS1 dependent:

To understand whether adaptation to different diet modulates SGLT1 substrate affinity and to explore the involvement of RS1 in this regulation, K_m values for sodium dependent AMG uptake rates were calculated. The radioactive uptake measurements of 2 seconds were made for different AMG concentrations (0.02 – 5 mM) in the presence and absence of 100 mM sodium in BBMVs isolated from small intestine of wild type and RS1-/- mice kept for 2 months with different diets. The radioactivity measured was calculated in Picomoles/mg/2 seconds and the values were plotted using Michaelis-Menten equation in a nonlinear regression curve to obtain *Km* values. Comparing the K_m values the results (Figure 17)

showed that diet does not alter affinity in wild type mice. However RS1-/- mice fed with HFHGD (K_m value 2.232 ± 0.471 mM) showed a borderline significance for difference in K_m value when compared with mice fed with ND (K_m value 0.8717 ± 0.197) and GGRD (K_m value 0.8290 ± 0.0315 mM).

Figure 17

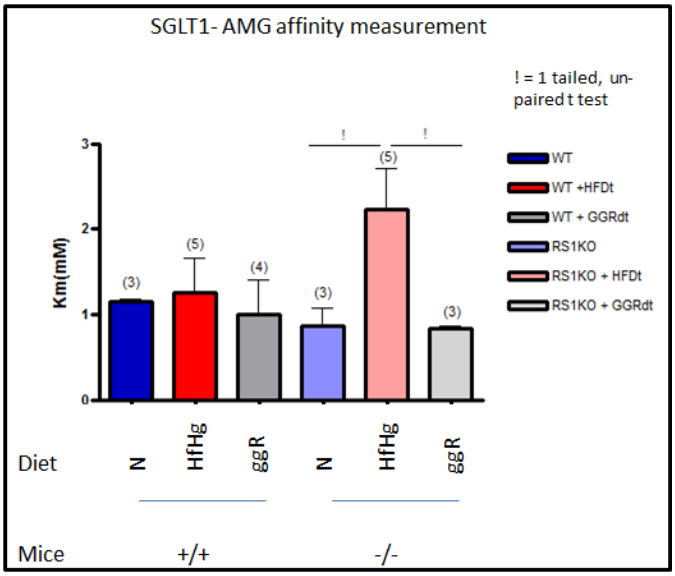

Figure 17: RS1 might alter SGLT1 substrate affinity for diet adaptation: Mice were fed with different diets for 2 months and starved for 24 hours prior to preparing BBMVs from mice small intestine. Sodium dependent uptake measurements were performed for 2 seconds in BBMVs incubated in Na^+ containing buffer and K^+ containing buffer at different radioactive AMG concentrations (0.02 mM – 5 mM). Radioactivity was measured in terms of Picomoles/mg/2 seconds and plotted to fit in non-linear regression curve using Michaelis – Menten equation to determine K_m value. Significant difference was observed between K_m value of RS1-/- mice fed with HFHGD to mice fed with ND (P=0.0395) and GGRD (P = 0.0339) by1 sided un-paired t test (!).

7. Summary:

The Na^+-D-glucose cotransporter in small intestine is regulated in response to food composition. Short term regulation of SGLT1 occurs post-transcriptionally in response to changes in luminal glucose. Adaptation to dietary carbohydrate involves long term regulation at the transcriptional level. The intracellular protein RS1 (gene *RSC1A1*) is involved in transcriptional and post-transcriptional regulation of SGLT1. RS1 contains an N-terminal domain with many putative phosphorylation sites. By Expressing SGLT1 in oocytes of *Xenopus laevis* it was previously demonstrated that the post-transcriptional down-regulation of SGLT1 by RS1 was dependent on the intracellular glucose concentration and activated by protein kinase C (PKC). The role of RS1 for short term regulation of SGLT1 in mouse small intestine in response to glucose and PKC was investigated comparing effects in RS1-/- mice and wildtype mice. Effects on SGLT1 activity were determined by measuring phlorizin inhibited uptake of α-methylglucoside (AMG). The involvement of RS1 in glucose dependent short term regulation could not be elucidated for technical reasons. However, evidence for RS1 independent short-term downregulation of SGLT1 after stimulation of PKC could be provided. It was shown that this downregulation includes decrease in the amount and/or in turnover of SGLT1 in the brush-border membrane as well as an increase of substrate affinity for AMG transport. Trying to elucidate the role of RS1 in long term regulation of SGLT1 in small intestine in response to glucose and fat content of the diet, wildtype and RS1-/- mice were kept for 2 months on a normo-caloric standard diet with high glucose and low fat content (ND), on a hyper-caloric glucose-galactose reduced diet with high fat content (GGRD) or on a hyper-caloric diet with a high fat and high glucose content (HFHGD). Thereafter the animals were starved overnight and SGLT1 mediated AMG uptake was measured. Independent of diet AMG uptake in ileum was smaller compared to duodenum and jejunum. In jejunum of wildtype and RS1-/- mice kept on the fat rich diets (GGRD and HFHGH) transport activity of SGLT1 was lower compared to mice kept on ND with low fat content. This result suggests an RS1 independent downregulation due to fat content of diet. Different to RS1-/- mice, the duodenum of wildtype mice showed transport activity of SGLT1 smaller in mice kept on glucose galactose reduced diet (GGRD) compared to the glucose galactose rich diets (ND and HFHGG). These data indicate that RS1 is involved in glucose dependent long term regulation in duodenum.

8. Zusammenfassung:

Der Na^+-Glukose-Cotransporter SGLT1 im Dünndarm wird in Abhängigkeit zur Nahrungszusammensetzung reguliert. Kurzzeitregulation von SGLT1 tritt posttranskritionell als Antwort zu sich ändernden Glukosekonzentrationen im Darmlumen auf. Anpassung an Nahrungskohlenhydrate beinhaltet die Langzeitregulation auf transkripionellem Level. Das intrazelluläre Protein RS1 (Gen RSC1A1) ist an der transkriptionellen und post-transkriptionellen Regulation von SGLT1 beteiligt. Es enthält eine N-terminale Domäne mit vielen putativen Phosphorylierungsstellen. Bei der Expression von SGLT1 im Xenopus leavis Oocytensystem wurde gezeigt, dass die posttranskriptionelle Herunterregulation von SGLT1 durch RS1 von der intrazelluläre Glukosekonzentration abhängt und durch Proteinkinase C (PKC) aktiviert wird. Die Rolle von RS1 in der Kurzzeitregulation von SGLT1 im Dünndarm der Maus als Antwort auf Glukose und PKC wurde durch vergleichende Studien zwischen RS1- knockout (RS1-/-)- Mäusen und Wildtyp-Mäusen untersucht. Effekte auf die SGLT1-Aktivität wurden durch Messung der durch Phlorizin inhibierbaren Aufnahme des SGLT1-spezifischen Substrats α-Methyl-Glycopyranosid (AMG) bestimmt. Der Einfluss von RS1 in der Glukose-abhängigen Kurzzeitregulation konnte aus technischen Gründen nicht untersucht werden, jedoch gab es Anzeichen für eine von RS1 unabhängige Kurzzeitregulation von SGLT1 durch PKC. Es wurde gezeigt, dass diese Herunterregulation sowohl eine Abnahme der Menge und/oder der Umsatzrate von SGLT1 in der Bürstensaummembran wie auch eine Zunahme der Substrat-Affinität für den AMG-Transport beinhaltet. Um die Rolle von RS1 auf die Langzeitregulation von SGLT1 in Dünndarm als Antwort auf den Glukose- und Fettgehalt der Nahrung zu untersuchen, wurden Wildtyp- und RS1-/- Mäuse für 2 Monate entweder auf einer normalenergetischen Standarddiät mit hohem Glukose- und niedrigem Fettgehalt (ND), auf einer hochenergetischen Diät mit reduziertem Glukose und Galaktose-Gehalt (GGRD) oder auf einer hochenergetischen Diät mit hohem Fett- und Glukosegehalt (HFHGD) gehalten. Danach wurden die Tiere über Nacht gefastet und die durch SGLT1 vermittelte AMG –Aufnahme gemessen. Unabhängig der Diät war die AMG-Aufnahme im Ileum geringer als in Duodenum und Jejunum. Im Jejunum von Wildtyp- und RS1-/- Mäusen die auf einer fettreichen Diät (GGRD und HFHGD) gehalten wurden war die Transportaktivität von SGLT1 geringer verglichen mit der Aktivität von Mäusen auf ND. Dieses Ergebnis lässt eine RS1-unabhängige Herunterregulation die durch den Fettgehalt hervorgerufen wird vermuten. Anders als in RS1-/- Mäusen war die Transportaktivität von SGLT1 im Duodenum von Wildtypmäusen bei der Glukose-Galaktose- reduzierten Diät

niedriger verglichen mit den Glukose-Galaktose-reichen Diäten (ND und HFHGD). Diese Daten legend die Vermutung nahen, das RS1 an der Glukose-abhängigen Langzeitregulation im Duodenum beteiligt ist.

9. Abbreviations:

AM	Adrenomedullin
AMG	Alpha Methyl D-Glucopyranoside
AMPK	5’ – Adenosine Mono phosphate activated protein kinase
BBMV	Brush border membrane vesicles
cRNA	Complementary Ribonucleic acid
cAMP	cyclic Adenosine Mono phosphate
cPLA2	cytosolic Phospholipase A2
CCK	Cholecystokinin
ERK	Extracellular signal regulated kinase
EGF	Epidermal growth factor
GGRD	Glucose-galactose reduced diet
GLP-2	Glucagon like peptide-2
GLUT	Facilitated glucose transporters
GSK3β	Glycogen Synthase kinase 3 beta
HFHGD	High fat high glucose diet
HNF1 α	Hepatocyte nuclear factor 1 alpha
HNF1 β	Hepatocyte nuclear factor 1 beta
IL-6	Interleukin 6
JNK	Jun – N – Terminal kinase
LLCPK1	Porcine kidney epithelial cell line
mTOR	Mammalian target of rapamycin
MAPK	Mitogen activated protein kinase
ND	Normal diet / high polysaccharide diet /Standard control diet
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
p38 MAPK	p38 Mitogen activated protein kinase
PAMP	Proadrenomedullin N – terminal 20 peptide

PI3K	Phosphotidylinositol 3 Kinase
PIP5K3	Phosphotidylinositol 3 phosphate 5 kinase
PI3,5P2	Phosphotidylinositol 3,5 biphophate
PKA	Protein Kinase A
PKB	Protein Kinase B/ Akt
PKC	Protein Kinase C
PMA	Phorbol – 12 – Myristate – 13 – Acetate
PME n	Plasma membrane enriched
PTCs	Renal proximal tubule cells
QEP	Gln-Glu-Pro
QSP	Gln-Ser-Pro
RAS	renin – angiotensin system
RNS	21 residue long nuclear shuttling domain
RELMβ	Resistin like molecule β
SMIT	SGLT1 homologous Na^+ myoinositol co-transporter
SEM	Standard Error of Mean ± standard error
SLC5A	Solute carrier family
SGK-1	Serum and Gluco-corticoid inducible kinase
SGLT1	Sodium dependent glucose transporter – 1
TGN	Trans Golgi Network
TGN-Reg	Post-transcriptional regulatory domain of RS1
TTK2	Tau tubulin kinase 2
OCT1	Organic Cation transporter 1
OCT2	Organic Cation transporter 2
UBA	Ubiquitin associated domain
URE	Uridine rich sequence
UTR	3’ Un-translated Region

Vmax	Maximum velocity of transport

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Publications

Filatova A, Leyerer M, Gorboulev V, Chintalapati C, Reinders Y, Müller TD, Srinivasan A, Hübner S, Koepsell H. **Novel shuttling domain in a regulator (*RSC1A1*) of transporter SGLT1 steers cell cycle-dependent nuclear location**. Traffic. 2009 Nov;10(11):1599-618. doi: 10.1111/j.1600-0854.2009.00982.x. Epub 2009 Sep 2. PubMed PMID: 19765263.

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Participation in scientific international conferences

Srinivasan A., Koepsell H. Involvement of regulator protein RS1 and Sodium Dependent glucose Co-transporter 1 (SGLT1) in short term regulation of glucose absorption in small intestine.
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Graduate School of Life Sciences, Wuerzburg, Germany.

Affidavit

I hereby confirm that my thesis entitled "**RS1 protein dependent and independent short term and long term regulation of sodium dependent glucose transporter 1**" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.
Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation **RS1 Protein abhängige und unabhängige Kurz- und Langzeitregulation des Natrium-abhängigen glukosetransporter 1** eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.
Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

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