The Journal of Nutritional Biochemistry
October 2010, Volume 21, Issue 10, Pages 914-921
http://dx.doi.org/10.1016/j.jnutbio.2009.07.004

A moderate threonine deficiency affects gene expression profile, paracellular permeability and glucose absorption capacity in the ileum of piglets ☆, ☆☆

Alice Hamard[a, b], David Mazurais[c], Gaëlle Boudry[a, b], Isabelle Le Huërou-Luron[a, b], Bernard Sève[a, b] and Nathalie Le Floc'h[a, b, *]

[a] INRA, UMR1079, SENAH, F-35590 Saint-Gilles, France
[b] Agrocampus Rennes, UMR1079, F-35000 Rennes, France
[c] Ifremer, Département PFOM, centre de Brest, 29280 Brest, France

☆ Part of the results was presented at the Xth International Symposium on Digestive Physiology in Pigs, May 2006, Vejle, Denmark. It was published as a short paper (Hamard A, Mazurais D, Boudry G, Le Huërou-Luron I, Sève B, Le Floc'h, N. Physiological aspects and ileal gene expression profile of early-weaned piglets fed a low threonine diet. Livestock Science, 2007;108:17–19).

☆☆ Alice Hamard is funded by INRA and Région Bretagne grants.

*: Corresponding author : N. Le Floc'h, email address : nathalie.lefloch@rennes.inra.fr

Abstract:

High dietary threonine extraction by the digestive tract suggests that threonine contributes to maintain gut physiology. In the present study, we evaluated the impact of a low (6.5 g of threonine/kg diet; LT group) or a control well-balanced threonine diet (9.3 g of threonine/kg diet; C group) given to piglets for 2 weeks on ileal permeability and Na+-dependant glucose absorption capacity in Ussing chambers. The paracellular permeability was significantly increased in the ileum of LT compared to C piglets (*P*=.017). The Na+-dependent glucose absorption capacity showed a nonsignificant increase in the LT piglets. In addition, we analysed ileal gene expression profiles in the LT and C groups using porcine multitissue cDNA microarrays. Compared to the C piglets, the expression of 324 genes was significantly modified in the ileum of the LT piglets: 214 genes were overexpressed (145 annotated) and 110 were down-expressed (79 annotated). Among them, some are involved in immune and defense responses, energy metabolism and protein synthesis. Furthermore, microarray analysis highlights changes in the expression of the gene encoding for the sodium/glucose cotransporter (SGLT1) and of genes involved in the regulation of paracellular permeability (ZO-1, cingulin and myosin light chain kinase). In conclusion, our results indicate that a moderate threonine deficiency affects intestinal functionality.

Keywords: Threonine; Small intestine; Paracellular permeability; Gene expression

INTRODUCTION

Although small intestine represents less than 5% of whole-body mass, it accounts for 25% of whole-body energy expenditure and for 20-50% of total protein turnover (7). This high metabolic activity generates important amino acid (AA) requirements. In order to meet its requirement, the small intestine extracts part of dietary AA (40, 41). Among essential AA, threonine is extracted in greater proportion by the small intestine (28, 41, 43), suggesting that threonine is involved in intestinal functionality and maintenance. However, the metabolic fate and the functional role of threonine in the small intestine are still unclear.

The high rate of intestinal threonine extraction could be associated with protein synthesis (28) and especially to the synthesis of mucins (17, 27, 45) which threonine content ranges from 13% to 26% of total AA (29, 30, 37). Threonine deficiency could also impact on other functions of the small intestine. We previously demonstrated that feeding young piglets with a low threonine supply (70% of recommendations), that corresponds to a moderate deficiency, for two weeks induced a villous atrophy associated with a reduction in aminopeptidase N activity in the ileum (20). Because villous atrophy is frequently associated with functional disturbances, further work was needed to determine the effect of threonine deficiency on small intestine physiology.

The objective of the present study was to identify biological functions affected by a moderate threonine deficiency, which corresponds to a deficiency that remains within nutritional range. We focused on the distal part of the small intestine where we observed structural modifications. To do so, we evaluated the effect of the dietary content of threonine on ileal paracellular permeability and glucose absorption capacity in Ussing chambers. In addition, we used porcine cDNA microarrays to evaluate the impact of the dietary threonine supply on global gene expression profile in the piglet ileum. This is particularly interesting

considering the scarcity of knowledge about the implication of this AA in the physiology of the small intestine.

Materials and Methods

Animals and feeding. The experiment was conducted under the guidelines of the French Ministry of Agriculture for animal care. Seven pairs of Pietrain x (Large White x Landrace) piglets from the INRA experimental herd (Saint-Gilles, France) were weaned at 7 days of age. These pairs were constituted of littermates with close body weights (2.5 ± 0.06 kg). From weaning, piglets were placed into individual stainless-steel cages in a room maintained at 30°C.

Within each pair, one piglet received a control well-balanced diet (C group) and the other one a low threonine diet (LT group). The composition of the diets is presented in Table 1. Protein was supplied by skimmed-milk powder and a soluble fish protein concentrate. Those raw materials set the basal threonine content in both diets. A free AA mixture was added according to the recommendations of Chung and Baker (9) for weaned piglets. Free threonine was added only in the C diet. The nitrogen content of the LT diet was adjusted by addition of aspartic acid and ammonium citrate. Threonine content was 9.3 g per kg in the C diet and 6.5 g per kg in the LT diet. Diets provided 250 g / kg of protein (Nx6.25) and 15 MJ of digestible energy (DE) per kg.

The meals were prepared as a mash (powdered diet-warm water, 2:1) just before distribution. The daily amount of diet was adjusted to the metabolic weight (600 kJ/kg body weight $^{0.75}$) and given in four equal meals. The piglets were offered 50% of this daily intake the first two days. Water was offered *ad libitum* throughout the experiment. Piglets were weighed on experimental days 1, 4, 6, 8, 11, and 13.

100 *Slaughter procedure.* After two weeks of experiment and 3 h after the last meal, piglets were
101 killed with a lethal dose of pentobarbital immediately followed by exsanguination. The
102 gastrointestinal tract was quickly removed. The small intestine, from the Treitz ligament to
103 the ileo-caecal junction, was weighed empty of contents and the length was measured. It was
104 divided in three parts of equal length, the proximal jejunum, the distal jejunum and the ileum.
105 In the middle of each part, 3 cm-segments were collected in phosphate-buffered formalin
106 (10%, pH7.6) for morphometric measurements. A 20-cm segment of the ileum was sampled
107 in bicarbonate Ringer's solution (in mmol/L: 145 Na^+, 128 Cl^-, 0.32 PO_4^{3-}, 2 Ca^{2+}, 1 Mg^{2+}, 25
108 HCO_3^-, 1 SO_4^{2-}, 6.3 K^+; pH 7.4) for measurements made in Ussing chambers. Small (1cm)
109 pieces of the ileum were collected, rinsed with sterile saline and stored in RNAlater®
110 (Ambion, USA) at -20°C until RNA extraction.
111
112 *Ileal morphometry.* After fixation in phosphate-buffered formalin during 24 hours at 4°C,
113 samples were washed and stored in ethanol:water (75:25, v:v). They were stained with
114 Schiff's reagent after dehydration according to the technique of Goodlad et al. (19).
115 Villous/crypt units were isolated from intestinal samples by microdissection and mounted on
116 a glass slide in acetic acid (45%). Villous height and crypt length, width and surface were
117 measured using image analysis (Lucia software, Laboratory Imaging, Czech Republic). Mean
118 values of these parameters were determined on 30 villi and crypts per sample.
119
120 *Measurements of ileal glucose absorption capacity and paracellular permeability in Ussing*
121 *chambers.* Immediately after sampling, ileal segments were stripped of their seromuscular
122 layers and mounted in Ussing chambers with an exposed area of 1.13 cm^2. They were bathed
123 on each side with a bicarbonate Ringer's solution with 16 mM glucose and 16mM mannitol
124 on the serosal and mucosal sides, respectively and maintained at 38°C (6). The short-circuit

current (I_{SC}) and the transepithelial resistance were measured as already described (6). A first set of Ussing chambers was used to estimate paracellular permeability through measuring the flux of fluorescein isothiocyanate dextran 4000Da (FD4) as a model molecule. This molecule was added on the mucosal side at the final concentration of 0.375 mg/mL. Its transport was monitored by sampling 500µl of bathing solution from the serosal side at 30-min intervals for 120 minutes. The solution was replaced by fresh medium to maintain a constant volume within the chamber. The concentrations of FD4 in the serosal side were measured by fluorometry. In a second set of Ussing chambers, Na^+-dependent glucose absorption capacity was evaluated. Increasing amounts of D-glucose were added to mucosal buffer every 5 minutes, resulting in final concentrations of 2, 4, 8, 16, and 32 mM. The addition of glucose on the mucosal side was osmotically balanced by the addition of mannitol on the serosal side. Maximal variation of the short-circuit current (Delta I_{SC}) was recorded at each concentration and V_{max} and K_m for Na^+-dependent glucose absorption were then calculated.

RNA extraction. Total RNA was extracted from ileal samples using Trizol reagent (Invitrogen corporation, USA) according to manufacturer's instructions. Concentration of RNA was quantified by measuring absorbance at 260 nm (Multiskan spectrum, Thermo Labsystems, France) and RNA integrity was checked using Agilent 2100 bioanalyser (Agilent technologies, Germany).

Microarray analysis and data processing. Transcriptomic analyses were performed on the 7 pairs of piglets using nylon microarrays obtained from the Resource Center GADIE (UMR LREG, INRA, France) and encompassing 8960 clones from a multi-tissue porcine cDNA library (AGENAE, INRA, France). The 8960 clones spotted on the arrays represented 8800

genes, of which 60% are annotated. These arrays are recorded on the GEO Platform under the accession number GPL3729.

Labelling of cDNA complex probes, hybridization and washes were performed according to the procedures described by Mazurais et al. (31). Briefly, after their extraction from ileum samples, total purified RNA was retro-transcripted in the presence of [α-^{33}P]dCTP for labelling. After array image acquisition (BAS 5000, Fuji), quantification of hybridization signals revealed the expression level of each 8960 clones (BZ Scan). Then, the expression level of each clone was first log-transformed to yield normal distribution and then median-centred to minimize technical variability. We selected clones which displayed differential expression between C and LT groups using variance analysis (P ‹ 0.01, GeneANOVA, CNRS, UPRESA 8087, France) (14). The selected clones were submitted to hierarchical clustering with the Gene Cluster software (16).

Real-time PCR. Reverse transcription was performed with 2 µg of total DNAse-treated RNA (High capacity cDNA archive kit; Applied Biosystems, USA). The primers were designed using Primer Express Software (Applied Biosystems, USA) based on *sus scrofa* published nucleotides sequences (Iccare) and are described in Table 2. Real-time PCR was carried out on an ABI PRISM 7000 SDS thermal cycler (Applied Biosystem, USA). Real-time PCR was performed in 25 µL of PCR buffer (SYBRGreenTM PCR Master Mix, Applied Biosystems, USA) with 500 nM of each primer, 5 µl of optimized concentration of the RT reaction and 2U of Uracyl DNA Glycosylase (Invitrogen, France). Forty cycles of PCR consisting of denaturation at 95°C for 15 sec and annealing and extension at 60°C for 1 min were performed. Amplification product specificity was checked by dissociation curve analyses. To determine the efficiency of each primer set, a standard curve was done with serial dilutions of a pool of samples' RT products. Then for each sample, the amount of the target RNA was

174 determined by comparison with the corresponding standard curve (3). Finally the amount of
175 the target RNA was calculated relative to the GAPDH transcript level of the same sample.
176
177 *Statistical analysis.* For all measurements, except for transcriptomic analysis (*see Microarray*
178 *analysis and data processing*), analysis of variance was performed using General Linear
179 Model procedure of Statistical Analysis System (SAS Institute, Cary, NC, USA). The effects
180 of pair (litter) and dietary threonine supply were tested using the residual variation between
181 piglets as the error. All the results are presented as Least square means (LSmeans) ± sem.
182 Differences were considered significant when $p < 0.05$. Trends ($0.1 < p < 0.05$) were
183 presented for discussion.
184
185 **Results**
186 *A moderate threonine deficiency did not affect growth rate.* As expected the average feed
187 intakes were not significantly different between pair-fed C and LT piglets (Table 3).
188 Threonine intake was significantly reduced by 29% in the LT piglets compared to the C
189 piglets ($p < 0.0001$). The low threonine supply affected neither final body weight, nor body
190 weight gain.
191
192 *A moderate threonine deficiency induced ileal villous hypotrophy.* The weight and length of
193 the small intestine were not altered by the low threonine supply (data not shown). In the
194 proximal and distal jejunum, no modification of the mucosa morphology was observed (Table
195 4). In the ileum, villous height tended to be reduced in LT piglets compared to C piglets ($p =$
196 0.06). In accordance with this result, villous surface was reduced by 18% in LT piglets
197 compared to C piglets ($p < 0.01$).
198

A moderate threonine deficiency increased glucose absorption capacity. Measurements performed in Ussing chambers showed a trend to an increased Na+-dependent glucose absorption capacity, measured as the delta I_{SC} to graded glucose addition, in LT piglets as illustrated by a higher dose-response curve (Figure 1): V_{max} tended to increase by 81% in the ileum of LT piglets compared to C piglets ($p = 0.1$; Table 5), and K_m did not change between LT and C groups.

A moderate threonine deficiency modified epithelial barrier function. The paracellular permeability measured in Ussing chambers was 89% increased in the ileum of LT piglets compared to C piglets ($p = 0.017$; Figure 2). Moreover, despite no statistical significance, the reduced threonine supply decreased transepithelial resistance by 30% (Figure 3).

A moderate threonine deficiency affected ileal transcriptome. A 30% reduction of dietary threonine supply significantly affected the expression of 324 genes ($p < 0.01$): 214 genes were over expressed (145 annotated) and 110 were down expressed (79 annotated) in LT piglets. Differentially expressed genes are listed in Supplemental Tables 1 and 2. The fold changes of down expressed genes in LT piglets ranged between 0.42 and 0.78. For over expressed genes, they ranged between 1.51 and 3.00 except for SGLT-1 which expression was 4.9-fold increased in LT group.

Differentially expressed genes were classified according to their biological process ontology determined from Uniprot/Swiss-Prot database and the QuickGO Gene Ontology browser (http://www.ebi.ac.uk/ego/). Some genes were not classified in a functional group and for some others no informative annotation was available (Supplemental Tables 1 and 2).

Feeding a reduced threonine supply for two weeks increased the expression of genes involved in immune and inflammatory responses such as the complement C1s subcomponent

(C1S), the MHC class I antigen (HLA-B), the T-cell differentiation antigen CD6 (CD6), the C-C motif chemokine 16 (CCL16) and chemokine receptors (IL17RB, CCR4, DARC). We also noted the overexpression of genes coding the selenoprotein W (SEPW1), the beta-defensin 129 (DEFB129), the microsomal glutathione S-transferase 1 (MGST1) and the mucin 1 (MUC1), these proteins playing a crucial role in antimicrobial or antioxidative defenses.

Feeding a low threonine diet also affected the expression of genes involved in cell turnover. The gene encoding IGF2 was overexpressed whereas several genes acting as inhibitor of cell proliferation (BTG1 protein, BTG1; Pin2-interacting protein X1, PINX1; Forkhead box protein C1, FOXC1) were downexpressed in the ileum of LT piglets. The expression of two genes involved in the induction of apoptosis, the BH3 interacting domain death agonist (BID) and the death-associated protein kinase 1 (DAPK1), was increased.

The expression of genes coding the sodium/potassium/calcium exchanger 4 (SLC24A4), the phospholemnan (PXYD1), the amiloride-sensitive sodium channel beta-subunit (SCNN1B) as well the Y+L amino acid transporter 1 (SLC7A7) and the sodium/glucose cotransporter 1 (SGLT-1) was significantly increased in the ileum of LT piglets. The increase in SGLT-1 mRNA expression was confirmed by RT-PCR (2.04-fold, $P < 0.05$) (Figure 4). This could indicate modifications in the transport of ions and nutrients. Modifications in the expression of genes involved in the intracellular protein transport were also observed. For example, genes encoding the kinectin (KTN1), the centractin (ACTR1B), the transmembrane protein 9 precursor (TMEM9), the Golgin subfamily A member 5 (GOLGA5), the importin alpha-1 subunit (KPNA1) were overexpressed whereas genes coding the adapter-related protein complex 3 delta 1 subunit (AP3D1), the charged multivesicular body protein 1a (PCOLN3), the vacuolar protein sorting-associated protein

33B (VPS33B) or the kinesin-like protein KIF2 (KIF2A) were downexpressed in the ileum of LT piglets.

Piglets fed the LT diet exhibited increased ileal expression of genes involved in cell adhesion (tight junction protein ZO-1, TJP1; cingulin, CGN; paxillin, PXN; cadherin EGF LAG seven-pass G-type receptor 2, CELSR2; plectin 1, PLEC1; collagen alpha 1, CO9A1; integrin α5, ITGA5) and communication (ephrin A-4, EFNA4; gap junction β5, GJB5) as well as in cytoskeleton organisation (neurofilament triplet M protein, NEFM; tropomodulin, TMOD1; tropomyosin 1, TPM1; Wiskott-Aldrich syndrome protein interacting protein homolog, WASIP). The significant increase in the expression of ZO-1 and cingulin (CGN) was confirmed by RT-PCR analysis: the relative levels of ZO-1 and CGN mRNA were 26% and 36% higher in LT piglets (Figure 4) although differences did not reach significance. Lack of significance could be explained by a high variability.

LT piglets displayed also modifications in the expression of genes involved in transcriptional and translational processes of protein synthesis. For example, genes coding the DNA directed RNA polymerase II 140 kDa polypeptide (POLR2B), the RNA polymerase-associated protein 1 (PAF1), the transcription initiation factor IIE alpha subunit (GTF2E1), and the transcription initiation factor IIB (GTF2B) were overexpressed. On the contrary, the eukaryotic translation initiation factor 2-alpha kinase 4 (GCN2), known to inactivate eIF2, and the eukaryotic translation initiation factor 4A-binding protein 1 (EIF4EBP1), known to inactivate eIF4, were downexpressed. The expression profile of these genes could be indicative of an increase in protein synthesis rate. The LT diet also induced modifications of transcription factors regulating expression of specific target genes (KLF9, ZNF644, ZNF169, ZFP161, ZFP37, ZNF429). Most of genes involved in mRNA splicing were downregulated (PRMT5, RBM9, SF1, SFRS5, SRRM1, STRAP, LSM2). Genes involved in RNA

metabolism such as mRNA stability (SERBP1) or mRNA degradation (EDC3) were also differentially expressed in the ileum of LT piglets.

The LT diet altered the ileal expression of genes involved in the cellular protein metabolism. Apart from genes involved in regulation of translation (noticed above), we identified genes involved in protein folding (Dnaj homolog subfamily B member 9, DNJB9; peptidyl-prolyl cis-trans isomerase, PPIF; prefoldin subunit 2, PFDN2; torsin A, TOR1A) and protein catabolism (STIP1 homolog and U box-containing protein 1, STUB1; mitochondrial processing peptidase beta subunit, MPPB; F-box/wd-repeat protein 4, FBXW4; CAAX prenyl protease 1 homolog, ZMPSTE24; ubiquitin carboxyl-terminal hydrolase BAP1, BAP1; proteasome subunit beta type 3, PSMB3; probable E3 ubiquitin-protein ligase TRIP12, ubiquilin, UBQLN1…). Nevertheless, the expression profile of these genes did not allow us to conclude about the impact of the LT diet on these biological processes.

Finally, we also showed differential expression of genes involved in fatty acid metabolic process (carnitine O-acetyl transferase, CACP; carnitine O-palmitoyltransferase I, CPT1B, peroxisomal-coenzyme A synthase, FAT2; peroxisomal 3,2-trans-enoyl-coenzyme A isomerase, PECI; fatty acid-binding protein, epidermal, FABP5; dihydroxyacetone phosphate acyltransferase, GNPAT), in generation of energy (ATP synthase O subunit, ATP5O; NADH-ubiquinone oxidoreductase 13kDa-B subunit, NDUFA5) or in signal transduction (calcitonin receptor precursor, CALCR; GTPase-activating protein GAP, GAP; calcium/calmodulin-dependent protein kinase type II beta chain, CAMK2B; insulin receptor substrate 1, IRS1; phosphatidylinositol 4-kinase alpha, PIK4CA; phosphatidylinositol-4phosphate 5-kinase type I gamma, PIP5K1C; tyrosine-protein kinase JAK1, mitogen-activated protein kinase 8, JNK1…).

Discussion

As previously shown, a low threonine supply induced ileal villous hypotrophy (20). It was associated with alterations of functionality. Indeed, a novel finding of the present study is that a 30% reduced threonine supply induced increased ileal paracellular permeability as measured by the mucosa-serosa FD4 flux. Such an increase was previously reported in piglets encountering non optimal nutritional conditions, receiving total parenteral nutrition (24), submitted to 48 h fasting (8) or in response to undernutrition associated with weaning (5).

Increased paracellular permeability reflects a reduction in epithelial barrier selectivity and consequently a greater susceptibility to antigens passage across the intestinal epithelium even if not associated with clinical signs (19). Piglets fed the LT diet presented neither diarrhea nor feverish episode. They consumed all their feed and their weight gain was not affected. The good sanitary and nutritional conditions have probably minimized the incidence of gut permeability and morphology modifications. Analyses perfomed with cDNA microarrays showed that genes coding the complement C1s subcomponent (C1S), the MHC class I antigen (HLA-B), the T-cell differentiation antigen CD6 (CD6), the C-C motif chemokine 16 (CCL16) or chemokines receptors (IL17RB, CCR4, DARC) were overexpressed in the ileum of LT piglets. This might reflect immune response to the passage of antigens through the intestinal epithelium. For example, the overexpression of genes coding chemokines and chemokine receptors characterises an inflammatory state (1). CCL16 is known to be a powerful proinflammatory chemokine that is expressed in ulcerative colitis (36). Moreover, feeding the LT diet induced increased expression of genes encoded for mucins, S-glutathione-transferase 1, the selenoprotein W or a defensin. These proteins play a crucial role in intestinal protection (18, 35, 46). Overexpression of MUC1 mRNA is of particular interest because threonine utilisation by the gut is generally associated with mucins synthesis (MUC2 and MUC3 were not represented on our microarrays). Mucins production is increased during infection (13) or inadequate nutritional conditions (33).

Microarray analysis revealed transcriptional modifications of factors controlling the paracellular permeability (ZO1, cingulin and MLCK). Changes in the expression of these genes are expected to be associated with decreased paracellular permeability in the ileum of LT piglets, which is apparently inconsistent with the physiological data we obtained with Ussing chambers. Indeed, genes encoded for ZO1 and cingulin were up expressed in LT piglets. Cingulin and ZO1 are important components of the tight junction which is the major element of the paracellular pathway. These two proteins belong to the complex structure coupling the transmembrane sealing protein (occludin and claudins) and the actin network (32). They play a pivotal role in the structural and functional organization of the tight junction. Impaired intestinal permeability has been associated with lower expression of ZO-1 in pathophysiological conditions (34, 38, 39). The role of cingulin in the regulation of paracellular permeability remains to be confirmed. Myosin light chain kinase (MLCK) allows the phosphorylation and the contraction of the perijunctional actomyosin ring leading to increased paracellular permeability (42). We hypothesized that cingulin and ZO1 over expression and MLCK down expression observed in the ileum of LT piglets could indicate an attempt to restore barrier function in response to functional changes.

Restoration of barrier function implied different processes such as cell proliferation and migration (4). Integrins play a crucial role in these processes. In our experiment, several genes encoding for actors of the integrin signalling pathway (PAK4, MLCK and WIP, integrin α5, paxillin) were differentially expressed in the ileum of LT piglets compared to C piglets. The gene coding the integrin α5 was overexpressed in the ileum of LT piglets. The increase in mRNA expression of integrin α5 promotes cell adhesion to fibronectin and cell migration in various cell types (10, 11, 22, 44). In the intestine, the role of integrin α5 in cell proliferation, notably during repetitive deformation (26, 47) has been explored. The fixation between the integrin and extracellular matrix proteins leads to the recruitment of proteins such

as the paxillin to the cellular membrane and the subsequent activation of p21-activated kinases such as PAKs involved in cytoskeletal rearrangement (23). Genes coding the paxillin and the PAK4 isoform were overexpressed in the ileum of LT piglets. Finally the gene coding the WIP, an important actin-binding protein that participates in the deformation of the actin network for migration (2) was overexpressed. Overall, the expression profile of these genes may prefigure the activation of the integrin pathway and supports the hypothesis of barrier restoration.

The over expression of SGLT-1 gene associated with the increased glucose absorption capacity measured in Ussing chambers demonstrated that threonine deficiency stimulated glucose absorption via an increase of SGLT-1 transporter. Indeed, the lack of an effect on the Km indicated no change in the affinity of the transporter for its substrate. The trend for an increase in V_{max} could be due to either an increase in SGLT-1 activity and/or an increase in Na^{+}-K^{+}-ATPase activity. An increase in glucose absorption has already been observed in other situations such as a 48h fasting (8) or undernutrition associated with weaning (5). Glucose is a major source of energy for body tissues and notably for the small intestine (15). So we hypothesized that an increase in glucose absorption capacity reflects an increase energy demand in the small intestine, or peripheral tissues, or both in LT piglets. Supporting our hypothesis two genes involved in energy generation were also differentially expressed: the gene coding the ATP synthase O subunit, a component of the mitochondrial proton-translocating ATP synthase complex and the gene coding the NADH-ubuquinone oxidoreductase 13kDa-B subunit from the mitochondrial respiratory chain complex I. Additionally or otherwise, it appears that the contribution of glucose to intestinal energy production depends on age. Darcy-Vrillon et al. (12) showed that the capacity of cultured porcine enterocytes to use glucose was high during the first week of life and decreased the

second week when the small intestine used mainly AA. Therefore that change in energy supplier may have been delayed in LT piglets.

We showed that a low threonine supply induced structural and functional alterations. These modifications could result from an alteration in protein synthesis rate. In accordance with this hypothesis, Wang et al. (45) demonstrated that protein synthesis rate was reduced in the small intestine of piglets receiving less than 50% of daily threonine recommendations. Our results did not confirm this observation since intestinal protein synthesis rate was not altered by a 30% reduced threonine supply (21). Using transcriptomic analysis, we identified genes coding regulatory factors of protein synthesis that were differentially expressed in the ileum of LT piglets. The downregulation of genes coding the eukaryotic translation initiation factor 2-alpha kinase 4 (GCN2) and the eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) is of particular interest. These genes are implicated in the down regulation of mRNA translation. Firstly, GCN2 prevents the formation of the 43S pre-initiation complex (Met-tRNA, GTP and eIF2) by phosphorylating the translation initiation factor eIF2α. Secondly, 4E-BP1 inhibits the assembly of the eIF4E-mRNA complex to the 40S ribosomal subunit by binding to the eukaryotic initiation factor 4E (eIF4E). These two factors are assumed to be implicated in the downregulation of protein synthesis by AA starvation. For example, *in vitro* leucine deprivation induced activation of these factors and consequently inhibition of the initiation phase of mRNA translation (25). In our study, the down regulation of these genes was expected to be associated with an increase or an attempt to increase protein synthesis rate. Regarding the lack of effect on fractional synthesis rate (21), we hypothesized that the downexpression of GCN2 and 4E-BP1 in the ileum of pigs fed the LT diet could be a mechanism for preserving protein synthesis in condition of moderate threonine deficiency.

In conclusion, this study demonstrates for the first time that a 30% reduced threonine supply for two weeks induced increased paracellular permeability and glucose absorption

capacity. Moreover transcriptomic analysis showed that a moderate threonine deficiency altered ileal gene expression profiles. These transcriptional modifications opened new pathways of investigation. Notably, the increase in the expression of genes involved in immune and defence functions associated with the increased paracellular permeability suggest that threonine may be essential to preserve intestinal integrity. Therefore the response of the piglets to a reduced threonine supply should be evaluated in aggression situations in order to provide irrefutable evidence for a protective role of this amino acid on a stressed intestine.

Acknowledgments

We would like to thank the GADIE Center (UMR314, LREG INRA-CEA, Jouy-en-Josas Cedex, France) for producing the porcine microarray. We also thank Veronique Romé, Cécile Perrier and Romain d'Inca for technical assistance, and Yves Lebreton, Francis Legouevec and Vincent Piedvache for animal care.

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Table 1: Ingredients and nutritional values of the experimental diets

	Diet	
	Low threonine (LT)	Control (C)
Ingredients, g /kg diet		
Skimmed milk powder	250	250
Soluble fish protein concentrate	74.3	74.3
Free amino acids mix[1]	54.9	54.9
Maltodextrins	430.15	430.44
Sunflower oil	62.37	62.37
Ammonium citrate tribasic	30	30
Bicalcium phosphate	49	49
Trace element and vitamin premix[2]	10	10
L-aspartic acid	39.28	36.48
L-threonine	-	2.51
Chemical analysis		
Dry matter, *%*	92.9	92.8
Crude protein (N x 6.25), *%*	24.4	25
Digestible energy, MJ/kg diet	15	15

[1] Supplying the following amount of free amino acids (g / kg diet): L-lysine HCl, 3.53; L-tryptophane, 0.85; L-leucine, 1.86; L-isoleucine, 1.35; L-valine, 1.39; L-phenylalanine, 1.42; L-glutamate monoNa /A. glutamique (50/50), 35.3; glycine, 9.2.

[2] Supplying the following amount of vitamins and minerals (per kg diet): Ca, 1.82 g; Fe, 200 mg; Cu, 40 mg; Zn, 200 mg; Mn, 80 mg; Co, 4 mg; Se 0.6 mg; I, 2 mg; vitamin A, 30,000 UI;

vitamin D3, 6000 UI; vitamin E, 80 UI; vitamin B1, 4 mg; vitamin B2, 20 mg; panthotenic acid, 30 mg; vitamin B6, 20 mg; vitamin B12, 0.1 mg; vitamin PP, 60 mg; folic acid, 4 mg; vitamin K3, 4 mg; biotin, 0.4 mg; choline, 1600 mg; vitamin C, 200 mg.

Table 2: Forward and reverse primers used in RT-PCR reactions.

Gene	Protein name	Forward primer	Reverse primer	Accession no.
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	CATCCATGACAACTTCGGCA	GCATGGACTGTGGTCATGAGTC	AF017079
TJP1	Tight junction protein ZO-1	AGGCGATGTTGTATTGAAGATAAATG	TTTTTGCATCCGTCAATGACA	CK453343
SGLT1	Sodium/glucose cotransporter 1	CCCAAATCAGAGCATTCCATTCA	AAGTATGGTGTGGTGGCCGGTT	DY417361
CGN	Cingulin	GTTAAAGAGCTGTCCATCCAGATTG	CTTAGCTGGTCTTTCTGGTCATTG	DN116728

The primers were designed using Primer Express Software (Applied Biosystems) based on *sus scrofa* published nucleotide sequences (Iccare; http://bioinfo.genopole-toulouse.prd.fr/Iccare/).

Table 3: Growth performance of piglets pair-fed either a well balanced control diet (C: 9.3 g threonine / kg diet) or a low threonine diet (LT: 6.5 g threonine / kg diet) for 2 weeks.

	Diet			
	C	LT	sem	p
Initial weight, kg(day 0)	2.57	2.56	0.01	NS
Final weight, kg (day 14)	4.54	4.52	0.06	NS
BW gain, kg / d	0.130	0.131	0.004	NS
Feed intake, g / kg $BW^{0.75}.d^{-1}$	51.7	51.8	0.74	NS
Thr intake, g / kg $BW^{0.75}.d^{-1}$	0.48	0.34	0.006	< 0.0001

Values are LSmeans for n = 7 piglets. sem are standard error of the mean.

Table 4: Small intestinal morphology of piglets pair-fed either a well balanced control diet (C: 9.3 g threonine / kg diet) or a low threonine diet (LT: 6.5 g threonine / kg diet) for 2 weeks.

	Diet			
	C	LT	sem	p
Jejunum proximal				
villous height, µm	623	653	36	NS
villous surface, µm²	105008	99303	7389	NS
crypt depth, µm	149	145	6	NS
Jejunum distal				
villous height, µm	568	586	39	NS
villous surface, µm²	89384	86907	6189	NS
crypt depth, µm	161	156	7	NS
Ileum				
villous height, µm	591	518	23	0.06
villous surface, µm²	81668	67197	2589	0.007
crypt depth, µm	150	146	4	NS

Values are LSmeans for n = 7. sem are standard error of the mean.

Table 5: Glucose-induced changes in short-circuit current in the ileum of early weaned piglets pair-fed either a well balanced control diet (C: 9.3 g threonine / kg diet) or a low threonine diet (LT: 6.5 g threonine / kg diet) for 2 weeks.

	Diet			
	C	LT	sem	P
V_{max}, µA / cm^{-2}	68.98	124.83	19.54	0.10
K_m, mM	4.93	4.10	0.91	NS

Values are LSmeans for n = 7. sem are standard error of the mean.

Figure titles and legends

Figure 1: Variation of delta $I_{SC,}$ in response to increasing dose of glucose, in the ileum of piglets pair-fed either a well balanced control diet (C: 9.3 g threonine / kg diet; dotted line) or a low threonine diet (LT: 6.5 g threonine / kg diet; full line) for 2 weeks. Tissues were mounted in Ussing chambers and graded doses of glucose were added to the mucosal side every 5 min, osmotically balanced on the serosal side by mannitol. The maximal increase in I_{SC} (delta I_{SC}) after addition of each dose of glucose was recorded. Values are LSmeans ± sem, n = 7. * difference between LT and C piglets, $p < 0.05$.

Figure 2: FITC dextran 4000 Da flux (ng / $cm^{-2}.h^{-1}$) across the ileum of piglets pair-fed either a well balanced control diet (C: 9.3 g threonine / kg diet, white bar) or a low threonine diet (LT: 6.5 g threonine/kg diet, black bar) for 2 weeks. Tissues were mounted in Ussing chambers. FITC dextran 4000 (FD4) was added on the mucosal side at the final concentration of 0.375 mg/mL. Its transport was monitored by sampling solution from the serosal side at 30-min intervals for 120 minutes. After measuring FD4 concentrations in the samples, the flux over the 120 min period was calculated. Values are LSmeans ± sem, n = 7. * difference between LT and C piglets, $p < 0.05$.

Figure 3: Transepithelial resistance (ohms / cm^{-2}) in the ileum of piglets pair-fed either a well balanced control diet (C: 9.3 g threonine/kg diet, white bar) or a low threonine diet (LT: 6.5 g threonine/kg diet) for 2 weeks. Tissues were mounted in Ussing chambers and the transepithelial resistance measured after 20 min-equilibrium. Values are LSmeans ± sem, n = 7.

Figure 4: Relative mRNA abundance of the sodium/glucose cotransporter 1 (SGLT-1, A), the tight junction protein (ZO-1, B) and cingulin (CGN, C) in ileum of piglets pair-fed either a well balanced control diet (C: 9.3 g threonine / kg diet, white bar) or a low threonine diet (LT: 6.5 g threonine/kg diet, black bar) for 2 weeks. Target gene was expressed relatively to GAPDH level. Values are LSmeans ± sem, n = 7. * difference between LT and C piglets, $p<0.05$.

Supplemental Table 1 Genes overexpressed in the ileum of piglets fed a low threonine diet (LT: 6.5 g threonine / kg diet) for two weeks (n = 7).

***Supplemental Table* 2** Genes downexpressed in the ileum of piglets fed a low threonine diet (LT: 6.5 g threonine/kg diet) for two weeks (n = 7).

Figure 1

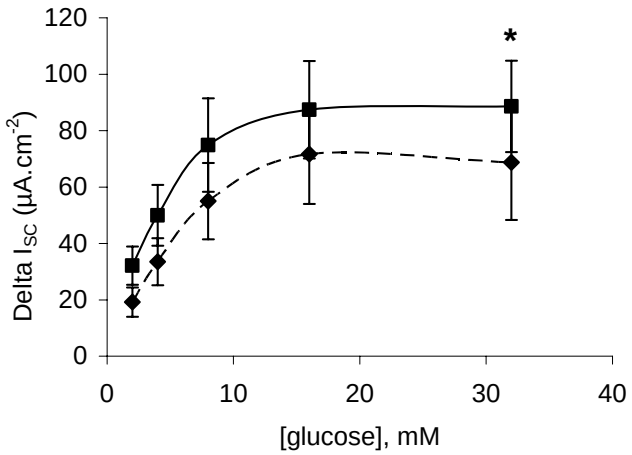


Figure 2

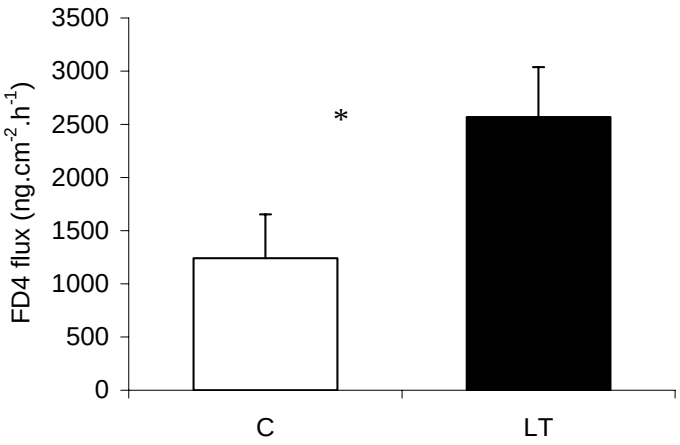


Figure 3

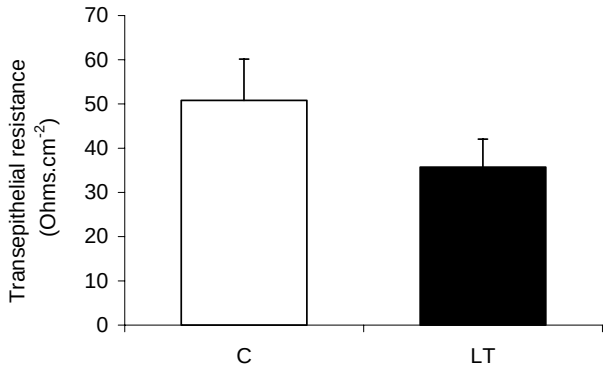
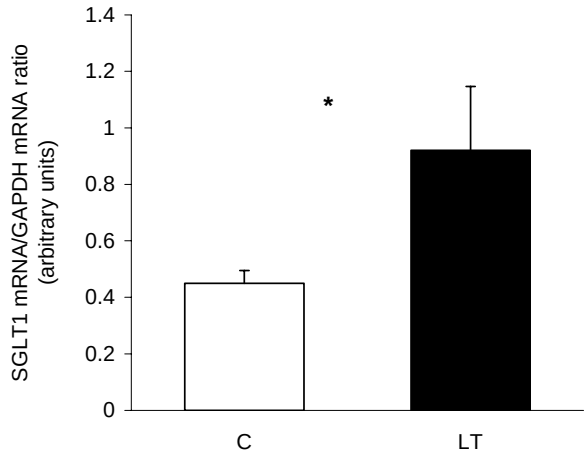
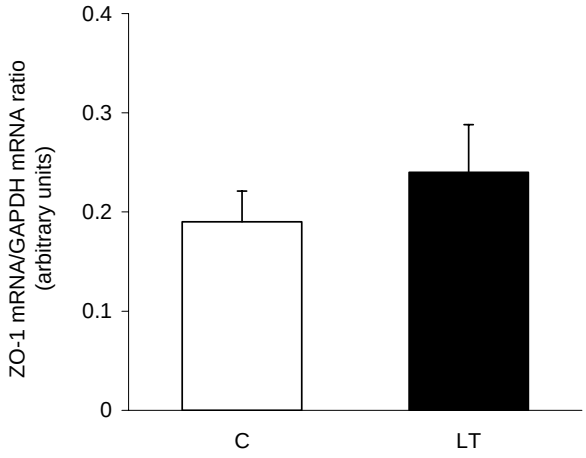

Transepithelial resistance
(Ohms.cm-2)
70
60
50
40
30
20
10
0
C
LT

Figure 4

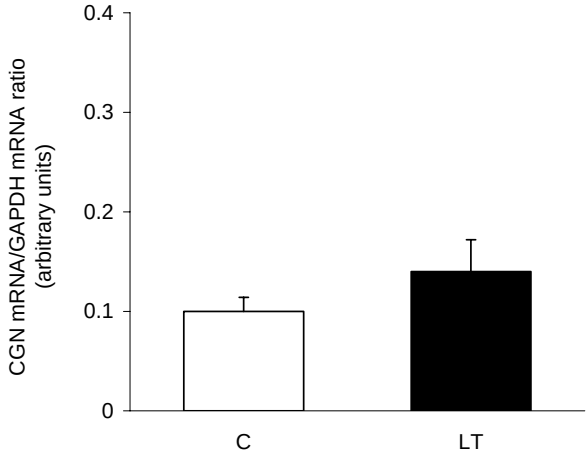
A


SGLT1 mRNA/GAPDH mRNA ratio
(arbitrary units)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
C
LT

B


ZO-1 mRNA/GAPDH mRNA ratio
(arbitrary units)
0.4
0.3
0.2
0.1
0
C
LT

C


CGN mRNA/GAPDH mRNA ratio
(arbitrary units)
0.4
0.3
0.2
0.1
0
C
LT

Supplemental Table 1: Genes overexpressed in the ileum of piglets fed a low threonine diet (LT: 6.5 g threonine/kg diet) for two weeks

ss	CONTIG	GENE	Ratio (LT/C)	SWISS PROT TENTATIVE DESCRIPTION (highest similarity)	BIOLOGICAL PROCESS GO
Immune and defense responses (13)					
scan0016.e.02	BM484902	*C1S*	1.85	Complement C1s subcomponent precursor	Complement activation
scab0141.i.24	BG384365	*IL17RB*	2.64	Interleukin-17 receptor B precursor	Defense response
scab0055.b.04	BF081123	*CCR4*	2.14	C-C chemokine receptor type 4	Inflammatory response; Chemotaxis
scan0030.g.11	BX916389	*CCL16*	2.07	C-C motif chemokine 16 (precursor)	Inflammatory response ; Chemotaxis
scac0025.o.07	CB097354	*DARC*	1.73	Duffy antigen/chemokine receptor	Defence response
scan0003.l.18	CB286296	*PTPRCAP*	2.39	Protein tyrosine phosphatase receptor type C-associated protein	Defence response
scan0007.b.20	BP156850	*CD6*	2.07	T-cell differentiation antigen CD6 precursor	Immune response
scac0025.p.05	BM658975	*HLA-B*	1.87	MHC class I antigen	Antigen processing and presentation of peptide antigen
scab0109.b.13	CF362072	*FCER1A*	1.71	High affinity immunoglobulin epsilon receptor alpha-subunit precursor	Immune response
scan0013.l.17	BM659897	*SEPW1*	1.95	Selenoprotein W	Cell redox homeostasis
scan0021.g.16	CA780101	*DEFB129*	2.72	Beta-defensin 129	Antimicrobial response
scaj0003.d.05	AJ275263	*MGST1*	2.66	Microsomal glutathione S-transferase 1 (EC 2.5.1.18)	Gluthatione metabolic process
scaa0081.l.15	CO994920	*MUC1*	2.03	Mucin-1 precursor	Defence response
Cell cycle, proliferation, differentiation and death (4)					
scan001.j.10	CK460804	*BID*	2.61	BH3 interacting domain death agonist	Induction of apoptosis
scab0053.l.23	CF361784	*DAPK1*	1.93	Death-associated protein kinase 1 (EC 2.7.1.37)	Induction of apoptosis
scan0002.b.07	CF793806	*IGF2**	1.56	Insulin-like growth factor II precursor (IGF-II)	Cell proliferation
scaj0001.d.04	CF176213	*CDK5RAP2*	1.80	CDK5 regulatory-subunit associated protein 2	Regulation of neuron differentiation
Transport (13)					
Scan0037.g.15	BX917979	*SLC24A4*	2.03	Sodium/potassium/calcium exchanger 4 precursor	Ion transport
Scan0002.a.16	BX921422	*PXYD1*	1.71	Phospholemnan precursor (FXYD domain-containing ion transport regulator 1)	Chloride transport
Scac0036.g.11	CA679461	*SGLT-1*	4.9	Sodium/glucose cotransporter 1	Glucose cotransport
Scan0002.b.09	BQ598790	*SLC7A7*	2.60	Y+L amino acid transporter 1 (y+LAT-1)	AA transport
scaa0081.i.16	BP435185	*SCNN1B*	2.01	Amiloride-sensitive sodium channel beta-subunit	Sodium transport
scac0036.g.05	BQ597494	*ATP6V0A2*	2.19	Vacuolar protein translocating ATPase 116 kDA subunit A isoform 2	Ion transport
Scac0040.g.18	BQ603902	*TMEM9*	2.10	Transmembrane protein 9 precursor	Intracellular transport
Scan0018.j.21	BP450608	*KTN1*	1.78	Kinectin (Kinesin receptor)	Cytoskeleton-dependent intracellular transport
Scan0009.c.23	CF791942	*ACTR1B*	1.63	Beta-centractin (Actin-related protein 1B)	Cytoskeleton-dependent intracellular transport
scan0021.i.09	CK465736	*GOLGA5*	2.10	Golgin subfamily A member 5	Golgi vesicle transport
scan0023.m.02	BQ605161	*KPNA1*	1.53	Importin alpha-1 subunit	Import into nucleus
scag0003.c.04	CF179098	*SLC33A1*	2.02	Acetyl-coenzyme A transporter 1 (AT-1)	Acetyl CoA transport
Scan0018.j.06	BM658973	*MTCH2*	1.95	Mitochondrial carrier homolog 2	Transport

Cell communication, cell adhesion and cytoskeleton (13)					
scab0083.n.09	BP152573	*EFNA4*	2.28	Ephrin-A4 precursor	Cell-cell signalling
scaa0084.o.07	AW311973	*GJB5*	2.60	Gap junction beta-5 protein (Connexin-31.1)	Connexon channel activity
scan0012.p.21	CF367574	*TJP1**	2.22	Tight junction protein ZO-1 (Zonula occludens 1 protein)	Cell-cell junction assembly
scab0007.b.16	CF791490	*CGN**	1.93	Cingulin	Cell-cell junction assembly
scac0028.p.11	CF176162	*PXN*	1.53	Paxillin	Cell-matrix adhesion
scan0006.d.21	BX920748	*CELSR2*	1.84	Cadherin EGF LAG seven-pass G-type receptor 2	Cell-cell adhesion
scac0033.g.12	BX670372	*PLEC1*	1.70	Plectin 1(Hemidesmosal protein 1)	Cell adhesion
scaa0085.g.04	BE236040	*CO9A1*	1.91	Collagen alpha 1 (IX)	Cell adhesion
scan0016.c.12	CF177583	*ITGA5*	2.12	Integrin alpha-5 precursor (Fibronectin receptor subunit alpha)	Cell adhesion
scan0005.k.13	BP439633	*NEFM*	2.43	Neurofilament triplet M protein (160 kDa neurofilament protein)	Cytoskeleton organisation
scac0028.g.19	BQ605009	*TMOD1*	1.70	Tropomodulin (Erythrocyte tropomodulin) (E-Tmod)	Cytoskeleton organisation
scan0001.c.12	BX914440	*WIPF1*	2.43	Wiskott-Aldrich syndrome protein interacting protein homolog	Cytoskeleton organization
scan0016.d.21	BX924036	*TPM1*	2.85	Tropomyosin 1	Cytoskeleton organisation
Regulation of transcription and RNA metabolism (15)					
scag0003.c.05	BQ603934	*POLR2B*	1.82	DNA_directed RNA polymerase II 140 kDa polypeptide (EC 2.7.7.6)	Transcription initiation
scac0031.i.17	CB480365	*PAF1*	1.38	RNA polymerase-associated protein 1	Transcription initiation
scaa0084.b.16	CB483014	*GTF2E1*	1.73	Transcription initiation factor IIE alpha subunit (General transcription factor TFIIE-alpha)	Transcription initiation
scan0013.a.02	BQ599964	*GTF2B*	1.81	Transcription initiation factor IIB (General transcription factor TFIIB)	Transcription initiation
scan0024.i.12	BX923131	*KLF9*	2.67	Transcription factor BTEB1 (Basic transcription element binding protein 1)	Regulation of transcription
scan0012.d.08	BQ605150	*SMARCA2*	2.07	Possible global transcription activator SNF2L2	Regulation of transcription
scab0085.k.15	BP153501	*MTF2*	1.81	Metal-response element-binding transcription factor 2	Regulation of transcription
scac0025.g.18	CF787149	*ZNF644*	3.00	Zinc finger protein 644	Regulation of transcription
scan0006.d.14	BX920743	*ZNF169*	1.74	Zinc finger protein 169	Regulation of transcription
scag0003.c.03	BX665395	*ZFP161*	1.89	Zinc finger protein 161	Regulation of transcription
scan0028.f.03	BQ597361	*TRIP4*	1.72	Thyroid hormone receptor interactor 4	Regulation of transcription
scan0011.j.19	BQ599264	*SF3B14*	1.85	Pre-mRNA branch site protein p14 (SF3B 14 kDa subunit)	RNA splicing
Scac0027.p.17	CF788806	*CWF18*	1.94	Cell cycle control protein cwf18	RNA splicing
scac0032.l.09	BX671553	*ADARB2*	1.73	Double-stranded RNA-specific editase B2	RNA processing
scac0032.n.01	BQ601512	*SERBP1*	2.22	Plasminogen activator inhibitor 1 RNA-binding protein	Regulation of mRNA stability
Cellular protein metabolism (10)					
scan0003.c.10	CA779705	*DNJB9*	1.86	DnaJ homolog subfamily B member 9	Protein folding
scan0036.a.17	CA778605	*PPIF*	1.66	Peptidyl-prolyl cis-trans isomerase, mitochondrial precursor (EC 5.2.1.8)	Protein folding
Scac0026.o.24	BX666928	*STUB1*	2.00	STIP1 homology and U box-containing protein 1 (EC 6.3.2.-)	Positive regulation of protein ubiquitination
Scac0034.g.21	BM675718	*MPPB*	1.63	Mitochondrial processing peptidase beta subunit	Proteolysis
Scac0033.p.20	CB472986	*FBXW4*	1.61	F-box/wd-repeat protein 4 (Dactylin)	Ubiquitin-dependent protein catabolic process

scan0022.f.09	BQ599441	*ZMPSTE24*	2.38	CAAX prenyl protease 1 homolog (EC 3.4.24.84)	Proteolysis
scan0023.a.05	BM659723	*VCP*	2.20	Transitional endoplasmic reticulum ATPase	ER-associated protein catabolic process
scan0031.a.12	CB471256	*TSSC1*	1.83	Protein TSSC1	Protein binding
scan0020.a.05	BX921900	*MTIF2*	1.90	Translation initiation factor IF-2, mitochondrial precursor	Regulation of translational initiation
Scac0027.g.18	BQ597589	*RT10*	1.63	Mitochondrial 28S ribosomal protein S10 (S10mt)	Translation

Signal transduction (10)

scac0034.i.03	CA780698	*APLP2*	2.30	Amyloid-like protein 2 precursor	G-protein coupled receptor protein signalling pathway
scac0033.g.11	BQ597942	*CALCR*	1.84	Calcitonin receptor precursor	G-protein coupled receptor protein signalling pathway
scan0010.b.03	BX922704	*GPR124*	1.98	Probable G-protein coupled receptor 124 precursor	G-protein coupled receptor protein signalling pathway
scan0025.c.02	BX921688	*PAK4*	2.60	Serine/threonine-protein kinase PAK 4 (EC 2.7.1.37)	Signal transduction
scan0003.b.04	BQ602864	*RAP1A*	1.62	Ras-related protein Rap-1A (Ras-related protein Krev-1)	Signal transduction
scaj0013.m.20		*GAP*	2.10	GTPase-activating protein GAP	Signal transduction
scan0025.c.08	CF364431	*CAMK2B*	2.37	Calcium/calmodulin-dependent protein kinase type II beta chain (EC 2.7.1.123)	Signal transduction
scan0006.g.02	BQ598573	*IRS1*	1.87	Insulin receptor substrate 1	Insulin receptor signalling pathway
scaa0064.k.04	AW485812	*LTBP2*	2.76	Latent transforming growth factor-beta-binding protein 2 precursor	TGFβ receptor signalling pathway
scaa0113.l.01	AU296045	*ARHGEF4*	1.88	Rho guanine nucleotide exchange factor 4 (APC-stimulated guanine nucleotide exchange factor) (Asef)	Regulation of Rho protein signal transduction

Other biological process (22)

scan0035.i.17	BX916635	*ATP5O*	1.81	ATP synthase O subunit	ATP biosynthetic process
scan0018.j.07	CF792524	*NUFM*	2.18	NADH-ubiquinone oxidoreductase 13 kDa-B subunit (EC 1.6.99.3)	ATP biosynthetic process
scab0081.d.15	CF175249	*CACP*	2.12	Carnitine O-acetyl transferase (EC 2.3.1.7)	Fatty acid metabolism
scac0036.n.17	CF364016	*CPT1B*	1.66	Carnitine O-palmitoyltransferase I	Fatty acid beta-oxidation
scan0012.o.24	BX919932	*FAT2*	2.00	Peroxisomal-coenzyme A synthetase	Fatty acid metabolism
Scac0040.e.22	BQ600082	*PECI*	1.83	Peroxisomal 3,2-trans-enoyl-CoA isomerase (EC 5.3.3.8)	Fatty acid metabolism
scan0005.k.19	CB286764	*CHKB*	2.24	Choline/ethanolamine kinase	Phospholipid biosynthetic process
scac0038.e.23	BM658676	*CP11A*	2.15	Cytochrome P450 11A1, mitochondrial precursor (EC 1.14.15.6)	Steroid biosynthetic process
scan0029.k.16	BX916139	*CYP21*	1.88	Cytochrome P450 XXI (EC 1.14.99.10)	Steroid biosynthetic process
scac0034.a.19	BX670680	*GAMT*	1.78	Guanidinoacetate N-methyltransferase (EC 2.1.1.2)	Creatine biosynthetic process
scan0005.k.05	BX917589	*MAN2B2*	2.28	Mannosidase alpha class 2B member 2 (EC 3.2.1.24)	Mannose metabolic process
scan0012.f.03	BM190280	*PHS2*	2.07	Glycogen phsopshorylase, muscle form (EC 2.4.1.1)	Glycogen metabolic process
scan0036.m.17	BX918235	*PCYOX1*	1.86	Prenylcysteine oxidase precursor (EC 1.8.3.5)	Prenylcystein catabolic process
scan0008.b.02	BX919941	*LZTR1*	2.19	Leucine-zipper-like transcriptional regulator 1	Anatomical structure morphogenesis
scaa0064.h.24	CN029176	*POU5F1*	1.76	POU domain, class 5, transcription factor 1	Anatomical structure morphogenesis
scag0004.c.10	BM445302	*ANKRD2*	2.33	Ankyrin repeat domain protein 2	Muscle development
scan0038.e.18	BM190067	*MB*	1.72	Myoglobin	Muscle oxygenation
scan0011.k.02	BQ598464	*AHSP*	1.77	Alpha-hemoglobin stabilizing protein (Erythroid-associated factor)	Hemoglobin metabolic process
scan0012.j.07	BX921917	*F12*	1.51	Coagulation factor XII precursor (EC 3.4.21.38)	Blood coagulation
scan0015.f.06	AJ429264	*NARG1*	2.63	NMDA receptor regulated protein 1	
scan0034.m.16	BX917536	*NDST1*	1.97	Heparan sulfate N-deacetylase/N-sulfotransferase (EC 2.8.2.8)	Heparan sulphate proteoglycan process

scaa.0085.f.12	CK454646	*PTGER3*	1.80	Prostaglandin E2 receptor, EP3 subtype	Cell homeostasis

Unknown biological process (45)

scan0013.l.08	CF181520	*UTX*	1.61	Ubiquitously transcribed X chromosome tetratricopeptide repeat protein
scac0044.d.24	BM659499	*POMC*	1.60	Corticotropin-lipotropin precursor (Pro-opiomelanocortin) (POMC)
scan0005.i.07	BX664905	*UPL2*	1.83	E3 ubiquitin protein ligase UPL2 (EC 6.3.2.-)
scac0038.g.16	BX674115	*CRYBA4*	1.85	Beta crystallin A4
scac0036.m.12	BX671984	*ADAM7*	1.83	A disintegrin and metalloproteinase domain 7
scan0033.m.17	BX917829	*REC8L*	2.91	Meiotic recombination protein REC8-like1 (Cohesin Rec8p)
scan0027.k.07	BM659681	*Serp1*	1.96	Stress-associated endoplasmic reticulum protein 1
scac0031.j.21	BX668576		1.93	Tsga10ip protein (Fragment)
scan0012.m.20	BX923588		2.05	UPF0472 protein C16orf72 homolog
scan0031.c.11	BX915954		1.95	Scavenger receptor cysteine-rich domain-containing protein LOC284297 homolog
scac0035.c.15	CB287682	*LYSMD1*	1.76	LysM and putative peptidoglycan-binding domain-containing protein 1
scac0033.i.01	CA778419	*FJX1*	1.99	Four-jointed box protein 1
scan0035.k.05	BP452343	*SGF3*	1.84	Silk gland factor 3
scan0035.k.04	BX915677	*SYNPO2L*	1.74	Synaptopodin 2-like protein
scan0028.f.20	BX914945	*Srst*	2.47	Octapeptide-repeat protein T2
scan0001.n.22	BX914369	*VPS39*	2.38	Vam6/Vps39-like protein
scac0030.b.14	CA780947	*ZFP64*	1.94	Zinc finger protein 64, isoforms 1 and 2
scan0016.a.19	BX924017	*CMYA5*	2.22	Myospryn
scag0006.g.10	BX665429	*PLXND1*	2.10	Plexin D1 precursor
scab0080.e.08	CF793796	*DMWD*	2.34	Dystrophia myotonica-containing WD repeat motif protein
scan0027.k.19	CB471599	*UBXD2*	1.63	UBX domain-containing protein 2
Scan0003.n.19	CB478819	*WDR13*	2.08	WD-repeat protein 13
Scac0030.i.21	BX676540	*MBRL*	1.51	Membralin
Scac0028.p.19	CF361271	*CU059*	1.75	Protein C21orf59
Scac0033.i.08	BQ599533	*Q3SYV1*	1.87	Hypothetical protein MGC127570
Scac0025.g.24	CF177974	*Q6K322*	1.74	Putative vegetative cell wall protein gp1precursor
Scac0031.j.15	BX668567	*Q9BGV3*	2.82	Hypothetical protein
Scan0012.m.20	BX923588	*Q6PAX8*	2.05	MGC68553 protein
Scag0009.c.04	BQ597597	*Q86v52*	1.99	Hypothetical protein MGC39606
Scan0034.l.21	BQ601418	*U327*	1.80	Hypothetical UPF0327 protein
Scag0003.c.07	CB479247	*RIM9*	1.76	pH-response regulator protein palI/RIM9
Scan0004.l.22	BX920964	*YPD8*	2.40	Hypothetical protein C05D11.8 in chromosome III
Scac0036.O.15	CA778597	*YMA7*	1.99	Hypothetical protein F54F2.7 in chromosome III
Scan0003.n.03	BX920856	*VE4*	2.42	Probable protein E4
Scan0026.a.22	CB474178	*MUTA*	2.01	Methylmalonyl-CoA mutase (EC 5.4.99.2)
Scac0041.n.17	BX674830	*Q4ITL4*	2.71	Lipopolysaccharide kinase
scan0007.a.06	BP149772	*C9orf9*	1.99	Uncharacterized protein C9orf9
scab0108.i.02	AW435883	*CMTM5*	1.70	CKLF-like MARVEL transmembrane domain-containing protein 5 (Chemokine-like factor superfamily member 5)

scan0022.d.05	CB287200	*MS4A8B*	2.27	Membrane-spanning 4-domains subfamily A member 8B
Scac0029.p.23	BM659898	*BRD2*	2.11	Bromodomain containing protein 2
scan0022.e.23	BX922153	*HS3ST2*	2.39	Heparan sulfate glucosamine 3-O-sulfotransferase 2 (EC 2.8.2.29)
scan0008.j.15	CB462875	*Hsp67Bb*	1.68	Heat shock protein 67B2
scan0017.m.20	BX923731		1.87	Hibernation-associated plasma protein HP-27 precursor
scan0016.c.03	BX923065	*GOLGA2*	2.56	Golgin subfamily A member 2
Scac0040.g.10	CF788497	*SPASR*	1.96	Spastin7

Clones without informative annotation (69): scaa0115.k.03; scac0025.h.05; scac0026.h.18; scac0026.p.12; scac0027.p.16; scac0029.h.18; scac0029.h.23; scac0029.i.04; scac0029.i.24; scac0030.i.18; scac0030.j.10; scac0031.j.20; scac0031.j.22; scac0031.k.01; scac0032.d.11; scac0032.l.10; scac0033.o.21; scac0036.e.16; scac0036.f.07; scac0036.f.17; scac0036.n.07; scac0040.e.07; scac0041.l.21; scac0042.m.12; scac0043.a.01; scag0002.c.04; scag0003.c.10; scag0003.h.12; scag0004.b.09; scag0005.g.02; scag0006.g.12; scag0011.g.04; scaj0012.k.12; scan0003.b.17; scan0003.n.07; scan0003.o.10; scan0004.n.08; scan0005.j.10; scan0006.g.20; scan0007.b.24; scan0008.j.09; scan0009.n.17; scan0009.o.16; scan0010.a.09; scan0010.n.02; scan0011.i.01; scan0011.j.20; scan0012.f.06; scan0012.m.14; scan0012.n.24; scan0012.p.04; scan0016.b.06; scan0016.d.23; scan0018.j.05; scan0018.j.08; scan0018.j.16; scan0019.b.07; scan0019.c.09; scan0021.f.20; scan0024.f.21; scan0024.j.16; scan0025.b.06; scan0025.b.15; scan0025.d.19; scan0027.m.14; scan0030.f.07; scan0032.a.02; scan0033.n.08; scan0035.j.07.

Supplemental Table 2: Genes downexpressed in the ileum of piglets fed a low threonine diet (LT: 6.5 g threonine / kg diet) for two weeks.

Clone	Contig	Gene	Ratio (LT/C)	Swiss Prot tentative description (highest similarity)	Biological process GO
Cell cycle, proliferation, differentiation and death (11)					
scan0026.h.21	BQ599726	*BTG1*	*0.59*	BTG1 protein (B-cell translocation gene 1 protein)	Negative regulation of cell growth ; Negative regulation of cell proliferation
scan0019.g.11	BP164036	*PINX1*	*0.59*	Pin2-interacting protein X1	Negative regulation of cell proliferation
scan0003.h.05	BX920295	*FOXC1*	*0.71*	Forkhead box protein C1	Negative regulation of mitotic cell cycle
scan0025.i.18	AU296654	*POLL*	*0.42*	DNA polymerase lambda (EC 2.7.7.7) (EC 4.2.99.-)	DNA repair
scan0009.j.16	BX919593	*Smg1*	*0.63*	Serine/threonine-protein kinase	DNA repair
scan0011.k.16	BX918925	*TBCB*	*0.60*	Tubulin-specific chaperone B	Nervous system development
scac0043.e.12	CB477020	*CDK5RAP3*	*0.67*	CDK5 regulatory subunit-associated protein 3	Regulation of neuron differentiation
scan0014.k.10	CF791957	*NTRK2*	*0.61*	BDNF/NT-3 growth factors receptor (EC 2.7.10.1)	Nervous system development
scan0020.b.08	BX924367	*COL1A2*	*0.52*	Collagen alpha 2(I) chain precursor	Skeletal development
scac0025.l.17	BM484811	*COL9A2*	*0.63*	Collagen alpha 2(IX) chain precursor	Skeletal development
scac0026.c.06	CF178392	*DLL4*	*0.58*	Delta-like protein 4 (precursor)	Angiogenesis
Transport (10)					
scan0020.e.04	CB287365	*AP3D1*	*0.65*	Adapter-related protein complex 3 delta 1 subunit	Vesicle-mediated transport
scac0033.k.01	CB477797	*PCOLN3*	*0.57*	Charged multivesicular body protein 1a	Vesicle-mediated transport
scan0032.i.13	BQ604596	*GGA3*	*0.52*	ADP-ribosylation factor-binding protein GGA3	Vesicle-mediated transport
scan0036.g.11	BQ600225	*VPS33B*	*0.55*	Vacuolar protein sorting-associated protein 33B	Vesicle-mediated transport
scan0004.p.03	BX918993	*KIF2A*	*0.55*	Kinesin-like protein KIF2 (Kinesin-2)	Microtubule-dependent intracellular transport
scan0028.k.11	CF179877	*TRAPPC5*	*0.59*	Trafficking protein particle complex subunit 5	ER to Golgi vesicle-mediated transport
scan0034.b.11	BQ602404	*SEC63*	*0.61*	Translocation protein SEC63 homolog	Protein targeting to membrane ; Protein folding
scac0033.k.04	CF794880	*SYS1*	*0.62*	Protein SYS1 homolog	Protein transport
scan0024.k.17	BQ599032	*NUTF2*	*0.49*	Nuclear transport factor 2	Protein transport
scan0017.b.17	BQ601586	*CDC42SE1*	*0.59*	CDC42 small effector protein 1	Phagocytosis
Cell communication, cell adhesion and cytoskeleton (1)					
scac0033.a.06	BX671826	*RELN*	*0.65*	Reelin precursor (EC 3.4.21.-)	Cell communication
Regulation of transcription and RNA metabolism (13)					
scan0035.b.12	CF795871	*ZFP37*	*0.63*	Zinc finger protein 37	Regulation of transcription
scan0030.k.23	BX916479	*ZNF429*	*0.49*	Zinc finger protein 429	Regulation of transcription
scan0010.k.05	BX918916	*BAZ2A*	*0.72*	Bromodomain adjacent to zinc finger domain 2A	Chromatin remodelling
scan0020.o.05	CB480127	*PRMT5*	*0.67*	Protein arginine N-methyltransferase 5 (EC 2.1.1.-)	RNA splicing; Spliceosomal snRNP biogenesis
scac0030.d.09	BX669065	*RBM9*	*0.62*	RNA-binding protein 9	RNA splicing; Regulation of cell proliferation
scaa0090.o.15	BP439412	*SF1*	*0.72*	Splicing factor 1	RNA splicing ; Spliceosome assembly
scac0025.i.06	CF364599	*SFRS5*	*0.55*	Splicing factor, arginine/serine-rich 5	RNA splicing ; mRNA splice site selection

scaj0016.i.05	CF361092	*SRRM1*	*0.60*	Serine/arginine repetitive matrix protein 1	RNA splicing
scac0033.l.01	BM658825	*STRAP*	*0.62*	Serine-threonine kinase receptor-associated protein	RNA splicing
scan0005.o.02	BX920377	*LSM2*	*0.71*	U6 snRNA-associated Sm-like protein LSm2	RNA splicing
scac0043.l.01	BM190144	*HIST1H2BD*	*0.74*	Histone H2B.b (H2B.1 B)	Nucleosome assembly
scaa0004.m.17	BE234098	*ELAC2*	*0.59*	Zinc phosphodiesterase ELAC protein 2 (EC 3.1.26.11)	tRNA processing
scan0027.c.09	CF176007	*EDC3*	*0.52*	Enhancer of mRNA-decapping protein 3	mRNA degradation

Cellular protein metabolism (10)

scan0031.d.16	BQ600874	*PFDN2*	*0.45*	Prefoldin subunit 2	Protein folding
scan0022.i.02	BX926209	*TOR1A*	0.49	Torsin A precursor	Protein folding
scac0033.l.16	CB473763	*BAP1*	0.78	Ubiquitin carboxyl-terminal hydrolase BAP1 (EC 3.4.19.12)	Ubiquitin-dependent protein catabolic process ; Negative regulation of cell proliferation
scan0020.b.19	CF787985	*PSMB3*	*0.50*	Proteasome subunit beta type 3 (EC 3.4.25.1)	Ubiquitin-dependent protein catabolic process
scac0029.d.19	BM658988	*TRIP12*	*0.59*	Probable E3 ubiquitin-protein ligase TRIP12 (EC 6.3.2.-)	Protein ubiquitination
scac0035.h.13	CB477405	*FBXO22*	*0.61*	F-box only protein 22	Ubiquitin-dependent protein catabolic proces
scan0038.j.06	BQ604222	*BAT3*	0.54	Large proline-rich protein BAT3	Protein modification process
scac0039.b.02	BM484008	*UBQLN1*	*0.67*	Ubiquilin-1	Protein modification process
scac0038.k.18	CF181697	*GCN2*	*0.56*	Eukaryotic translation initiation factor 2-alpha kinase 4 (EC 2.7.11.1)	Regulation of translational initiation
scan0020.d.09	BQ605065	*EIF4EBP1*	*0.52*	Eukaryotic translation initiation factor 4A-binding protein 1	Regulation of translational initiation

Signal transduction (6)

scan0016.f.21	CF795279	*PIK4CA*	*0.55*	Phosphatidylinositol 4-kinase alpha (EC 2.7.1.67)	Signal Transduction
scan0009.f.20	AU296611	*PIP5K1C*	*0.51*	Phosphatidylinositol-4-phosphate 5-kinase type I gamma (EC 2.7.1.68)	Signal transduction
scan0006.j.01	BQ603969	*IRF2*	*0.63*	Interferon regulatory factor 2	Signal transduction
scab0038.h.18	AW486143	*MAPK8*	*0.39*	Mitogen-activated protein kinase 8 (EC 2.7.1.37)	Signal transduction
scan0039.f.24	BX918476	*BCR*	*0.52*	Breakpoint cluster region protein (EC 2.7.1.-)	Signal transduction
scag0006.b.06	BQ601055	*JAK1*	*0.68*	Tyrosine-protein kinase JAK1 (EC 2.7.1.112)	Signal transduction

Other biological process (7)

scan0037.n.06	CB468944	*FABP5*	*0.51*	Fatty acid-binding protein, epidermal	Lipid metabolic process
scan0020.o.14	BQ597572	*GNPAT*	*0.59*	Dihydroxyacetone phosphate acyltransferase (EC 2.3.1.42)	Fatty acid metabolic process
scag0004.e.03	BX665123	*CYB561*	*0.52*	Cytochrome b561 (Cytochrome b-561)	Generation of precursors metabolites and energy
scac0028.c.01	BX676542	*NMNAT1*	*0.52*	Nicotinamide mononucleotide adenylyltransferase 1 (EC 2.7.1.1)	NAD biosynthetic process
scan0011.l.22	CF366445	*NDST2*	*0.60*	Heparin sulfate N-deacetylase/N-sulfotransferase (EC 2.8.2.-)	Heparan sulphate proteoglycan biosynthetic process
scan0020.e.14	BM083159	*MYLK*	*0.61*	Myosin light chain kinase, smooth muscle and non-muscle isozymes (EC 2.7.11.18)	Protein amino acid phosphorylation
scan0018.l.09	BM658572	*ARS2*	*0.44*	Arsenite-resistance protein 2	Response to arsenic

Unknown biological process (21)

scac0027.a.20	CF366739	*B6404.2*	*0.57*	Hypothetical protein B0464.2 in chromosome III	

scac0034.n.21	BQ602036	*EFEMP1*	*0.59*	EGF-containing fibulin-like extracellular matrix protein 1
scan0013.o.02	CA781095	*KIAA1542*	*0.51*	RING and PHD-finger domain-containing protein KIAA1542
scan0018.b.18	BP166641	*TBC1D13*	*0.51*	TBC1 domain family member 13
scan0008.l.23	BQ597366	*ARGLU1*	*0.55*	Arginine and glutamate-rich protein 1
scaa0090.k.14	BM484348	*Pi4k2a*	*0.59*	Adult male spinal cord cDNA, RIKEN full-length enriched library, clone:A330095A06 product:inferred: 55 kDa type II phosphatidylinositol 4-kinase (Rattus norvegicus), full insert sequence
scan0013.b.22	CB285603	*BSDC1*	*0.63*	BSD domain-containing protein 1
scac0028.j.18	BQ604045	*OBTP*	*0.64*	Overexpressed breast tumor protein homolog
scan0022.h.10	CK454312	*MEGF9*	*0.47*	Multiple EGF-like-domain protein 5 precursor
scag0007.b.08	BQ604208	*Arl4c*	*0.53*	ADP-ribosylation factor-like protein 4C
scan0023.d.06	CB469449	*ATP13A2*	*0.61*	Probable cation-transporting ATPase 13A2 (EC 3.6.3.-)
scan0016.i.07	CA779229	*KIAA0737*	*0.71*	Epidermal Langerhans cell protein LCP1
scac0027.a.17	BX672648		*0.51*	Dpy-30-like protein
scan0035.c.15	BX671049	*HIGD2A*	*0.54*	HIG1 domain family member 2A
scac0038.l.24	BQ602258	*MAPBPIP*	*0.57*	Late endosomal/lysosomal Mp1 interacting protein
scan0008.n.07	CA778984	*MRPL46*	*0.63*	39S ribosomal protein L46, mitochondrial precursor
scac0036.h.23	BQ600174	*MRPS23*	*0.68*	Mitochondrial ribosomal protein S23 (S23mt)
scan0002.d.01	BQ605112	*SGTA*	*0.58*	Small glutamine-rich tetratricopeptide repeat-containing protein A
scac0035.n.08	BX670521	*FBXL12*	*0.57*	F-box/LRR-repeat protein 12
scan0027.n.16	BX915058	*HELZ*	*0.59*	Probable helicase with zinc-finger domain (EC 3.6.1.-)
scac0035.p.20	BQ601210	*FLOT1*	*0.67*	Flotillin-1

Clones without informative annotation (31): scaa0016.c.10; scac0026.b.06; scac0026.c.15; scac0030.f.02; scac0031.d.15; scac0037.a.17; scac0043.e.19; scaj0009.c.15; scan0004.p.15; scan0005.l.13; scan0008.c.24; scan0008.d.05; scan0008.l.18; scan0012.h.19; scan0014.c.07; scan0014.j.07; scan0016.f.11; scan0016.f.14; scan0017.o.14; scan0018.k.20; scan0019.i.05; scan0021.l.22; scan0023.d.20; scan0023.p.09; scan0024.b.12; scan0026.g.18; scan0027.n.09; scan0030.j.05; scan0030.l.01; scan0037.a.24; scan0039.f.11.