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Downregulation of two novel genes in Sl/Sld and W^{LacZ}/Wv mouse jejunum ☆,☆☆

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Abstract

Interstitial cells of Cajal (ICC) are the so-called pacemaker cells of the gut. W^{LacZ}/Wv and Sl/Sld mice lack ICC surrounding the myenteric plexus (MP) in the jejunum. We compared the gene expression profile of wild type (WT) and W^{LacZ}/Wv and Sl/Sld mice using suppression subtractive hybridization (SSH), generating a cDNA library of 1303 clones from which 48 unique sequences were differentially expressed with Southern blot. Among them, we identified heme oxygenase2, TROY, and phospholamban in ICC using immunohistochemistry. Using RT-qPCR, c-Kit and two new transcripts Dithp and prenylcysteine oxidase1 were significantly lower expressed in Sl/Sld and W^{LacZ}/Wv versus WT. Prenylcysteine oxidase1 appeared cytotoxic for COS-7 cells and was highly expressed in liver while Dithp was mainly expressed in small intestine. The combination of SSH, Southern blot, RT-qPCR, and immunohistochemistry turned out to be a useful approach to identify rarely expressed genes and genes with small differences in expression.

Keywords: Interstitial cells of Cajal; Dithp; Prenylcysteine oxidase1; Heme oxygenase2; Phospholamban; TROY

The role of the interstitial cells of Cajal (ICC) in the generation of the slow waves involved in proper transit of the gut content has been established in recent years [1–3]. Depending on their location, ICC are associated with several functions. ICC at the level of the deep muscular plexus (ICC-DMP) and intramuscular ICC (ICC-IM) are believed to mediate neurotransmission [4–6], while ICC surrounding the myenteric plexus (ICC-MP) are involved in the generation of the slow waves [2,5,7].

In humans, disturbed ICC networks have been reported in several disorders of the GI transit, e.g. chronic intestinal pseudo-obstruction [8–10], infantile pyloric stenosis [11], Hirschsprung's disease [12,13], slow transit constipation [14–17], and diabetic gastroparesis [18]. Alterations of ICC function might also occur in other conditions where ICC distribution appears unaffected [19].

In mice carrying partial loss of function mutations in the c-Kit gene (W/Wv) or its ligand stem cell factor (Sl/Sld), ICC-DMP are preserved while ICC-MP are lacking, resulting in the loss of slow waves and in a disturbed intestinal transit [5,20–22]. Although the loss of ICC in the jejunum

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is restricted to ICC-MP, these two models, W/Wv and Sl/Sld, will further be referred to as ''ICC-deficient'' mice. The selective loss of ICC-MP in the jejunum of these models compared to their WT littermates offered the opportunity to identify genes or proteins that are restricted to ICC-MP. The identification of previously unrecognized proteins in ICC, and elucidation of their function might be a way to find innovative, new therapeutic developments in GI motility disorders.

Suppression subtractive hybridization (SSH), a PCR-based method of subtractive cloning, compares two populations of mRNA in order to identify transcripts that are expressed in one population but not in the other. Here, SSH was performed in combination with Southern blotting, followed by RT-qPCR and immunohistochemistry in order to identify genes less expressed in the ICC-deficient models Sl/Sld and W^{LacZ}/Wv mice compared to WT. In the present study, the isolation and characterization of c-Kit and 2 uncharacterized genes Dithp and prenylcysteine oxidase1 less expressed in jejunal tissues of W^{LacZ}/Wv and Sl/Sld mice is reported. Furthermore, we could confirm the expression of KIT, phosphorylated phospholamban, heme oxygenase2, and TROY in ICC.

Materials and methods

Animals. Mice were maintained and experiments performed in agreement with the Local Ethical committees of the Université Libre de Bruxelles, Brussels, Belgium and Janssen Pharmaceutica NV, Beerse, Belgium. Heterozygous Sl/+ and Sld/+ mice were obtained from Jackson Laboratory (Bar Harbor, ME). Wv/+ and W^{LacZ}/+ mice were a generous gift from Prof. J.J. Panthier, Ecole Nationale Vétérinaire, Maisons-Alfort, France. The product of the W^{LacZ} transgene lacks kinase activity and is functionally equivalent to the spontaneous null allele W [23]. As both W^{LacZ} and Sl homozygous mutations are lethal, heterozygous mice were bred to obtain viable Sl/Sld, W^{LacZ}/Wv and WT littermates. One-month-old mice were killed by cervical dislocation. The jejunum was quickly dissected out, flushed with ice-cold Krebs solution, and pinned onto a Petri dish cooled at 4 °C. Under a binocular microscope the mesentery was carefully removed.

PCR suppression subtractive hybridization (SSH) and differential screening. SSH was performed as described in detail elsewhere [26]. Briefly, mucosa was removed from the muscle layers of WT and ICC-deficient mouse jejunum, and total RNA was extracted from the muscle layers using the Rneasy™ Fibrous Tissue Mini kit (Qiagen, Westburg BV, Leusden, Netherland) and treated with DNAse I (On Column Dnase, Qiagen).

Poly(A)$^+$ RNA was isolated from total RNA using Oligotex™ mRNA spin-columns (Qiagen).

Two micrograms of poly(A)$^+$ RNA was reverse-transcribed using Powerscript™ Reverse Transcriptase (BD Biosciences, Erembodegem, Belgium), and PCR Select™ cDNA subtraction (SSH) was performed according to the manufacturer's instructions (Clontech, Palo Alto, CA).

The subtraction was carried out in two ways: (1) the forward subtraction, WT minus ICC-deficient, contained cDNA enriched for ICC related genes while (2) the reverse subtraction, ICC-deficient minus WT, served as negative control. The subtraction procedure was performed on both models of ICC-deficient mice, Sl/Sld and W^{LacZ}/Wv.

From the forward-subtracted cDNA library 1344 clones were isolated, and used for colony PCR and DNA sequencing using the ABI PRISM BigDye Terminators v3.0 Cycling Sequencing Kit (Applied Biosystems, Foster City, CA).

Southern blotting. PCR products of the clones from the forward-subtracted cDNA library were spotted identically on two positive-charge nylon membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) and hybridized overnight at 65 °C to ^{32}P-labeled (Megaprime™ DNA labeling system, Amersham) forward- and reverse-subtracted cDNAs from both subtractions (Sl/Sld and W^{LacZ}/Wv), respectively. Analysis of the Southern blots was performed using Imagene™ 4.2 software (BioDiscovery Inc., El Segundo, CA).

Bio-informatics (in silico analysis). Sequences (1344) were compared to the Universal Protein Resource (Uniprot) sequence database [24] using BLASTX [25] and were annotated as the hit sequence when the sequence and the BLAST hit contained at least 95% similar amino acids, the overlap between query and hit of at least 20 residues and the proportion of overlap between query and hit was at least 25% of the total sequence length. The sequences were also compared to each other using BLASTN [25] and considered to be identical if they contained at least 95% identical nucleotides, an overlap between query and hit of at least 20 nucleotides, and the proportion of overlap between query and hit is at least 25% of the total length of the sequence.

Real time quantitative PCR (RT-qPCR). Expression levels of genes having a difference larger than 2 in hybridization signal with the forward versus the reverse probe from both ICC-deficient models were examined by SybrGreen™ QPCR (Applied Biosystems, Warrington, UK), on reverse-transcribed total RNA isolated from jejunum muscle layers.

Amplification reactions were performed in triplicate with 1× PCR master mix (Applied Biosystems), 200 nmol/L of gene specific primers, and 25 ng of sample DNA in a 50 µl final volume. The primers were designed with Primer Express 1.5 (Table 1) and RT-qPCR was performed on an ABI Prism 1700 Sequence detector. Identical thermal profile conditions, namely 95 °C for 10 min, 45 cycles of 95 °C for 15 s, and 60° for 1 min, were used for all primer sets. Emitted fluorescence was measured during the annealing/extension phase, and amplification plots were generated using the Sequence Detection System (Applied Biosystems). To differentiate specific amplicons from nonspecific products using SybrGreen, a DNA dissociation curve was generated after each reaction with the ABI Prism sequence detection system.

All mRNAs were quantified relative to GAPDH mRNA using the comparative threshold cycle number (Ct) method. The Ct difference ($\delta Ct = Ct_{gene} - Ct_{GAPDH}$) was taken as a relative quantity of the transcript. To ascertain the validity of the δδCt calculation ($\delta\delta Ct = \delta Ct_{ICCdeficient} - \delta Ct_{WT}$), the amplification efficiency was checked and found to be identical for all the genes measured. Statistical analysis was performed with the unpaired *t* test and a *p*-value smaller than 0.05 was considered to represent a statistically significant difference.

cDNA of different mouse adult and embryonic tissues (MTCI, Clonetech) was analyzed with oligos for the uncharacterized genes Dithp and prenylcysteine oxidase1 using RT-qPCR. The absolute amount of mRNA was calculated based on standard curves.

Rapid amplification of cDNA ends (RACE). The full-length clones of two EST transcripts were identified using the SMART-RACE™ cDNA amplification kit (Clontech) according to the manufacturer's specifications (primers in Table 1). Assembling the cDNA sequences, the entire coding sequence (cds) was determined and sequencing was performed as described above. Similarity to known genes was investigated using the BLAST program and the translated protein sequence was searched for protein specific domains using SMART analysis (http://smart.embl-heidelberg.de/).

Transfections and cellular localization studies. HeLa cells were seeded into Lab-Tek II™ chamber #1.5 German coverglass (Nalge Nunc International Corp., Naperville, IL, USA) at 10^5 cells/ml. Cells were transfected at 60% confluency (16–24 h after plating) using 0.2 µg of each plasmid DNA (coding sequence cloned in pZsGreen vector, Clontech) and FuGene 6 ™ transfection Reagent (Roche Diagnostics Corp., Germany), at a ratio of 1:3 µg/µl, for 24 h.

Immunohistochemistry. HeLa cells grown on coverslips were washed in PBS and fixed with 4% paraformaldehyde (PFA, Sigma, Bornem, Belgium) in PBS for 10 min and kept at 4 °C until use. Nuclear staining was performed during 5 min with 1/10,000 TOPRO-3 (Molecular Probes,

Table 1
List of primer sequences for RT-qPCR and SMART-RACE

Gene	Protein	Sequence primer 5′–3′	Sequence primer 3′–5′
c-Kit	KIT	TGGGAGCTCTTCTCCTTAGGAA	TGCTCCGGGCTGACCAT
Dithp	DITHP	AGTGCTATGACCCGGAACAGA	CACACTCCATCCTCCTTTCCA
PCYOX1.	Prenylcysteine oxidase1	CCTTCTGATTAAACTATGAATTTAATTTCTGT	TCCCTACTCTAGCTGTAGGAATTAATAATG
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA
SMART-RACE	Sequence primer 3′–5′	T_m (°C)	
Dithp	GTGCTGTTTTGTAGAGGTAGAGAAGGTTCCCTC	73	
Prenylcysteine oxidase1	CACCATATCAAATATATCACCGCTGCTTTCTCC	76	

Oregon, USA) in PBS. After 3 washes in PBS, cells were mounted with the Slow Fade Light Anti-fade kit (Molecular Probes).

For tissue sections, samples were cryopreserved in graded solutions of sucrose (10%, 20%, and 30%; overnight each), embedded in Tissue-Tek OCT compound (Miles, Elkhart, IND.), snap-frozen in 2-methyl butane that had been cooled on dry ice, and stored at −80 °C. Sections (15 μm) were mounted on glass slides coated with 0.1% poly-L-lysine, rinsed three times in 10 mM Tris in 0.15 M sodium chloride, pH 7.4 (Tris-buffered saline, TBS), containing 0.1% (v/v) Triton X-100 (TBS-TX), incubated for 1 h in 10% NHS in TBS-TX to reduce background staining and incubated overnight with the primary antisera (Table 2) diluted in TBS-TX containing 2% NHS, rinsed in TBS, and incubated in the dark for 2 h at RT in TBS containing the secondary antibody (Jackson Immunoresearch Laboraties, West Grove, PA, USA) coupled to FITC and Cy3.

The blue fluorescent Hoechst 33342 (further referred to as Hoechst) (H3570, 1/1000, Molecular Probes, Eugene, OR, USA) was used as nuclear counterstaining.

Fixed preparations were mounted with Slow Fade Light™ Anti-fade (Molecular Probes). Omission of one of the primary or of one of the secondary antibodies resulted in the absence of immunoreactivity. The protocol used for double-immunofluorescence staining did not modify the distribution or the intensity of each individual labeling observed in corresponding single procedures.

Preparations were observed on an Axiovert LSM 510 NLO META™ confocal microscope (Zeiss, Jena, Germany) equipped with C-Apochromat 40×/1.2 NA and 63×/1.2 NA water immersion objectives.

For visual inspection, phase contrast transmitted light and standard fluorescence filter sets for the visualization of green (FITC), red (Cy3, TOPRO), and blue (Hoechst) fluorescence were used.

In confocal mode, the 488 nm excitation wavelength of the Argon2 laser and a band-pass emission filter (BP505-530 nm) were used for detection of the FITC fluorescence while the 543 nm excitation beam of the HeNe1 laser and a long-pass emission filter (LP560 nm) were used for detection of the red (Cy3) fluorochrome. TOPRO-3 was excited at 630 nm and a LP emission filter (LP630) was used for detection. The blue nuclear stain (Hoechst) was excited in multiphoton mode at 740 nm with a Mai Tai™ tunable broad-band laser (Spectra-Physics, Darmstad, Germany) and emitted light was detected with a short-pass emission filter (KP685 nm).

Optical sections through regions of interest in each preparation (X–Y: 512 × 512 pixels), 1 μm apart (Z step), were sequentially collected for each fluorochrome at each Z step.

Images were analyzed with the Zeiss LSM 510 Image Examiner™ software.

Results

Screening for genes less expressed in the jejunum muscularis propria of ICC-deficient mice compared to WT littermates

cDNA of ICC-deficient Sl/Sld mice and of WT mice was subjected to forward (WT-Sl/Sld) and reverse (Sl/Sld-WT) subtraction and amplified by PCR. The forward subtracted library (WT-Sl/Sld) was cloned and 1344 clones were sequenced. The sequences of 1303 clones with an insert gave BLAST hits for 495 unique mouse or human genes and for 255 expressed sequence tags (EST). Housekeeping and ribosomal genes were not identified, indicating that the subtractive hybridization had been effective.

Using Southern blotting with the WT versus Sl/Sld subtracted cDNA probes, 139 out of 1303 clones were at least two times less expressed in Sl/Sld versus WT (Fig. 1). As a control and to reduce the influence of genetic background variation, the same analysis of the 1303 clones was

Table 2
List of primary antibodies

Primary antibody	Company	Dilution
Goat anti-KIT (M14)	Santa Cruz Biotechnologies, Santa Cruz, CA	1/900
Rabbit anti-heme oxygenase2	Stressgen Biotechnologies, Victoria, Canada	1/500
Rabbit anti-phospho-phospholamban	Research Diagnostics, Flanders, NJ	1/250
Rabbit anti-TROY	Active Motif, Rixensart, Belgium	1/250

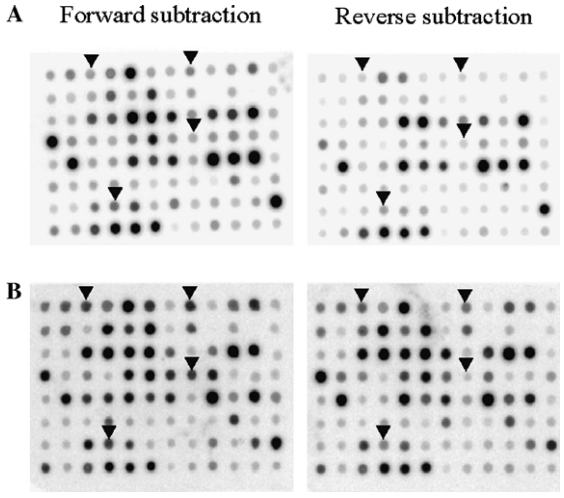

Fig. 1. Subtraction efficiency analysis using subtracted versus unsubtracted libraries. Clones (1344) of the forward subtraction, WT minus Sl/Sld, were spotted in duplicates in 96-well configuration on hybridization filters. They were hybridized with ^{32}P-labeled forward and reverse subtracted, Sl/Sld minus WT (A) and W^{LacZ}/Wv minus WT (B), probes. Fifty-three clones that were at least two times less expressed with the reverse than with the forward subtracted probe in the two models (arrowhead) were selected for further analysis with RT-qPCR.

performed using subtracted probes for a different ICC-deficient model, W^{LacZ}/Wv, resulting in 146 sequences that were at least two times less expressed versus WT. Forty-eight unique sequences were less expressed in both ICC-deficient models (Table 3). As indicated in Table 3, some of these unique sequences were represented by more than one clone in the cDNA library. Sometimes all clones were shown to be more than 2-fold higher in WT versus Sl/Sld, while for some genes only one of the clones reached the cut-off of 2-fold difference. Among the 48 sequences identified, 17 corresponded to EST clones and 31 to unique genes. All were subjected to further analysis by RT-qPCR.

Confirmation of differential expression using RT-qPCR

Using RT-qPCR, only four out of the 48 sequences were significantly differentially expressed in Sl/Sld versus WT ($n = 14$ pairs) including c-Kit (Y00864) and two novel, uncharacterized genes mouse Dithp (AL845261), and prenylcysteine oxidase1 (BC006056) (Fig. 2). A fourth gene Slcl2a2 (U13174, not shown) is described elsewhere [26]. These results were confirmed in W^{LacZ}/Wv versus WT animals ($n = 3$ pairs) (Fig. 2). The expression of prenylcysteine oxidase1 is 1.48 ± 0.02 and 1.67 ± 0.06 less in Sl/Sld and W^{LacZ}/Wv than in WT, respectively, while mDITHP is decreased 2.11 ± 0.06- and 2.30 ± 0.09-fold, respectively. c-Kit expression is 5.25 ± 0.07- and 4.50 ± 0.10-fold less in Sl/Sld and W^{LacZ}/Wv than in WT, respectively.

Further characterization of the two differentially expressed genes prenylcysteine oxidase1 and Dithp and their expression in COS-7 cells

The full-length mouse cDNA prenylcysteine oxidase1 obtained by RACE matched EMBL clone BC006056 which belongs to the prenylcysteine oxidase precursor gene family. Analysis of the prenylcysteine oxidase1 protein sequence (511 AA) revealed a signal peptide (AA 1–33) and two overlapping domains: an amino oxidase (AA 48–486) and a prenylcysteine lyase domain. The mouse protein was for 80% identical to a protein available as Accession No. ADG63582 in the DGENE patented protein sequence database (Thomson Scientific, UK) representing a human secreted/transmembrane polypeptide PRO1183 for AA 33–509.

The other identified full-length mouse cDNA sequence derived from cDNA clone 6B3 matched EMBL clone AL845261. Analysis of the translated protein sequence (558 AA) revealed one broad complex, tramtrack, and bric à brac motif (BTB) (AA 36–134) followed by six Kelch repeats (AA 274–319, 321–369, 370–416, 417–466, 467–508, and 509–557). The murine protein sequence was 99% similar to the human diagnostic and therapeutic polypeptide DITHP #181 (Fig. 3), available as Entry No. ABG60123 in the DGENE patented protein sequence database. We propose therefore to name the cDNA sequence mouse Dithp, and the corresponding protein mouse DITHP. Other proteins, e.g. mouse ENC-1 (Uniprot Accession No. O35709), mouse actinfilin (Uniprot Accession No. Q8K430), mouse actin binding protein IPP (Uniprot Accession No. P28575), and human gigaxonin (Uniprot Accession No. Q9H2C0), contained a comparable BTB motif followed by six Kelch repeats, but were only 34–44% identical to DITHP (Fig. 3).

In order to reveal their subcellular distribution, prenylcysteine oxidase1 and Dithp were cloned into a green fluorescent reporter construct ZsGreen and transfected into COS-7. Prenylcysteine oxidase1 proved highly toxic and transfected COS-7 cells died within 24 h. Transfected cells rounded up and demonstrated membrane blebbing indicative for cell death as soon as the protein was expressed (Fig. 4A). DITHP localized in the cytoplasm but not in the nucleus (Fig. 4B) while the empty ZsGreen reporter

Table 3
List of unique sequences established as downregulated in Sl/Sld and W^{LacZ}/Wv animal models using Southern blotting

#	FS/RS 1	FS/RS 2	Gene description	EMBL Accession number	SwissProt/SPtrembl Accession number
1	14.18		Mouse prenylcysteine oxidase1	BC006056	Q99JK4
1	9.97		Mouse stannin	AF030522	P61807
1	8.44		Mouse phospholamban	AF214653	
2 Ident. Seq	5.76	1.54	Human diagnostic and therapeutic protein DITHP	AL845261	
1	5.68		Human colon cancer antigen protein	AK010376	Q9CWU6
1	5.11		Mouse naked cuticle-2	AF358136	Q91Y45
2 Ident. Seq	4.41	1.41	Proto-oncogene tyrosine-protein kinase KIT (C-KIT)	Y00864	P05532
2 Ident. Seq	4.22	5.05	Mouse neurocalcin δ	BC026979	Q91X97
3 Diff. Seq.	4.13	1.3;1.4	Mouse mast cell growth factor	U44725	P20826
2 Ident. Seq	4.05	1.23	UNKN	AA239254	
1	3.96		UNKN	AW121683	
1	3.96		Mouse syntaxin 3A	D29797	Q64704
1	3.87		nonmuscle caldesmon	U18419	Q62736
1	3.87		Mouse SNAP25 interacting protein	AK008144	Q9CQU5
1	3.78		UNKN	AK009543	Q9CV53
2 Ident. Seq	3.69	1.64	Rat pheromone receptor VN6	U36898	Q62855
1	3.66		UNKN	AF132207	Q9H350
1	3.62		Human calcium-activated chloride channel protein 2	AF127035	Q9UNF7
1	3.54		Mouse heme oxygenase2	AA035816	
1	3.49		Mouse basolateral Na-K-2Cl cotransporter	U13174	P55012
1	3.46		UNKN	AA221682	
1	3.27		Mouse cardiac calsequestrin	AF068244	O09161
1	3.24		Mouse lysosome-associated protein, transmembane-4α	U34259	Q60961
1	3.18		Mouse direct IAP binding protein with low PI	AF203914	Q9JIQ3
1	3.15		Rat Na^+,K^+-ATPase α(+) isoform catalytic subunit	M14512	P06686
1	3.10		Mouse TROY	AB040432	Q9JLL3
1	3.09		UNKN	AA117277	
2 Ident. Seq	2.83	4.35	UNKN	AA466515	
1	2.81		UNKN	BE380475	
1	2.79		UNKN	AI194283	
1	2.75		Human oxoglutarate dehydrogenase (lipoamide)	BC004964	Q9UDX0
1	2.74		UNKN	AA143945	
2 Ident. Seq	2.70	2.68	UNKN	AI587911	
1	2.56		Mouse hypothetical FAD/NAD(P)-binding domain containing protein	AK002851	Q9CW85
1	2.52		Human chemokine receptor CCR5	AF011503	P51681
2 Ident. Seq	2.50	1.02	Mouse caveolin-1 β isoform	AB029930	P49817
1	2.47		UNKN	AK002408	
2 Ident. Seq	2.38	2.6	UNKN	U02094	
2 Ident. Seq	2.32	1.37	UNK	L04739	P11505
1	2.25		UNKN	AY007793	Q99M95
1	2.23		Rat extracellular signal-related kinase (ERK2)	M64300	P63086
2 Ident. Seq	2.22	1.41	UNKN	AK052525	Q80YF6
1	2.20		UNKN	X77567	Q14642
2 Ident. Seq	2.19	2.17	Mouse cAMP-dependent protein kinase C β subunit	X61434	P68181
4 Ident. Seq	2.12		Mouse mitochondrial DNA	AB042432	P03899
1	2.08		Mouse interferon inducible protein 1	AA985781	
1	2.08		Mouse RAB23, member RAS family	AK047068	Q9D4I9
1	2.05		Mouse monocarboxylate transporter 1	BC014777	P53986

#: Number of clones representing the same gene.
Ident. Seq: # identical sequences are present in the forward subtracted library; Diff. Seq.: # different sequences represent the same gene in the forward subtracted library.
FS/RS 1: ratio forward versus reverse subtraction (WT versus Sl/Sld model), FS/RS 2: ratio forward versus reverse subtraction (WT versus Sl/Sld model) of the identical sequence or different sequences representing the same gene.

construct abundantly expressed ZsGreen in the whole cell, including the nucleus (Fig. 4C). RT-qPCR on cDNA of various adult mouse tissues revealed that prenylcysteine oxidase1 was abundantly expressed in liver, kidney, and heart (Fig. 5). Interestingly, Dithp expression was highest in adult small intestine (Fig. 5).

Localization of the differentially expressed genes in the jejunum using immunohistochemistry

In WT animals (Fig. 6A), ICC-MP and ICC-DMP displayed immunoreactivity for KIT, the established marker for ICC, while in ICC-deficient animals (Fig. 6B), KIT-ir

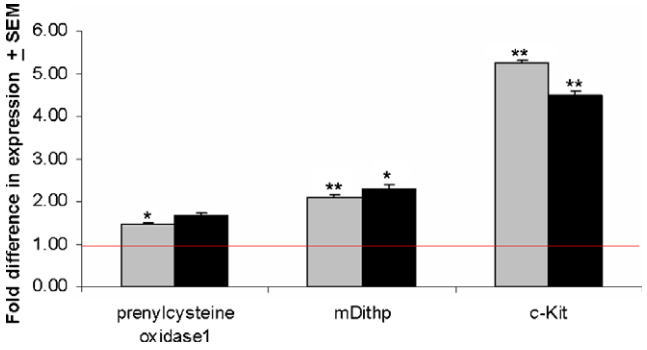

Fig. 2. Confirmation of differentially expressed genes using RT-qPCR. The difference in gene expression in jejunum muscle strips of Sl/Sld ($n = 14$ pairs) and W^{Lacz}/Wv ($n = 3$ pairs) versus WT mice is indicated in grey and black, respectively. Data are expressed as δδ value means ± SEM and statistical analysis was performed using an unpaired t-test. $*p < 0.05$ $**p < 0.001$. RT-qPCR was performed on 25 ng cDNA from muscle strips. c-Kit and prenylcysteine oxidase1 expression reached the threshold 6.5 cycles later (~90× less expressed) than GAPDH while Dithp was 4.7 cycles later (~13× less expressed) than the housekeeping gene.

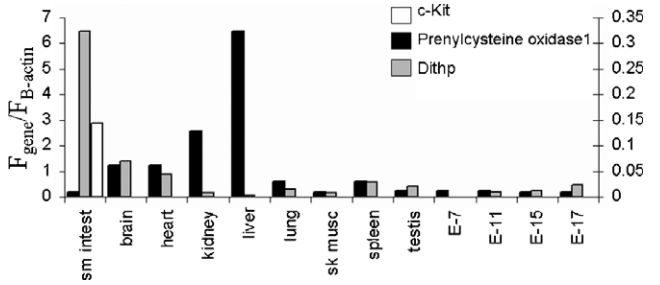

Fig. 5. Quantification of mRNA content for prenylcysteine oxidase1 and Dithp, normalized against β-actin in various mouse tissues. Scale for prenylcysteine oxidase1 and c-Kit is on the left Y-axis. Prenylcysteine oxidase1 was abundantly expressed in adult liver. Dithp expression (scale on the right Y-axis) was very low compared to prenylcysteine oxidase1. Its highest expression level was detected in adult small intestine although it did not reach the level of c-Kit, the ICC marker. sm intest, small intestine with mucosa; sk musc, skeletal muscle; E, embryonic day; F, fluorescence intensity.

was only present in ICC-DMP. Unfortunately, all custom-made polyclonal antibodies for DITHP failed to deliver any signal by immunohistochemistry on mouse tissue as well as on transfected COS cells (data not shown).

In order to further confirm the validity of the Southern blot results at the protein level, we performed immunohistochemistry for a selection of proteins for which SSH and Southern blot indicated a more than 3-fold difference and for which validated antibodies were available. Proteins for IHC were further prioritized based on their function

A

	DITHP_Human	MIPP_Mouse	Actinfilin_Mouse	ENC1_Mouse	Gigaxonin_Human
6B3_Mouse	99%	37%	37%	46%	34%
DITHP_Human		38%	37%	46%	34%
MIPP_Mouse			44%	40%	38%
Actinfilin_Mouse				40%	36%
ENC1_Mouse					37%

B

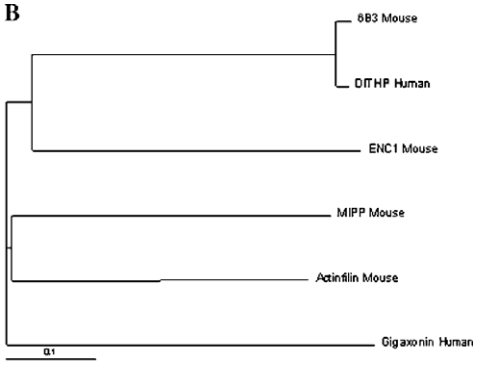

Fig. 3. Comparison of the protein sequence of clone 6B3 with human DITHP (DGENE Accession No. ABG60123), mouse ENC-1 (Uniprot Accession No. O35709), mouse actinfilin (Uniprot Accession No. Q8K430), mouse actin binding protein IPP (Uniprot Accession No. P28575), and human gigaxonin (Uniprot Accession No. Q9H2C0). Sequence alignments were performed with CLUSTALW [69]. Sequence similarity shown in (A) was calculated with GeneDoc [70] (http://www.psc.edu/biomed/genedoc) and the dendrogram shown in (B) was produced with TreeView [71] (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The scale bar indicates 0.1 distance or 10% dissimilarity.

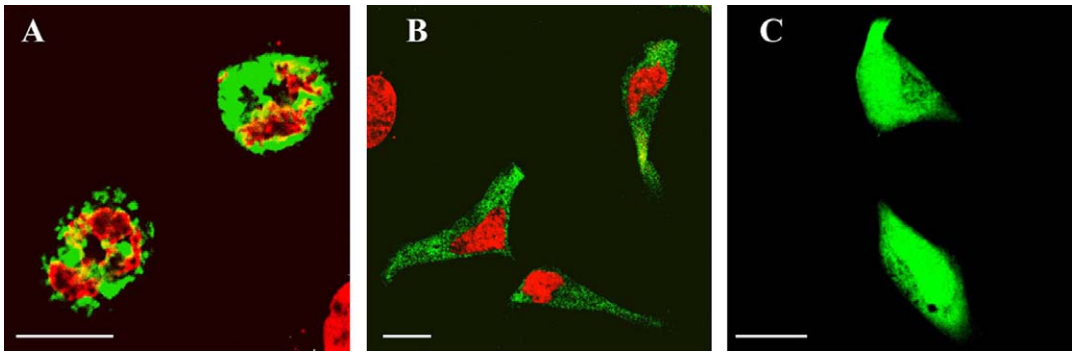

Fig. 4. Transient transfection of the uncharacterized genes prenylcysteine oxidase1 and Dithp into COS-7 cells at 24 h. Nuclei are red (Hoechst) and the construct is in green (ZsGreen). Prenylcysteine oxidase1 was expressed near the nucleus (A) while DITHP was expressed in the cytoplasm but not in the nucleus (B). The control (empty) ZsGreen vector localized in cytoplasm and nucleus (C). Scale bars, 20 μm.

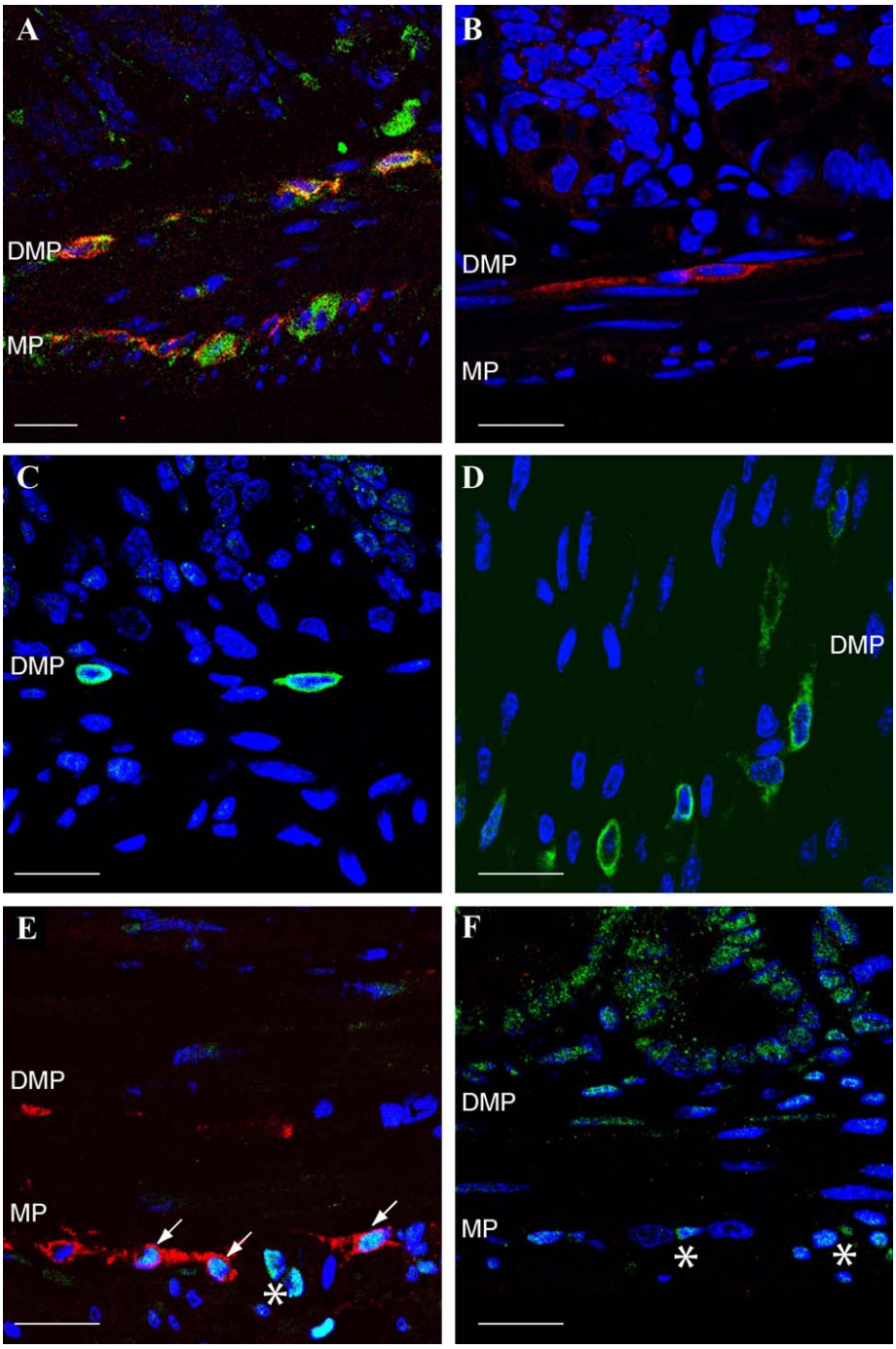

Fig. 6. Expression of heme oxygenase2, phosphorylated-phospholamban (ser16), and TROY in the mouse jejunum. In WT jejunum, heme oxygenase 2-immunoreactivity was present in ICC-MP and ICC-DMP as well as in neurons in myenteric and submucosal plexus (A). In W^{LacZ}/Wv, only a faint heme oxygenase 2-immunoreactivity was detected in SMC (B). Phosphorylated-phospholamban (ser16) immunoreactivity was detected in ICC-DMP in both WT (C) and W^{LacZ}/Wv animals (D). TROY-immunoreactivity was present in nuclei of ICC-P and ICC-DMP and in myenteric neurons of WT animals (E) while it was only expressed in myenteric neurons in ICC-deficient animals (F). KIT (M14)-immunoreactivity in red; nuclei (Hoechst) in blue; gene of interest in green. MP, myenteric plexus layer; DMP, deep muscular plexus. (E–F) Arrowheads, KIT-ir ICC; ∗, myenteric neurons. Scale bars, 20 μm.

in other cell types that could indicate a potentially interesting function in pacemaking ICC (Table 2).

Heme oxygenase2 (Fig. 6A) immunoreactivity was clearly detectable in WT mice in ICC-MP, ICC-DMP, and myenteric neurons. An antibody raised to total phospholamban (Table 2) failed to deliver any staining in immunohistochemistry. However, an antibody raised to the active, phosphorylated, form of phospholamban on serine 16, exhibited a strong perinuclear immunoreactivity in ICC-DMP (Fig. 6C and D). TROY, also known as TAJ, was

strongly expressed in the nuclei of ICC-MP and ICC-DMP (Fig. 6E). Myenteric neurons were also faintly stained. In ICC-deficient animals only the myenteric neurons and ICC-DMP exhibited Troy immunoreactivity (Fig. 6F).

Discussion

The use of ICC-deficient animals offered the opportunity to gain novel insight into the gene expression profile of ICC-MP and their possible functions in the GI tract. However, ICC only represent a small fraction of all the cells present in the wall of the mouse jejunum. This would make the isolation of genes differentially expressed between WT and ICC-deficient jejunum difficult when using commonly used screening strategies like differential display, representational difference analysis, conventional (non-PCR based) subtractive hybridization or microarray [27–33]. A limited number of studies have been described that attempted to identify ICC related genes using micro array or differential display. So far, confirmed localization in ICC or function remains obscure for all of the identified genes, except for ACLP1 [32] and G28K [30] which were confirmed to be expressed by ICC using single cell PCR.

In contrast to these strategies known to poorly perform for rare transcripts [34–37], SSH is a PCR based method which was claimed to combine equalization of abundantly expressed cDNAs with an over 1000-fold enrichment of rare sequences [38]. Therefore, SSH was selected to identify genes less expressed in ICC-deficient models compared to WT. To limit the false positive rate, differential hybridization was performed using two independent ICC-deficient models Sl/Sld and W^{LacZ}/Wv. Only genes differentially expressed in both models were further analyzed.

Upon subtraction (WT–Sl/Sld) 1303 clones were obtained, representing 495 unique genes and 255 ESTs. No housekeeping and ribosomal genes were isolated, indicating that the subtractive hybridization had been largely effective. Therefore, the prevalence of multiple clones for the same genes suggested that most differentially expressed clones were covered in the subtracted library, although it cannot be ruled out that in some cases multiple copies of a clone might also originate from mRNA species with extremely high abundance in the starting material and subsequent incomplete normalization, or differences in the efficiency of PCR amplification of the subtracted cDNA.

Differential screening of the subtracted libraries by southern blotting yielded 53 sequences that showed more than 2-fold difference in expression in the two models, of which five sequences were identified twice. In some cases multiple clones of the same sequence were present in the cDNA library, but not each clone reached the threshold of 2 with the differential screening. One of the novel identified genes Dithp showed a 5.8- and 1.5-fold differential expression for two different clones, while this gene was clearly significantly differently expressed by RT-qPCR (Table 3). This indicates not only that false positives may be generated at the subtractive amplification step but also suggests a high false negative rate at the hybridization step and points out that other genes of interest may be missed in the screening.

In the list of 48 unique sequences, interestingly, some sequences were already described in the literature to be related to ICC: c-Kit [7], stem cell factor [39,40], and caveolin [41,42] appeared to be less expressed in both W^{LacZ}/Wv and Sl/Sld animals compared to WT. Also mitochondrial DNA was identified four times in the forward subtracted library, in line with the observation that mitochondria are abundantly present in ICC [43,44].

RT-qPCR was used to confirm quantitative differences in gene expression. Four of the 48 genes, namely c-Kit, Slc12a2, prenylcysteine oxidase1, and Dithp, were expressed at significantly lower levels in both ICC-deficient models. c-Kit, coding for the receptor tyrosine kinase KIT, has been extensively used as the reference ICC marker by immunohistochemistry [19,52,53]. Although KIT is apparently not directly involved in the generation of pacemaker currents, the KIT-SCF signaling pathway is vital for the differentiation and maintenance of several populations of ICC, including the pacemaking ICC in the jejunum, which are lacking in the W/Wv and Sl/Sld mouse models [22,53,54]. The identification of c-Kit as a gene less expressed in ICC-deficient than in WT mice confirmed the ability of SSH to pick up ICC related genes. Slc12a2 codes for the $Na^+K^+2Cl^-$ cotransporter NKCC1 and is expressed by the pacemaking ICC but not by the ICC at the level of the deep muscular plexus. Moreover, the modulating role of NKCC1 on the amplitude and frequency of the slow wave has been established and is described in detail elsewhere [26].

If and how the two novel, uncharacterized genes, provisionally referred to as prenylcysteine oxidase1 and Dithp, play a role in ICC in the jejunum remains to be demonstrated. The significantly different expression of Dithp and prenylcysteine oxidase1 in ICC-deficient models compared to WT using RT-qPCR raises the hypothesis that the corresponding proteins would be more abundant in WT than in ICC-deficient mouse jejunum but suitable antibodies are still lacking. Using RT-qPCR, prenylcysteine oxidase1 was detected in embryonic tissue and in various adult organs. It was particularly abundantly expressed in liver, in line with patent WO2003064624-A2 describing this gene sequence as a toxicity marker in the liver. Moreover, the induction of cell death of cells transfected with prenylcysteine oxidase1 raises the possibility that like TROY it might play a role in cell death in the intestine. Interestingly, one of the novel genes, Dithp, seemed to be mainly expressed in adult small intestine, and was not detectable in embryonic tissue suggesting the same temporal distribution as ICC, which only become differentiated post-natal. The DITHP protein contains 1 BTB motif and 6 Kelch motifs with low homology to MIPP [45,46], Actinfilin [47], Mayven [48], ENC-1 [49,50], and gigaxonin [51], which are all actin binding proteins that might be involved in the dynamic organization of the actin cytoskeleton.

Although the homology with these proteins is low, the presence of the BTB domain that can interact with other BTB/POZ containing proteins, and of the Kelch motif repeats that can interact with the actin cytoskeleton, would suggest a similar function in the cytoskeleton organization for DITHP.

Although phospholamban, Hmox-2, and Troy were not significantly different with RT-qPCR, immunohistochemistry was also performed for the corresponding proteins, since the Southern blot results suggested them to be differentially expressed. They were indeed all identified in ICC, but were present in neurons as well. The small differences at RNA level for heme oxygenase2, phospholamban, and Troy most likely were not detectable by RT-qPCR due to overshadowing of RNA in nerves. Clearly, the small number of ICC present in a complex tissue like the jejunum makes it difficult to confirm differences at the mRNA level without the enrichment step used in SSH. Ideally therefore, the RT-qPCR should be performed on purified ICC or, for tissues with various cell types packed, immunohistochemistry might represent the method of choice to confirm their expression and precise localization.

The three proteins identified in ICC also raised special interest. It has been established that Ca^{2+} oscillations underlie the slow wave generation [43,55]. Phospholamban has been extensively studied as a regulator of cardiac contractility [56]. Phospholamban phosphorylation relieves its inhibition on the SERCA pump, hence activating Ca^{2+} uptake into the SR [57–59]. Phospholamban can be phosphorylated at three distinct sites by various protein kinases, namely on serine 10 by protein kinase C, on serine 16 by cAMP- and cGMP-dependent protein kinase, and on threonine 17 by Ca-calmodulin-dependent protein kinase and by Ca^{2+} store depletion [60].

Antibodies against the phosphorylated site Ser16 revealed its expression on ICC-DMP but not on the pacemaking ICC. Further experiments with antibodies against the other phosphorylation sites will be required to identify whether other activated forms of phospholamban are present in other cell types.

Secondly, Hmox2, coding for heme oxygenase2, was detected in the cytoplasm of myenteric neurons and in ICC-MP and ICC-DMP, in line with published results [61]. Activation of heme oxygenase2 in ICC results in an endogenous production of the atypical gaseous neuromodulator carbon monoxide (CO) [62–65], which plays a role in the maintenance of the intestinal SMC membrane potential and its gradient in the muscle coat [66,67]. The SMC depolarization observed in ICC-deficient animals (hence lacking heme oxygenase2 normally associated with ICC) appears in line with this putative function for heme oxygenase2.

Lastly, expression of TROY was detected in the GI-tract. TROY, an orphan TNF receptor family member, interacts with TRAF family members and activates the Janus kinase (JNK) signaling pathway when overexpressed in cells [68]. TROY induces apoptosis by a caspase independent mechanism [68]. This study was the first to identify TROY in the GI tract. TROY-immunoreactivity in nuclei of myenteric neurons and ICC-MP and ICC-DMP raised the hypothesis that it might be involved in ICC cell death in the normal GI tract. TROY may also have other specific roles in ICC in the GI tract that remain to be unraveled.

Altogether, this study exemplifies the difficulties encountered when studying a specific cell type present in low abundance in a highly complex tissue like the muscularis propria of the GI tract. We here demonstrated that the use of SSH and RT-qPCR in two independent mouse models combined with immunohistochemistry is a useful technique to identify rarely expressed genes.

Combination of subtractive hybridization, Southern blotting, and RT-qPCR revealed c-Kit, Slc12a2, and 2 uncharacterized genes to be significantly lower expressed in Sl/Sld and W^{LacZ}/Wv animals compared to WT. Although differences at the RNA level were sometimes too small to detect with RT-qPCR, immunohistochemistry allowed for specific identification in ICC of KIT, NKCC1, heme oxygenase2, phospholamban, and TROY. Further experiments will be required to identify the role of the two uncharacterized genes Dithp and prenylcysteine oxidase1 in the mouse small intestine.

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