RESEARCH ARTICLE

Four selenoproteins, protein biosynthesis, and Wnt signalling are particularly sensitive to limited selenium intake in mouse colon

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Selenium is an essential micronutrient. Its recommended daily allowance is not attained by a significant proportion of the population in many countries and its intake has been suggested to affect colorectal carcinogenesis. Therefore, microarrays were used to determine how both selenoprotein and global gene expression patterns in the mouse colon were affected by marginal selenium deficiency comparable to variations in human dietary intakes. Two groups of 12 mice each were fed a selenium-deficient (0.086 mg Se/kg) or a selenium-adequate (0.15 mg Se/kg) diet. After 6 wk, plasma selenium level, liver, and colon glutathione peroxidase (GPx) activity in the deficient group was 12, 34, and 50%, respectively, of that of the adequate group. Differential gene expression was analysed with mouse 44K whole genome microarrays. Pathway analysis by GenMAPP identified the protein biosynthesis pathway as most significantly affected, followed by inflammation, Delta-Notch and Wnt pathways. Selected gene expression changes were confirmed by quantitative real-time PCR. GPx1 and the selenoproteins W, H, and M, responded significantly to selenium intake making them candidates as biomarkers for selenium status. Thus, feeding a marginal selenium-deficient diet resulted in distinct changes in global gene expression in the mouse colon. Modulation of cancer-related pathways may contribute to the higher susceptibility to colon carcinogenesis in low selenium status.

1 Introduction

The current recommended daily intake for the essential micronutrient selenium ranges between 40 µg/day (WHO)

and 85 µg/day (Australia) for adult males, depending on the country; the value for females is normally 10 µg less [1]. These intakes are not achieved in many countries [1] and sub-optimal selenium intake may have impact on immune function and susceptibility to viral disease and cancers [2]. Both observational and intervention studies suggest that a low selenium status is inversely correlated with an increased colorectal cancer incidence [3–5]. Furthermore, results of the Nutritional Prevention of Cancer Trial in USA indicated that a daily supplement of 200 µg Se reduced mortality from colorectal cancer [6].

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Abbreviations: Dvl, dishevelled; **FC,** fold change; **FDR,** False discovery rate; **Sec,** selenocysteine; **qPCR,** quantitative real-time PCR

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Selenium is incorporated into selenoproteins as seleno-cysteine (Sec). In humans, 25 selenoprotein-encoding genes have been identified, whereas the number is 24 in mice [7]. Selenoprotein levels respond differently to selenium deficiency, a phenomenon called the "hierarchy of selenoproteins". Glutathione peroxidase-1 (GPx1) is considered to rank lowest in this hierarchy, since it rapidly declines upon selenium deprivation and is re-synthesized with considerable delay upon selenium repletion [8, 9]. Others, such as GPx4, GPx2, deiodinases, or thioredoxin reductases rank high in the hierarchy. In general, the ranking of selenoproteins parallels the stability of the pertinent mRNA. However, the ranking of all selenoproteins is not known so far, mainly due to lack of both systematic feeding experiments and data on the response of all selenoproteins.

Selenium intake also affects expression of non-selenoproteins. Organo-selenium compounds increase the expression of phase II enzymes and pro-apoptotic genes and decrease expression of genes regulating cell growth [10, 11]. In mice, stress response, cell cycle/growth control, and angiogenesis/cell adhesion genes are affected by severe selenium deficiency [12]. Xenobiotic metabolizing enzymes are modulated by selenium deficiency [13] *via* the activation of Nrf2 and Nrf2-independent pathways [14]. Thus, either supranutritional selenium intake or forms of selenium not usually present in the diet alter the expression of a range of non-selenoprotein genes. In addition, nutritionally relevant amounts of selenium have been shown recently to increase expression of protein biosynthesis genes in lymphocytes of human volunteers [15].

Since selenium intake has been implicated in colon carcinogenesis [6], this study used microarray and quantitative real-time PCR (qPCR) analysis of the mouse colon to identify both selenoproteins and novel targets affected by a selenium depletion comparable to those found in humans. We focussed on the colon because it is one of the organs in which selenium status affects development of cancer [6]. Levels of SelW, GPx1, SelH, and SelM mRNAs were down-regulated in selenium deficiency. Non-selenoprotein pathways were also affected, notably those involved in protein biosynthesis, inflammatory pathways, and Wnt signalling, which might contribute to higher cancer risk in selenium deficiency.

2   Materials and methods

2.1   Animals, feeding protocol, and sampling

The animal study was approved by the Governmental Animal Ethics Committee (MLUV 32-44457+41). Male C57BL/6J mice (3–4 wk of age) from Charles River (Sulzfeld, Germany) were randomly assigned to the selenium-deficient or selenium-adequate group (12 mice *per* group) with free access to food and water. The selenium-adequate diet was produced by mixing selenomethionine (Acros, Geel, Belgium) into the selenium-deficient diet (No. C1045 with 50% carbohydrates, 17% protein, 5% fat, 4% fibre, and mixture of micronutrients; Altromin, Lage, Germany) containing 0.086 mg Se/kg [16] to yield a selenium content of 0.15 mg/kg corresponding to the dietary reference intake for mice [17]. Diets were fed as powder for 6 wk until mice were killed in the non-fasted state. Animals were anesthetized with isofluran and blood was withdrawn from the retro-orbital plexus. Anesthetized animals were killed by cervical dislocation. Plasma and tissues freeze clamped in liquid nitrogen were stored at -80°C.

2.2   RNA isolation

Colon tissue was ground under liquid nitrogen. In total, 20–30 mg powder were suspended in 800 µL of cold Trizol (Invitrogen, Karlsruhe, Germany), homogenized with a tissue lyzer (Qiagen, Hilden, Germany), and RNA isolated using the Trizol protocol and RNeasy mini columns (Qiagen). RNA quality was checked using a Bioanalyzer 2100 (Agilent Technologies, Böblingen, Germany). RNA samples subsequently used for microarray analysis had a RIN-number ≥ 9.0.

2.3   Microarray analysis

Differential gene expression was analysed using one RNA sample *per* mouse 44K microarray (Agilent Technologies) [18]. Briefly, the microarray (G4122A) contains ~41 000 probes that cover all known genes in the mouse genome and the primary resulting transcripts. A total of 500 ng RNA was subjected to cDNA synthesis and subsequent cRNA amplification and labelling, using the Agilent Low RNA Input Fluorescent Linear Amplification Kit. Each cDNA sample was split in two aliquots: one (sample) was labelled with Cyanine 5-CTP (Cy5), the other (reference) with Cyanine 3-CTP (Cy3). Cy5 and Cy3 were obtained from PerkinElmer (Boston, MA, USA). Cy-labelled cRNAs were purified using RNeasy mini columns (Qiagen). A reference pool was generated with equimolar amounts of each Cy3-labelled cRNA. Hybridization of 1 µg Cy5-labelled sample cRNA and 1 µg of the Cy3-labelled reference pool cRNA and subsequent washing were performed by following the Agilent hybridization procedure with modifications [18]. Microarrays were scanned with a Scanarray Express HT scanner (PerkinElmer) and signal intensity of each spot determined with ArrayVision 8.0 (Imaging Research, Ont., Canada). *Per* spot signal over background intensity was calculated *per* array for both channels and each averaged over all arrays; those with a threshold above 2.0 were processed further. Raw data quality of the microarrays was assessed using the software "R" [19] and Microsoft Excel (Microsoft, Redwood, Maryland, MD, USA), based on intensity ratios (M) against the average intensity (A) plots (MA plots), scatter plots, and NP plots (sample size n times the

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proportion of non-forming items P). All arrays passed. Subsequently, data were normalized [20] using GeneMaths XT 1.6 (Applied Maths, Sint-Martens-Latem, Belgium). False discovery rate (FDR) analysis at a cut-off of 5% was carried out according to the Benjamini–Hochberg procedure [21]. Significantly, regulated genes were also identified with Student's *t*-test ($p < 0.05$).

2.4 Pathway analysis

Probes were annotated to UniProt and Ensembl IDs to make them compatible with the program GenMAPP (Gene Map Annotator and Pathway Profiler) [22] that was used for pathway analysis. Pathways were selected using the MAPPFinder based on the amount of regulated genes present [23]. Genes were considered to be regulated when meeting the criterion: absolute fold change (FC) ≥ 1.2 and *p*-value ≤ 0.05. An FC of 1.2 was chosen not to overlook small changes caused by dietary intervention and more importantly to find multiple altered genes in one pathway reflecting its physiological relevance [24]. Per pathway *z*-score was calculated [23].

2.5 qPCR

RNA (3 μg) was reversely transcribed with 150 fmol oligo(dT)15 primers and 180 U moloney murine leukemia virus reverse transcriptase (Promega, Mannheim, Germany) in a total volume of 45 μL. qPCRs (Mx3005P[TM] qPCR System, Stratagene, Amsterdam, Netherlands) were performed in triplicates with 1 μL of tenfold diluted cDNA in 25 μL reaction mixtures using SYBR Green I (Molecular Probes, Eugene, OR, USA) as fluorescent reporter. PCR products were quantified with a standard curve. Primers (Table 1) were designed to be specific for cDNA with PerlPrimer v1.1.14 [25]. Hprt and Rpl13a were used as reference genes based on criteria: least variation in microarray data between all samples (%CV < 5%), mean expression level ratio of deficient over adequate group close to 1, and an expression level of at least ten times the background. The mean of reference genes was used for normalization [26].

2.6 PCR arrays

Mouse Wnt Signaling Pathway RT[2] Profiler[TM] PCR Arrays and RT[2] Real-Timer SyBR Green/ROX qPCR Master Mix were purchased from SuperArray Bioscience (Frederick, MD, USA). cDNA of the selenium-deficient and -adequate group was pooled to equal amounts to provide two samples for PCR array analysis. Raw data of 84 analyzed genes were normalized and analysed by the RT[2] Profiler[TM] PCR Array Data Analysis Web Portal using the ΔΔCt method.

2.7 Liver GPx activity and plasma selenium content

In total, 20 mg of tissue powder were homogenized in 500 μL 100 mM Tris/HCl, 300 mM KCl, 0.1% Triton X-100, pH 7.6, containing 4 μL of protease inhibitor cocktail (Calbiochem, Bad Soden, Germany). Cellular debris was removed at $20\,000 \times g$, 15 min, 4°C. Protein content was estimated according to Bradford [27]. GPx activity was measured in the glutathione reductase-coupled test optimized for mouse tissue [28] and expressed as mU/mg protein. The amount of plasma selenium was measured fluorimetrically as described [29] and modified previously [30].

2.8 Statistics

Student's *t*-test was used to compare differences between group (12 animals each) means. $p < 0.05$ was considered significant. Statistical analysis was performed using GraphPad Prism version 5 (La Jolla, San Diego, CA, USA).

3 Results

3.1 Plasma selenium concentration and liver GPx activity

Plasma selenium in mice fed the selenium-deficient diet was significantly lower than in those on selenium-adequate diet (Fig. 1A) indicating the success of the feeding. The plasma concentrations observed here were consistent with the previous study [16]. GPx activity, a classical functional marker of Se status, was about one-third of the adequate group in the liver and half in the colon of Se-deficient mice (Figs. 1B and C, respectively). Maximal differences between feeding groups were usually reached after 5 wk and did not further change after 6 wk. Food intake, weight gain, and behaviour were unaffected by the diets with different selenium contents. Histological monitoring of the colons did not show any changes in cell pattern in selenium deficiency. Especially, no infiltration of inflammatory cells was observed (data not shown). Thus, any change in gene expression described in the following does not result from a change in composition of colon cells.

3.2 Selenium-responsive genes

Colon RNA samples from 12 selenium-deficient and 12 selenium-adequate mice individually hybridized to whole genome microarrays yielded a total of 21 008 transcripts (13 656 unique and annotated genes) with levels twice above background and were used for subsequent data analyses. Mean gene expression ratios of selenium-deficient and selenium-adequate samples were calculated. By using an FDR $\leq 5\%$ a total of 952 genes (722 down- and 230 up-regulated in

Table 1. Primer sequences (5′→3′)

Gene	Acc. Number	Primer sequence	Product size (bp)
14-3-3 (Ywhaq)	NM_011739	fwd CACGGTCCTGGAATTGTTGG rev ATCGCCACAAGCTACTTCAGC	125
β-Catenin	NM_007614.2	fwd AGCTGGCCTGGTTTGATACTGAC rev CTAAAACCATTCCCACCCTACCAA	130
c-Myc	NM_010849.4	fwd AATCCTGTACCTCGTCCGATTCC rev TCTTGCTCTTCTTCAGAGTCGCT	150
Dio 1	NM_007860	fwd GGGATTTCATTCAAGGCAGCAGG rev TGTGGAGGCAAAGTCATCTACGA	115
Dvl2	NM_007888.3	fwd GGTTGTCTCCTGGCTTGTGTC rev CCCAATTCCACTGGTCCTTTCTG	135
GPx1	NM_008160	fwd GAAGAGATTCTGAATTCCCTCAA rev CACACCAGGAGAATGGCAAGA	256
GPx2	NM_030677	fwd GTGCTGATTGAGAATGTGGC rev AGGATGCTCGTTCTGCCCA	252
GPx3	NM_001083929	fwd CCATTTGGCTTGGTCATTCTGGG rev CACCTGGTCGAACATACTTGAGAC	105
GPx4	NM_001037741	fwd GCTGGGAAATGCCATCAAATGGA rev ACGGCAGGTCCTTCTCTATCAC	115
Gsk3β	NM_19827	fwd ATCAGCTGTTCAGAAGTCTAGCCT rev AGCTGCTTTGCACTTCCAAAGTC	132
Hmox1 (heme oxygenase-1)	NM_010442.1	fwd CCTGGTGCAAGATACTGCCC rev GAAGCTGAGAGTGAGGACCCA	105
Lef1	NM_010703.3	fwd CCGAGATCAGTCATCCCGAAGAG rev TTGTCTGACCACCTCATGCCC	109
Nqo1	NM_008706.4	fwd ATGTACGACAACGGTCCTTTCCAG rev GATGCCACTCTGAATCGGCCA	134
Selenoprotein H	NM_001037279	fwd CCTTATTCCACCAACGCGCCA rev GCGTCAGCTCGTACAATGCTC	154
Selenoprotein I	NM_027652	fwd ACTGGTTACTGCTTCCTCTCCTC rev CTGCTTCACCACTTGTACGCC	145
Selenoprotein K	NM_019979	fwd ATGGAAGAGGGCCACCAGGA rev TTACCTTCCTCATCCACCAGCC	100
Selenoprotein M	NM_053267	fwd GGACATTCAACTGTACCACAACCT rev TAGAAGCCGAGCTCCTGTACCA	158
Selenoprotein O	NM_027905	fwd TGACACTGAGTTCCAAAGGCAC rev GTTAGTGAAGTCAGCACCAGTCAG	138
Selenoprotein P	NM_001042613	fwd CTCATCTATGACAGATGTGGCCGT rev AAGACTCGTGAGATTGCAGTTTCC	137
Selenoprotein S	NM_024439.3	fwd GAAGGCCTCAGGAAGAAGATGGT rev GTCTCCAGGAGCAGGTTCCA	137
Selenoprotein T	NM_001040396	fwd CTTTAAATGATGTGCCAGTGTGGT rev GGTAGGGCTATGATCGATGATGTG	140
Selenoprotein W	NM_009156	fwd ATGCCTGGACATTTGTGGCGA rev GCAGCTTTGATGGCGGTCAC	152
Selenoprotein X	NM_013759	fwd ACTTCGAGCCAGGTGTCTACG rev GGCACTTGGTCACACTGTCTG	129
Sep15	NM_053102	fwd GTTTCAAGCGGCGTCTGCTC rev TGCTTCTTCCTGACAGCACCC	159
Sephs2	NM_009266.3	fwd CAAGTACGGAGAGGGTCACCA rev CGTTGGAATTATCAGGAGCAGCAG	148
Smad4	NM_008540	fwd CACACCTAATTTGCCTCACCACC rev ACTCAGGAGCAGGATGATTGGA	136
Stat3	NM_213659	fwd AAAGGACATCAGTGGCAAGACCC rev TCGCATCCATGATCTTATAGCCCA	112
Tle2	NM_019725.1	fwd CTGGATTGCCTGAACCGAGAC rev CTCTGCTTTGATCCGTGGTGTG	137
Trspap1	NM_027925.3	fwd AGCACCATGCAGACATATGAAGAG rev GTGACAGTCCATCAGAGCATCGT	134
Txnrd 1	NM_015762.1	fwd TACTGCATCAGCAGTGATGATC rev CCATGTTCTTCCATGTGTTCAC	206

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Table 1. Continued

| Gene	Acc. Number	Primer sequence	Product size (bp)
Txnrd 2	NM_013711.3	fwd TACAGCAATGTTCCCACAACTGTC rev CTATCCGCCACCGTGAACTC	148
Txnrd 3	NM_153162.3	fwd TCGACAACGAACGTGTGGTGG rev AGTAGCTGCTTCGTGAGCCC	111
Reference genes			
Hypoxanthine phosphoribosyl transferase (Hprt1)	NM_013556	fwd GCAGTCCCAGCGTCGTG rev GGCCTCCCATCTCCTTCAT	168
Ribosomal protein L13a (Rpl13a)	NM_009438	fwd GTTCGGCTGAAGCCTACCAG rev TTCCGTAACCTCAAGATCTGCT	157
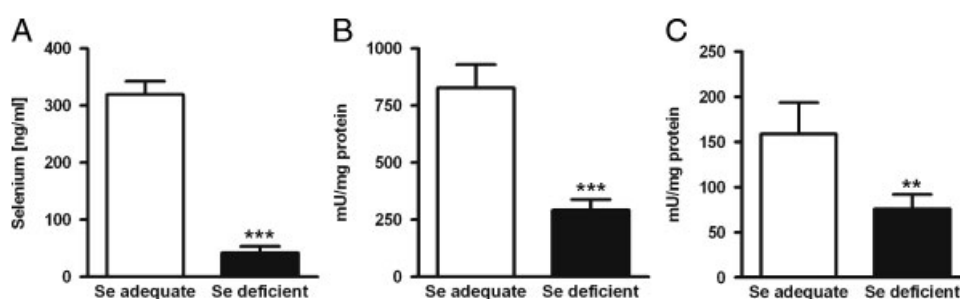
Figure 1. Plasma selenium concentration (A), liver (B), and colon (C) GPx activity in selenium-deficient and selenium-adequate mice. Values are means of 12 animals *per* group (five animals in case of colon) measured in triplicate $\pm$ SD. ** $p < 0.01$ and *** $p < 0.001$ using Student's *t*-test.

selenium deficiency) were identified. Using a less stringent criterium, *p*-value of ≤ 0.05, 3995 genes (1979 down- and 2016 up-regulated in selenium deficiency) were identified. This number clearly exceeds the number you might expect by chance (5% would be 1050 genes).

A complete list of 20 selenoprotein genes being expressed in the mouse colon is given in Table 2. Four selenoprotein genes were significantly lower expressed in selenium-deficient mice: *Selw*, *Gpx1*, *Selh*, and *Selm*. The remaining four known murine selenoprotein genes *Dio2*, *Dio3*, *Seln*, and S*elv* were not expressed at detectable levels in any dietary group.

To confirm the array data, qPCR was performed for all selenoprotein genes (Table 2). Significantly, lower expression after the marginal selenium-deficient diet was confirmed for *Selw*, *Gpx1*, *Selh*, and *Selm*. Expression of *Gpx3*, *Selk*, *Sels*, *Txnrd1*, *Sep15*, and *Selt* significantly responded to selenium supply, which was not obvious from the array data. Similarly, a small increase in the expression of *Txnrd2* and *Txnrd3* was observed in qPCR but not in arrays. qPCR showed expression levels of *Gpx2*, *Sephs2*, *Sepp1*, *Seli*, *Dio1*, *Sepx1*, *Selo*, and *Gpx4* to be unaffected by Se intake, as observed in microarrays. Thus, qPCR confirmed the major changes observed by microarray analysis. The gene for tRNA Sec-associated protein 1 (*Trspap1*, also known as *Secp43*), a factor required for selenoprotein synthesis [31], was slightly, but significantly, decreased in Se-deficient mice according to microarrays (FC: 0.84; *p*-value: 0.017) and qPCR (Fig. 2A).

3.3 Selenium-responsive pathways

Pathways were analyzed using GenMAPP. The data set consisted of 41 441 probes of which 28 208 were annotated to UniProt- and Ensembl-IDs. In total, 6536 genes of these 28 208 probes were linked to murine pathways present in GenMAPP (Table 3). A total of 2294 genes were regulated according to the criteria: absolute $FC \geq 1.2$ and *p*-value ≤ 0.05. Based on these regulated genes and the total amount of 6536 genes, pathways influenced by selenium intake were ranked by the *z*-score (see Section 2). Five of the top 15 pathways (translation factors, mRNA processing/binding, mTOR signalling pathway, regulation of eIF4e-and-p70-S6-Kinase, ribosomal proteins) are related to protein biosynthesis (Table 3). A complete list of genes regulated in protein biosynthesis pathways is provided in Supporting Information Table S1.

The remaining ten regulated pathways comprised stress response and regulatory phenomena, in particular related to inflammation (TNFα-NFκB, IL-2, IL-3) and carcinogenesis (Alpha6-Beta4-Integrin, Delta-Notch). These pathways contained one or more of the genes for Smad4, Stat3, Ywhaq (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein θ, also called 14-3-3 θ), and glycogen synthase kinase 3β (GSK3β). Due to their multiple appearances, these genes were selected for and confirmed by qPCR (Figs. 2B–D and Fig. 3B).

Table 2. Colon selenoprotein genes responding to selenium supply as measured by microarray analysis and qPCR

Gene name	ID	Description	Microarray ($n = 12$)			qPCR ($n = 12$)	
			FDR	*p*-Value	FC	*p*-Value	FC
Sepw1	A_51_P474078	Selenoprotein W	0.00004	2.90E−08	0.58	1.9×10^{-13}	0.42
Gpx1	A_52_P684378	Glutathione peroxidase 1	0.0277	0.0008	0.49	2.6×10^{-20}	0.45
Selh (2700094K13Rik)	A_52_P396774	Selenoprotein H	0.0289	0.0011	0.68	2.9×10^{-12}	0.32
Selm	A_52_P357133	Selenoprotein M	0.1669	0.027	0.81	1.9×10^{-10}	0.44
Gpx2	A_51_P486810	Glutathione peroxidase 2	0.2417	0.065	0.67	0.1503	0.89
Gpx3	A_51_P292008	Glutathione peroxidase 3	0.3688	0.1314	0.92	0.0035	0.72
Sep15	A_51_P111143	15kDa selenoprotein	0.454	0.2023	0.89	0.0028	0.85
Sephs2	A_51_P418765	Selenophosphate synthetase-2	0.4551	0.2108	0.83	0.3690	0.91
Selk	A_51_P290170	Selenoprotein K	0.5585	0.3028	1.18	0.0271	0.84
Txnrd2	A_52_P394561	Thioredoxin reductase-2	0.5631	0.3093	0.93	0.0126	1.12
Sels	A_51_P323610	Selenoprotein S (VIMP)	0.5631	0.3176	0.90	0.0005	0.55
Sepp1	A_51_P470328	Selenoprotein P	0.5815	0.3301	0.90	0.7490	1.03
Seli (D5Wsu178e)	A_52_P228171	Selenoprotein I	0.6813	0.4524	1.05	0.3850	0.93
Dio1	A_51_P403477	Deiodinase-1	0.6842	0.4571	0.92	0.118	0.87
Sepx1 (MsrB1)	A_51_P393634	Selenoprotein X, (Methionine sulfoxide reductase B1)	0.7311	0.5215	0.95	0.522	1.04
Selo (1300018J18Rik)	A_51_P340947	Selenoprotein O	0.7364	0.5303	0.96	0.303	1.08
Txnrd1	A_51_P320614	Thioredoxin reductase-1	0.7646	0.5722	0.94	0.001	0.65
Gpx4	A_51_P462448	Glutathione peroxidase 4 Phospholipid hydroperoxide GPx (PHGPx)	0.8049	0.6332	1.04	0.710	0.98
Txnrd3	A_51_P450365	Thioredoxin reductase-3	0.8674	0.7387	1.02	3.6×10^{-6}	1.25
Selt (2810407C02Rik)	A_52_P445969	Selenoprotein T	0.9911	0.9759	1.00	0.0295	0.87

Genes were sorted by the *p*-value by which gene expression in selenium deficiency differs from selenium adequacy by microarray analysis ($n = 12$ *per* diet). Sorting by either the *p*-value or the FDR resulted in identical ranking. FC, mean fold change in relation to the adequate diet. For ACC numbers see Table 1.
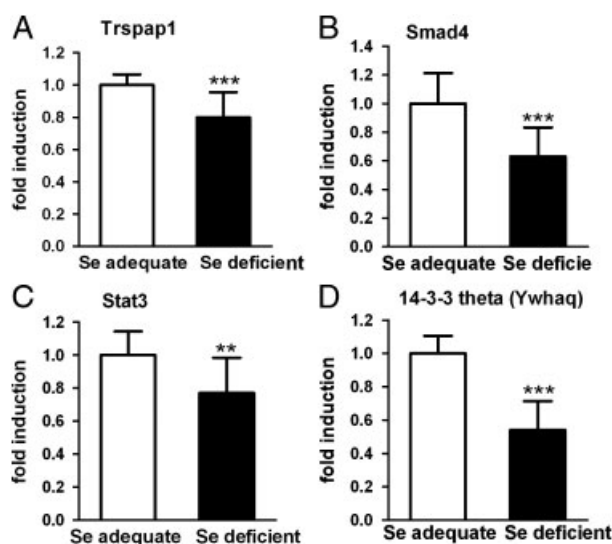
Figure 2. mRNA expression of non-selenoproteins in the colon of selenium-deficient relative to selenium-adequate mice. (A) tRNA Sec-associated protein (Trspap or Secp43), (B) Smad4, and (C) Stat3, (D) 14-3-3 θ (Ywhaq). Gene expression was analysed in triplicate by qPCR and normalized to the mean of the reference genes Hprt1 and Rpl13a. Values are means of 12 animals *per* group ± SD. ** $p < 0.01$ and *** $p < 0.001$ using Student's *t*-test.

classified as ''not expressed'' in the arrays and therefore this pathway was further analysed by comparing two pools of samples in a Wnt PCR array. In total, 37 genes responded to lowering the selenium status with an absolute FC ≥ 1.2, however, only 25 were expressed at a level that could be measured with sufficient sensitivity (c_T-value < 30, Table 4). Seven of these 25 genes were also significantly regulated when analysed in microarrays (microarray FC and *p*-value, Table 4). In total, 18 genes were ''not expressed'' in the microarray from which six are still in the table due to a distinct response in the PCR array. Changes in the expression of genes for β-catenin, GSK3β, dishevelled (Dvl), lymphocyte enhancer factor-1 (Lef1), transducin-like enhancer of split-2 (Tle2), and c-Myc as target of β-catenin were confirmed by qPCR (Fig. 3A–F).

4　Discussion

The aim of this study was to analyse not only the expression of selenoprotein genes but also the global expression patterns in the colon, an organ that responds to the availability of selenium. In contrast to most earlier studies relating gene expression to selenium status, animals were

Table 3. Pathways in which genes were regulated by selenium supply ranked by *z*-score

MAPP Name	Down	Up	z-score
Mm_Translation_Factors[a]	86	55	8.63
Mm_mRNA_processing_binding[b]	217	104	8.23
Mm_mTOR-Signaling-Pathway[c]	55	44	8.23
Mm_TNF-α-NF-κB[d]	165	88	8.06
Mm_Regulation-of-eIF4e-and-p70-S6-Kinase[c]	56	39	7.95
Mm_Ribosomal_Proteins	6	27	7.15
Mm_Proteasome_Degradation[a]	98	49	7.09
Mm_ADP-Ribosylation-Factor[c]	53	22	7.09
Mm_Gene-Expression[b]	78	58	7.02
Mm_Phosphoinositides-and-their-downstream-targets[c]	51	22	6.47
Mm_IL-2[d]	68	36	6.36
Mm_IL-3[d]	80	38	6.26
Mm_Insulin_Signaling[a]	91	34	6.23
Mm_α6-β4-Integrin[d]	61	34	6.22
Mm_Delta-Notch[d]	93	23	6.19
Mm_WNT-Signaling-Pathway[c]	73	13	4.37
Mm_Wnt[d]	105	28	4.02

Up and down indicate the number of genes which are up- or down-regulated in the respective pathway by selenium deficiency. Underlying database present in GeneMAPP: the break between the Delta-Notch and the Wnt pathway indicates that Wnt does not directly follow Notch.
a) COREG.
b) Reactome.
c) Biocarta.
d) Netpath.

Although the Wnt pathway was not within the top 15 pathways, it also showed a reasonably high *z*-score. However, a high number of genes in the Wnt pathway were

fed a selenium-adequate and a marginally deficient diet, the latter containing about half of the selenium considered to be adequate for mice [17, 32]. The selenium intake, thus,
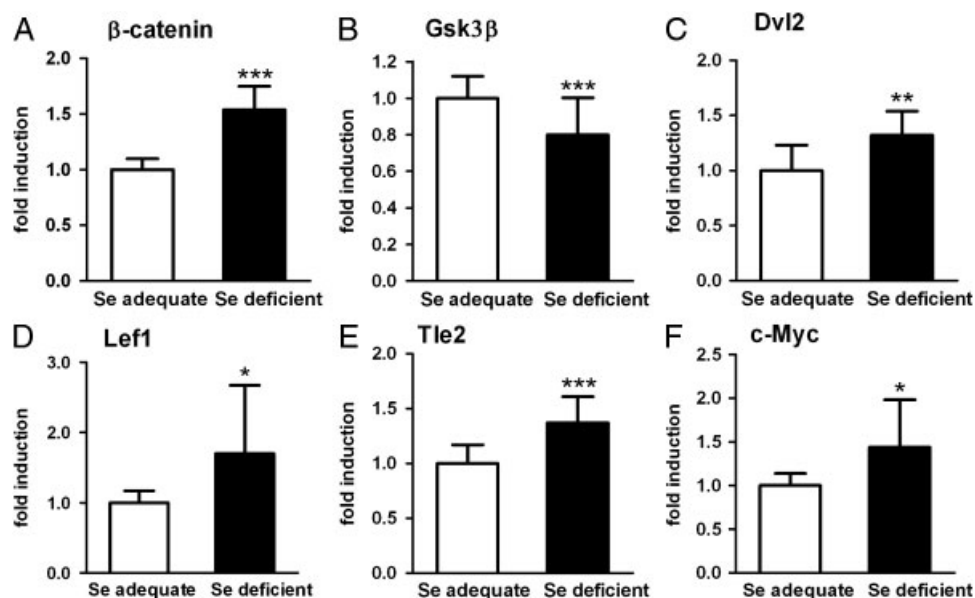
Figure 3. mRNA expression of non-selenoproteins involved in the Wnt pathway in the colon of selenium-deficient relative to selenium-adequate mice. (A) β-catenin, (B) glycogen synthase kinase 3β (Gsk3β), (C) Dvl2, (D) lymphocyte-enhancing factor-1 (Lef1), (E) transducin-like enhancer of split 2 (Tle2), and (F) c-Myc. Gene expression was analysed in triplicate by qPCR and normalized to the mean of the reference genes Hprt1 and Rpl13a. Values are means of 12 animals per group $\pm$ SD. $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$ using Student's *t*-test.

Table 4. Genes of the Wnt pathway changed in the colon of Se-deficient mice as identified in the microarray and/or the PCR array

Gene name	Agilent ID	Description	Acc. number	Microarray FC and (*p*-value)	PCR array FC
Ctnnb1	A_51_P272046	Catenin (cadherin associated protein), β1	NM_007614	0.84 (0.119)	1.28
Dixdc1	A_51_P468876	DIX domain containing 1	NM_178118	1.21 (0.011)	1.38
Dvl1	A_52_P234958	Dishevelled, dsh homolog 1 (Drosophila)	NM_010091	1.12 (0.173)	1.32
Dvl2	A_51_P113106	Dishevelled 2, dsh homolog (Drosophila)	NM_007888	n.d.	1.96
Ep300	A_52_P411601	E1A binding protein p300	NM_177821	0.98 (0.712)	1.30
Fbxw11	A_51_P141860	F-box and WD-40 domain protein 11	NM_134015	1.42 (0.038)	1.33
Fzd1	A_52_P597634	Frizzled homolog 1 (Drosophila)	NM_021457	1.03 (0.751)	1.73
Fzd3	A_51_P190863	Frizzled homolog 3 (Drosophila)	NM_021458	1.27 (0.148)	1.42
Fzd8	A_51P312517	Frizzled homolog 8 (Drosophila)	NM_008058	n.d.	1.45
Gsk3b	A_52_P641629	Glycogen synthase kinase 3β	NM_019827	0.75 ($5,4 \times 10^{-5}$)	1.06
Lef1	A_51_P420400	Lymphoid enhancer binding factor 1	NM_010703	n.d.	5.74
Lrp6	A_51_P169401	Low density lipoprotein receptor-related protein 6	NM_008514	n.d.	1.35
Myc	A_52_P278549	Myelocytomatosis oncogene	NM_010849	1.07 (0.267)	1.20
Nxn	A_52_P57013	Nucleoredoxin	NM_008750	1.20 (0.018)	1.14
Pitx2	A_52_P57013	Paired-like homeodomain transcription factor 2	NM_011098	1.31 (0.152)	1.68
Rhou	A_51_P227392	Ras homolog gene family, member U	NM_133955	1.12 (0.154)	1.36
Senp2	A_51_P492141	SUMO/sentrin specific peptidase 2	NM_029457	0.87 (0.015)	1.31
Sfrp2	A_51_P520849	Secreted frizzled-related protein 2	NM_009144	1.10 (0.163)	1.32
Sfrp4	A_51_P457196	Secreted frizzled-related protein 4	NM_016687	n.d.	0.59
Tle2	A_51_P175303	Transducin-like enhancer of split 2	NM_019725	1.13 (0.032)	0.30
Wif1	A_51_P484526	Wnt inhibitory factor 1	NM_011915	1.08 (0.180)	0.76
Wisp1	A_51_P220343	WNT1 inducible signaling pathway protein 1	NM_018865	1.21 (0.005)	1.37
Wnt4	A_51_P130475	Wingless-related MMTV integration site 4	NM_009523	n.d.	0.65
Wnt5b	A_51_P137991	Wingless-related MMTV integration site 5B	NM_009525	1.02 (0.648)	0.67
Wnt6	A_52_P415155	Wingless-related MMTV integration site 6	NM_009526	0.91 (0.063)	0.28

Genes are sorted based on gene name. The PCR array was performed once. Gene names in bold indicate significant change as measured by qPCR (see Fig. 3).

reflects situations attained by common variation in the human diet. The selenium status reached after 6 wk feeding of these diets is characterized by a drop of plasma selenium to 12%, and liver and colon GPx activity to 34 and 50%, respectively. The decrease, thus, corresponds to a real deficiency and is more severe than expected. Whether a constant reduction in the intake of selenium leads to the same deficiency in humans is not known. Definitely plasma selenium

levels in Europeans are not as low as 12% of those in the US. Consequences of a low intake, however, are obvious from the well-known increase in cancer incidence in selenium-deficient areas [33] and the decrease in cancer incidence by selenium supplementation only in groups entering respective studies with a low selenium status [34].

Transcripts of SelW, GPx1, SelH, and SelM responded significantly to selenium as measured by both microarray analysis and qPCR. Although the response of GPx1 was expected, the *in vivo* response of the other genes had so far been poorly (SelW) or not at all documented (SelH and SelM). The observed fall in SelW expression is consistent with the previously observed almost complete loss in SelW RNA expression in the colon of severely selenium-deficient rats [35] fed a diet containing 1/10 of the Se content in the deficient diet fed in this study. Moreover, SelW mRNA expression strongly decreased in brain and testes of SelP knockout mice [36] and increased in skeletal muscle of rats fed selenium in a range from 0 to 4 mg/kg diet [37]. Taken together, these data confirm the high sensitivity of SelW to selenium status. Additionally, we show here that expression of SelH, and SelM in the murine colon was also highly sensitive and this novel observation deserves further evaluation, especially in view of their largely unknown functions.

As selenoprotein expression is regulated both at a translational level and at the level of RNA stability [8, 38–40], thus assessment of selenoprotein RNA levels in response to changes in selenium supply provides information on RNA degradation and on the hierarchy of selenoproteins. To date, the mechanisms behind the hierarchical response of all selenoproteins to dietary selenium remain unclear. The tRNA[ser]sec plays a key role in this process. It is modified post-transcriptionally at two bases and one ribose moiety in the anticodon loop: A37 is modified to N^6-isopentenyladenosine (i^{6}A), U34 is methylated to methylcarboxyl-5′-methyluridine (mcm^{5}U) and further methylated at the ribosyl residue resulting in mcm^{5}Um, also called Um34 (reviewed in [41]). Um34 formation is increased with increasing selenium [42] and Um34 is responsible for the synthesis of so-called stress-response selenoproteins such as GPx1, GPx3, SelX (MsrB1), SelT, SelH, SelM, SelV, TrxR2, and SelW in murine liver [43, 44]. The tRNA[ser]sec lacking the 2′-methylribose at position 34 (mcm^{5}U) is responsible for the synthesis of housekeeping selenoproteins (such as TrxR1 and TrxR3) [44]. The observed sensitivity of SelW, GPx1, SelH and SelM to selenium deprivation suggests that the synthesis of these enzymes depends on the tRNA[ser]sec methylation in the colon in a similar way as in the liver. An absence or intermediate response to selenium was observed for GPx2, GPx4, SelP, and Sep15, suggesting that their synthesis was partially dependent on Um34 also in the colon. A more complex response was observed for the thioredoxin reductase genes that showed no response by microarray, as expected from their housekeeping function, but a moderate response when assessed by qPCR. The

dependency of the remaining selenoproteins (SPS2, SelK, SelS, SelI, Dio1) on Um34 has not been investigated so far.

Contrary to the previous observations of an effect on growth arrest and apoptosis in animals fed various selenium compounds [10–12, 45], no striking effects on these pathways were observed in the present report. This could reflect the fact that substantially higher doses of selenium than here were used in the previous studies. Interestingly, five of the top 15 regulated pathways are involved in protein biosynthesis, indicating a striking effect of selenium on protein biosynthesis in the colon. The net effect of selenium deficiency on protein biosynthesis is not clear since some genes are down-regulated and others up-regulated. However, consistent with this observation, the protein biosynthesis pathway was recently shown to be regulated by selenium supplementation in human lymphocytes too [15]. The comparable findings in two different organs in two different species identify protein synthesis as a pathway that is particularly sensitive to selenium availability. Whether selenoproteins are involved in the regulation of protein synthesis is not known, but SelW [46], SelH [47], and SelM [48] belong to the redoxin family of selenoproteins, which contain a thioredoxin-like fold and a CXXU motif, indicating a redox function. SelW is localized in the cytosol and has been identified as 14-3-3-binding partner [46, 49]. SelH is mainly localized in the nucleoli [47], a site for rRNA biosynthesis and modification [50]. It further has redox-responsive DNA-binding properties and can regulate genes that respond to changes in the redox status [51]. SelM and SelS/VIMP, for VCP/p97-interacting membrane protein [52], are localized in the endoplasmic reticulum, the site for protein folding and quality control [53]. They might act as chaperones and prevent protein misfolding. A putative redox-regulating capacity of all four above-mentioned selenoproteins influencing translational activity needs further evaluation.

Interestingly, the number of selenium-responsive genes in the Wnt pathway was surprisingly high. Wnt-inhibitory factors and GSK3β, were down-regulated, whereas Wnt receptors and the co-receptor (LRP), the stimulatory factors Dvl, β-catenin and TCF/LEF, as well as β-catenin targets, the cell type specific differentiation factor pitx2 [54] and c-myc [55], were up-regulated the former at least in qPCR (Fig. 4). By changing the expression of these genes, the predicted net effect of selenium deficiency would be to slightly stimulate the Wnt pathway, hence predisposing the tissue to further Wnt-stimulating agents ultimately leading to carcinogenesis. Recently, Wnt signalling has been linked to the mTOR pathway [56], in which mTOR functions as a central regulator of cell growth [57]. To elucidate whether and how selenium regulates this complex network is a challenging future task.

Selenium deficiency decreased the expression of Smad4 and STAT3 as observed in several cytokine pathways (inflammation) and in the Delta-Notch pathway (carcinogenesis). STATs are activated in response to many different
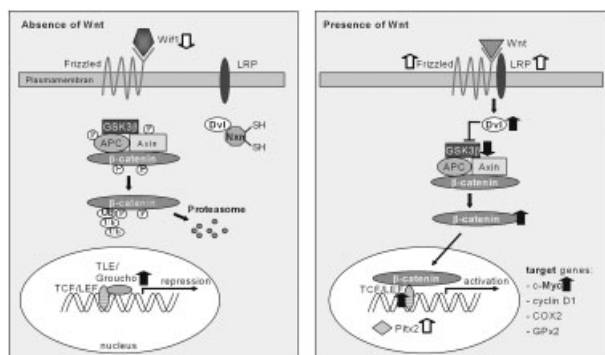
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Figure 4. Wnt signalling. (Left side) In the absence of Wnt signals the Wnt receptor frizzled is blocked by, *e.g.* the Wnt inhibitory factor Wif1 and cytoplasmic β-catenin is associated in the degradation complex consisting of adenomatous polyposis coli, axin, and glycogen synthase kinase 3β (GSK3β). Phosphorylation of β-catenin by GSK3β promotes ubiquitinylation (Ub) and subsequent proteasomal degradation of β-catenin. As a result, levels of free β-catenin remain low and the formation of the TCF/LEF(T-cell factor/lymphocyte enhancer factor)-β-catenin hetero-dimer in the nucleus is prevented. Instead, TCF/LEF is associated to the transcriptional co-repressor TLE/groucho (transducin-like enhancer of split 2) and acts as a transcriptional repressor. (Right side) Upon binding of Wnt proteins, the Wnt receptor frizzled interacts with its co-receptor LRP (low-density lipoprotein receptor-related protein), resulting in the activation of Dvl which in the silent status is bound to nucleoredoxin (Nxn) and released upon oxidation [58]. Dvl in turn inhibits GSK3β. Inactivation of GSK3β prevents phosphorylation and degradation of β-catenin, which now can translocate into the nucleus where it interacts with TCF/LEF transcription factors and promote expression of specific genes such as c-Myc and Pitx2 [59]. Genes up- and down-regulated by selenium deficiency are indicated by respective arrows. Empty arrows indicate genes found in the PCR array, filled arrows indicate confirmation of microarrays, and/or PCR arrays by real-time PCR.

cytokines and growth factors, whereas Smads transfer TGFβ signals. TGFβ is a multifunctional growth factor and has both tumour suppressor and tumour-promoting activities. TGFβ-mediated cell growth is triggered by Smad 2, 3, and 4, which are part of the transcriptional repression complexes, *e.g.* at the c-myc promoter. Since TGFβ serves as tumour suppressor in the normal intestinal epithelium, the down-regulation of signal transducers such as Smad4 in selenium deficiency might contribute to the generally higher cancer risk in selenium deficiency. How and if, however, selenium interferes with these pathways awaits further investigations.

In conclusion, feeding a marginally selenium-deficient diet to mice led to a change in the global gene expression in the colon. Expression of four selenoproteins (SelW, H, M, and GPx1) dramatically decreased in selenium-deficient status, suggesting that these selenoproteins rank low in the selenoprotein hierarchy in the colon. Interestingly, this correlates with the dependence of synthesis of these sele-noproteins on Um34, highlighting the role of this methy-lated tRNA in the hierarchy. These four selenoproteins

should be further investigated as markers for selenium status. Two of them are directly, or indirectly, involved in the regulation of protein folding (SelM, SelW *via* 14-3-3) and may contribute to the change in genes functioning in protein biosynthesis, which was the pathway most signifi-cantly affected by selenium intake. Low selenium intake also modulated cancer-relevant pathways, such as the Wnt, mTOR, and TGF-pathways, and this may contribute to the higher susceptibility to colon carcinogenesis in a selenium-deficient status.

The authors have declared no conflict of interest.

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