cancers

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Supplementary Materials and Methods

Immunity Depletion, Telomere Imbalance, and Cancer-associated Metabolism Pathway Aberrations in Intestinal Mucosa upon Short-Term Caloric Restriction

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1. Database Usage

Table S1. The collection of databases used to carefully manually curate and re-annotate differentially expressed duodenum mucosa CR-responded genes.

Database	Reference
UniProtKB/UniProt Tissue Annotation (https://www.uniprot.org/) DAVID Bioinformatics 6.7 Functional Annotation Tool (https://david-d.ncifcrf.gov/)	[1]
Ingenuity Pathway Analysis (IPA) Disease and Biological Function Annotations (https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis)	[2]
STRING v11 Gene Ontology (GO) (https://string-db.org/)	[3]
Cancer Cell Metabolism Gene (CCM) Database (https://bioinfo.uth.edu/ccmGDB/)	[4]
Cancer Predisposition Gene (CPG) Database	[5]
The Tumor Suppressor Gene (TSG) Database (https://bioinfo.uth.edu/TSGene1.0/)	[6]
TelNet Database (www.cancertelsys.org/telnet/)	[7]
Cancer Stem Cell Database (CSCdb)	[8]
Enrichr Database (https://amp.pharm.mssm.edu/Enrichr/)	[9]
Mouse Genome Informatics (MGI) Batch Query tool (http://www.informatics.jax.org/batch)	[10]
Genome Browser (http://genome.ucsc.edu/) with mouse Dec. 2011 (GRCm38/mm10)	[11]
The telomerase RNA component (TERC) Path Designer via IPA	[2]
NCBI Reference Sequence (RefSeq) (https://www.ncbi.nlm.nih.gov/refseq/)	[12]
OncoMX (http://oncomx.org/)	[13]
dbEMT (http://dbemt.bioinfo-minzhao.org/) Epithelial-Mesenchymal Transition Gene Database	[14]
ImmPort (https://www.immport.org)	[15]

2. Gene Symbols Associated with Multiple Probesets (p.s.)

If more than one p.s. was associated with a gene symbol, the directionality of the regulation was checked. When the p.s. associated with a specific gene symbol had either all upregulation or all downregulation expression data, the p.s. with the lowest adj. p-value was chosen (Table S12). Only one gene associated with multiple p.s. had both upregulation and downregulation data. This led to the distinction of *Armc2* having isoform A (upregulated) and isoform B (downregulated). Isoform A is associated with p.s. 10368881, is located at chr10:42008653-42008725, and is *Armc2's* second longest exon isoform. Isoform B is associated with p.s. 10368859, is located at chr10:41914993-42007709, and is *Armc2's* longest isoform. The UCSC Genome Browser (http://genome.ucsc.edu/) with mouse Dec. 2011 (GRCm38/mm10) assembly was utilized to annotate Armc2's isoforms [11].

3. Gene Enrichment Analysis

Enrichment analysis of tissue-associated proteins in mice was completed using the Uniprot tissue (UP_tissue) annotation database through DAVID Bioinformatics 6.7. and its Functional Annotation Tool [1]. Through selection of tissues with Benjamini < 0.05,

Enriched Immune System Gene (ISG) subset was represented by the proteins connected to the jejunal and colic lymph nodes (at 87.14-fold enrichment), spleen, activated spleen and thymus. Additionally, Epithelial Cell-Enriched Genes (ECG) were represented by proteins that referred to the colon, liver, kidney, and SI. Next, both the ISGs and ECGs were excluded from the 505 DEGs and returned the "Other DEGs" category. Genes related to the immune system were identified in the Other DEGs list and ECG list; the classification system was strengthened using immune resources and IPA's Disease and Biological Function annotations [16].

4. Gene Subset Recourses

The Mouse Genome Informatics (MGI) Batch Query tool (http://www.informatics.jax.org/batch) was used for ortholog conversions [10]. Additionally, gene BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) determined the best homologous genes in the mouse genome for lost or re-solve conflict annotations [17].

5. Expression Data Analysis

Transcriptional profiling using Affymetrix Human Transcriptome Array 2.0 of duodenal biopsies from normal tissue of 12 familial adenomatous polyposis (FAP) patients who developed cancer was assessed [18]. The NCBI GEO accession for this dataset is #GSE111156. This microarray was normal human duodenum expression and had 26,869 p.s. after removal of control p.s. and p.s. without annotation data from Affymetrix. For each p.s. across the 12 samples, the median expression value was utilized to represent the p.s. signal. We determined the overlap with the microarray from the CR DM scraping; the mean value was used to represent each p.s. signal. The cutoff for both datasets was 3 for log microarray signal intensity as defined from the frequency distribution expression functions; this separated expressed genes for both datasets. MGI batch query converted human gene symbols to mouse orthologs. Of the 467 mouse CR DEGs, 382 had human orthologs. When one mouse gene symbol was associated with multiple human orthologs, the protein sequences were aligned with the Clustal Omega program through UniProt [19].

6. qPCR

RNA was isolated from intestinal scrapings using the RNeasy mini kit (Qiagen). Samples were thawed in lysis buffer, disrupted using a syringe and needle, and processed following the manufacturer's recommendations. RNA isolation from other tissues was done with Trizol according manufacturers recommendations (Life Technologies, Carlsbad, California, USA). SuperScript® II Reverse Transcriptase (Invitrogen™, Life Technologies, Carlsbad, California, USA) and random primers (Promega, Madison, WI, USA) were used for the reverse transcription step. Quantitative real-time PCR (qRT-PCR) reactions were carried out using the Applied Biosystems 7900HT, Life Technologies, Carlsbad, California, USA) with the SYBR green PCR Master Mix (Applied Biosystems 7900HT, Life Technologies, Carlsbad, California, USA). The primers used are listed below.

In our previous study [20] in Figure 4 we validated the CR microarray-based duodenum observations of inflammatory genes (*Stat1, Tlr3, Irf1, Reg3b, Reg3g, Oas1a*) and metabolic genes (*Acot4, Acox2, Scd1, Cd36, Pparα, Vldlr*) across samples from the stomach, jejunum, ileum, and proximal and distal colon. In [21] we validated glutathione metabolism related genes *Gsta3, Gsta4, Mgst1,* and *Mgst2* upon CR in mice. Here, we show qPCR validation for *Mt2* and *Myd88*. Thus, listed below are the 18 genes which are microarray-defined DM DEGs, validated by RT-qPCR across six GI tract tissues.

7. RT-qPCR Primer List:

	Forward (5′-3′)	**Reverse (5′-3′)**
MyD88	GCACCTGTGTCTGGTCCATT	TGTTGGACACCTGGAGACAG
Gsta3	GAATGGAGCCTATCCGGTGG	GCATGGCGGTACAAGCCTTT
Gsta4	ACTTTAATGGCAGGGGACGG	GCAGGTGTCCATCCTTTTGC

Mgst1	CCTTCTCCCTGGATTCAGTCAT	TCGGCCATGCTTCCAATCTT
Mgst2	GAAAGAAAGATGGCCGGGGA	CCGCCAAGCGAAATAACTTTG
Cd36	TGATACTATGCCCGCCTCTCC	TTTCCCACACTCCTTTCTCCTCTA
Scd1	GCCCACATGCTCCAAGAGAT	GGGCACTGTCTTCACCTTCT
Acot4	TGCGGTACATGCTTCGACAT	TGGAAACTGTGGCTGAGACAT
Vldlr	GGTTACCAAGTATCTGTA	CATAGAAATATCTTCAAAGTG
Oas1a	ATGGAGCACGGACTCAGGA	TCACACACGACATTGACGGC
Stat1	CAGTATGATGAGCACAGTA	AAGTCCTTCAGAGTAACAG
Tlr3	GTATTGCCTGGTTTGTTAATTGG	AAGAGTTCAAAGGGGGCACT
Irf1	CCCAGCTCTTGCTTTCGGA	AAGCCCAGTAGTTCACGACC
Reg3b	TGGGAATGGAGTAACAATG	GGCAACTTCACCTCACAT
Reg3g	TTCCTGTCCTCCATGATCAAAA	CATCCACCTCTGTTGGGTTCA
Acox2	GCCTCATGCAATACTCTGGC	GGTACCAAGAACCTCTGTCCTG
Pparα	TACTGCCGTTTTCACAAGTGC	AGGTCGTGTTCACAGGTAAGA
Mt2	AGATCTCGGAATGGACCCCA	AGGAGCAGCAGCTCTTCTTG

8. qPCR Statistics

The differences in the intestine mucosa PCR expression data between the experimental groups were used to calculate the sample size. Within the study, data sets with two groups (CR and ad libitum) of seven to nine biological replicates were compared using a two-sided student's t-test to verify statistical significance.

9. Gene Ontology, Network Analysis, and Gene Set Enrichment

STRING v11 (https://string-db.org/) generated networks for mouse and orthologous human genes [3]. Interaction sources were experiments and databases, with the minimum required interaction score at high confidence (0.700). The statistical background was the whole mouse or human genome. Gene Ontology (GO) information was generated by STRING for each network as defined by the COG database. Functional GO enrichments (FDR < 0.05) were compared between human and mouse.

DAVID Bioinformatics 6.7 was utilized for additional GO of all mouse CR DEGs [1]. The background set was the whole mouse genome. Functionally enriched DAVID terms were defined as those with a Benjamini-corrected *p*-value of less than 0.05.

Ranked Gene Set Enrichment Analysis (GSEA) was performed with software v4.1.0 [22]. The gene set annotations were downloaded from: http://download.baderlab.org/EM_Genesets/current_release/Mouse/symbol/Mouse_GO_AllPathways_no_GO_iea_September_01_2020_symbol.gmt. The file excludes GO annotation evidence codes 'IEA' (inferred from electronic annotation), 'ND' (No biological data available), 'RCA' (inferred from reviewed computational analysis). Default settings were used. "Collapse dataset to gene symbols" was set to "false". The ranked list prior to analysis was calculated by the p-value and direction of the log fold-change for mouse CR DEGs with adjusted *p*-value < 0.05 at |FC| > 1.2. Significant gene sets (with $p < 0.05$ and FDR < 0.25) were visualized in Cytoscape v3.8.0 EnrichmentMap [23].

10. Immune Cell Type Enriched Genes

Enriched genes of immune cells types (>2-fold difference) from human and mouse expression profiles were identified from Table 1 [16]. Human genes were converted to mouse orthologs and compared against the 505 CR DEGs. To extend our curation and mining, further classification of the CR DEGs was completed using ImmPort gene lists available at https://www.immport.org/shared/genelists, including chemokines, TNF family members, interleukin receptors, and NK cell markers. We profiled the CR-responded immune DEGs using the Single Cell Portal (https://singlecell.broadinstitute.org/) from the Broad Institute. We visualized sin-gle-cell RNA-seq data of the murine small intestine epithelium (SCP241, GSE106510). This strengthened our classification system of the immune system genes by the cell-types: macrophage, CD8, DC, epithelial, plasma cell, inflammatory monocyte, neutrophil, CD4, NK, B cell, and pDC.

11. Curation of Cancer-Associated Genes and Selection Criteria

We curated genes lists from colorectal adenoma and duodenal adenoma/adenocarcinomas, the Cancer Cell Metabolism Gene Database, Apc knockout and APCMin/+ mouse model of colorectal cancer studies, and literature searches. Next, these gene lists were compared to the 505 CR DEGs for significance (adj. p-value <0.05 at |FC|>1.5).

Three supplementary files were utilized that indicated gene transcript under/over expression in human adenoma versus normal mucosa [24-26]. These included duodenal adenoma/adenocarcinomas tumor-normal tissue pairs (FC ≥ 1.25 compared with matched normal mucosa) from Table S2 [26], colorectal adenoma samples of 44k Whole Human Genome microarrays (Agilent) (≥2.0 FC, p -value ≤ 0.01 by t-test with FDR) from Supplementary Table 2 [24], and colorectal adenomas (FC ≥ 4.0 compared with normal mucosa and Mann-Whitney test using a 1% FDR) from Supplementary Table 4 [25]. Additionally, DEGs from human duodenal cancer-normal comparisons in familial adenomatous polyposis (FAP) cases ($p < 0.05$ and FC > 2) were used; FAP is caused by loss of *APC* function [18]. Human transcripts were converted to mouse orthologs with MGI batch query [10]. Only matching directionality gene expression changes were included when comparing to the 505 CR DEGs (upregulated in both CR and adenomas or downregulated in both CR and adenomas).

The Cancer Cell Metabolism Gene Database maintains a comprehensive resource of human cancer cell metabolism (CCM) genes (https://bioinfo.uth.edu/ccmGDB/) [4]. Their 514 CCM gene list (curated from five other cancer gene databases) was converted to mouse orthologs using MGI batch query and compared to our 505 CR DEGs [10]. The directionality of the CC gene expression changes were not indicated.

Top regulated genes following Apc loss at days four and five (FDR of 5%) from Table S1a–c [27] were compared to our 505 CR DEGs. Additionally, transcription profiles of $APC^{Min/+}$ adenomas and carcinomas compared to wild-type epithelial and $APC^{Min/+}$ normal epithelial cells (Mann-Whitney pairwise comparison test, $p \leq 0.05$) were overlapped with our 505 CR DEGs [28]. Only matching directionality gene expression changes were included (Apc knockout/$APC^{Min/+}$ upregulated and CR downregulated or Apc knockout/$APC^{Min/+}$ downregulated and CR upregulated).

11. Curation of Telomeric Maintenance Genes and Selection Criteria

After preliminary network analysis of the upregulated ECGs using STRING v11, we hypothesized that CR may dysregulate telomere maintenance [3]. We curated genes from databases *TelNet* and Ingenuity Pathway Analysis (IPA), literature searches, and manual curation. Human genes were converted to mouse orthologs using MGI Batch Query [10]. Next, these gene lists were compared to the 26,966 p.s. and were compiled using statistical criteria of adjusted p-value < 0.05 at |FC| > 1.2.

The *TelNet* database included a list of 132 significant human genes in Table S1 involved in telomere maintenance after pan-cancer analysis of tumor compared to normal cells (p-value < 0.01, log2 ratio below − 0.782 or above 0.852) or an (anti-) correlation of gene expression and telomere length ratios (p-value < 0.01, Rho below − 0. 295 or above 0.186) [7]. The TERC Path Designer via IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis) generated a list of 45 human genes involved in the telomerase RNA component (TERC) pathway [2]. RNA-seq analysis of TERRA knockdown in mouse embryonic stem cell gene expression (log 2 (FC) > +/− 1 and p < 0.05) identified 199 genes in Table S1 [29]. In addition, the protein interactome of TERRA was characterized with 134 protein partners, which are involved in chromatin modification/transcription in Table S2 [29]. PubMed was used to search for articles relating to telomeres, their maintenance, and CR gene expression. Lastly, genes were curated via manual search in the 26,966 p.s. for Telo*.

12. Interferon-Inducible GTPase Family Annotations

Multiple alignment and blat/blast mapping of the Affymetrix micro-array individual probe sets (p.s.) (designed in UTR and exons) showed many p.s. have multiple alignment

across the IFN-inducible GTPase genes. It is almost impossible to unambiguously assign to one gene or the other certain gene pairs (*9930111J21Rik1*, *9930111J21Rik2* and *Tgt1*, *Tgtp2*) based on sum and individual p.s. expression signals that is used as endpoint mRNA abundance signal. However, for other genes, microarray p.s. design allows us to develop bioinformatics strategies that reduces or fully excludes such uncertainty.

Chromosome coordinates of the Chr11 qB(1.2) locus IFN-inducible GTPase family are provided in Supplementary Data 5 Table B. Exon sequence data was exported from the UCSC genome browser (https://genome.ucsc.edu/) track NCBI RefSeq for the genes of interest (selecting for 5'UTR exons, CDS exons, 3' UTR exons): *Irgm1*, *Gm5431*, *Gm12185*, *Gm12186*, *9930111J21Rik1*, *9930111J21Rik2*, *Tgtp1*, *Tgtp2*. Next, we built a custom BLAST database using the makeblastdb application. Within the NCBI Genome Workbench, all probe set sequences (ProbeIDs) associated with a Transcript Cluster ID were ran against the local exon sequence database with BLASTn (using default parameters wordsize =11, E-value = −10.00). For each Transcript Cluster ID, only ProbeIDs with greater than 90% in query coverage and identity match were included in the 8x8 matrix in Supplementary Data 5 Table F. Figure S6 contains UCSC genome browser tracks defining paralogs of the interferon-inducible GTPase family located at other loci than Chr11 including F830016B08Rik, Iigp1, Irgm2, Igtp, and Irgc1.

13. Network Analysis

A core expression analysis was run in IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis) based on the expression fold changes of our p.s. for the 467 mouse CR DEGs [2]. The reference set was the Mouse Gene 1.0 ST Array and the confidence was experimentally observed. Fischer's exact test in IPA determined enriched canonical pathways for each subset. The Disease and Biological Function annotations within IPA strengthened the curation of gene category subsets for the cancer-associated and ISGs and identified additional immune genes in the Other category. Lastly, core expression analyses were run in IPA separately on each mouse subset (tumor/cancer, ISG, ECG, telomere) using the same criteria as the 467 CR DEGs. After manual curation and inspection, the IPA Networks were imported into Cytoscape v3.8.0 [30]. Unconnected nodes were removed from the analysis. The network analyzer tool calculated Cytoscape network statistics using directed analysis. This identified the number of proteins, edges (interactions), average number of neighbors, clustering coefficient, and network density. Lastly, network data were imported into STRING v11 to determine protein-protein interaction (PPI) enrichment p-values.

14. Supplementary Discussion

Table 2. Selected CR DEGs in tumor suppressive, oncogenic, immune, epithelial stem-like, anti-cancer, and detoxifying pathways for future validation and consideration in pre-cancer and metabolic reprogramming states.

Gene Name	CR	FC	Annotation	References
Fkbp5	Up	8.54	most over-expressed CR-response gene, androgen-responsive gene with high expression in esophageal adenocarcinoma (EAC) tissues and this is associated with decreased patient survival, pro-oncogenic role in EAC	[31-33]
Vldlr	Up	2.81	pro-oncogene involved in both lipid metabolisms and proliferation, pathogenesis of gastric cancer, breast cancer, and involvement in cancer cell growth	[34-37]
Tlr4	Down	-1.77	Innate immune system, pathogen recognition, therapeutic target, reduced expression associated with metastatic status of CRC	[38-41]
Arntl	Down	-1.87	tumor suppressor, circadian rhythms	[42-45]
Plau	Up	1.78	cancer stem-like, poor pancreatic ductal adenocarcinoma prognosis	[46]
Ly6a, Ly6e, Ly6c1	Down	-2.67, -2.18, -1.63	immune cell differentiation, cancer stem cell biology	[47,48]

Sirt3	Down	-1.38	oxidative stress, protection of DNA damage, chromosome maintenance	[49-52]
Rnasel	Down	-1.74	tumor suppressor, predisposition to prostate cancer, antiviral pathways	[53-59]
Cxcl9, Cxcl10	Down	-1.79, -2.03	proinflammatory chemokines, therapeutic target	[60,61]
Ndrg1	Up	2.65	tumor suppressor, EMT, therapeutic target	[62-65]
Ugt2b5, Ugt2b35, Ugt2b36	Up	2.5, 1.7, 2.56	detoxifying enzymes, reduce risk of carcinogenesis and toxicities by inactivating aromatic-like metabolites	[66]
Cyp2c55 (and family)	Up	2.70	metabolizing endogenous compounds, detoxifying exogenous chemicals, drug metabolism	[67]
Lamc2	Up	1.75	promotes proliferation, cell migration, and invasion in cancers including colorectal and malignant metastases	[68-70]
Rrm2	Up	4.02	cell cycle, therapeutic target, oncogene playing a key role in tumorigenesis and cancer progression, poor prognostic factor for colon, breast, and pancreatic cancers, cancer driver	[71-75]
Aldh1a1	Up	2.45	EMT-related oncogenic, cancer-stem like, upregulated in $APC^{Min/+}$ mouse model of colorectal cancer	[76]
Casp4	Down	-1.87	apoptosis, CASP4-deficicient mice exhibit a defect in autophagy	[77]
Cemip	Up	4.25	overexpression correlates with poorer colon cancer patient survival and facilitates colorectal and stomach tumor growth, cancer driver	[68,78]

Tlr4 was downregulated upon CR. *Tlr4* interacted with the hubs *Stat1, Stat2, Cxcl10, Irf1, Nos2,* and *Pml*. *Tlr1/3* were also downregulated. Toll-like receptor 4 (TLR4) promotes neoplasia through activation of the β-catenin pathway and 40% of sporadic CRC and 20% of colon adenomas over-express TLR4 [38]. TLR4 signalling in the colon induces epithelial proliferation and blocking TLR4 may reduce tumor development [39]. TLRs are involved in the progression from precancerous polyps to tumors [40]. However, studies have also demonstrated strong reduced expression of TLR4 is associated with increased metastatic potential of CRC [41].

CR-regulated DM DEGs overlapped with TERRA knockdown and protein interactome data indicating chromosomal instability and telomere maintenance imbalance was likely. In single telomere depletion of telomeric repeat-containing RNA (TERRA) transcripts in cancer cells, DNA damage was induced at telomeres and extratelomeric sites in the genome [79]. TERRA molecules bind chromatin throughout the genome [29]. Thus, in CR response DM DNA damage due to TERRA depletion may alter the genomic integrity of chromatin sites normally bound to TERRA [79].

Arntl was suppressed upon CR. The *BMAL1*/*ARNTL* gene acts as a TSG. Brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1 (*BMAL1*/*ARNTL)* maintains circadian rhythms and inhibits growth and metastasis of tumor cells in lung, ovarian, and breast cancer and tongue squamous cell carcinoma [42-45]. *BMAL1* alters the proliferation, migration, and invasion of cancer cells [42]. Therefore, *BMAL1* could be a potential therapeutic target for cancer treatment. Significant *Arntl* suppression found in our work suggests that treatment of *Arntl* expression may be a perspective strategy for reducing risk of cancerous effects in CR conditions.

Irf1 was suppressed upon CR. IRF1 expression was lower in CRC than in normal mucosa and inversely associated with CRC proliferation rate and metastasis [80]. Ras/MEK activation in cancer cells downregulates IFN-inducible gene transcription by targeting IRF1 expression, increasing susceptibility to viral oncolysis [81]. Wnt signaling pathway impairment is involved in CRC development via activating anti-apoptotic properties of tumors associated with IRF1 degradation [82]. However, pro-oncogenic function(s) of IRF1 is possible; IRF1-deficient tumor cells lost the ability to upregulate PD-L1 expression in vivo in cancer that enhances tumor-infiltrating T-cell cytotoxicity [83].

All CSC genes are multifunctional and context-dependent; this allows alternative pathways. For instance, *Aldh1a1, Ll2rg*, and *Dll4* are also involved in telomerase maintaining and signalling. CSCs have been implicated in chemoresistance properties and

ALDH1A1 expression is involved in resistance to gemcitabine chemotherapy for pancreatic cancer treatment [84]. Urokinase plasminogen activator (PLAU) was upregulated upon CR in our study; poor pancreatic ductal adenocarcinoma prognosis has been correlated with increased expression of PLAU and following its suppression, tumorigenicity, and gemcitabine resistance decreases [46]. The Lymphocyte antigen-6 (Ly6) gene family members *Ly6a, Ly6e, Ly6c1*—which are involved in immune cell differentiation—were suppressed upon CR. Expression of human LY6E is required for tumor immune escape and its knockdown reduced PDl1 (immune checkpoint molecule) expression after IFN-γ treatment [47]. Upregulation of Stem Cells Antigen-1 (Sca-1) encoded by the Ly6a gene enriches tumorigenicity, metastasis, and chemoresistance in mouse gastric cancer [48].

Sirt3 was suppressed upon CR. Current data highlights recent advances and controversies regarding the yin and yang functions of SIRT3 in cancer. Sirt3 over-expression is associated with the reduction of oxidative stress, protection of DNA damage, chromosome maintenance, acetylation of histones, tumor suppression, and metabolic control of cellular homeostasis [49,50]. SIRT1, SIRT3, and SIRT6 mediate hnRNP A1 deacetylation and inhibit both proliferation and tumorigenesis [51]. However, murine tumors lacking Sirt3 induce genome instability and SIRT3 can act as a tumor suppressor by reducing reactive oxygen species (ROS) and regulating HIF-1α [49,50]. SIRT3 protein levels are decreased in human breast cancers, and its knockout in mice has led to age-related diseases [85]. Sirt3 expression downregulation is functionally connected to telomere dysfunction. Telomere shortening in livers of telomerase knockout mice repressed all seven sirtuins [52].

A key tumor suppressor, RNase L, is strongly suppressed in response to CR. The RNase L gene (RNASEL) encodes a component of the interferon-regulated 2-5A system that functions through antiviral, antibacterial, and anti-proliferative activities [53,54]. Interferon-activated 2', 5'-oligoadenylate synthetase (OAS) induces RNaseL to cleave cellular and viral mRNA resulting in apoptosis, autophagy and inflammation [55]. *Oas1a, Oas1b, Oas1g, Oas2*, and *Oasl2* were downregulated upon CR, explaining a mechanism for increased pathogen susceptibility. RNase L induces STAT1 and BRAC1 dependent interferon-gamma (IFN-γ) responses [55] and promotes innate immune responses to intestinal damage, ameliorating murine colitis and colitis-associated cancer [53]. RNase L is a candidate for the hereditary prostate cancer 1 (HPC1) allele and its gene mutations are associated with predisposition to prostate cancer [55-59]. Functionally different variants of SNP in RNASEL are associated with the age of disease onset of hereditary non-polyposis CRC in Lynch syndrome patients [56,57]. RNase L suppression (or mutation) upon CR may play negative roles in the mucosa and therapeutic expression control may be essential.

Proinflammatory chemokines Cxcl9/10 were CR-downregulated. The type II interferon signalling molecule *Cxcl10* had enriched interactions with immunity network hubs (*Stat1/2, Tlr4, Irf*). The analysis noted an asymmetry in input/output connections for *Cxcl10* with 1 downstream and 16 upstream targets. Changes in CXCL10 expression are associated with infectious disease, immune dysfunction, and tumor development [60]. *Cxcl10* mediates leukocyte trafficking by activating T lymphocytes (Th1), NK cells, macrophages, dendritic and B cells [60]. CXCL9/10 aortic concentrations increase due to aging; however, CR prevented age-related increases in CXCL10 [86-89]. CXCL10 stable expression in vivo growing and metastasized colon carcinoma cells is controlled by recruiting and cytolytic functions of NK cells [61]. CR-mediated CXCL10 reduction in mucosa epithelial cells and NK cell numbers may increase cancer development risks. CXCL10 therapeutic implications have been identified [60].

Myd88 is a signaling adapter protein involved in the toll-like receptor and IL-1 receptor signaling pathway in the innate immune response and plays a role in intestinal host-microbial interface mediated by Paneth cells [90,91]. This gene is essential in colorectal cancer cell proliferation, migration, and invasion via NF-κB/AP-1 signaling pathway [92]. However, our microarray and PCR results suggest that this gene is not involved in the short-term CR response in all GI tract tissues. For example, of the six GI tract tissues we

studied, Myd88 was only significantly reduced in qPCR of the jejunum under CR. Interestingly, deficiency in MYD88 blocked colorectal tumor progression [90].

Metallothioneins (MTs) are small cysteine-rich proteins that play important roles in metal homeostasis and protection against heavy metal toxicity, DNA damage, and oxidative stress. However, emerging evidence shows that MTs may contribute to tumorigenesis, tumor growth-promoting effects, formation, progression, and drug resistance. *Mt2* overexpression is associated with proliferative markers (Ki-67) in several cancers (including large intestine adenocarcinoma). *Mt2* inhibits apoptosis and plays multiple roles in immunity, the adaptive immune response, and could drive tumor-immune evasion [93]. Upon CR, in the six GI tract tissues we studied using qPCR, *Mt2* was commonly upregulated in all tissues.

Predicted CR modulated therapeutic target, Ndrg1. Our results showed CR induces the expression of N-myc downstream regulated gene-1 (NDRG1), a marker of normal epithelial cells. Ndrg1 pharmacological targeting is a promising cancer therapeutic strategy because it plays potent metastasis suppressing roles by inducing apoptosis. More tumorigenic and stem-cell like properties were found in NDRG1-silenced CRC cells [62]. NDRG1 suppresses the stress-induced pro-survival autophagic pathway [63]. In colon cancer, NDRG1 expression declines as normal colonic epithelium progresses to carcinoma [64]; patients with lowered NDRG1 mRNA had a shorter 5-year survival rate. However, pro-oncogenic pleiotropic roles have been reported in other cancer-types [65]. NDRG1 anti-tumor activities may be crucial in CR conditions.

Regulation of detoxification enzymes upon CR. UDP-glucuronosyltransferases (UGTs) reduce the risk of mutagenesis, carcinogenesis, and toxicities by inactivating aromatic-like metabolites [94]. This detoxifying system (*Ugt2b5, Ugt2b35, Ugt2b36)* is upregulated upon CR. UGT enzymes are suppressed in several cancers, including bladder cancer by allowing carcinogen accumulation [66]. The negative modifications in tumor suppressors and pro-oncogenic upregulation in CR mice may be combatted by the increases in detoxifying enzymes. The cytochrome P450 (CYP) superfamily is involved in metabolizing endogenous compounds, detoxifying exogenous chemicals, and drug metabolism; furthermore, deficiencies in several P450s have been demonstrated in human diseases [67]. CYP genes upregulated in CR mice were *Cyp2b10, Cyp2c55, Cyp2d10, Cyp2d9, Cyp2j6, Cyp3a44,* and *Cyp4b1*. According to CR-induced expression profiles and network analysis, the glutathione pathway and chemical carcinogenesis rewiring processes are interconnected, but in some contexts, their balance might be lost.

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