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Meta-analysis of global transcriptomics suggests that conserved genetic pathways are responsible for Quercetin and Tannic acid mediated longevity in *C. elegans*

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Recent research has highlighted that the polyphenols Quercetin and Tannic acid are capable of extending the lifespan of *Caenorhabditis elegans*. To gain a deep understanding of the underlying molecular genetics, we analyzed the global transcriptional patterns of nematodes exposed to three concentrations of Quercetin or Tannic acid, respectively. By means of an intricate meta-analysis it was possible to compare the transcriptomes of polyphenol exposure to recently published datasets derived from (i) longevity mutants or (ii) infection. This detailed comparative *in silico* analysis facilitated the identification of compound specific and overlapping transcriptional profiles and allowed the prediction of putative mechanistic models of Quercetin and Tannic acid mediated longevity. Lifespan extension due to Quercetin was predominantly driven by the metabolome, TGF-beta signaling, Insulin-like signaling, and the p38 MAPK pathway and Tannic acid's impact involved, in part, the amino acid metabolism and was modulated by the TGF-beta and the p38 MAPK pathways. DAF-12, which integrates TGF-beta and Insulin-like downstream signaling, and genetic players of the p38 MAPK pathway therefore seem to be crucial regulators for both polyphenols. Taken together, this study underlines how meta-analyses can provide an insight of molecular events that go beyond the traditional categorization into gene ontology-terms and Kyoto encyclopedia of genes and genomes-pathways. It also supports the call to expand the generation of comparative and integrative databases, an effort that is currently still in its infancy.

Keywords: Quercetin, Tannic acid, TGF-beta, ILS, DAF-12, p38 MAPK, *C. elegans*

INTRODUCTION

Although numerous molecules of herbal origin have been hailed as powerful decelerators of aging, few studies have focused on the precise physiological and genetic mechanisms that drive this process. The nematode *Caenorhabditis elegans* is ideally suited for biogerontological research, not only because of its short life cycle (Gami and Wolkow, 2006; Gill, 2006; Kaletta and Hengartner, 2006) but also due to the considerable conservation of basic cellular and molecular principles (The *C. elegans* Sequencing Consortium, 1998).

Of the many bioactive polyphenols (PPs) previously shown to extend the lifespan of *C. elegans* we selected two well characterized compounds, namely Quercetin (Q; work in *C. elegans*: Kampkötter et al., 2007a,b, 2008; Saul et al., 2008; Pietsch et al., 2009, 2011; Surco-Laos et al., 2011; Grünz et al., 2012; for a detailed review about general beneficial effects of Q, tested *in vivo* and *ex vivo*, be referred to Boots et al., 2008) and Tannic acid (TA; work in *C. elegans*: Saul et al., 2010, 2011; Lublin et al., 2011; for a detailed review be referred to Koleckar et al., 2008). Both PPs are characterized by inverted J-shaped concentration–response curves, typical for a hormetic effect (Calabrese and Baldwin, 2001). Moreover, the exposure to PPs enhances the oxidative and thermal stress resistance. Both PPs exert no major negative impact on the reproductive output but result in a reduction of body length and fat

content, two phenotypes that align well with the disposable soma patterns described by Kirkwood (1977, 1988). Utilizing various nematode mutant strains, preliminary genetic analyses identified *daf-2*, *age-1*, *sek-1*, and *unc-43* as mediators of Q induced longevity and stress resistance (Pietsch et al., 2009) and *sek-1* was shown to be important for TA mediated extension of lifespan (Saul et al., 2010). Notwithstanding these differences, some of the data obtained suggests that Q and TA induce longevity by similar genetic mechanisms, e.g., the involvement of *sek-1*, altering the fat metabolism, or hormesis-based dose–responses. An overview of results from previous studies can be found in Table S1 in Supplementary Material 1.

To pinpoint molecular genetic pathways, we performed genome-wide DNA microarray experiments spanning different concentrations of Q and TA, including at least two lifespan prolonging concentrations for each PP. In addition, we tested a low dose of Q (50 μM) because (i) the limitation of solubility precluded the use of doses above 200 μM Q and (ii) this concentration was shown to enhance thermal tolerance (unpublished data) possibly indicative of elevated stress resistance. For TA we included a post-effective, in single trials already toxic (Saul et al., 2010) concentration. Initially, this identified differentially expressed genes (DEGs), which were found to be significantly up- or down-regulated in response to Q and/or TA, many displayed a concentration dependent change in expression. A second layer of analysis included gene ontology (GO), Kyoto encyclopedia of genes and genomes (KEGG) pathways, as well as gene expression mountain algorithms (as introduced by Kim et al., 2001). By analyzing the overlapping transcripts identified in the respective lifespan prolonging concentrations of each PP (Q 100 and 200 μM = $Q_{longevity}$; TA 100 and 200 μM = $TA_{longevity}$), we were able to predict putative master regulators involved in PP mediated longevity. A final meta-analysis compared these core-genes with recently published transcriptional profile data linked to age-related gene expression, the genetic background of longevity mutants or infection. The alignment of common (condition-overlapping) DEGs facilitated the identification of genes and associated pathways that may act as master switches of longevity.

The primary goal of this study was to highlight that the meta-analysis of large datasets is not restricted to complement a simplistic database evaluation but has the potential to uncover compound overlapping molecular switches.

MATERIALS AND METHODS

STRAINS AND SAMPLE GENERATION

N2 wild type *C. elegans* were maintained on nematode growth medium (NGM) plates using *Escherichia coli* OP50 as food source (Brenner, 1974; Sulston and Hodgkin, 1988; Lewis and Fleming, 1995). Untreated nematodes (the P0 generation) were chunked onto control and treatment plates (50, 100, and 200 μM Q; 100, 200, and 300 μM TA) and incubated at 20°C for 4 days. A synchronous culture was generated through egg preparation (Strange et al., 2007) with sodium hypochlorite (Sigma, Germany). Eggs were rotated over night (20 rpm) and the resultant hatched L1s subsequently transferred to plates containing the respective doses of Q or TA. Worms were grown to the young (pre-reproductive) adult stage, harvested by rinsing off with M9 buffer, washed at least three times with M9, shock frozen in liquid nitrogen and stored at −80°C. For each condition, samples were cultivated in triplicate.

RNA PREPARATION

RNA was isolated following the standard procedures as defined by the Trizol protocol (Invitrogen, Germany) but modified to include a homogenization step with 0.5 mm glass beads to maximize cell breakage. The resultant RNA was further processed by means of a RNeasy purification followed by DNase digestion (Qiagen, Germany). All samples were stored at −80°C until further use.

RNA AMPLIFICATION, BIOTIN LABELING, AND DNA MICROARRAY ANALYSIS

RNA was processed with the MessageAMP™Premier RNA Amplification Kit (Ambion, Austin, TX, USA) which relies on the T7 *in vitro* transcription (IVT) amplification technology (Van Gelder et al., 1990). First- and second-strand cDNA synthesis, cRNA synthesis, labeling, fragmentation, GeneChip hybridization, and scanning were performed according to the manufacturer's specifications (Affymetrix, Santa Clara, CA, USA). We utilized the *C. elegans* array chip (Affymetrix) which covers the whole genome (22,548 transcripts). Triplicate chips were run for each condition (designated as Q0, Q50, Q100, Q200 and TA0, TA100, TA200, TA300). Whole RNA, cDNA, and cRNA qualities and quantities were assessed at each step using capillary electrophoresis (Bioanalyzer, Agilent Technologies, UK) and micro volume spectrophotometry (NanoDrop1000, Thermo Scientific, UK).

DATA INTERPRETATION AND STATISTICAL ANALYSIS

Processing of global transcription expression values (DNA microarray)

Pre-processing of raw microarray data included probe-specific background correction, summation of probe set values, and normalization using the GCRMA algorithm with CARMAweb 1.4, an R- and Bioconductor-based web service for microarray data analysis[1] (Rainer et al., 2006). The quality of normalization was assessed by box plot and MA-graph analyses. Differences between treatments were visualized by principal component analysis (PCA) plotting with multiexperiment viewer (MeV)[2] (Saeed et al., 2003). Data was initially filtered for missing values and then subjected to a CLEAR-test that combines differential expression and variability using the GEPAS web server[3] (Tárraga et al., 2008). An unpaired *t*-test was followed by a significance analysis of microarray (SAM) test including a calculation that estimates the false discovery rate (FDR). The FDR was set to a non-stringent level of <12.5%. DEGs showing a fold change of at least 1.25 were analyzed for their molecular functions, biological processes, and cellular components using the software packages GoMiner (Zeeberg et al., 2005) and DAVID[4]. Due to the sound technical and experimental quality of the data, which returned strong statistically significant signal intensities, the chosen fold-cut-off values were deemed to be biologically meaningful and aligned to previous data analyses (Grigoryev et al., 2004; McCarthy and Smyth, 2009).

[1] https://carmaweb.genome.tugraz.at/carma
[2] http://www.tm4.org/mev
[3] http://www.gepas.org
[4] http://david.abcc.ncifcrf.gov/

Comparison of datasets to screen for significant overlaps: representation factor

The overlap between differing conditions (e.g. PPs and gene expression mountains, gene classes, or datasets taken from the literature) was determined by computing the representation factors (RFs). The RFs define the fold enrichment between gene lists (Kim et al., 2001; Evans et al., 2008). The choice of N (genome) was based on the values recommended by the authors. Intersection p-values were calculated from the hypergeometric distribution. RFs were considered significant when RF >1 and $^{*}p < 0.05$, $^{**}p < 0.005$ or $^{***}p < 0.001$.

RESULTS

THE RELATIONSHIP BETWEEN EXPOSURE TO Q OR TA, LIFESPAN EXTENSION AND THE OUTPUT OF TRANSCRIPTIONAL RESPONSES

Previous reports have stated that, whilst 50 μM of Q evoked no significant change in lifespan, 100 and 200 μM Q prolonged the mean lifespan by 11 and 10%, respectively (Pietsch et al., 2009, 2011). However, at 250 μM of Q the lifespan was reduced by −7% (see Table S1 in Supplementary Material 1) and therefore can be considered to be a toxic concentration. In addition, because concentrations above 200 μM of Q did not dissolve completely in the agar and the bacteria, we refrained from using doses above 200 μM Q for the microarray experiment. Thus, for Q, we utilized two lifespan prolonging concentrations (100 and 200 μM), and a non-effective (50 μM) pre-longevity dose for global transcriptomics.

For TA, the most effective concentration was 100 μM, which resulted in an increase in mean lifespan of 18%, an effect that was still significant but notably less pronounced at 200 μM (8%) and was absent at 300 μM (Saul et al., 2010; see Table S1 in Supplementary Material 1). Hence, we used two life-extending concentrations (100 and 200 μM of TA), and in addition one post-effective (borderline toxic) concentration (300 μM TA).

We chose this test design to cover a large dose-range: from pre-effective to effective in the case of Q (Q0, Q50, Q100, Q200), and from effective to post-effective/toxic for TA (TA0, TA100, TA200, TA300; Note: the numerical identifiers represent the concentration in micromolar). Pre-processing of raw microarray data included probe-specific background correction, summation of probe set values, and normalization using the GCRMA algorithm with 93 CARMAweb 1.4, an R- and Bioconductor-based web service for microarray data analysis (see text footnote 1) (Rainer et al., 2006). The CARMAweb reports can be viewed in Supplementary Material 2 and 3.

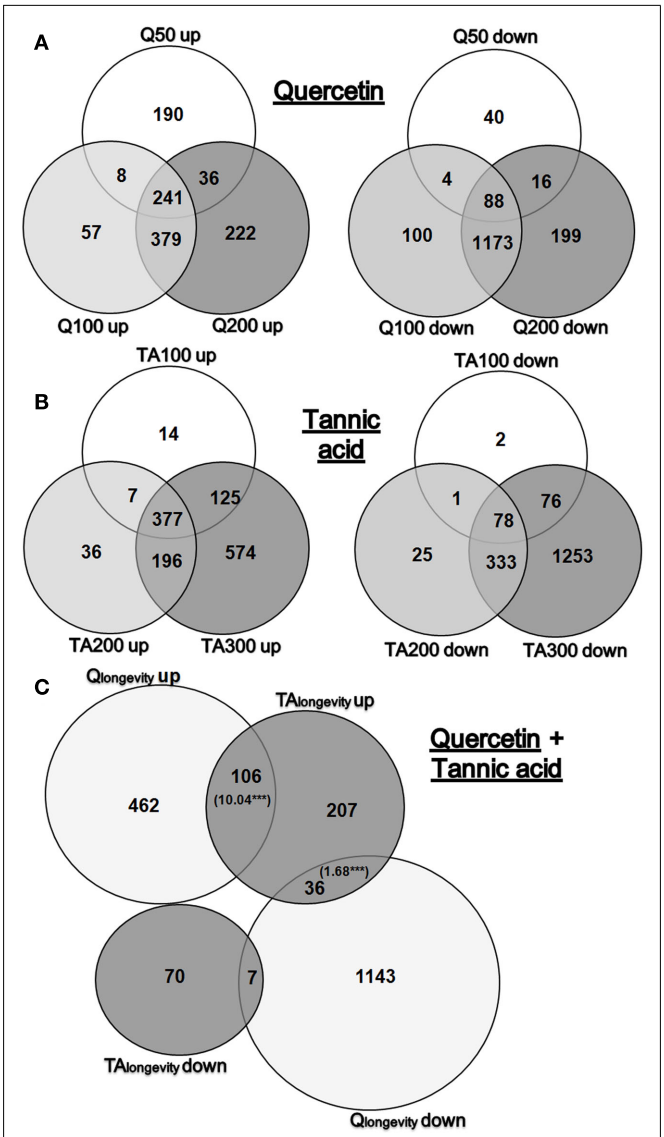


FIGURE 1 | Venn diagrams of DEGs. Overview of significant DEGs (fold change >1.25 or <0.8) in response to Q50, Q100, Q200 or TA100, TA200, TA300. Shown is the overlap of either up- or down-regulated genes in Q **(A)** or TA **(B)** treated nematodes. **(C)** Displays the overlap of significantly up- and down-regulated genes in $Q_{longevity}$ and $TA_{longevity}$. The bracketed numbers in the intersections display significant overrepresented RFs, $^{***}p < 0.001$.

Venn diagrams (**Figure 1**) were compiled to summarize the level of overlap between doses (statistics for significant DEGs can be found in Supplementary Material 4: sheet "Statistics"; full genome fold change (FC-) values are listed in Supplementary Material 5). Investigating the transcriptional response of Q treatment (**Figure 1A**), revealed that the number of DEGs increased markedly at the lifespan modulating doses of Q100 and Q200 (3.3- and 3.8-fold compared to the non-longevity dose Q50). Interestingly, more genes were up-regulated than down-regulated at Q50, but the opposite was observed at Q100 and Q200. It is worthy of note that the majority of DEGs overlapped in Q100 and Q200 exposures, thus suggesting the presence of common response pathways.

As with Q, exposure to TA resulted in a dose dependent increase in DEGs which was most pronounced at the highest dose tested. More genes were up-regulated than down-regulated in the lifespan prolonging conditions TA100 and TA200, a ratio that was reversed at TA300 where more genes were down-regulated (**Figure 1B**). Striking was the proportion of DEGs that responded exclusively to TA300 (61%), a substantial proportion compared to TA100 (2%) or TA200 (6%).

By taking the intersection of DEGs shared by the lifespan prolonging concentrations of each PP, it was possible to extract gene lists for $Q_{longevity}$ (Q100 and Q200 overlap), $TA_{longevity}$ (T100 and T200 overlap) and $Q\&TA_{longevity}$ (overlap of all four groups). A Venn diagram that incorporates $Q_{longevity}$ and $TA_{longevity}$ identified a significant overlap (**Figure 1C**). As our main focus was the identification of mechanisms involved in PP triggered lifespan extension, downstream data processing focused primarily on $Q_{longevity}$ and $TA_{longevity}$ gene lists, however, individual GO-, KEGG-, gene expression mountains-, and gene class-analyses are provided for all concentrations in the Supplementary Material 4.

OVERREPRESENTED GO-TERMS

The summary results of the GO-cluster analysis with DEGs from the $Q_{longevity}$, $TA_{longevity}$, and $Q\&TA_{longevity}$ gene lists are presented in Table S2 in Supplementary Material 1. The complete analyses for all single concentrations are provided in the Supplementary Material 4: sheet "GO-Analysis." The $Q_{longevity}$ analysis returned, due to the large input list of DEGs, more overrepresented GO-terms than the equivalent analysis with $TA_{longevity}$. Overrepresented GO-terms derived from the up-regulated transcripts in $Q_{longevity}$ included *chromatin assembly*, *lipid metabolic process*, *monooxygenase activity*, and *nucleosome*. GO-terms from down-regulated genes included, besides others, *nervous system development*, *regulation of multicellular organism growth*, *Dauer entry*, *regulation -of transcription*, *-of response to stimulus*, *-of cell communication*, *-of biological quality*, *-of locomotion*, and *-of programmed cell death*. In $TA_{longevity}$, significant GO-terms were linked to *muscle contraction*, *neurotransmitter transporter activity and cytoskeleton*, *embryonic development ending in birth or egg hatching*, *positive regulation of biological process*, and *chromatin*. GO-terms in $Q\&TA_{longevity}$ were restricted to Cellular Components (*pseudopodium*).

Scrutinizing the GO-analysis of single concentrations (Supplementary Material 4: sheet "GO-Analysis") revealed, for example, that the GO-terms in the pre-lifespan extending concentration Q50 (*lysozyme activity and oxidoreductase activity acting on the CH–CH group of donors*) may reflect an early onset of induced immunity and stress resistance. In contrast, a striking accumulation of overrepresented GO-terms were observed in TA300, which included numerous categories indicative of an unfavorable condition (*DNA damage response*, *signal transduction*, *mismatch DNA binding*, *cell death*, and others).

OVERREPRESENTED KEGGs

Whilst the assignment of GO-terms is defined by automatic/electronic annotation that is based on sequence homology, KEGG-pathways are manually curated from the literature. As with the GO-ontology, KEGG-analysis on the Q treatment lists returned a multitude of pathways (**Table 1**). For further details about regulated genes in the respective KEGG-terms see Table S3 in Supplementary Material 1 for $Q_{longevity}$ and $TA_{longevity}$, and Supplementary Material 4: sheet "KEGG-Analysis" for single concentrations. Overrepresented KEGGs in Q50 included pathways involved in the metabolism of amino acids, glutathione, and xenobiotics as well as fatty acid elongation. At higher exposures seven (Q100) and eight (Q200) KEGG-pathways were found to be overrepresented, notably most (six) were present at both concentrations (see $Q_{longevity}$, **Table 1**) with an analogous mode/direction of regulation, i.e., either repressed or induced (Table S3 in Supplementary Material 1). Pathways include metabolism of amino acids, xenobiotics or drugs, transport processes (*Lysosome*), and signal transduction processing (*Wnt* and *TGF-beta signaling*). Whilst DEGs from amino acid metabolism and the *lysosome* displayed heterogeneous expression levels, transcripts belonging to the signaling pathways were predominantly repressed, whereas almost all genes associated with *drug/xenobiotic metabolism* were found to be induced.

The dynamic response patterns of Q exposure were not mirrored by the TA treatments. No KEGG-pathways could be linked to the most potent lifespan extending concentration, TA100; hence, none were assigned to $TA_{longevity}$. For TA200 only two KEGG-pathways were found to be overrepresented, both derived from amino acid metabolism. Analysis of the TA300 gene list returned six KEGG-pathways, notably *DNA replication*, *mismatch repair*, and *Ubiquitin-mediated proteolysis*.

META-ANALYSIS: COMPARISON OF GLOBAL TRANSCRIPTIONAL PATTERNS IN $Q_{LONGEVITY}$ AND $TA_{LONGEVITY}$ TO SELECTED DATASETS IN THE LITERATURE

To date, several microarray studies have identified age-related transcriptional changes in the nematode *C. elegans* (summarized in Golden and Melov, 2007). To investigate if a systematic comparison would reveal common age-related pathways, we correlated our datasets with the published expression profiles obtained from long-lived *daf-12(rh273)* (Fisher and Lithgow, 2006), *daf-2* mutants (Evans et al., 2008), TGF-beta mutants (Shaw et al., 2007), and immune-responsive *Pseudomonas aeruginosa* infected nematodes (Evans et al., 2008). The dataset from Evans et al. (2008) includes a meta-analysis of different *daf-2* alleles (Murphy et al., 2003; McElwee et al., 2004) and immune-challenged nematodes (Shapira et al., 2006; Troemel et al., 2006).

The meta-analysis aimed to indentify similar molecular mechanisms of longevity in $Q_{longevity}$, as well as $TA_{longevity}$ and define (if possible) the correlation to long-living mutants. Datasets were analyzed by focusing on (i) overrepresented gene expression mountains (according to Kim et al., 2001), which characterize the global patterns of a transcriptional response and (ii) DEGs, to identify new target genes. The significance of overlap between two gene lists were assigned by calculating the RF, and significances were determined by hypergeometric probability scoring. Supplementary Material 5 provides Gene Expression Mountains, Gene Classes and Groups, and further studies in a table. By using the filter function in Excel, all results that are described in the following sections can be reconstructed.

Overrepresented expression mountains

Kim et al. (2001) assembled data from several hundred *C. elegans* DNA microarray experiments. This allowed a three-dimensional gene expression map to be computed consisting of 44 co-regulated gene-groups (mountains), 30 of which could be assigned to specific gene classes and therefore to a potential physiological importance.

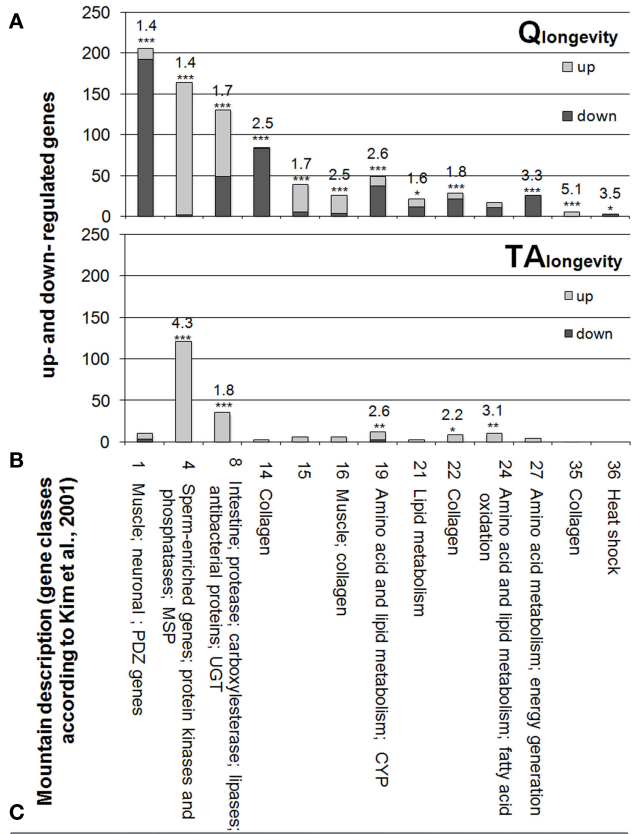
Figure 2 displays all gene expression mountains, in which DEGs of $Q_{longevity}$ and/or $TA_{longevity}$ (**Figures 2A,B**) are overrepresented

Table 1 | Overview of significant KEGG-pathways (see Table S3 in Supplementary Material 1 for pooled groups and Supplementary Material 4: sheet "KEGG-Analysis" for genes with FC-values).

	KEGG-pathway	No. DEGs/listed genes	*p*-Value
Q50	cel00330: arginine and proline metabolism	6/30	0.002
	cel00062: fatty acid elongation in mitochondria	4/13	0.007
	cel00480: glutathione metabolism	5/35	0.022
	cel00270: cysteine and methionine metabolism	4/25	0.041
	cel00980: metabolism of xenobiotics by cytochrome P450	4/26	0.045
Q100	cel04142: lysosome	15/68	0.000
	cel04350: TGF-beta signaling pathway	11/36	0.001
	cel00600: sphingolipid metabolism	8/20	0.001
	cel04310: Wnt signaling pathway	14/60	0.001
	cel00980: metabolism of xenobiotics by cytochrome P450	8/26	0.005
	cel00982: drug metabolism	8/29	0.009
	cel00330: arginine and proline metabolism	8/30	0.011
Q200	cel04350: TGF-beta signaling pathway	13/36	0.000
	cel04310: Wnt signaling pathway	15/60	0.001
	cel00982: drug metabolism	9/29	0.005
	cel00480: glutathione metabolism	9/35	0.015
	cel00330: arginine and proline metabolism	8/30	0.020
	cel04142: lysosome	14/68	0.026
	cel00980: metabolism of xenobiotics by cytochrome P450	7/26	0.033
	cel00340: histidine metabolism	3/9	0.048
$Q_{longevity}$	cel00330: arginine and proline metabolism	9/30	0.000
	cel04350: TGF-beta signaling pathway	9/36	0.001
	cel04310: Wnt signaling pathway	11/60	0.004
	cel00982: drug metabolism	7/29	0.008
	cel04142: lysosome	11/68	0.009
	cel00980: metabolism of xenobiotics by cytochrome P450	6/26	0.022
TA100	x	x	x
TA200	cel00450: selenoamino acid metabolism	6/20	0.006
	cel00270: cysteine and methionine metabolism	6/25	0.015
TA300	cel04914: progesterone-mediated oocyte maturation	15/38	0.004
	cel04120: ubiquitin-mediated proteolysis	26/86	0.006
	cel00534: heparan sulfate biosynthesis	7/8	0.006
	cel03430: mismatch repair	9/18	0.008
	cel03030: DNA replication	13/33	0.008
	cel00500: starch and sucrose metabolism	9/24	0.050
$TA_{longevity}$	x	x	x
$Q\&TA_{longevity}$	x	x	x

(additional RF values for single concentrations in all 44 gene expression mountains can be found in Supplementary Material 4: sheet "Gene Expression Mountains" and "Gene Classes and Groups"). DEGs from $Q_{longevity}$ and $TA_{longevity}$ could both be assigned to mounts 4 (sperm-enriched genes, protein kinases and phosphatases, MSPs), 8 (enriched for intestine-, protease-, carboxylesterase-, lipase-genes, antibacterial proteins and UGTs), 19 (enriched for genes from amino acid and lipid metabolism and CYPs), 22 (collagen enriched), and 24 (also enriched for amino acid and lipid metabolism genes, as well as fatty acid oxidation). In addition, $Q_{longevity}$ was significantly linked to mounts 1 (enriched for muscle, neuronal, and PDZ genes), 14 and 35 (enriched for collagen), 15 (no specific gene-groups), 21 (enriched for lipid metabolism genes), and 32 (enriched for nucleosomal histones). Comparing the direction of regulation, it was noticeable that in $TA_{longevity}$ the majority of DEGs in the respective mounts were up-regulated, whereas no trend was observed in the $Q_{longevity}$ gene list (with the exception of mountain 4).

The comparison of PP treatments to overrepresented mounts from experiments with long-lived mutants and *P. aeruginosa* infected nematodes (**Figure 2C**; Supplementary Material 4: sheet "Gene Expression Mountains") revealed large overlaps. For example, Fisher and Lithgow (2006) reported for *daf-12(rh273)* an enrichment of induced DEGs in mount 4, a finding that matches well with both PPs tested. Further differentially regulated mounts in *daf-12(rh273)* are also overrepresented in $Q_{longevity}$ and $TA_{longevity,}$ suggesting parallels in molecular mechanisms to



mountain	1	4	8	14	15	16	19	21	22	24	27	35	36
daf-12(rh273) **(Fisher and Lithgow, 2006)**													
up		x											
down			x				x		x	x	x		
daf-2 **(metaanalysis Evans et al., 2008)**													
up			x		x	x		x	x				
down			x				x	x	x	x	x		
TGFbeta adults (Shaw et al., 2007)													
up			x	x	x	x	x	x	x	x	x		
down													
P. aeruginosa **infection (metaanalysis Evans et al., 2008)**													
up			x		x		x	x		x	x		x
down			x	x		x	x	x	x	x	x		

FIGURE 2 | Overrepresented gene expression mountains. Identification of overrepresented gene expression mountains (based on Kim et al., 2001) from $Q_{longevity}$ **(A)** and $TA_{longevity}$ **(B)**, as well as from datasets of longevity mutants and *P. aeruginosa* infected *C. elegans* (Fisher and Lithgow, 2006; Shaw et al., 2007; Evans et al., 2008) **(C)**. Depicted are only RFs from mounts which were significantly affected by at least either $Q_{longevity}$ or $TA_{longevity}$. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$. (The complete dataset and RFs for all 44 gene expression mountains in each and pooled PP concentrations, as well as RFs for previously published datasets can be found in Supplementary Material 4: sheet "Gene Expression Mountains"; results can be reconstructed using the filter function in Supplementary Material 5.)

daf-12(rh273) and possibly DAF-12's involvement in Q and TA mediated longevity.

Similarly, distinct mounts of $Q_{longevity}$ are highly similar to *daf-2* (Evans et al., 2008) and TGF-beta mutants (Shaw et al., 2007), as well as *P. aeruginosa* infected nematodes (Evans et al., 2008). The pattern of overrepresented mounts in $TA_{longevity}$ best resembles the results from adult TGF-beta mutants (Shaw et al., 2007), but also overlaps to some extent with the other conditions. These marked similarities will be probed in more detail in the next section.

Overlapping DEGs from selected microarray studies in C. elegans

Figure 3 demonstrates the relationship between $Q_{longevity}$ (left section), $TA_{longevity}$ (right section), and $Q\&TA_{longevity}$ (middle section) to *daf-12(rh273)* mutants (**Figure 3A**), *daf-2* mutants (**Figure 3B**), adult TGF-beta mutants (**Figure 3C**), and *P. aeruginosa* infected nematodes, respectively. [A comparison to further microarray studies (Hill et al., 2000; Wang and Kim, 2003; Viswanathan et al., 2005) and single concentration results can be found in Supplementary Material 4: sheet "Meta-Analysis."] To gain a deep functional insight into aging-related genes, significantly overlapping DEGs were examined further with a GO- and InterPro-cluster analysis (Table S4 in Supplementary Material 1).

A comparison to daf-12(rh273). The RFs for overlapping genes (both up- and down-regulated) of $Q_{longevity}$ and *daf-12(rh273)* were significantly enriched (**Figure 3A**), suggesting that they share similar genetic mechanisms. Interestingly, eight DEGs (*bre-1*, *clec-4*, *clec-66*, *dod-22*, F35E12.5, F55G11.5, M02F4.7, *pho-1*) were down-regulated in both conditions, transcripts which were also repressed in *daf-2* and previously thought to be down-regulated in several longevity backgrounds (Fisher and Lithgow, 2006). The downstream analysis revealed one prominent cluster in each intersection (*major sperm protein* for commonly up-regulated genes and *C-type lectin-like* for commonly down-regulated genes). It is however noteworthy that a group of eight genes of the *CUB-like region* proteins were up-regulated in $Q_{longevity}$ but down-regulated in *daf-12(rh273)* mutants.

In $TA_{longevity}$, the overlap with *daf-12(rh273)* was significant for common up-regulated DEGs and also the category "up in $TA_{longevity}$/down in *daf-12*" (**Figure 3A**). Cluster analyses for these intersections returned transcripts involved in *major sperm protein* and *hydrolase activity*, respectively. Comparing DEGs of $Q\&TA_{longevity}$ and *daf-12(rh273)* revealed a strong overrepresentation of common up- and down-regulated transcripts. All up-regulated genes, 18 in total, assign to the gene-groups *msp*, cell structure, and/or sperm- and male enriched (notably 17 are members of gene expression mountain 4).

A comparison to daf-2. $Q_{longevity}$ and *daf-2* mutants share a significant proportion of DEGs (**Figure 3B**). The GO- and InterPro-cluster analyses of the intersection of up-regulated genes revealed five statistically significant clusters, including *determination of adult lifespan*, *lipid metabolic process*, and *oxidoreductase activity* (Table S4 in Supplementary Material 1). The intersection of down-regulated transcripts returned four clusters, again *determination of adult lifespan* and *UDP-glycosyltransferase activity*. In contrast, $TA_{longevity}$ differs from $Q_{longevity}$ when compared to *daf-2* mutants; only the section "up in $TA_{longevity}$/down in *daf-2*" revealed an overrepresentation (**Figure 3B**).

Since a substantial part of the category "up in $TA_{longevity}$/down in *daf-2*" are also up-regulated in TGF-beta mutants (17 genes)

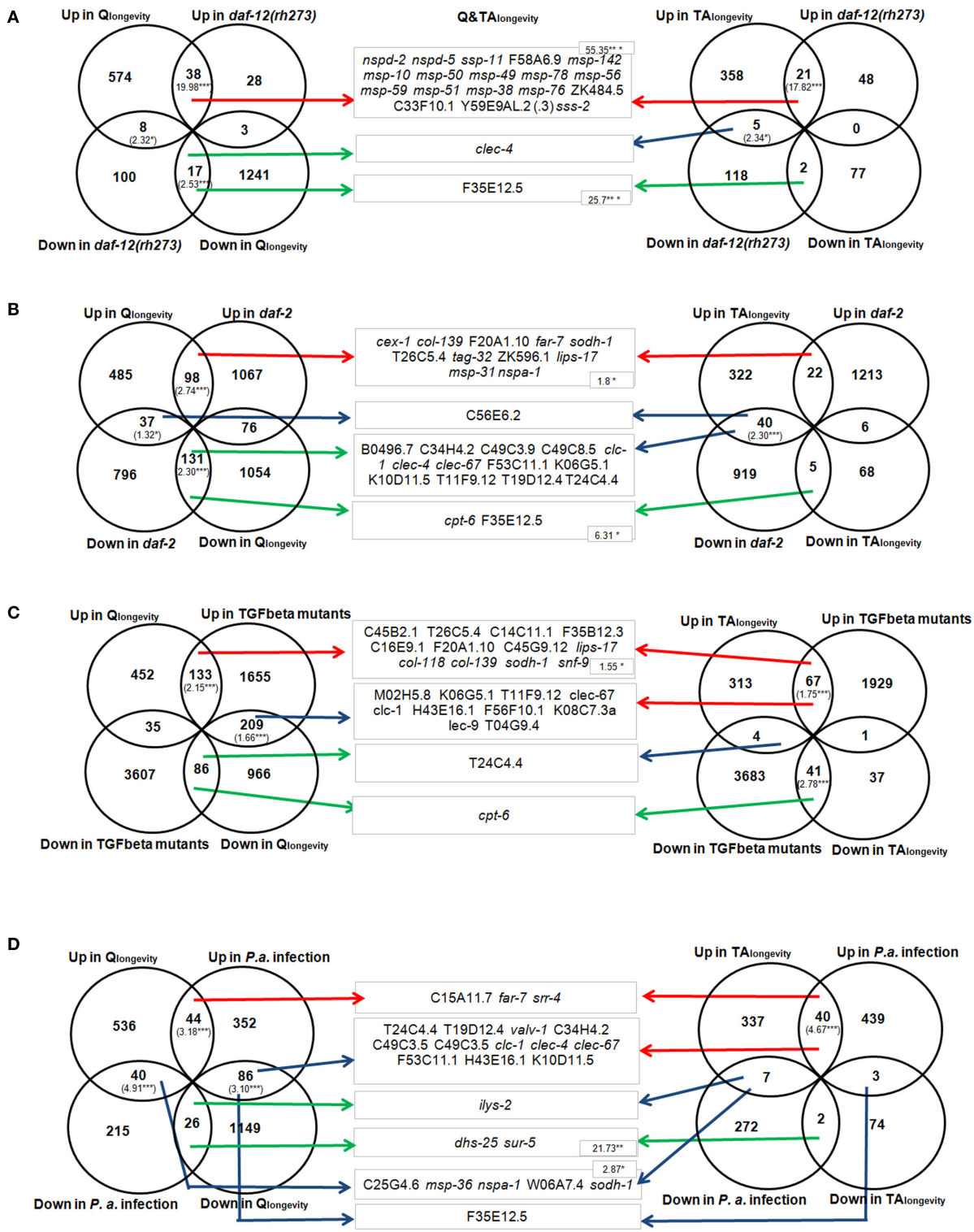


FIGURE 3 | Comparison of DEGs derived from $Q_{longevity}$, $TA_{longevity}$, and *daf-12(rh273)* mutants (Fisher and Lithgow, 2006) (A); *daf-2* mutants (Evans et al., 2008) (B); TGF-beta adults (Shaw et al., 2007) (C); and *P. aeruginosa* challenged *C. elegans* (Evans et al., 2008) (D). Significant overlap is indicated by RF values in brackets. The middle section represents the overlap of pooled DEGs in $Q\&TA_{longevity}$ and respective conditions. Arrow colors define the mode of regulation, where red defines intersections containing jointly up-regulated DEGs, green down-regulated DEGs and blue DEGs that are regulated in opposite directions. The overlap is significant where RF values (numbers in brackets) are shown. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$. (Additional information about all RF values in single PP concentrations and published data can be found in Supplementary Material 4: sheet "Meta-Analysis." A GO- and InterPro-cluster analysis of overlapping DEGs in respective intersections can be found in Table S4 in Supplementary Material 1. All results can be reconstructed using the filter function in Supplementary Material 5.)

and in *P. aeruginosa* infected nematodes (18 genes), suggests that a network of DEGs are present in all three conditions. Four clusters emerge from this intersection, notably as in $Q_{longevity}$, the term *lipid metabolic process like* stands out. The fact, that no gene of this GO-term was present in the intersection $Q_{longevity}$/*daf-2* confirms that both PPs modulate lipid metabolism, but via differing routes. Overall, $Q\&TA_{longevity}$ and *daf-2* share 11 up-regulated genes and two down-regulated genes (**Figure 3B**, middle) but 13 genes are expressed in opposite direction (two CUB-like genes (F53C11.1, K10D11.5), two belonging to the defense response category (C49C3.9, T19D12.4) and two coding for proteins with a von Willebrand factor type A (vWA) domain).

A comparison to adult TGF-beta mutants. The overlap between the gene lists for $Q_{longevity}$ and TGF-beta mutants is significant both in the up-regulated section and the section "$Q_{longevity}$ down/TGF-beta mutants up" (**Figure 3C**). The cluster analysis revealed, e.g., the terms *catalytic activity*, *active transmembrane transporter activity*, and *peptidase 28*, *carbohydrate metabolic process*, respectively (Table S4 in Supplementary Material 1). Indeed, 45 genes of the "$Q_{longevity}$ down/TGF-beta mutants up" section are also down-regulated in *daf-2*, suggesting that some overlap of TGF-beta and ILS signaling prevails, albeit with contrasting regulation patterns.

The comparison of $TA_{longevity}$ and TGF-beta mutants (**Figure 3C**) revealed that approximately 25% of the DEGs overlapped. The cluster analysis highlighted two clusters for each intersection: *peptidase 28* and *lipid metabolic process* in the section comprising induced DEGs and *chromatin organization* and *embryonic development ending in birth or egg hatching* in the group of repressed DEGs. Comparing DEGs from $Q\&TA_{longevity}$ and TGF-beta mutants (**Figure 3C**, middle) returned only 12 genes that were marginally overrepresented in the up-regulated intersection which were also up-regulated in *daf-2* mutants.

A comparison to P. aeruginosa infected nematodes. $Q_{longevity}$ or $TA_{longevity}$ were compared to the transcriptional response induced by the infection with the pathogenic bacteria *P. aeruginosa* (Shapira et al., 2006; Troemel et al., 2006; summarized in Evans et al., 2008). Venn diagrams (**Figure 3D**) illustrate the presence of significant intersections of DEGs in $Q_{longevity}$ and immune-challenged nematodes (both up; up in $Q_{longevity}$/down in infection; both down; up in infection/down in $Q_{longevity}$). The cluster analysis revealed a common up-regulation in, for example, *lipid metabolic processes*, *monooxygenase activity*, and genes coding for *UDP-glucuronosyl/UDP-glucosyltransferase* as well as a concurrent down-regulation in particular parts of *catalytic activity*. The intersections of oppositely responding DEGs contain genes coding for *oxidoreductases* and *serine-type peptidase activity* ($Q_{longevity}$ up/infection down) and for structural constitutes of cuticle ($Q_{longevity}$ down/infection up), respectively. A large proportion of DEGs from the intersection "up in infection/down in $Q_{longevity}$" is down-regulated in TGF-beta, *daf-2* and *daf-12(rh273)* (for exact numbers and genes see Supplementary Material 5 in respective filter condition), and 21 genes of "up in $Q_{longevity}$/down in infection" are up-regulated in TGF-beta mutants.

$TA_{longevity}$ shares highly significant patterns with the list of up-regulated DEGs from infected nematodes. The cluster analysis returned only one term, genes with a *CUB-like region*, however, interestingly, CUB-like genes were previously found to be activated in the induced immune response pathway modulated by PMK-1 (Troemel et al., 2006).

Only five genes are regulated in $Q\&TA_{longevity}$ as well as in nematodes infected with *P. aeruginosa* (up-regulated: C15A11.7, *far-7* and *srr-4*; down-regulated: *dhs-25*, *sur-5*). Of the 15 common up-regulated DEGs in "$TA_{longevity}$ and *P. aeruginosa* infection and down-regulated in $Q_{longevity}$," 11 are also down-regulated in *daf-2* mutants, including two C-type lectins (*clec-4*, *clec-67*), two genes coding for proteins with a CUB-like region (F53C11.1, K10D11.5) and two defense response genes (C49C3.9, T19D12.4).

Finally, we evaluated the overlap of DEGs in Q and TA treated nematodes with the DEGs regulated by PMK-1 (Troemel et al., 2006; Supplementary Material 4: sheet "Meta-Analysis") and found a significant overlap for $Q_{longevity}$ and $TA_{longevity}$ in the up-regulated intersection and the "down-regulated by PP/up due to PMK-1." These results underline the involvement of the p38 MAP kinase pathway in both PP actions.

DISCUSSION

Polyphenols transform the transcriptional output and thereby alter the physiological status of an animal. By performing DNA microarray experiments and comparing the resulting data with previously published gene expression screens, we revealed an interlaced interplay of genetic pathways affected by Q and TA. Moreover, our findings support results from previous studies (Pietsch et al., 2009, 2011; Saul et al., 2010).

CONCENTRATION DEPENDENT VARIATIONS IN GLOBAL TRANSCRIPTIONAL RESPONSES

Low concentrations of Quercetin (Q50) modulate processes which may contribute to the wellbeing of the nematode (e.g. the gene classes *gst*s, peroxidases, lysozymes; GO-terms: *oxidoreductase activity*; KEGG-analysis: *glutathione metabolism* and *metabolism of xenobiotics by cytochrome P450*), but not sufficiently to significantly extend lifespan (Pietsch et al., 2011). In contrast, Q100 and Q200 impact significantly on the global transcriptome which manifests itself in a strong longevity phenotype. This effect is, at least in part, driven by transcriptional repression mechanisms as indicated by the high number of down-regulated genes. As shown recently, increasing the concentration to Q250 reverts the beneficial effects on longevity (Pietsch et al., 2011). High dose toxicity is particularly apparent at, for example, TA300 (GO-terms: *DNA repair*, *DNA damage response*, *response to stress*, and *cell death* and KEGG-analysis: *Ubiquitin-mediated proteolysis*, *mismatch repair*, *DNA replication)*.

GENETIC BASIS OF $Q_{LONGEVITY}$ AND $TA_{LONGEVITY}$

$Q_{longevity}$ shares transcriptional patterns with long-lived *daf-12(rh273)*, *daf-2* mutants, TGF-beta mutants, as well as *P. aeruginosa* challenged nematodes (as identified by the meta-analysis and overlapping gene expression mountains). These results suggest that Q operates through a complex network of interlinked pathways. Combining the microarray analysis with lifespan data from Q exposed nematode mutants (Pietsch et al., 2009) allowed

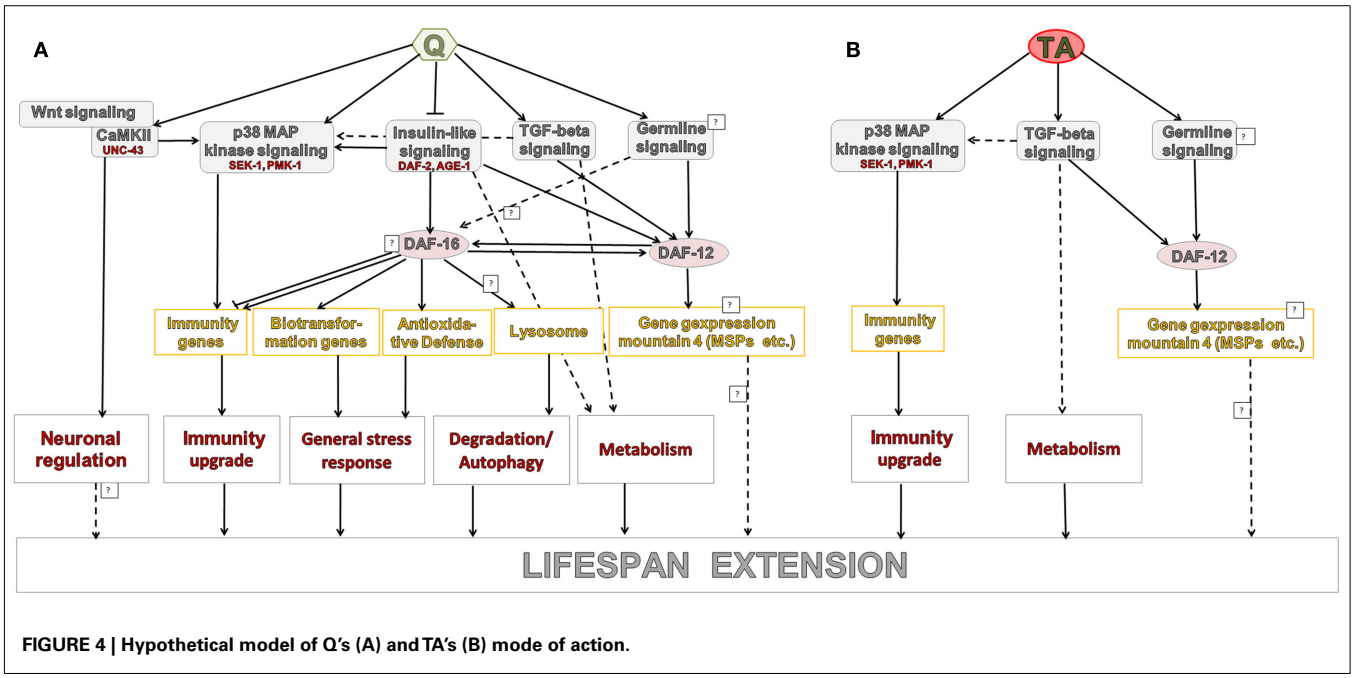


FIGURE 4 | Hypothetical model of Q's (A) and TA's (B) mode of action.

the construction of a hypothetical model that describes the mode of action of Q induced longevity (**Figure 4A**).

The overlap between $TA_{longevity}$ and the conditions assessed as part of the meta-analysis revealed a less pronounced, yet still significant correlation. Beside activation of amino acid metabolism pathways, the strongest overlap was with data from the TGF-beta mutants. This suggests that the TGF-beta pathway plays a prominent role in the TA mediated life extension which is summarized in **Figure 4B**.

It is intriguing to note that TA treatment has, compared to Q treatment, a lesser effect on the transcriptome (as defined by the number of significant DEGs) but a more marked life-extending property. It is currently not known if this is an indirect effect or whether transcriptional changes diminish/suppress the positive output of Q action. Notwithstanding the observed differences in genetic action modes, TGF-beta and p38 MAPK pathways, as well as the nuclear hormone receptor DAF-12 seem to be involved in both PPs. DAF-12, which is downstream of the TGF-beta- and ILS pathways, is a member of the steroid hormone receptor superfamily. It is linked to Dauer formation and, together with DAF-16, also influences gonad-dependent adult longevity. Given that neither Blueberry polyphenols (Wilson et al., 2006), Caffeic acid nor Rosmarinic acid (Pietsch et al., 2011) could extend the lifespan of *sek-1* mutants (*sek-1* is a genetic player in the p38 MAPK pathway) suggests that the innate immunity may act as a prominent downstream effector of PPs.

Based on the meta-analysis with $Q\&TA_{longevity}$ and relevant microarray studies (Hill et al., 2000; Wang and Kim, 2003; Viswanathan et al., 2005; Fisher and Lithgow, 2006; Shaw et al., 2007; Evans et al., 2008), we were able to identify a subset of transcripts that are possibly relevant for aging (see Table S5 in Supplementary Material 1). Clearly further investigations into their aging-modulating activities are warranted. Likewise, we call for further experimentation to establish the regulatory interlink between TGF-beta signaling and DAF-12, as well as p38 MAPK.

CONCLUSION

The meta-analysis displayed an extensive overlap between PP treatment and numerous mutants as well as immunity challenging conditions, however, frequently the mode of regulation was in opposite direction. This strongly suggests the presence of a complex regulatory interplay between the input and multiple downstream targets. Clearly, PPs action cannot be reduced to the activation or inhibition of single genes and pathways; nevertheless it is apparent that TGF-beta, ILS, and p38 MAPK play a prominent role in PPs' mode of action. Furthermore, we were able to demonstrate that an extensive comparison with data from the literature can provide a deep insight into the transcriptome to a level that goes beyond a simple GO- and KEGG-analysis. Given the convolution observed with single gene knockout alleles and exposures to pure compounds, one can envisage the complexity that will arise with multidimensional mixture toxicity experiments. The development of comparative databases and most importantly powerful, yet intuitive, bioinformatic tools will undoubtedly aid in the streamlining of large datasets. Overall, our results strengthen the notion that both PPs act in conserved genetic pathways that overlap, or at least correlate, with the longevity phenotypes and transcriptional fingerprints of certain mutant strains. Clearly, further future tests are needed to confirm single genetic players and specify the interplay of conserved pathways.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Toxicogenomics_/10.3389/fgene.2012.00048/abstract

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