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Fish oil improves gene targets of Down syndrome in C57BL and BALB/c mice

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ABSTRACT

We have considered a novel gene targeting approach for treating pathologies and conditions whose genetic bases are defined using diet and nutrition. One such condition is Down syndrome, which is linked to overexpression of RCAN1 on human chromosome 21 for some phenotypes. We hypothesize that a decrease in RCAN1 expression with dietary supplements in individuals with Down syndrome represents a potential treatment. Toward this, we used in vivo studies and bioinformatic analysis to identify potential healthy dietary RCAN1 expression modulators. We observed Rcan1 isoform 1 (Rcan1-1) protein reduction in mice pup hippocampus after a 4-week curcumin and fish oil supplementation, with only fish oil reduction being statistically significant. Focusing on fish oil, we observed a 17% *Rcan1-1* messenger RNA (mRNA) and 19% Rcan1-1 protein reduction in BALB/c mice after 5 weeks of fish oil supplementation. Fish oil supplementation starting at conception and in a different mouse strain (C57BL) led to a 27% reduction in hippocampal *Rcan1-1* mRNA and a 34% reduction in spleen *Rcan1-1* mRNA at 6 weeks of age. Hippocampal protein results revealed a modest 11% reduction in RCAN1-1, suggesting translational compensation. Bioinformatic mining of human fish oil studies also revealed reduced RCAN1 mRNA expression, consistent with the above studies. These results suggest the potential use of fish oil in treating Down syndrome and support our strategy of using select healthy dietary agents to treat genetically defined pathologies, an approach that we believe is simple, healthy, and cost-effective.

1. Introduction

We have considered a novel "rational gene targeting" approach for treating pathologies whose genetic bases are defined using diet and nutrition [1]. We hypothesize that certain healthy dietary agents, or nutraceuticals, can modulate the expression of disease-causing genes back toward the normal, slowing the disease process while lowering treatment cost, toxicity, and

Abbreviations: DHA, docosahexaenoic acid; DYRK1A, dual-specificity tyrosine phosphorylation-regulated kinase 1A; EPA, eicosapentaenoic acid; FO, fish oil; GEO, Gene Expression Omnibus; NCBI, National Center for Biotechnology Information; RCAN1, regulator of calcineurin 1.

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long-term risks, and improving compliance as compared with currently used traditional drugs. An important caveat to this approach is the need for identified risk genes that are clearly causative to the given pathology, as these, in turn, become targets for modulation with nutraceuticals.

Because our approach focuses on select nutraceuticals, it has the major advantages of using dietary agents with known health benefits. This is in contrast to existing and future experimental drugs with unknown long-term effects and usually high cost. By "healthy," we mean that reports of a dietary agent's benefits to health have been published in respected peer-reviewed journals, and that the agent has been used for health benefit for a long period. Many studies have demonstrated the beneficial health effects of nutraceuticals, including those tested here because of these benefits, due to their content of numerous and diverse nutrients such as polyphenols, antioxidants, omega-3 fatty acids, vitamins, phase II inducers, and potent chemoprotectants [1–12].

A potential example of a condition that can be targeted with our rational gene targeting approach is Down syndrome. Down syndrome (trisomy 21) affects 1 in 800 humans and is the most prevalent genetic-based cause of mental retardation [13–16]. It is characterized by a number of phenotypic abnormalities including reduced brain size and altered gyral configuration, mental retardation, facial abnormalities, early-onset dementia, muscle weakness, thymic and skeletal deformities, congenital heart disease, and more [15–18]. Genetically, it is characterized by an extra copy, or partial copy, of chromosome 21. A major focus of research in treating this condition has concentrated on identifying the chromosome 21 genes that are most critical to its etiology. One such chromosome 21 gene is *RCAN1* (regulator of calcineurin 1). This gene has become one of the leading candidates for contributing to some (but not all) phenotypes of Down syndrome [19–24]. RCAN1 has also been implicated in Alzheimer disease, and early-onset Alzheimer disease is a much higher risk in Down syndrome [19,23,24]. Mechanistically, embryonic overexpression of both *RCAN1* and a second chromosome 21 gene, *DYRK1A*, has been shown to block NF-AT–related gene expression in a synergistic manner [25]. Importantly, *NF-AT* knockout mice exhibit Down syndrome–like phenotypes. This study implicates both *RCAN1* and *DYRK1A* as major contributors to some phenotypes of Down syndrome, and possibly early-onset Alzheimer as well.

The postulated involvement of RCAN1 in at least some cases of Down syndrome, and in at least some tissues affected by Down syndrome, is shown in Fig. 1. RCAN1 protein inhibits calcineurin [26–30], a calcium-activated protein phosphatase that mediates many cellular functions, especially in the brain where it comprises more than 1% of the total protein and regulates neuronal apoptosis, N-methyl-D-aspartate receptor channels, neurite outgrowth, long-term memory and potentiation, neurotransmitter release, and tau dephosphorylation [30–34]. Under normal conditions, agonists that elevate intracellular calcium lead to activation of calcineurin, which dephosphorylates NF-AT, leading to its nuclear migration and activation of target genes. Both RCAN1 isoforms inhibit this calcineurin signaling. In Down syndrome, an extra copy of the *RCAN1* gene occurs due to trisomy 21. Subsequent elevated expression of RCAN1 protein leads to inhibition of calcineurin signaling, thereby reducing NF-AT dephosphorylation and subsequent nuclear migration. In addition, DYRK1A, a kinase whose gene is also located on chromosome 21, is also increased in trisomy 21, leading to elevated nuclear phosphorylation of NF-AT and subsequent efflux. Combined, there is an overall loss of nuclear NF-AT that is postulated to be an important contributor to some phenotypes of Down syndrome. It is important to note here that Down syndrome is a complex and multifactorial disorder, with different phenotypes likely caused by different combinations of chromosome 21 genes [35,36]. Thus, it is more appropriate to state that the combined overexpression of RCAN1 and DYRK1A has now been identified as a possible key contributor to some phenotypes of Down syndrome etiology.

To date, no scientifically proven drug is available to treat Down syndrome. However, the recent observation that RCAN1 and DYRK1A appear to be important contributors to at least some phenotypes of Down syndrome identifies these 2 genes/proteins as potentially valuable therapeutic targets. In evaluating this, we hypothesize that our rational gene targeting has potential application to treating Down syndrome, that is, by identifying nutraceuticals that can reduce RCAN1 (and DYRK1A) levels, in turn, activating NF-AT signaling. Importantly, DYRK1A enzymatic reduction with green tea has already been reported including a pilot human study [37].

2. Methods and materials

2.1. *Mice*

All analyses were performed on female mice. BALB/c mice were maintained under 12-hour light/dark cycles with water and special diet food provided ad libitum at Albany Medical College. C57BL mice studies were initiated at Jackson Labs (Bar Harbor, ME, USA). Here, the mice were fed a diet supplemented with and without fish oil (1.9%) starting at mating (1-2 days before a copulatory plug). Impregnated mice were then continued on the same diet throughout gestation, and pups were weaned during their third week of age. The mice were transferred to Albany Medical College at 4½ weeks of age where 8 per treatment group were maintained on the same diet and cared for as above for BALB/c mice until the end of the study at 6 weeks of age. All animal studies were approved by the Albany Medical College Institutional Animal Care and Use Committee (Dr Crawford, PI; Protocol: 11-01002), and animals handled and treated according to the regulations set forth in the approved Albany Medical College Institutional Animal Care and Use Protocol.

2.2. *Mouse dietary treatments*

Food was placed in dry-diet feeder jars to avoid significant food loss that often accompanies overhead pellet feeding, and the jars were weighed daily to determine the amount of food consumed. General animal health and water intake were also monitored during the test periods. Diets consisted of defined mouse diet AIN-93 M (Test Diet, Richmond, IN, USA) supplemented with nutraceuticals. Fish oil–supplemented diets were prepared by Test Diet, with the only difference being the amount of the 2 fat sources present (medium-chain triglyceride and fish oil). The fish

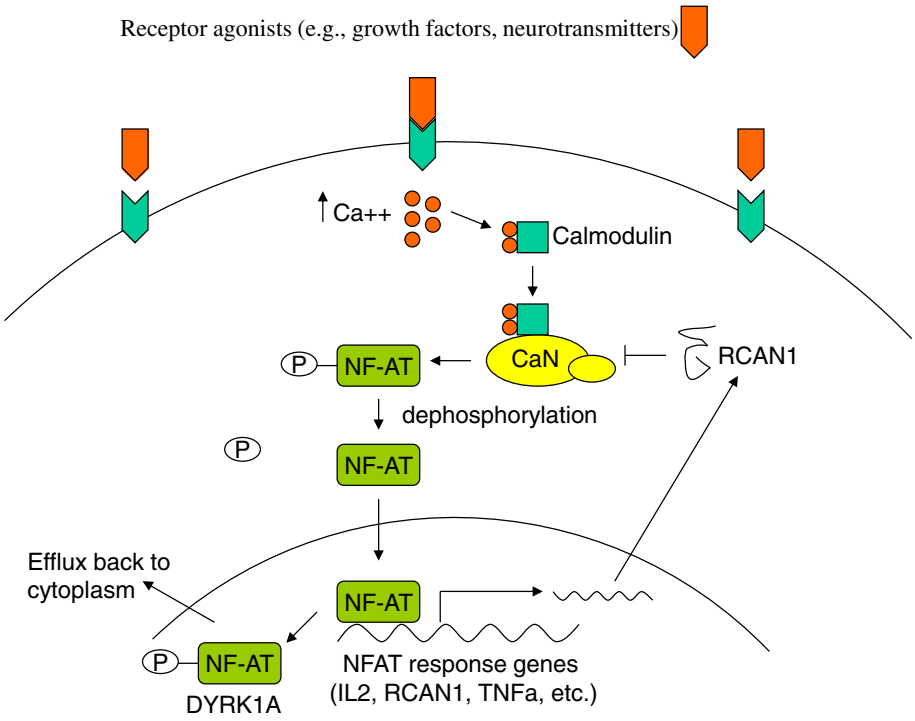

Fig. 1 – Postulated relationship between RCAN1 signaling and Down syndrome. Elevated intracellular calcium leads to activation of calcineurin, which dephosphorylates NF-AT leading to its nuclear migration and activation of target genes. RCAN1 inhibits this calcineurin signaling. In Down syndrome, there is an extra copy of the chromosome 21 *RCAN1* gene leading to elevated RCAN1 protein expression and inhibition of calcineurin signaling, reducing NF-AT dephosphorylation and subsequent nuclear migration. In addition, chromosome 21 DYRK1A kinase is also increased in Down syndrome, promoting nuclear phosphorylation of NF-AT and subsequent efflux. Combined, there is an overall loss of nuclear NF-AT that is postulated to be an important contributor to some cases of Down syndrome.

oil source in this diet was anchovy, sardines, and mackerel, and contained equal parts docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). These diets were isocaloric, isonitrogenous, 60.1% to 60.2% carbohydrate, 18.2% to 18.2% protein, 10.1% to 10.2% fat, and approximately 0.30% ash and 90% dry matter.

For the first BALB/c mice study, 5 pups aged 4 weeks were treated with each nutraceutical added to a defined mouse diet. The nutraceuticals included the botanicals curcumin (Cayman Chemical, Ann Arbor, MI, USA; minimum 90% purity), quercetin (Sigma, St Louis, MO, USA; minimum 98% purity), gingko (24% ginkgo flavonglycosides content), garlic, grape extract (95% polyphenol content), Echinacea (4% Echinacea content), and the botanical extract mixture Maximum greens (General Nutrition Corporation, Pittsburg, PA, USA), all at 0.25% to 0.50% (wt/wt), with the exception of gingko (0.05 wt/wt). Additional groups included fish oil (0.84% omega-3 final), corn oil (0.06% omega-3 as a control for fish oil), or unsupplemented control diet. After 4 weeks, brain extracts were prepared from the hippocampus by sonicating and centrifuging. Western immunoblotting was then performed on supernatants using antibody to Rcan1-1 protein, then α-tubulin as a loading control as described below. For the second BALB/c mice study, 8 pups aged 4 weeks were treated with either omega-3 elevated to 1.46% or soybean oil as a control (0.13% omega-3). Supplementation time was extended to 5 weeks, and both protein and messenger RNA (mRNA) were extracted from the hippocampus and mRNA from the spleen for analysis, as described below.

2.3. *Protein and mRNA preparation*

Mice were euthanized by CO_2 inhalation followed by decapitation, and brains and spleens (where applicable) were carefully removed. Dissections were then performed on an inverted Petri dish overlaying a mixture of ice and dry ice, and dissected left and right hippocampi and spleen were placed into Eppendorf tubes and froze immediately in liquid nitrogen. For protein, left hemisphere hippocampi were sonicated in ice-cold extraction buffer (Cell Signaling, Danvers, MA, USA) containing phosphatase and protease inhibitors and were centrifuged (12 000*g* for 10 minutes), and the supernatant extract was analyzed as below. For mRNA, frozen right hemisphere hippocampus and spleen were homogenized in Qiazol (Qiagen, Valencia, CA, USA) using an electric-driven Polytron homogenizer followed by mRNA purification using the RNeasy kit according to the manufacturer (Qiagen).

2.4. *Western blot analyses*

Each extract was mixed with an equal volume of 2× sodium dodecyl sulfate sample buffer and was boiled for 4 minutes. Equal protein amounts of the boiled cell lysates were then

electrophoresed on an sodium dodecyl sulfate–polyacrylamide gel, electroblotted to nitrocellulose, and incubated with primary antibody followed by peroxidase-conjugated secondary antibody and signal development with Western light chemiluminescent substrate (Perkin Elmer, Boston, MA, USA) [28,34]. All signals were captured on film and quantified using the ImageJ program (National Institutes of Health, Bethesda, MD, USA). Primary antibodies included those to Rcan1 (generously provided by Dr Sandra Ryeom and Dr Frank McKeon), and α-tubulin (Sigma Chemical Co) as a loading control.

2.5. *Real-time polymerase chain reaction*

Messenger RNA samples prepared as above were converted to complementary DNAs, and real-time polymerase chain reaction (PCR) was performed using primers specific for *Rcan1* isoform 1 and GAPDH (for normalization) using a CFX96 Real Time PCR machine, all according to the manufacturer (BioRad, Hercules, CA, UAS). Primer sequences were 5′-AGCTTCATCGACTGCGAG ATGGA-3′ (forward) and 5′-ACTGGAAGGTGGTGTCCTTGTCAT-3′ (reverse) for *Rcan1-1*, and 5′-TGTGTCCGTCGTGGATCTGA-3′ (forward) and 5′-TTGCTGTTGAAGTCGCAGGAG-3′ (reverse) for *GADPH*. Relative gene expression analysis was performed using the Livak $2^{-\Delta\Delta CT}$ method, also as described by BioRad [38].

2.6. *Bioinformatics*

Bioinformatic searches were performed using PubMed of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/pubmed), ClinicalTrials.gov, and the Gene Expression Omnibus (GEO) data set repository, also an NCBI resource (http://www.ncbi.nlm.nih.gov/geo). For the analysis of the effects of fish oil supplementation on human *RCAN1* levels, the NCBI program GEO2R was used to analyze noncurated data gene expression changes using GEO raw data files.

2.7. *Statistical analyses*

Western blot–determined protein levels (normalized to tubulin after ImageJ program quantitation) and real-time PCR mRNA levels (normalized to GAPDH) were expressed as percent control diet in response to each dietary supplement. Data are reported as the means ± SEM with statistical significance defined as $P < .05$ using a 2-tailed Student *t* test. For the 8 mice per group in 2 groups tested using an equal variance Student *t* test (2-tailed α of .05), there is a power of 80% for detecting a statistically significant difference between groups that is at least as large as 1.5 within group SDs.

3. Results

3.1. *Effect of various nutraceuticals on BALB/c mouse hippocampus Rcan1-1 protein levels*

Four-week-old normal disomic BALB/c mice were fed a defined mouse diet (AIN-93 M) supplemented with 0.25%-0.50% (wt/wt) curcumin, garlic, grape extract, Echinacea, quercetin, and Maximum greens (GNC); 0.05 wt/wt ginkgo; fish oil (0.84% omega-3 final); corn oil (0.06% omega-3 as a control for fish oil); or unsupplemented control diet for 4 weeks. No statistically significant differences in relative food intakes over this time or mouse weights (starting from a common mouse pool of equal weights) at the end of the study were observed for all supplemented diets (Table). After 4 weeks of test diet feeding, brain extracts were prepared and Western immunoblotting was performed using antibody to Rcan1, then tubulin as a control. As shown in Fig. 2, fish oil and curcumin reduced Rcan1 isoform 1 levels, with fish oil suppression being statistically significant. These data suggest that RCAN1-1 levels can be reduced by healthy nutraceuticals—in this case fish oil—as a potential therapy for Down syndrome and Alzheimer disease.

3.2. *Bioinformatic analysis*

Based on the above result, we performed a bioinformatic search in PubMed, ClinicalTrials.gov, and GEO using the search phrases "fish oil," "gene," "expression," and "human" or "mice." Two studies of note were found. In male Rj:NMRI mice, Berger et al [39] assessed hippocampal gene expression changes after 57 days of dietary fish oil administration. Although not focused on *Rcan1* expression or Down syndrome per se, these authors reported a major 2.2-fold reduction of *Dscr1* (*Rcan1*) mRNA in the hippocampus, which is consistent with our observation that at least some Rcan1 reduction was observed after fish oil administration. In addition, a human study was found in which healthy elderly subjects were given fish oil supplements for 26 weeks [40]. For such human studies, brain gene expression analysis is not possible, but these authors did analyze peripheral blood mononuclear cells. From the raw data provided, we used the NCBI program GEO2R and calculated an 8% reduction in *RCAN1* mRNA level. Two later studies were found that were reported in GEO at the time we were conducting our studies: GSE29678 and GSE20114. In vivo, a 13% RCAN1 mRNA reduction was calculated in human peripheral blood cells in patients with early-stage chronic lymphocytic leukemia after a high-dose omega 3 supplementation (GSE29678). In addition, in vitro

Table – Food intake and body weights for each study

Study	Mice	Supplement	Food intake (g/d)	Body weight (g)
1	BALB/c	Control	3.06 ± 0.14	17.61 ± 0.94
		Grape	2.97 ± 0.15	17.48 ± 0.40
		Garlic	2.83 ± 0.17	17.42 ± 0.51
		Quercetin	2.90 ± 0.19	17.85 ± 0.72
		Echinacea	3.13 ± 0.14	17.67 ± 0.77
		Curcumin	3.21 ± 0.16	18.07 ± 0.95
		Max Greens	2.57 ± 0.18	17.12 ± 1.35
		Gingko	2.87 ± 0.19	17.22 ± 0.48
		Corn oil control	2.92 ± 0.15	17.73 ± 1.03
		Fish oil	3.05 ± 0.17	18.11 ± 0.67
2	BALB/c	Control	3.43 ± 0.10	17.99 ± 0.18
		Fish oil	3.57 ± 0.12	18.74 ± 0.36
3	C57BL	Control	3.93 ± 0.13	20.83 ± 1.19
		Fish oil	3.74 ± 0.12	22.23 ± 0.81

Data are expressed as means ± SEM.

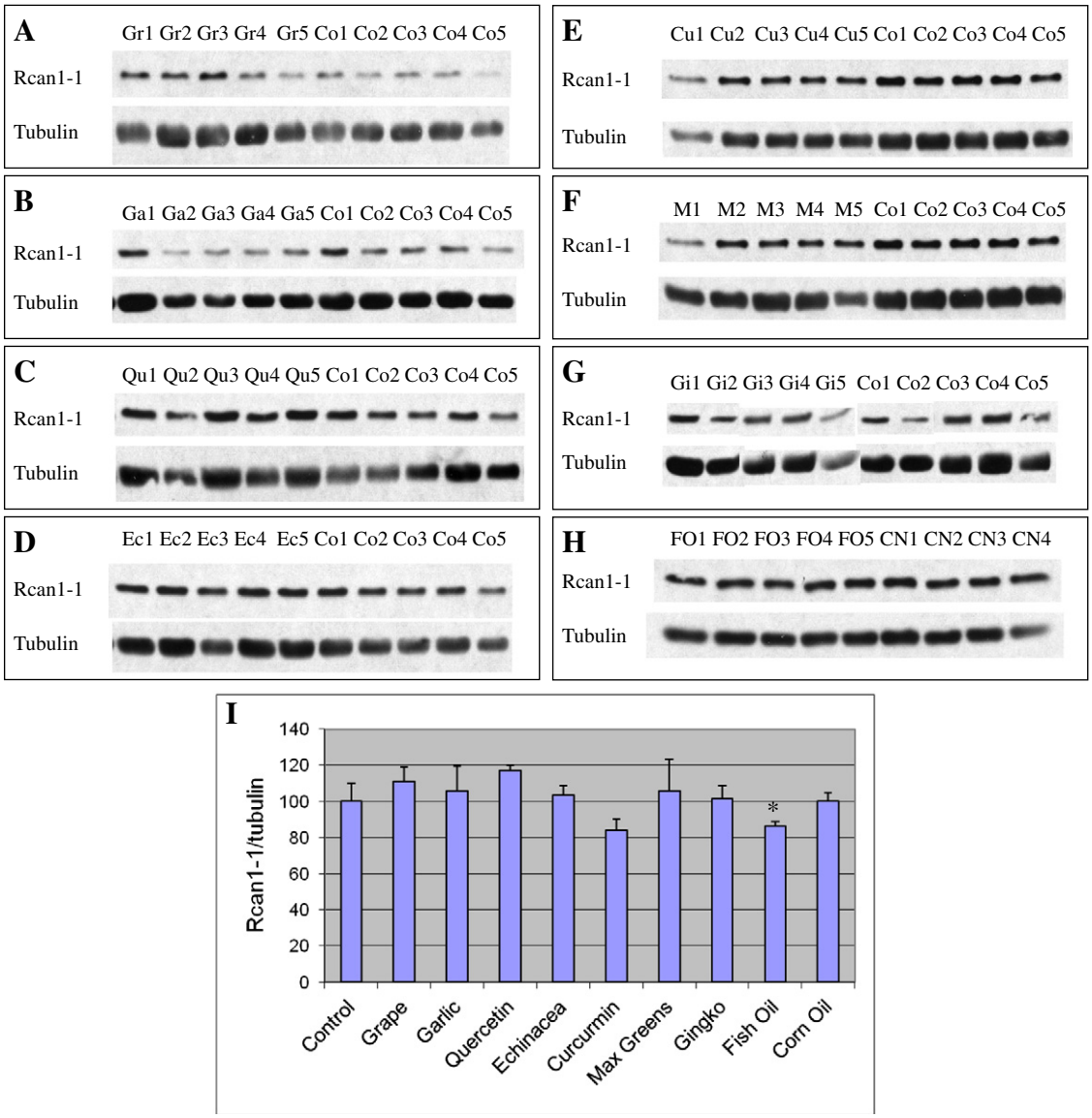

Fig. 2 – Dietary effect of various nutraceuticals on BALB/c mouse hippocampus Rcan1-1 protein levels. A-H, Four-week-old BALB/C mice were fed AIN-93 M supplemented with curcumin (Cu), garlic (Ga), grape extract (Gr), Echinacea (Ec), ginkgo (Gi), quercetin (Qu), Maximum greens (M), fish oil (FO; 0.84% omega-3), corn oil (CN; 0.06% omega-3), or unsupplemented control (Co; 0.13% omega-3) diet. After 4 weeks, hippocampal extracts were prepared by sonication and centrifugation and Western immunoblotting was performed using antibody to Rcan1-1, then α-tubulin as a loading control. I, Quantitation of A-H Western blot bands, normalizing Rcan1-1 values to tubulin and quantifying blot signals with ImageJ. Data are expressed as means ± SEM (*n* =5 except CN, n = 4). *P ≤ .05 compared with corn oil control using a 2-tailed Student *t* test.

treatment of cells from hypertriglycemic men treated with DHA led to a 12% RCAN1 mRNA reduction (GSE20114).

3.3. *Effect of fish oil on BALB/c mouse hippocampus Rcan1-1 protein level using increased concentration and time*

Based on our Fig. 2 results and the study by Berger et al [39], we decided to pursue fish oil as a down-regulator of Rcan1. A second in vivo study was performed where the percent omega-3 was elevated to 1.46%; soybean oil was used as a control (0.13% omega-3); supplementation time was extended to 5 weeks; and mRNA was also analyzed. No statistically significant difference in relative food intake over this time or mouse weight (starting from a common mouse pool of equal weights) at the end of the study was observed for control vs fish oil diets (Table). As shown in Fig. 3, the high omega-3 diet reduced BALB/c mice *Rcan1* mRNA expression by 17%. Parallel analysis of the same brains revealed a 19% reduction in Rcan1 protein (Fig. 3, including Western blots).

3.4. *Effect of fish oil on C57BL mouse hippocampus Rcan1-1 protein level starting at conception*

Because Down syndrome is a developmental disorder, we decided to assess the effect of fish oil supplementation

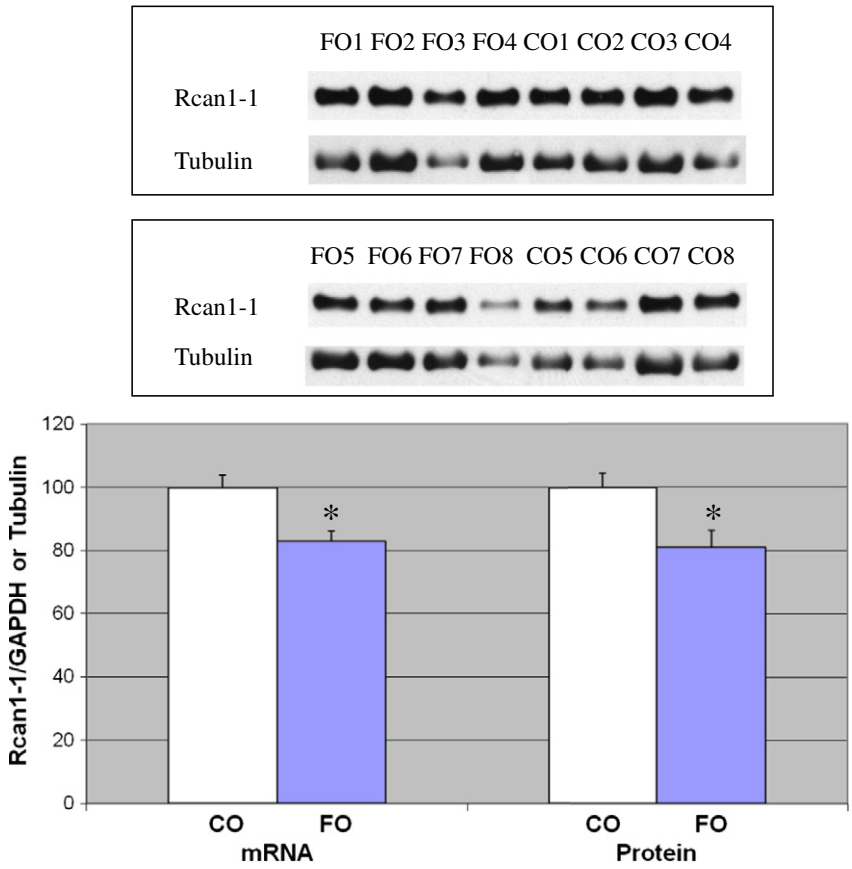

Fig. 3 – Effect of elevated fish oil on BALB/c mouse hippocampus *Rcan1* mRNA and Rcan1-1 protein levels. Four-week-old BALB/C mice were fed AIN-93 M supplemented with (FO; 1.46% omega-3) and without (CO control; 0.13% omega-3) fish oil. After 5 weeks, hippocampal protein extracts were prepared as above, and mRNA extracts prepared after homogenization and RNeasy kit purification. Top panel: Western blot protein signals. Bottom panel: quantitation of Rcan1-1 protein using ImageJ analysis of Western blots and *Rcan1*-1 mRNA using real-time PCR. Data are expressed as means ± SEM (n = 8). *$P \leq .05$ compared with control using a 2-tailed Student *t* test.

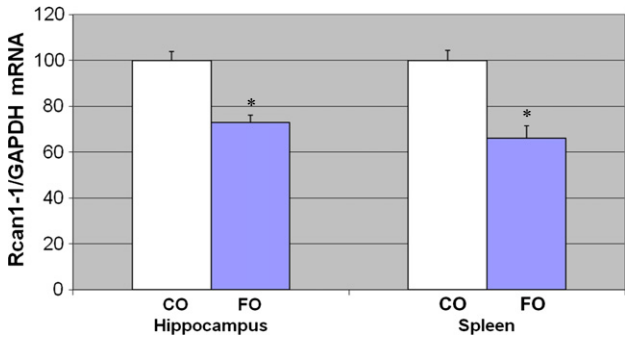

Fig. 4 – Effect of fish oil diet on C57BL *Rcan1*-1 mRNA levels in hippocampus and spleen when supplemented starting at conception. C57BL mice were supplemented with dietary fish oil starting at conception. At 6 weeks of age, pup hippocampal and spleen RNA were extracted as above and *Rcan1* isoform 1 was analyzed by real-time PCR and normalized to GAPDH. Data are expressed as means ± SEM (n = 8). *$P \leq .05$ compared with control using a 2-tailed Student *t* test.

beginning at conception. This type of approach has been used in other experimental models (e.g., Ref. [41]). Pups were fed either a normal (0.13% omega-3) or high-fish-oil (1.46% omega-3) diet starting at conception through a contractual arrangement with Jackson labs. No statistically significant difference in relative food intake, which was measured starting after weaning and shipment to Albany Medical College, over this time or mouse weight at the end of the study was observed for control vs fish oil diets (Table).

At the end of the study (6 weeks of age), the hippocampi and spleens were removed and analyzed for *Rcan1* isoform 1 mRNA using real-time PCR and normalized to *GAPDH* (mRNA). Results (Fig. 4) show a 27% reduction in *Rcan1* mRNA in the hippocampus and a 34% reduction in spleen in the fish oil–supplemented mice, suggesting the potential use of a high-fish-oil diet as a healthy treatment for Down syndrome and underscoring the importance of early dietary intervention. Protein was also analyzed in the brain hippocampus. In fish oil–supplemented mice, a modest but significantly less reduction in Rcan1-1 protein was observed as compared with mRNA, suggesting translational compensation (Fig. 5).

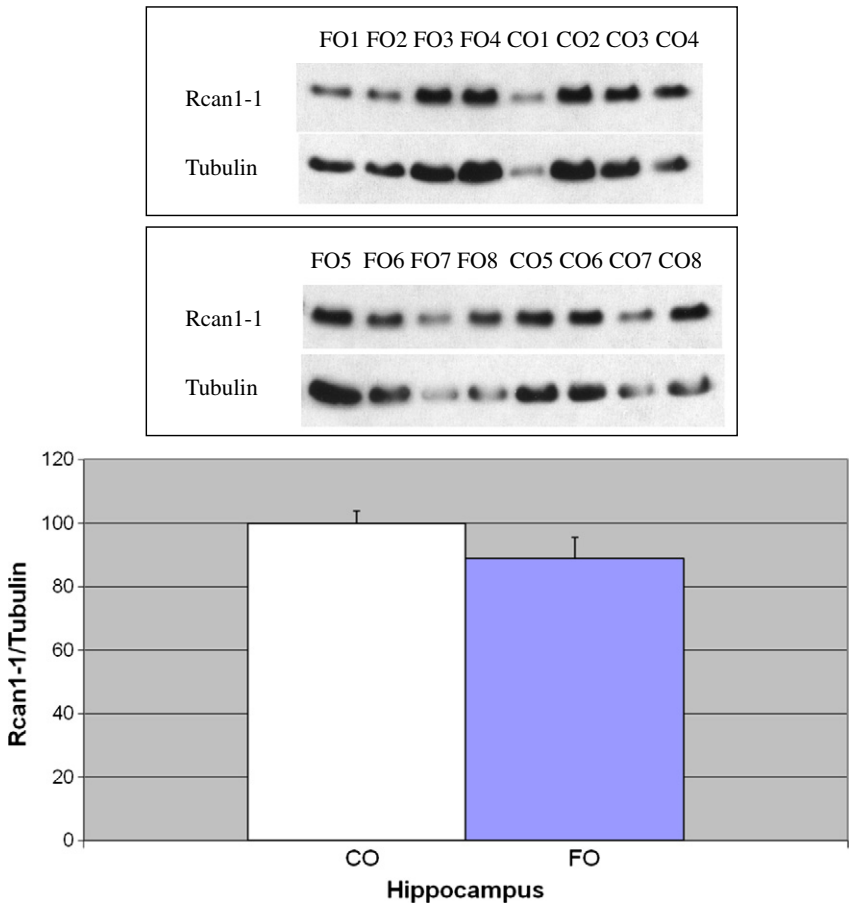

Fig. 5 – Effect of fish oil diet on C57BL Rcan1-1 protein levels in the hippocampus and spleen when supplemented starting at conception. C57BL mice were supplemented with dietary fish oil starting at conception. At 6 weeks of age, pup hippocampal and spleen protein were extracted as above, and Rcan1-1 protein was analyzed by immunoblotting. Top panel: Western blot protein signals. Bottom panel: quantitation of Rcan1-1 protein using ImageJ analysis of Western blots. Data are expressed as means ± SEM (n = 8).

4. Discussion

Down syndrome is a complex and multifactorial disorder [35,36]. Nonetheless, the combined overexpression of RCAN1 and DYRK1A has now been identified as a possible key contributor to some phenotypes of Down syndrome etiology [20,22–25]. We believe that a simple, healthy, and cost-effective approach to treating Down syndrome (and possibly associated Alzheimer) is to identify nutraceuticals that reduce RCAN1 and DYRK1A levels, in turn, activating NF-AT signaling (usually represented by NF-AT dephosphorylation). In this study, we screened a number of healthy nutraceuticals and observed that omega-3–containing fish oil can modestly suppress Rcan1-1 levels in mice. These results support our hypothesis that fish oil represents a potential complementary approach to treating Down syndrome and associated early-onset Alzheimer disease, as the latter also involves increased RCAN1 levels [22,24,34,42].

Others have reported a reduction in DYRK1A kinase activity with green tea, including a recent human pilot study that provides evidence for reversal of some cognitive deficits in individuals with Down syndrome [37]. This suggests that a combination of fish oil and green tea in individuals with Down syndrome may lead to a significant reversal of the reduced NF-AT signaling that is thought to underlie this condition. At this stage, however, it is unclear whether our modest reduction in Rcan1-1 will be sufficient to affect NF-AT signaling. In addition, as presented above, a bioinformatic analysis in humans taking fish oil supplements revealed a 8% to 13% reduction in *RCAN1-1* mRNA level in peripheral blood mononuclear cells [40] (GSE29678). Nonetheless, it is possible that even these modest reductions may be effective because the original article by Arron et al [25] reported strong synergy between Rcan1 and Dyrk1a. In other words, a modest reduction in RCAN1-1 with fish oil combined with a reduction in DYRK1A kinase activity with green tea may still lead to a significant synergistic combined effect in activating NF-AT signaling. It should also be noted that green tea is considered a liver toxin at higher levels [43,44], and the study by De la Torre [37] reported increased levels of the disease risk factor homocysteine over the 3-month course of their study. Thus, lower levels of green tea as a long-term treatment for Down syndrome may be desirable. Furthermore, such modest DYRK1A reduction with green tea combined with modest

RCAN1-1 reduction with fish oil may still potentially lead to a significant synergistic impact on NF-AT signaling. Also, it is conceivable that future studies will identify a more potent fish oil supplementation regimen that will maximize RCAN1-1 reduction. Although differences between DHA and EPA effects are generally well documented (eg, Ref. [45]), the relative effects of each on gene expression has not yet been carefully assessed as evidenced by a paucity of such comparative studies in GEO. Nonetheless, studies that empirically test the effects of both DHA and EPA as well as the ratio of n-3/n-6 PUFA on RCAN1-1 expression are a reasonable future strategy.

The reduction of Rcan1-1 protein levels to a statistically insignificant 11% reduction from a statistically significant reduction of 27% at the mRNA levels in the C57BL studies suggests that a translational compensation may occur here. That is, to compensate for the mRNA reduction, some increase in the efficiency of translation of that mRNA occurs. It is also likely that this is reflective of high experimental variability from model to model, as 8 mice were analyzed here per group, and more may be needed to achieve statistically significant changes. Another possibility is that this is a strain effect, as a closer correlation between mRNA and protein expression was observed in BALB/c mice. Relative to this, it is worth noting that the above-mentioned study by Berger et al [39] identified through our bioinformatics search reported a dramatic 2.2-fold reduction in mouse hippocampus *Rcan1* (*Dscr1*) mRNA after fish oil supplementation, further underscoring the variability issue. There, a different mouse strain and somewhat different diet were used. Combined, these results suggest a suppressive effect of fish oil on RCAN1-1 expression, but one that exhibits wide variability depending on organism, strain, exact diet, and other likely factors.

Commercial dietary supplements such as Warner's Hap Caps have long been available as treatments for Down syndrome. Unfortunately, it has been concluded "to date there has been no consistent or rigorous proof that any form of nutritional supplementation improves the outcome of Down syndrome" [46]. We believe that this is because these supplements have not been evaluated for their effect on the main trisomic genes contributing to Down syndrome, now known to include RCAN1 and DYRK1A. In fact, despite the healthy supplement make-up of Hap Caps and others, it is even possible that they elevate RCAN1 and/or DYRK1A, exacerbating Down syndrome. The novel aspect of our rational approach is that although it contains healthy dietary components (as does Hap Caps), it also targets modulation of the expression of key disease-causing genes toward the normal (or in this case, disomic). Thus, it has significant potential impact in improving cognitive (and other) function in Down syndrome and Alzheimer disease. Our approach is also highly translatable, requiring only a simple dietary modification using readily available commercial dietary supplements. All supplements for our rational gene targeting study are reportedly healthy; that is, multiple reports of their health benefits have been published in respected peer-reviewed journals, and they have been associated with health benefit for a long period of time.

The potential use of rational gene targeting with healthy nutraceuticals to treat Down syndrome and Alzheimer should also be considered in the context of conventional drug treatment. Although potentially effective, pharmaceutical drug treatments can lead to high costs and adverse effects. Because our approach focuses on healthy dietary nutrients, it is more likely safer and healthier than newly identified drugs whose long-term health risks are not yet known. Thus, one potential future approach would be the combined use of both, with effective nutraceutical approaches complementing conventional drug treatments, effectively leading to a reduction in their dosage.

At this stage, there are 2 key limitations to our studies. First, it is unclear whether our modest reduction in Rcan1-1 will be sufficient to affect NF-AT signaling. Such determination would be best assessed by in vivo studies. Second, it would ultimately be best to test our hypothesis using a Down syndrome mouse model, most notably Ts65Dn. These limitations will hopefully be addressed in future studies.

In conclusion, the potential use of healthy nutraceuticals as a strategy to reduce the expression of Down syndrome–associated RCAN1-1 was evaluated. Multiple nutraceuticals were tested in mice with fish oil exerting a statistically significant reduction. Subsequent studies in BALB/c pups and C57BL pups starting at conception also revealed reduced Rcan1-1 protein and *Rcan1-1* mRNA levels. However, these reductions were variable, and in the case of early intervention starting at conception, they were less at the protein level suggesting translational compensation. These results suggest the potential use of fish oil as a healthy treatment for Down syndrome and associated early-onset Alzheimer disease and support our strategy of using select healthy dietary agents to treat genetically defined pathologies, an approach that we believe is simple, healthy, and cost-effective.

Acknowledgment

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