PROGRESS REPORT

Summary

We have utilized an *in vitro C. elegans* model of hyperglycemia-induced cell dysfunction and shortened life span, as a model to study the complications of Type 1 and Type 2 diabetes. Using this model we conducted a whole organism-based screening of (1) a library of clinically used drugs and drug-like small molecules and (2) a focused siRNA library. The goal of the study was to generate proof-of-concept data to show that this approach can be utilized to identify small molecules and genes, which beneficially affect the survival of worms exposed to elevated extracellular glucose. In the first part of our studies, we have set up an automated system (Microtracker) to monitor the motion/viability of *C. elegans* in a 96-well format to enable medium-throughput screening. The system has been validated using both hyperglycemia (which reduced the motion/viability) compared to normoglycemia and with oxidative stress (hydrogen peroxide), causing a concentration-dependent decrease in motion/viability of *C. elegans*. In **Specific Aim 1** we have conducted a pilot small molecule screening campaign of clinically used and pharmacologically active compounds to identify clinically used drugs in the hyperglycemic *C. elegans* model of diabetic complications. Confirmed hit compounds that protected against the loss of motility/viability included nelfinavir and indinavir (two HIV protease inhibitors of different chemical classes), benidipine (a calcium channel blocker), taxaifolin (a flavanolol antiproliferative agent) duloxetine (a serotonin/norepinephrine reuptake inhibitor anxiolytic) and the antibiotic gemifloxacine. All of these compounds are likely related to targets/mechanisms that have previously not been connected to the pathogenesis of diabetic complications. In **Specific Aim 2,** using a focused library of genes targeting transcription factors, phosphatases and chromatin, we conducted a pilot siRNA screening campaign to identify pathways, which extend life span in an oxidative stress *C. elegans* model of diabetic complications. Because of the lack of availability of glucose uptake deficient bacteria, which would have been required for hyperglycemic siRNA experiments, the design of these latter studies had to be refined such that they were conducted against hydrogen peroxide mediated oxidative stress, rather than the background of the prolonged hyperglycemia used for small molecule screening. Confirmed genes, the silencing of which protected against oxidative stress-mediated loss of motion/viability in *C. elegans* included pir-1 (an RNA-phosphatase homolog), C34D4.2 (a serine/threonine phosphatase), ceh-7 (a homeobox protein), and ces-2 (a bZIP protein). All of these proteins represent new targets both in the context of diabetic complications and in the context of oxidative stress-mediated cell injury. Work-up focusing on the mechanism of action of some of the above identified small molecules and protein targets is currently on-going and will include expansion into mammalian systems/mammalian homologs and diabetic endothelial cell dysfunction / vascular complication models. Taken together, our screening project has proven useful for the identification of compounds and novel therapeutic targets that counteract the complications of diabetes.

Background and Significance

The utility of metazoan model organisms to study complex diseases. Novel, creative experimental models are needed to improve our insight into the signaling pathways that govern cellular energy homeostasis, cell dysfunction, cell injury and cellular recovery. Three metazoan organisms, *Caenorhabditis elegans*, Drosophila, and zebrafish receive increasing attention as model organisms to study complex disease processes. Studies using genetically tractable lower organisms are now being recognized as innovative tools that help increase our understanding of the fundamental pathophysiology of a variety of complex diseases including neurodegeneration, autoimmunity, infectious diseases, metabolic diseases, obesity, vascular disease and diabetic complications[1-5].

The model of 'hyperglycemic'* C. elegans *to study the complications of diabetes. *C. elegans* has been an important resource for biological exploration since its adoption in the 1970's. *C. elegans* is simply propagated and maintained on agar plates or liquid cultures with non-pathogenic *Escherichia coli* as their food source. Each adult animal (~1 mm) produces ~300 genetically identical progeny in its 3-day life cycle, facilitating the establishment and maintenance of large populations of animals. *C. elegans* is diploid and hermaphroditic, which is an advantage in genetic analysis. Gene expression in *C. elegans* can be knocked down easily via RNAi by feeding worms live *E. coli* expressing double-stranded RNAs corresponding to *C. elegans* genes. Adult *C. elegans* have only 959 cells, the developmental lineages of which have been traced completely to the fertilized egg. The above listed properties lend substantial advantages to the use of these organisms for biological studies[6-10].

Typically, endothelial cells placed in elevated extracellular glucose are used to study the fundamental processes of diabetic complications, since the constant or intermittent elevation in extracellular glucose is widely recognized as the primary and fundamental trigger of the complications of diabetes. This elevated glucose serves as the proximal trigger, which then leads to cardiovascular dysfunction, accelerated aging, pro-inflammatory responses and many other clinical manifestations of diabetes[11-15]. However, over the last several years, the use of *C. elegans* has been introduced in the context of hyperglycemia and diabetic complications by several laboratories: enrichment of the diet of the worms with glucose was found to lead to a significant shortening of the life span[16-18]. A limited number of pathways that regulate these responses have also been identified, such as FOXO family member DAF-16, the heat shock factor HSF-1 and the aquaporin glycerol channel, aqp[16]. In another study, the high glucose-induced shortening of life span was linked to some of the pathways that are also operative in mammalian systems: reactive oxygen species (ROS) and the advanced glycation end product / methylglyoxal pathway[17]. In the third study, hyperglycemia-induced decreased life span has been attributed to ectopic apoptosis, with ced-3 and ced-4 playing important roles[18]. These three studies

represent only a few examples with respect to the molecular mechanisms involved in the processes that restrict the life spam of *C. elegans* subjected to elevated extracellular glucose and the subject remains to be further explored. *C. elegans* was also used to elucidate the molecular mechanisms of the antidiabetic drug metformin: the compound was found to act through a mechanism that resembles caloric restriction, and its effect involved the energy sensor AMPK and AMPK-activating kinase LKB1, as well as the SKN-1/Nrf2 pathway[19].

Cell-based screening: a tool to identify novel pharmacological modulators and cellular pathways of hyperglycemic injury. In order to identify novel molecules with potential therapeutic properties, a radically different system biology approach (sometimes also termed 'chemical genomics') is emerging. Cell-based screening generally focuses on global functional read-outs (e.g. improvement in cell viability, changes in cell differentiation, etc.), without *a priori* invoking a specific pathway or pharmacological target[20-25]. Using this approach compound libraries or siRNA libraries can be screened and molecules that trigger the desired phenotypic changes in the model system can be identified. Subsequently, the molecular mode of the action of such compounds can be delineated and the compounds can also undergo medicinal chemistry optimization. The PI is familiar with this approach using cultured mammalian cells and has identified compounds/pathways that ameliorate ischemic/hypoxic conditions[26-30]. One of our current screening campaigns uses hyperglycemic endothelial cells, as a model of diabetic vascular complications. Several novel compounds and pathways already emerging as part of this approach[30,31], while several other effective 'hit molecules' currently under detailed characterization.

Cell-based screening approaches in* C. elegans*: In recent years a number of groups have employed *C. elegans* models for the purpose of cell-based screening, focusing on simple but powerful primary outcome variables, such as survival or life span[32-37], with many advantages. First, this type of screening enables the rapid discovery of compounds/pathways that influence complex *in vivo* processes, that may not be apparent from screens involving a single target (enzyme, channel, receptor), or even a single cell in culture. Second, the approach identifies all *in vivo* active compounds including prodrugs that need *in vivo* metabolism to become effective. Third, (because the readout of this *in situ* assay is nematode survival), the approach decreases the large background of compounds that lack practical utility *in vivo* due to poor pharmacokinetics or mechanism-independent cytotoxicity. Recent *C. elegans*-based small molecule screening campaigns have led to the identification of novel antimicrobial compounds[33]; the identification of molecules that confer protection from thermal stress[34], identification of factors involved in the neuronal specification[35], and the identification of compounds that improve skeletal muscle function in a model of spinal muscular atrophy[36]. A comprehensive review of the use of the *C. elegans* model for cell-based screening has been recently published[37].

Results

We have set up the model of hyperglycemic *C. elegans* in our laboratory using an approach similar to recently published studies[16-18], with the exception being the utilization of liquid cultures (as opposed to solid agar plates), to expedite compound/gene target screening, coupled with continuous motility/viability measurements. Under our experimental conditions, elevation of the extracellular glucose to 10, 20 or 40 mM caused a concentration-dependent shortening of the life span, an effect, which is unrelated to any osmotic confounding effects.

Our overall screening approach is depicted in **Fig. 1**. In order to facilitate the screening of large numbers of compounds / siRNA silencing conditions, we have set up an 96-well based infrared locomotor tracking system, known as the Microtracker method[38] to detect the motion/viability of the worms on a continuous basis (**Fig. 1**). Under our liquid culture experimental conditions, both elevated extracellular glucose (**Fig. 2**) and oxidative stress induced by hydrogen peroxide (**Fig. 3**) induced a marked suppression of motility index; the dynamic range of the assay was suitable to enable 96-well-based screening.

Library name	Supplier	Compounds	Short description
LOPAC1280	Sigma-Aldrich	1280	Pharmacologically active compounds
NIH Clinical Collection	BioSource/NIH	450	Phase I-III trial compounds
International Drug Collection	MicroSource Discovery Systems Inc.	240	Compounds marketed in Europe or Asia but not in the US
US Drug Collection		1040	Clinical trial stage USP drugs
FDA Approved	Enzo	640	FDA approved bioactive compounds
Prestwick Chemical	Prestwick Chemical	1200	Marketed drugs in Europe

Table 1. *Compounds assessed in the hyperglycemic* C. elegans *model of diabetic complications. These libraries include drugs that either have reached clinical trial stage or are currently in clinical use.*

Specific Aim 1. We have conducted a molecule screening campaign of clinically used and pharmacologically active compounds to identify small molecules that protect in the hyperglycemic *C. elegans* model of diabetic complications. The libraries used are listed in **Table 1**. The list of identified hit compounds is shown in **Tables 2** and **3** from two separate screening campaigns (clinically used compounds, and the LOPAC library of pharmacologically active compounds, respectively). Confirmed protective compounds (i.e. 'hit compounds'), listed in their rank-order at Day 5 of hyperglycemia, are shown in **Fig. 4** and **Fig 5** (clinical compounds and pharmacologically active compounds, respectively). Clinically used compounds that protected against the loss of motility/viability included nelfinavir and indinavir (two HIV protease inhibitors of different chemical classes), benidipine (a calcium channel blocker), taxaifolin (a flavanolol antiproliferative agent) duloxetine (a serotonin/norepinephrine reuptake inhibitor anxiolytic) and the antibiotic gemifloxacine. From the LOPAC library of pharmacologically active compounds, the kinase inhibitor HA100, gabapentin, hydroquinone, tyrphostin 47, hydroxylurea and acetylsalicylic

acid extended motility/viability of the worms exposed to elevated glucose. None of these compounds relate to targets/mechanisms previously associated with the pathogenesis of diabetic complications.

Compound	Motility index	Known mechanism of action
Acarbose	16	Anti-diabetic drug, inhibits alpha glucosidase
Benidipine HCl	13	Dihydropyridine calcium channel blocker
Betaxolol hydrochloride	12	Beta1 receptor blocker
Caffeine	12	PDE inhibitor
Clarithromycin	20	Macrolide antibiotic
Delta1-hydrocortisone	18	Glucocorticoid
Dofetilide	12	Class III antiarrhythmic agent
Duloxetine	24	Serotonin/norepinephrine reuptake inhibitor
Gemifloxacin mesylate	22	Antibacterial
Indinavir sulphate	21	Antiretroviral, HIV protease inhibitor
Letrozol	18	Non-steroidal aromatase inhibitor
Maltol	26	Natural flavor enhancer
Milrinone	25	Phosphodiesterase 3 inhibitor
Nefazodone	38	Antidepressant
Nelfinavir mesylate	12	Antiretroviral, HIV protease inhibitor
Olopatadine HCl	18	Antihistamine
Phenelzine sulfate	12	Monoamine oxidase inhibitor
Taxifolin	16	Flavanonol antiproliferative agent
Vardenafil hydrochloride	18	Phosphodiesterase 5 inhibitor

Table 2. *Clinical drugs identified in the primary hyperglycemic* C. elegans *model of diabetic complications to protect against the hyperglycemia-induced loss of motility. All compounds that were found to be outside 3xSD are listed in alphabetical order.*

Compound	Motility index	Known mechanism of action
Atropine sulfate	39	Muscarinic acetylcholine receptor antagonist
Amiprilose hydrochloride	26	Immunomodulator
Acetylsalicylic acid	20	Cyclooxygenase inhibitor
4-Androsten-4-ol-3,17-one	19	Aromatase inhibitor
Methotrexate hydrate	15	Folic acid antagonist
Benzamil hydrochloride	16	Na^+/H^+ and Na^+/Ca^{2+} channel blocker
Chloroethylclonidine diHCl	20	Adrenoceptor alkylating agent
Bicuculline methbromide	18	GABA-A receptor antagonist
BRL 54443 maleate	40	5-HT1E/1F serotonin receptor agonist
L-Cycloserine	19	Inhibitor of ketosphinganine synthetase
Corticosterone	14	Glucocorticoid
Chlorpheniramine maleate	22	H1 histamine receptor antagonist
5-Carboxamidotryptamine	18	5-HT7 serotonin receptor agonist
N-Methyldopamine HCl	62	Dopamine receptor agonist
Daidzein	21	Inhibitor of aldehyde dehydrogenase
3-deazaadenosine	15	Antiviral agent
Icilin	33	CMR1 receptor agonist
3,5-Dinitrocatechol	19	Inhibitor of catechol O-methyltransferase

Genipin	24	Cross-linker
Gabapentin	28	Anticonvulsant, Ca^{2+} channel modulator
Hydroxyurea	28	Ribonucleoside reductase inhibitor
MHPG sulfate potassium	20	Norepinephrine metabolite
Hydroquinone	22	Arachidonate 12-Lipoxygenase inhibitor
R-(+)-7-Hydroxy-DPAT	15	D3 dopamine receptor agonist
HA-100	20	Inhibitor of PKA, PKC and MLCK
N-Oleoylethanolamine	14	Ceramidase inhibitor
Tyrphostin 47	24	EGFR tyrosine kinase inhibitor

Table 3. *Pharmacological compounds from the LOPAC library identified in the primary hyperglycemic* C. elegans *model of diabetic complications to protect against the hyperglycemia-induced loss of motility. All compounds that were found to be outside 3xSD are listed in alphabetical order.*

Sequence	Gene	Motility index
transcription factor library		
C34C6.8	ceh-7	20
ZK909.4	ces-2	26
T01G6.4	nhr-106	19
C03G6.12	NHR-149	20
C33G8.9	NHR-140	17
F46C8.5	CEH-14	14
F11C1.6	nhr-25	19
H01A20.1	nhr-3	14
phosphatase library		
T23G7.5	pir-1	28
C34D4.2	C34D4.2	30
chromatin library		
F22E12.4	egl-9	30
C39F7.2	trim-9	36
H02I12.7	his-65	32

Table 3. *Genes the transient silencing of which protects against oxidative stress-induced loss of viability/motility in* C. elegans*. Libraries were obtained from Source BioScience. The following 3 libraries were tested: Chromatin Library (257 clones), Phosphatase Library (166 clones) and Transcription Factors Library (387 clones). Genes that were found to be outside 3xSD are listed in the table.*

Specific Aim 2. Next, we conducted a pilot siRNA screening campaign to identify pathways, which extend life span in the hyperglycemic *C. elegans* model of diabetic complications. Because of the lack of availability of glucose uptake deficient bacteria, which would have been required for hyperglycemic siRNA experiments, the design of these latter studies had to be refined such that they were conducted against hydrogen peroxide mediated oxidative stress, rather than the background of the prolonged hyperglycemia used for small molecule screening. The gene cbs-1 (cystathionine-beta synthase) was selected to refine the conditions suitable for silencing. Silencing of cbs-1 was achieved with OP50 bacteria containing RNAi feeding vector (**Fig. 6**). The efficacy of the silencing was confirmed by Western blotting, as well as functional determination (CBS, and its product H_2S is known to act as a mitochondrial electron donor; accordingly, cbs-1 silencing was

expected to reduce basal and FCCP-uncoupled mitochondrial respiration in *C. elegans*, and, indeed, this suppression was experimentally confirmed by Extracellular Flux Analysis (**Fig. 6**). Using the same, efficient silencing conditions, screening was conducted in a focused library of >800 *C. elegans* gene targets modulating transcription factors, phosphatases and chromatin. The list of hit genes is presented in **Table 4**. Confirmed genes that protected against oxidative stress-mediated loss of motion/viability in *C. elegans* (as shown at 20 hours post H_2O_2 exposure) are shown according to their rank order of activity in the confirmatory assay in **Fig. 7**. Confirmed hit gene targets that afforded protection included pir-1 (an RNA-phosphatase homolog), C34D4.2 (a serine/threonine phosphatase), ceh-7 (a homeobox protein), ces-2 (a bZIP protein) and egl-9 (prolin hydroxylase). All of these proteins - except egl-9, which has previously been implicated in the regulation of HIF-1 and thereby the modulation of survival and life span of *C. elegans* in response to various stresses[39,40] - represent new targets: both in the context of diabetic complications and in the context of oxidative stress-mediated cell injury.

Discussion and Future Directions

Work-up focusing on the mechanism of action of some of the above identified small molecules and protein targets is currently on-going and will include expansion into mammalian systems/mammalian homologs and diabetic endothelial cell dysfunction / vascular complication models. We hope that this work, to be completed over the next 2 years, will identify further details of the mechanism of action, and will be reported in several papers.

Nevertheless, already from the data generated so far, we can conclude that the current screening project has proven useful as an innovative novel approach for the identification of compounds and novel therapeutic targets that counteract the complications of diabetes, as it has identified completely unexpected and previously not implicated drugs and pathways.

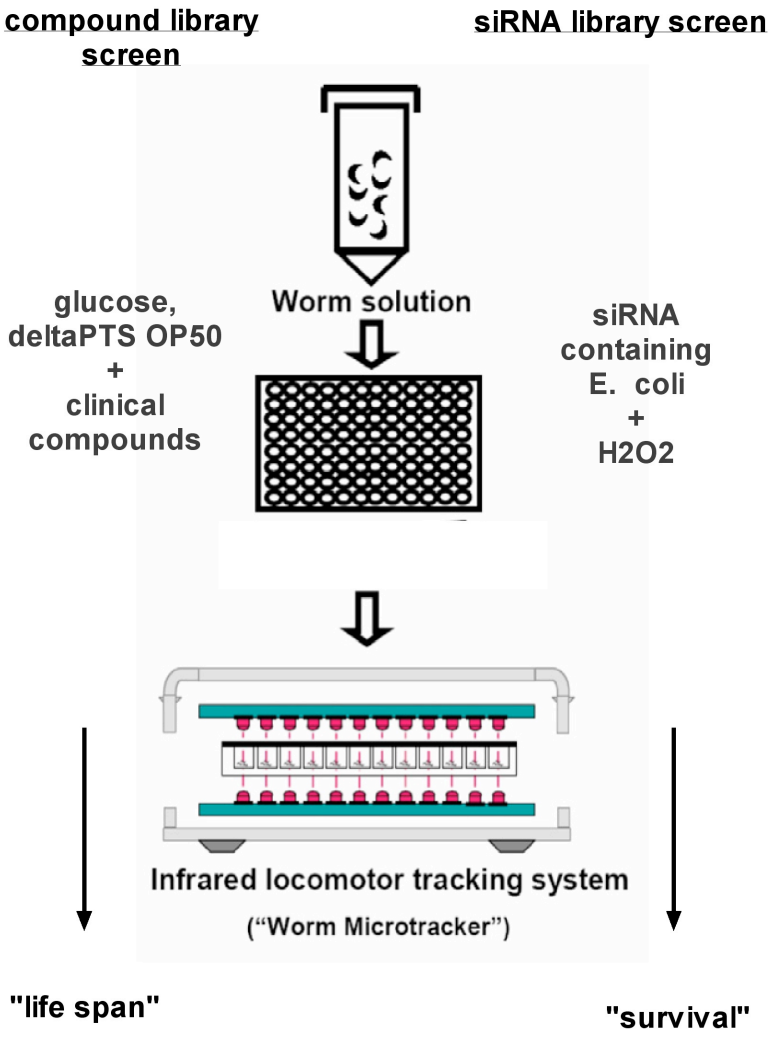

Fig. 1. ***Experimental design of compound library screen and siRNA screen.*** *Age-synchronous population of* C. elegans *was plated on 96-well plates. In the compound library screen the worms were treated in L4 stage with glucose and 100 μM final concentration of the test compounds. Locomotor activity/viability was recorded from zero to seven day, every day for 1 hour using the Microtracker method. In the siRNA library screen the worms were feeding with siRNA containing E. coli in L1 stage, after the siRNA transfection the worms were treated in L3-L4 stage with H_2O_2 for 2 hours, then locomotor activity/survival was detected with the Microtracker for 15 minutes.*

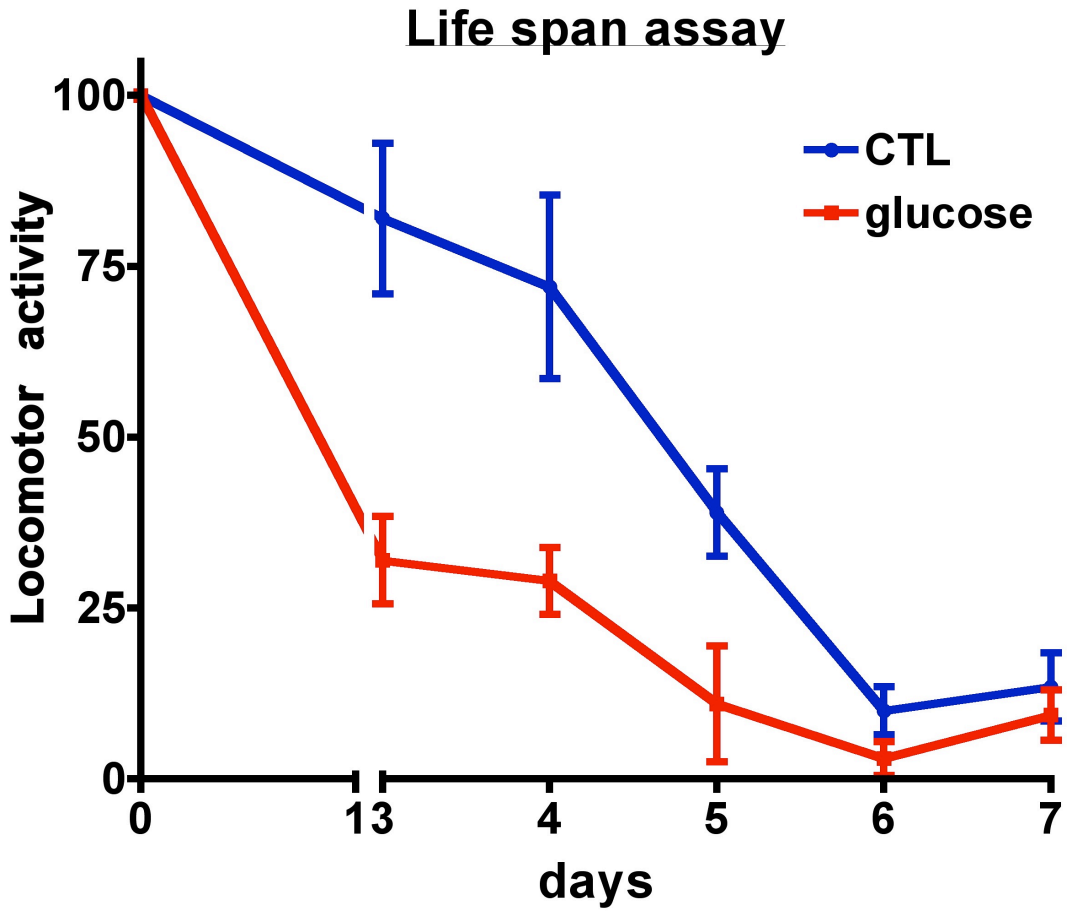

Fig. 2. ***Effect of high glucose on the life span of*** **C. elegans in liquid culture.** *The age-synchronous* C. elegans *population was treated with 40 mM glucose in L4 stage (or normal control, 5 mM glucose). Locomotor activity/viability of the two groups was detected during 7 days. Mean ± SEM values are shown.*

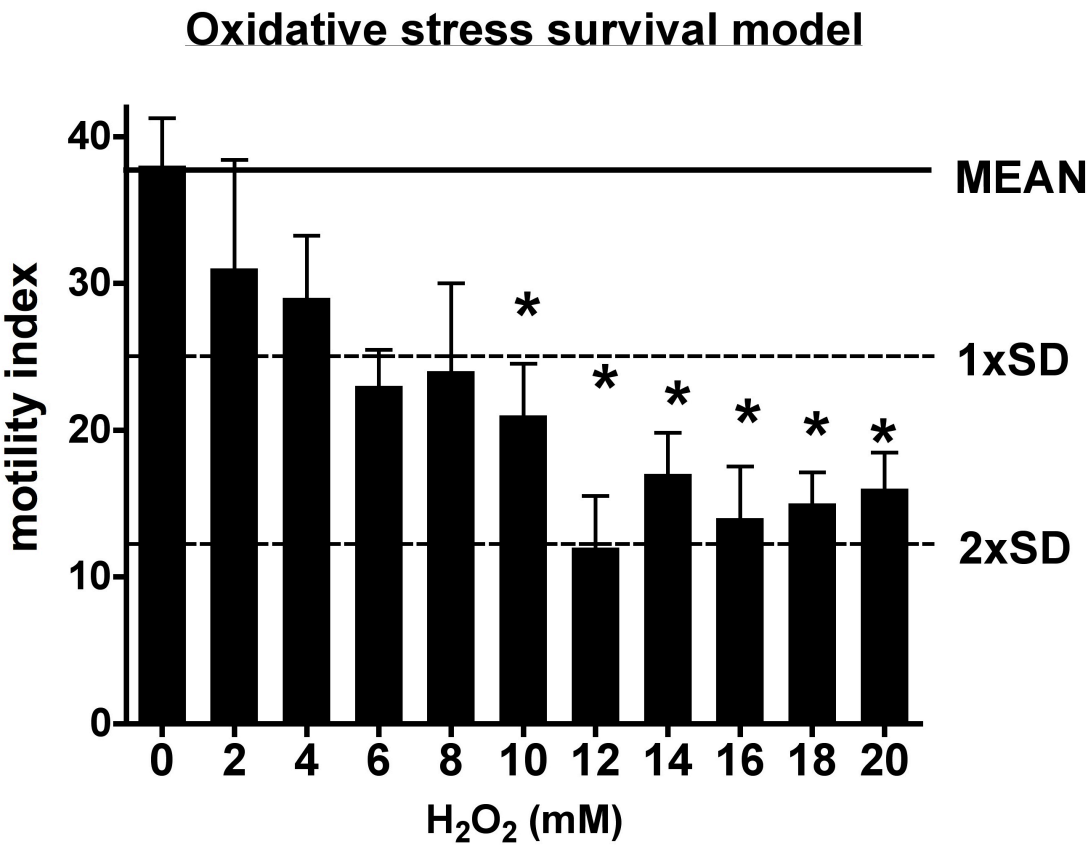

Fig. 3. ***Effect of H_2O_2 on siRNA transfected*** **C. elegans.** *The age-synchronous* C. elegans *population was treated with increasing concentrations of H_2O2 for 2 hours, then the locomotor activity/viability recorded for 15 minutes. Locomotor activity is shown as a motility index. The mean of non-treated group is labeled with solid line, and the 1xSD and 2XSD value is labeled as dashed line. Mean ± SEM values are shown.*

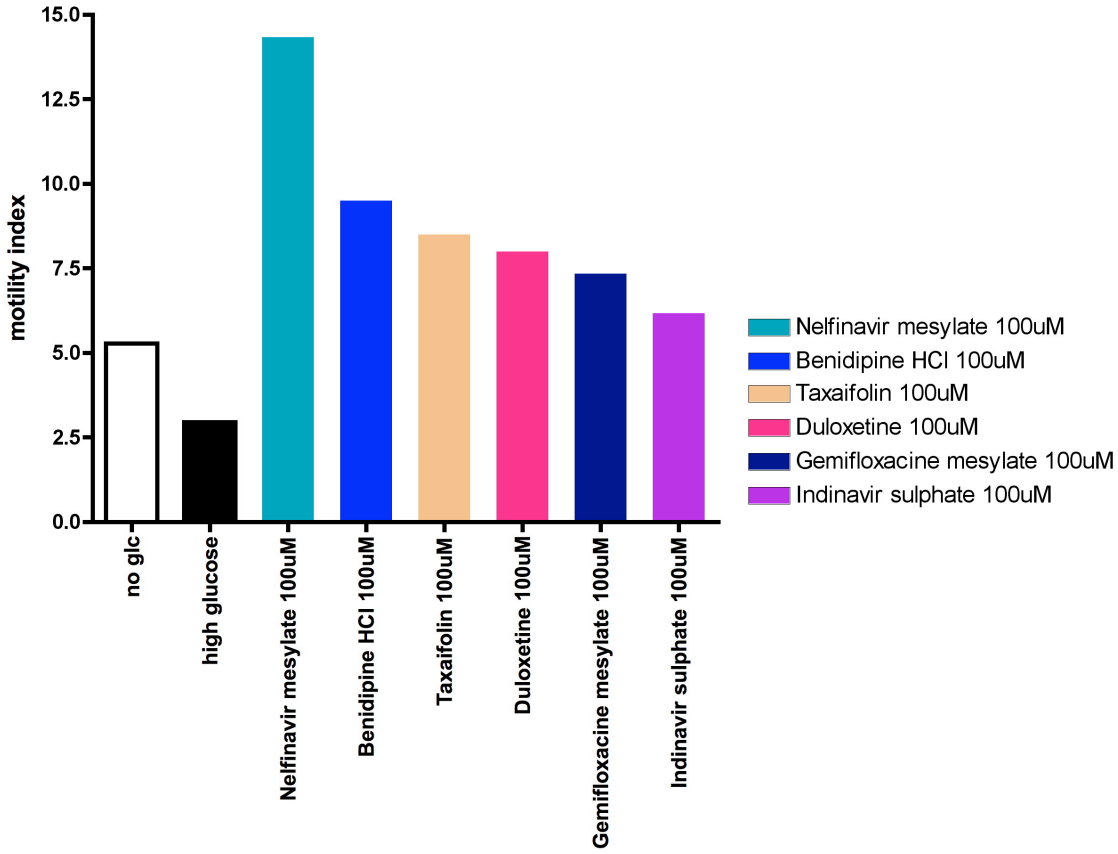

Fig. 4. ***Result of the hit confirmation of the screening of clinical libraries.*** *The age-synchronous C. elegans in L4 stage were treated with 40 mM glucose, and 100 uM concentration of the test compounds. The locomotor activity as motility index was recorded for 1 hour. Mean values are shown (n≥6).*

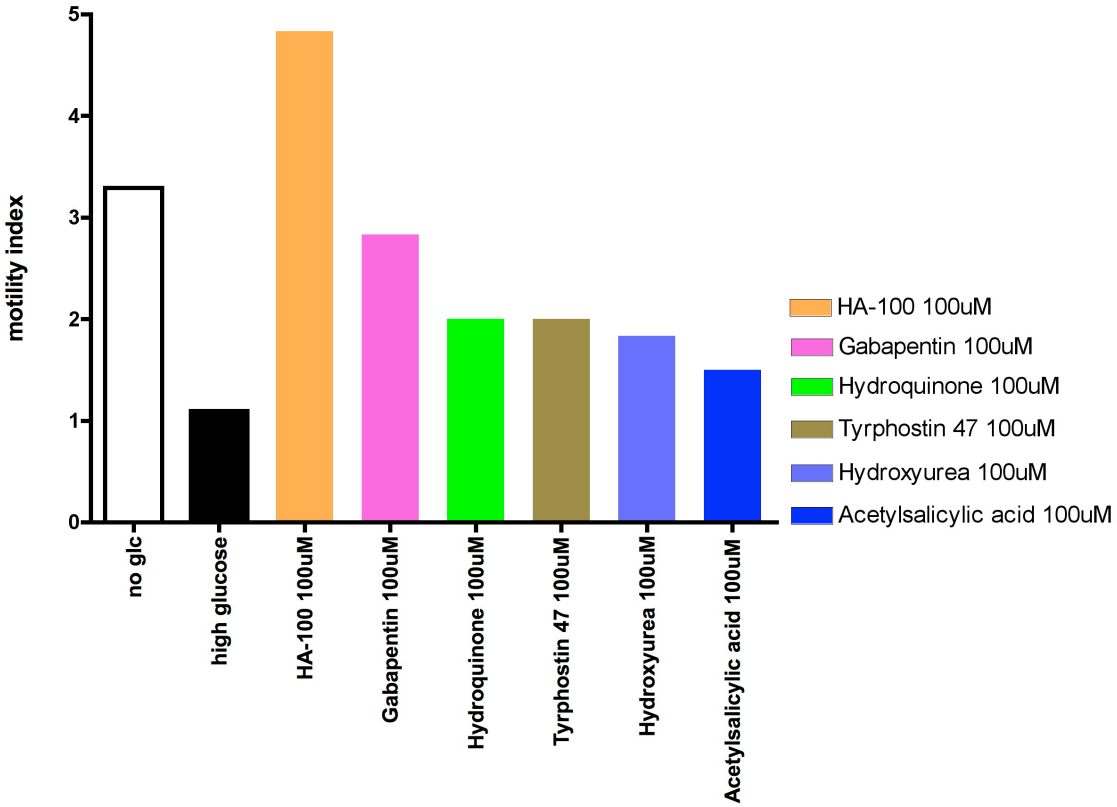

***Fig. 5.* Result of the hit confirmation of the screening of the Library of Pharmacologically Active Compounds (LOPAC).** *The age-synchronous* C. elegans *was treated with 40 mM glucose, and 100 uM concentration of the test compounds. Locomotor activity as motility index was recorded for 1 hour. Mean values are shown (n≥6).*

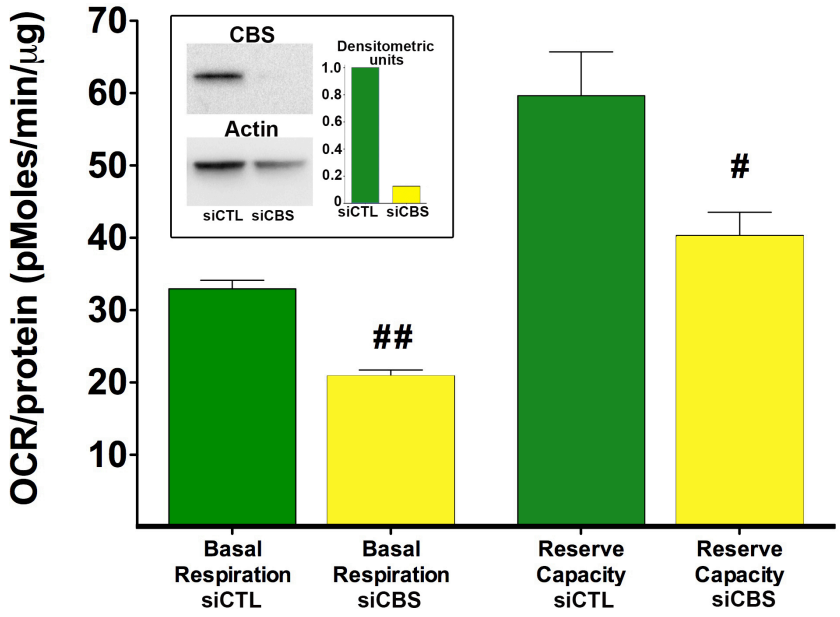

Fig. 6. ***CBS silencing attenuates the bioenergetics in*** **C. elegans**. *Nematode culturing, synchronization and RNAi feeding were performed according to standard techniques. Egg isolation was done using the bleaching method. The resulting embryos were placed in medium without OP50 bacteria, and allowed to hatch overnight at 20 ^{o}C with constant rotation. The L1 larvae were counted and supplemented with strain OP50 bacteria with pL4440-CBS or pL4440-scrambled RNAi feeding vector containing Ampicillin resistance marker to resume the life cycle. RNAi induction of bacteria was induced by IPTG. L1 larvae were transferred to a flask in a concentration of 80-100 worms/ml in S-complete. To sterilize the animals FUDR (5-Fluoro-2'-deoxyuridine) stock solution was added to each well 50 hours after hatching. 5 days after reaching adult stage, worms were washed with water and collected for subsequent western blot analysis. For western blot analysis followed by immuno-detection of CBS-1 a rabbit polyclonal antibody against recombinant CBS-1 was used. The general technique of the functional analysis of the metabolic activity of the worms was conducted using a 24-well Seahorse Extracellular Flux Analyzer with 50 worms per well under basal conditions and upon the addition of the uncoupling agent FCCP (10 μM). CBS silencing attenuated CBS-1 protein expression, and suppressed basal and FCCP-stimulated oxygen consumption. Data are shown as mean ± SEM of n=20 determinations collected from 3 experimental days. Oxygen consumption was normalized to protein content. #p<0.05 and ##p<0.01 show lower oxygen consumption in the CBS-1 silenced group, when compared to the respective sham-silenced controls.*

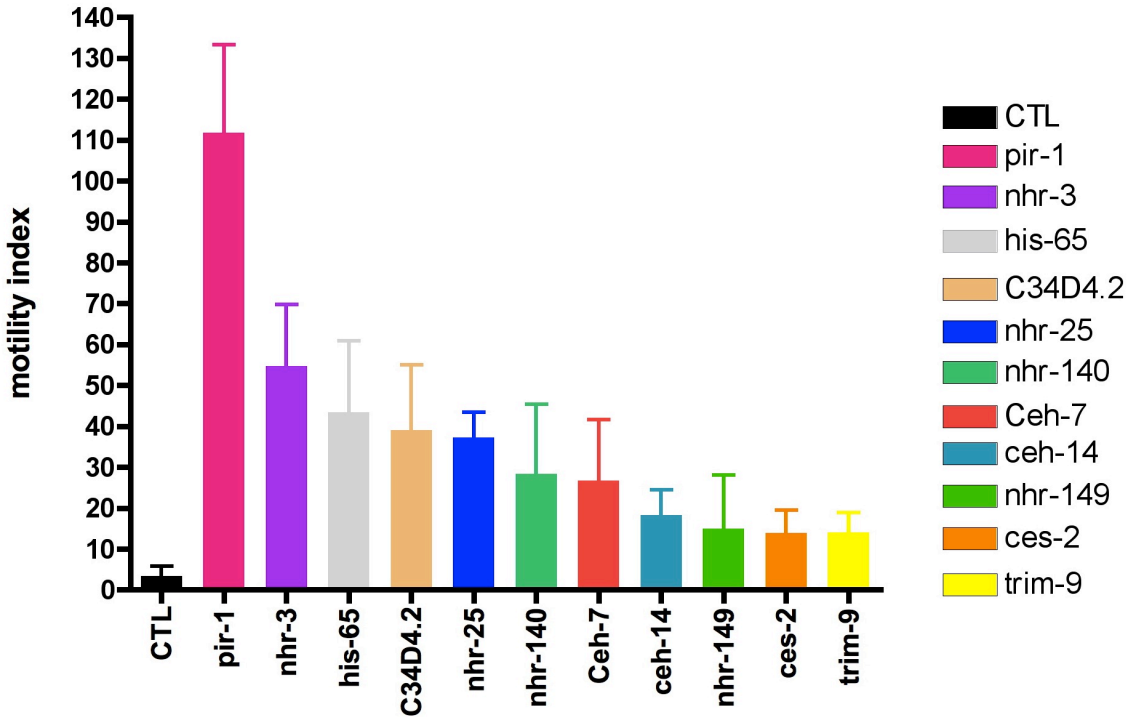

Fig. 7. ***Result of the hit confirmation of the results of the siRNA screen.*** C. elegans *were fed with siRNA containing E. coli in L1 stage. After the siRNA transfection worms were treated in L4 stage with* H_2O_2 *for 2 hours, and then locomotor activity/survival was recorded after 20 hours with the Microtracker method for 15 minutes. Fresh bacteria culture was inoculate of LB containing 50ug/ml ampicillin from overnight bacteria culture, siRNA production was inducted with IPTG treatment and incubated for 4 hours at 37 °C. Mean ± SEM values are shown (n=8).*

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