Role of the cystathionine β-synthase / H₂S pathway in the development of cellular metabolic dysfunction and pseudohypoxia in down syndrome

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1. Background

Down syndrome (DS, caused by trisomy of chromosome 21) is the most common human genetic disorder, with a high prevalence of 1:700 births in developed countries. DS presents with cognitive impairment, craniofacial dysmorphism and co-morbidities such as cardiovascular defects and amyloid plaque pathology. The available therapeutic options for DS are limited and the clinical need for new therapeutic approaches is significant [1]. Although DS is associated with the dysregulation of thousands of genes and the dysregulation of numerous biochemical pathways, one of the underlying common themes is pseudohypoxia, i.e. a suppression of mitochondrial ATP generation in DS cells [2].

One of the genes that chromosome 21 codes for is the gene for cystathionine β-synthase (CBS), and overproduction of its product, hydrogen sulfide (H₂S) are recognized as potential pathogenetic factors in Down syndrome (DS). The purpose of the study was to determine how the mitochondrial function and core metabolic pathways are affected by DS and how pharmacological inhibition of CBS affects these parameters.

Methods: 8 human control and 8 human DS fibroblast cell lines have been subjected to bioenergetic and fluxomic and proteomic analysis with and without treatment with a pharmacological inhibitor of CBS.

Results: DS cells exhibited a significantly higher CBS expression than control cells, and produced more H₂S. They also exhibited suppressed mitochondrial electron transport and oxygen consumption and suppressed Complex IV activity, impaired cell proliferation and increased ROS generation. Inhibition of H₂S biosynthesis with amino-oxyacetic acid reduced cellular H₂S, improved cellular bioenergetics, attenuated ROS and improved proliferation. 13C glucose fluxomic analysis revealed that DS cells exhibit a suppression of the Krebs cycle activity with a compensatory increase in glycolysis. CBS inhibition restored the flux from glycolysis to the Krebs cycle and reactivated oxidative phosphorylation. Proteomic analysis revealed no CBS-dependent alterations in the expression level of the enzymes involved in glycolysis, oxidative phosphorylation and the pentose phosphate pathway. DS was associated with the dysregulation of several components of the autophagy network; CBS inhibition normalized several of these parameters.

Conclusions: Increased H₂S generation in DS promotes pseudohypoxia and contributes to cellular metabolic dysfunction by causing a shift from oxidative phosphorylation to glycolysis.

Keywords: Down syndrome, Metabolism, Oxidative phosphorylation, Glycolysis, Hydrogen sulfide

Abstract

Background: Overexpression of the transsulfuration enzyme cystathionine-β-synthase (CBS), and overproduction of its product, hydrogen sulfide (H₂S) are recognized as potential pathogenetic factors in Down syndrome (DS). The purpose of the study was to determine how the mitochondrial function and core metabolic pathways are affected by DS and how pharmacological inhibition of CBS affects these parameters.

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Conclusions: Increased H₂S generation in DS promotes pseudohypoxia and contributes to cellular metabolic dysfunction by causing a shift from oxidative phosphorylation to glycolysis.
growth medium to a 10X working solution. From the latter, the added volume to the cell culture medium was adjusted to achieve the final concentration of 3 μM; control cells received the same volume of medium without AOAA. Treatment was initiated 2 h after cell seeding; measurements were made at 24 h.

Cell Physiology Assays. Cell Proliferation kit II (XTT), Cell Proliferation ELISA BrdU kit and Cytotoxicity Detection KitPLUS (LDH) were employed the quantification of fibroblast viability, proliferation, and necrosis, respectively. The assays were formatted in Corning® Costar® TC-Treated, flat-bottom, transparent 96-well microplates and performed as previously described [12,13].

Quantification of H$_2$S & ROS Levels in Live Cells. All assays were formatted in Thermo Fisher Nunod® 96-well black, optical-bottom plates. Fluorescent signals were read with Infinite® 200 PRO microplate reader and imaged with the upright laser scanning confocal LEICA TCS SP5 BIO microscope. AzMC and P3 fluorophores were employed for the quantification of the intracellular H$_2$S levels, as per our previously published methodology [12], while the cell-permeant reagent 2′,7′-dichloro-fluorescein diacetate (DCFDA) was utilized for measuring measures hydrogen, peroxyl, and other ROS species within the cell in accord to the manufacturer’s protocol. Briefly, 24 h post the treatment with AOAA, fibroblasts were incubated with 20 μM DCFDA, 100 nM Invitrogen™ MitoTracker™ Deep Red FM and 1 μg/ml DAPI in assay buffer supplemented with 10% FBS for 1h at 37 °C in a humidified incubator with 5% CO$_2$ and 95% air. The fluorescence signal of the DCFDA probe was detected at λ excitation = 485 nm and λ emission = 535 nm. A rise in the signal corresponds to increased oxidation of the intermediate deacytelated non-fluorescent product into the 2′, 7′-DCF catalyzed by ROS. MitoTracker™ signal was detected at λ excitation = 580 nm and λ emission = 644 nm. All fluorescent signals were double corrected to the total cell population of each condition (DAPI fluorescence; λ excitation = 341 nm, λ emission = 452 nm) and background (cell auto-fluorescence).

Measurement of Mitochondrial Respiration. Extracellular Flux (XF) analysis was employed for real-time quantification of oxygen consumption rate (OCR), proton efflux rate (PER) in intact cells; measurement of Complex IV activity was performed in permeabilized cells, using ascorbate and tetramethyl-p-phenylene diamine as Complex IV electron donors, according to our previously described methodology [9,12]. The assays were formatted in Seahorse XF24 cell-culture microplates, where diploid and 21-trisomic fibroblasts were seeded at a density of 2 × 10^4 cells/ml in total 5 ml in Corning® Costar® TC-Treated T25 flasks for 2 h. Cells were

### 2. Methods

**Materials.** Cell proliferation kit II (2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide) (XTT; Ref ID: 11465015001), Cell Proliferation 5-bromo-2-deoxyuridine (BrdU) colorimetric ELISA kit (Ref ID: 11647229001), and Cytotoxicity Detection KitPLUS (lactate dehydrogenase (LDH); Ref ID: 4744934001) were purchased from Roche Diagnostics Ltd (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). The fluorescent H$_2$S probes, 7-azido-4-methylcoumarin (AzMC) [10] and (E)-2-(3-(6-(2-hydroxyethylamino)naphthalen-2-yl)-3-oxopro-1-enyl)-3,5-dimethoxybenzaldehyde (P3) [11], bovine serum albumin (BSA), α-glucose,$^{13}$C$_6$, and lactalbumin hydrolysate were purchased by Sigma-Aldrich Chemie GmbH. Agilent Seahorse XF Glycolytic Rate Assay (Ref ID: 103344–100) and Cell Metostress Test (Ref ID: 103015–100) kits, Seahorse XF-24 cell culture microplates, and the corresponding Seahorse XF assay media and calibrant solution were purchased from Agilent Technologies (Bucher Biotech AG, Basel, Switzerland). The DCFDA/H$_2$DCFDA ROS assay kit was purchased from Abcam PLC (Cambridge, UK). The human real-time PCR mitochondrial DNA damage analysis and mitochondrial DNA copy number kits were purchased from Detroit R&D (Holzel Diagnostika Handels GmbH, Köln, Germany). All other materials and reagents for cell culture and Western blotting were from Thermo Fisher Scientific (Basel, Switzerland), unless otherwise stated.

**Cell Culture.** Human dermal fibroblasts from healthy control subjects (CC) and dermal fibroblasts from individuals with Down syndrome (DSC) were obtained from LGC Standards (Wesel, Germany), the Coriell Institute for Medical Research (New Jersey, USA) and the Jérôme Lejeune Institute (Paris, France), as summarized in Table 1. Fibroblasts were cultured as previously described [9].

**Cell Treatments.** Aminoxyacetic acid (AOAA) hemihydrochloride was purchased from Sigma-Aldrich Chemie GmbH and maintained in desiccated form till being used. AOAA was reconstituted in complete growth medium to a 10X working solution. From the latter, the added volume to the cell culture medium was adjusted to achieve the final concentration of 3 μM; control cells received the same volume of medium without AOAA. Treatment was initiated 2 h after cell seeding; measurements were made at 24 h.

**Table 1**

| Reference ID | Group ID | Dot/Blot ID | Origin | Description | Gender | Age at sampling |
|--------------|----------|-------------|--------|-------------|--------|-----------------|
| CC001044S   | CC       | C1          | CC     | LGC Standards | Diploid | Male            |
| 3-FCYPR10000280* | C1      | C2          | CC     | Diploid     | Male | 5 y            |
| GM00576     | CC       | C3          | CC     | Diploid     | Male | 1 y            |
| GM09569     | CC       | C4          | CC     | Diploid     | Male | <1 y           |
| Detroit 551 | CC       | C5          | CC     | Diploid     | Female| <1 y           |
| 1-FCYPR10000368* | CC     | C6          | CC     | Diploid     | Female| 12 y           |
| GM00041     | CC       | C7          | CC     | Diploid     | Female| <1 y           |
| GM09669     | CC       | C8          | CC     | Diploid     | Female| <1 y           |
| Detroit 532 | DSC      | D1          | DSC    | Trisomy 21  | Male | 1 y            |
| 3-FCYPR10000285* | DSC  | D2          | DSC    | Trisomy 21  | Male | 5 y            |
| AG07096     | DSC      | D3          | DSC    | Trisomy 21  | Male | <1 y           |
| AG05979     | DSC      | D4          | DSC    | Trisomy 21  | Male | 1 y            |
| Detroit 539 | DSC      | D5          | DSC    | Trisomy 21  | Female| 2 y            |
| 3-FCYPR10000369* | DSC  | D6          | DSC    | Trisomy 21  | Female| 9 y            |
| GM02571     | DSC      | D7          | DSC    | Trisomy 21  | Female| <1 y           |
| GM04616     | DSC      | D8          | DSC    | Trisomy 21  | Female| <1 y           |
subsequently treated with 0 or 3 μM AOAA for 24 h. Following, cells were washed twice in pre-warmed 1X phosphate-buffered saline (PBS) formulated without Ca\(^{2+}\) and Mg\(^{2+}\), detached with TrypLE™ Express Enzyme, and pelleted by centrifugation at 200 x g for 7 min. The cell pellet was used for DNA extraction with the DNAse Blood and Tissue Kit (Qiagen AG, Hombrechtikon, Switzerland) according to the manufacturer’s recommendations. Mitochondrial DNA integrity was addressed with the commercially available human real-time PCR mitochondrial DNA damage analysis kit for the damaged amplicon 8.8 kb. Results were normalized to mitochondrial copy number and DNA integrity was expressed as a percentage of the mean value for the diploid, control fibroblasts. Both PCR assays were conducted according to the manufacturer’s instructions.

Protein Extraction and Western Blotting. Diploid and 21-trisomic fibroblasts seeded at a density of 1 x 10^5 cells/ml in total 30 ml of SILAC Advanced DMEM/F-12 Flex specially supplemented with 3 g/l stable isotopic labeled α-Glucose-13C₆ (in addition to the other media supplements described above) in Corning® Costar® TC-Treated T75 flasks for 2 h. Cells were subsequently treated with 0 or 3 μM AOAA for 24 h. Following, cells were washed twice with ice-cold 1X PBS formulated without Ca\(^{2+}\) and Mg\(^{2+}\) and harvested in 1X PathScan® Sandwich ELISA cell lysis buffer (Cell Signaling Technology, BioConcept AG, Allschwil, Switzerland) supplemented with Halt™ protease/phosphatase inhibitor cocktail (1X). After two freeze/thaw cycles, the whole-cell lysate was collected and sonicated for 1 min (5 x ON/5 s OFF-cycles). Whole-cell proteins were extracted, quantified, handled and processed for Western blotting as previously published [9,12].

13C Metabolic Flux Analysis. CCs and DSCs were seeded at a density of 1 x 10^5 cells/ml in total 30 ml of SILAC Advanced DMEM/F-12 Flex specially supplemented with 3 g/l stable isotopic labeled α-Glucose-13C₆ in homogeneity. Differences among means were considered significant when p was less than 0.05. Statistical calculations were performed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, USA). No data were excluded from any analysis.

Proteomics Analysis. Proteomics data were then generated from a total of 32 samples using LC/MS labels with tandem mass tags at Charles River Laboratories (CRL) Little Chesterford, UK. The initial data matrix consisted of protein intensities for 7485 proteins in a total of 40 samples (8 pool samples + 32 human donor samples). Initial processing revealed that 1947 proteins had no intensity readings in at least 2 out of 8 replicates in any one of the four sample groups. These proteins were therefore excluded from analysis, leaving intensity values for 5538 proteins remaining for further analysis. Of the remaining proteins that still contained missing values, imputation was performed for these using the LOD2 approach. Automatic outlier tests using Euclidian distance, Kolmogorov-Smirnov, correlation, and Bonferroni’s D were performed on this batch corrected dataset. No samples were identified as outliers; therefore all samples were retained for inclusion in downstream analysis. Significantly differentially expressed proteins were determined using linear modelling, as implemented in the Bioconductor package limma. REACTOME pathway and Gene Ontology (GO) analyses were performed using genes encoding the differentially expressed proteins identified (at the false discovery rate-adjusted p ≤ 0.1 threshold). Protein values were log2 normalized. Four groups (n = 8 CC, n = 8 CC + AOAA, n = 8 DSC and n = 8 DSC + AOAA) were included in the analysis.

Data analysis and statistical procedures. For the design of the current study group sizes were deemed to be equal. Blinding was undertaken in the analysis of the data: cells were cultured and treated with pharmacological inhibitors by one investigator who has also performed randomization of the samples; functional (e.g. bioenergetic, fluxomic) analysis was conducted by other investigator(s) not aware of the groups and treatments. Results were expressed as mean ± standard error (SEM) of 8 independent pairs of diploid and 21-trisomic fibroblasts, as listed in Table 1. Exact group sizes (n) are provided in all figures and tables with group size referring to biological samples (i.e. each data point is a separate, different cell type from a different donor) – i.e. not a technical replicate. When justified, data pairs (i.e. the same cell with/without pharmacological inhibitor) are highlighted connected with a dotted line. Sex was considered as an experimental variable; the sex of the donors used in the study is shown in Table 1; no significant sex-dependent differences were observed in the study, consistent with the body of prior data showing that the biochemical alterations in DS are predominantly independent of sex. The fluxomic and proteomic sets were log2 transformed and quantile normalized; the main rationale for this transformation being heteroskedasticity: the variance of expression measurements on many platforms (arrays, etc.) depends on the expression level. By log-transformation, this dependence is reduced the data are more suitable for statistical testing. Two-way ANOVA analysis, followed by post-hoc Bonferroni’s multiple-comparison t-test was used to identify differences among groups of treated and untreated conditions. Post-hoc tests were conducted only if F was significant and there was no variance in homogeneity. Differences among means were considered significant when p was ≤ 0.05. Statistical calculations were performed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, USA). No data were excluded from any analysis.

3. Results and discussion

We have assembled a panel of 8 human control and 8 human DS fibroblast cell lines (Table 1) for the current investigation. First, we have assessed the potential overactivation of the CBS/H2S pathway in DS,
Based on the 'gene dosage' effect (since CBS is encoded on human Chromosome 21) [7], as expected, there was a marked upregulation of CBS in the DS cells, although there was significant variability in the degree of this upregulation (Fig. 1A, Fig. 1B). As previously reported [13], although not encoded on Chromosome 21, the expression of a second, major mammalian H$_2$S- and polysulfide-producing enzyme, 3-mercaptopyruvate sulfurtransferase (3-MST) was also higher in the DS fibroblasts than in the control cells (Fig. 1A and B). The H$_2$S-metabolizing enzymes SQR, ETHE-1 and TST did not show any difference between control and DS cells (Fig. 1A and B). There was a marked and uniform increase in H$_2$S generation in DS cells as demonstrated by live cell imaging by two structurally different H$_2$S fluoroprobes (Fig. 1C, D, Fig. 1E). There appeared to be a weak inverse correlation between the degree of CBS and 3-MST expression in DS cells (Fig. 1F).

In line with prior findings [9,13] DS cells proliferated slower than control fibroblasts (Fig. 2A), and exhibited a decreased ability to convert the dye XTT to formazan (a mitochondrion-dependent function) (Fig. 2B). However, no significant cell necrosis (breakdown of the cell membrane, and LDH release into the supernatant) was noted in the DS fibroblasts (Fig. 2C), indicating that these cells, even if their mitochondrial function is impaired, are able to produce sufficient amounts of ATP to maintain membrane integrity [14]. When treated with amino-oxyacetic acid (AOAA, 3 μM, 24 h), a commonly used pharmacological inhibitor of CBS [3], DS cell proliferation was stimulated; an effect that was uniform in its direction and magnitude across the 8 DS cells studied. In contrast, in control cells AOAA did not increase cell proliferation, but, instead, produced a slight decrease (Fig. 2A) while it slightly increased XTT conversion (Fig. 2B). Taken together, in control cells AOAA did not increase cell proliferation, but, instead, produced a slight decrease (Fig. 2A) while it slightly increased XTT conversion (Fig. 2B). In control cells AOAA did not increase cell proliferation, but, instead, produced a slight decrease (Fig. 2A) while it slightly increased XTT conversion (Fig. 2B). In control cells H$_2$S does not appear to play a large role in modulating mitochondrial function and proliferation, while in DS cells, the elevated H$_2$S levels exerts inhibitory effects on mitochondrial function and cell proliferation. Indeed, H$_2$S has a well-documented ability to inhibit mitochondrial Complex IV, leading to the suppression of cellular bioenergetics and cell viability; an action which has been well characterized by earlier environmental toxicological and biochemical studies [3,4,17,18].

Extracellular Flux Analysis studies showed that DS cells exhibited a marked decrease in mitochondrial electron transport, ATP generation and coupling efficiency compared to control cells (Fig. 3A,B,C). In line with the prior analysis [9], this effect is due to the inhibition of mitochondrial Complex IV activity in DS cells (Fig. 3D and E). In control cells AOAA did not affect the bioenergetic parameters, but in DS cells it increased various bioenergetic parameters related to mitochondrial oxidative phosphorylation (Fig. 3). These findings confirm and extend our earlier findings, obtained in comparison with a single pair of control and DS fibroblasts (Detroit 551 vs. 539) [9], and suggest that a reversible inhibition of Complex IV by increased H$_2$S contributes to the mitochondrial and overall bioenergetic deficit. Inhibition of aerobic O$_2$ consumption and coupling efficiency in DS fibroblasts was also reported recently in a study [19] conducted by Anderson and colleagues at the University of Colorado, by comparing 14 healthy and 14 DS fibroblasts (using DS populations that are different from those used in the current study). The effect of AOAA in reversing the bioenergetic suppression in DS cells was somewhat variable across all 8 DS cell lines investigated (Fig. 3), indicative that the CBS/H$_2$S component of metabolic suppression shows individual variation across DS, perhaps as a function of different degree of overexpression of H$_2$S-producing enzymes, and/or individually different counterregulatory and compensatory mechanisms.

When electron flux over the mitochondrial electron transport chain is blocked, electrons often “leak off” from these complexes to react with oxygen to produce various oxidants and free radicals [20]. Indeed, prior studies have already demonstrated an increase in ROS generation in DS cells [21-24]. In line with these observations, DS cells exhibited an intense 2',7’-dichlorofluorescein (DCF) signal brightly colocalized with Mitotracker™ staining, signaling for an increased mitochondrial ROS generation (Fig. 4A and B). There was more mitochondrial DNA damage in DS than in control cells (Fig. 4C), in accordance with prior studies [19, 25, 26] and in line with the sensitivity of the mitochondrial DNA to ROS-mediated oxidative damage [27-29]. In line with its beneficial effects on mitochondrial electron transport, AOAA normalized ROS generation (Fig. 4A and B) and improved mitochondrial DNA integrity (Fig. 4C) in DS cells.

In order to obtain further insight into the metabolic alterations associated with DS, we next employed fluxomics analysis. Addition of $^{13}$C-labeled glucose to cell culture medium leads to time-dependent incorporation of $^{13}$C into various metabolic intermediates and allows

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**Fig. 1.** DS is associated with CBS and 3-MST overexpression in human dermal fibroblasts. (A) & (B): Protein expression of the H$_2$S-producing enzymes – CBS and 3-MST, and of the H$_2$S-catabolizing enzymes ETNE1, SQR, and TST, as quantified by immunoblotting. β-actin served as loading control for densitometry. (C): Representative images of the fluorescent signal of the H$_2$S-specific probe, AzMC along with (D) its quantification under baseline (untreated) conditions and following 24-h treatment with 3 μM AOAA. (E): Quantification of cellular H$_2$S levels using a second H$_2$S probe, P3. (F): Correlation between CBS and 3-MST enzyme expression patterns in DS. Each line and bar graph represents the mean ± SEM of n = 8 human euploid control fibroblasts and n = 8 DS fibroblasts. C1–C8 and D1–D8 corresponds to the specific donors listed in Table 1. Dotted connecting lines in the bar graphs indicate the same cell from a specific donor with/without AOAA treatment. **p ≤ 0.01 DSC indicates significant differences between untreated vs. CC untreated; **p ≤ 0.01 indicates significant differences between DSC + AOAA vs. DSC untreated. The immunoblot for 3-MST along with the corresponding loading control has been previously published [13] and is reused with permission and according to the Open Access Policy of the Journal.
the detection of metabolic fluxes into various core bioenergetic pathways – including glycolysis, Krebs cycle and the pentose phosphate shunt [30]. We have observed marked differences between control and DS cells that are consistent with a suppression of mitochondrial electron transport demonstrated above. Importantly, DS cells, in comparison to control cells, exhibited a marked suppression of 13C fluxes into the Krebs cycle (Figs. 5–7). At the same time, a significant (likely compensatory) increase in carbon fluxes was observed into glycolysis metabolites (Figs. 5–7). The increased glycolytic activity of DS cells was evidenced by increased levels of the isotopologues of glycerol-3-phosphate (G3P), 3-phosphoglycerate (3-PG), dihydroxyacetone phosphate (DHAP), 2-phosphoglycerate (2-PG), phosphoenolpyruvate (PEP), and pyruvate.

In healthy cells with intact mitochondrial electron transport and in the presence of sufficient O2 supply, pyruvate enters the Krebs cycle. However, when the Krebs cycle is inhibited, it is reduced to lactate. The increased levels of lactate isotopologues in DS cells (Fig. 5) indicates that DS cells are in a pseudohypoxic state, a finding already predicted by a prior meta-analysis [3]. The increased flux of glucose-derived 13C carbons into lactate shown in the current study is consistent with biochemical and clinical observations demonstrating an increased lactate production and a decreased exercise tolerance in DS individuals [1,2].

In contrast to the enhanced glycolytic fluxes, many Krebs cycle metabolites (citrate, cis-aconitate, alpha-ketoglutarate, malate) exhibited reduced fluxes in DS cells (Fig. 7). These data indicate that in DS cells the mitochondrial electron transport chain’s activity is suppressed, and, consequently, the generation of electron donors to feed this activity (the fundamental function of the Krebs cycle) is consequently also decreased. Although glycolysis is a less efficient way to generate ATP than oxidative phosphorylation (2 molecules vs. 36 molecules of ATP per glucose consumed), the speed of glycolysis (i.e. the number of glucose molecules utilized over a given time) is substantially faster than that of oxidative phosphorylation [31,32]. We believe that this is the reason why cellular energy charge is not more drastically depleted in DS cells, and the likely reason while the DS cells, in fact, are able to maintain fundamental baseline cellular functions, such as the maintenance of membrane potential and cell division. We hypothesize that the above shift from oxidative phosphorylation to glycolysis may explain why DS individuals exhibit reduced exercise tolerance [1,2]; for a cell that is “fueled” by a large part by glycolysis, it is difficult to mobilize additional ATP generation to meet sudden increases in cellular metabolic demand.

Interestingly, DS was associated with a significant increase in 13C...
upregulation of the pentose-phosphate pathway (PPP) in DS (Fig. 6). Via fluxes into ribose/ribulose-5-phosphate (Ri/Ru5P), indicative of an

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or forced overexpression can induce a metabolic shift into the PPP [33, 34]. Moreover, the fluxomic analysis revealed a DS-associated increase in the
drial electron transport by excess H2S. Therefore, pyruvate does not enter the Krebs cycle and essentially shuts it off (thus, there is a decrease in all metabolites of the cycle). CBS inhibition “lifts” the H2S-mediated inhibition of Complex IV, restores mitochondrial electron transport; consequently, the Krebs-cycle-derived electron donors are, once again, utilized normally. Thus, the activity of the Krebs cycle, and the entry of pyruvate into the Krebs cycle are restored. Interestingly, in a previous study [35], in MIN6 cells subjected to the endoplasmic reticulum stress inducer thapsigargin, inhibition of cellular H2S biosynthesis also attenuated the stress-induced increases in 13C fluxes into lactate, while – again, similar to our findings – inhibition of cellular H2S biosynthesis also restored the suppressed 13C fluxes through the Krebs cycle.

AOAA treatment of DS cells also normalized the DS-associated upregulation of 13C flux into the proximal (oxidative) phase of PPP and normalized the DS-associated increase in the 13C fluxes into glycerol-3-phosphate (G3P) from DHAP (Fig. 6). Treatment of DS cells with AOAA produced an additional fluxomic response with potential biological significance: a partial restoration of the DS-associated suppression of the
donors of homocysteine from the methionine cycle, such as glutathione, in healthy control cells. While the biochemical mechanism underlying this effect remains to be further explored, we hypothesize that via this effect CBS inhibition may improve the activity of various NAD+-dependent enzymes in DS.

AOAA in DS cells increased 13C flux into glutamate, but a similar AOAA-induced increase was also observed in normal control cells (Fig. 7). Glutamate has important roles both as a metabolic factor, a building block of protein synthesis, as well as an excitatory amino acid,
and the depletion of which has been implicated in the pathogenesis of DS [36].

AOAA in DS cells also increased $^{13}$C flux into the pentose phosphate pathway intermediate sedoheptulose 7-phosphate (Fig. 8). DS cells exhibited increased $^{13}$C fluxes into hypoxanthine; an effect, which was normalized by AOAA treatment (Fig. 8). Interestingly, in DS cells, $^{13}$C flux into the oncometabolite 2-hydroxyglutarate (2-HG) was lower than in control cells; this alteration was also restored by AOAA (Fig. 8). Since 2-HG is produced by isocitrate dehydrogenase (ICDH) and other Krebs cycle enzymes, the inhibition of these enzymes in DS, and the reactivation of the Krebs cycle after CBS inhibition would be consistent with the observed alterations in cellular 2-HG levels in DS. The marked increase in ADP in DS (Fig. 8) was also corrected by AOAA; it is conceivable that DS cells are using ATP at a faster rate than the rate at which the cells are able to replenish them.

The above results indicate that DS cells exhibit a significant misalignment of biochemical pathways downstream from glucose, and CBS inhibition – with a consequent normalization of cellular H$_2$S levels – can prevent correct many of these biochemical alterations. Thus, the current findings would predict that CBS inhibition may provide multiple metabolic and cellular functional benefits in DS. The current fluxomic findings are in partial agreement with recent fluxomic studies recently reported by Anderson and colleagues [37] which were conducted predominantly in fibroblasts obtained from newborn or young donors [13]. Similar to our results, the Anderson study also reported (a) a lower $^{13}$C flux into alanine in DS than in control cells; (b) an increase in various pentose phosphate isobars, which is consistent with our observation of higher Ri/RuSy $^{13}$C flux in DS cells than in control cells, confirming a higher flux into the oxidative phase of the PPP in DS than in control (see also above); (c) a tendency for lower sedoheptulose 7-phosphate fluxes in DS cells than in control cells, (d) a tendency for lower malate levels in DS cells, perhaps indicative of alterations in the aspartate/malate shuttle in DS; (e) a tendency for lower a-ketoglutarate levels in DS cells than controls and (f) a lower alanine levels in DS cells than in controls. However, in contrast to our findings, in the adult DS fibroblast populations, the Anderson study revealed no significant differences betweens control and DS cells (but a tendency for higher fluxes in DS) for hexose phosphates, and no differences in the $^{13}$C fluxes into pyruvate were noted between control and DS cells. Importantly, the Anderson study did not directly evaluate carbon fluxes into lactate, and thus it did not directly assess potential shifts between glucose utilization by the Krebs cycle and glycolysis.

Another recent study, utilizing neurospheres derived from embryonic Ts1Cje mouse model of DS (vs. wild-type controls) also reported significant changes in fundamental cellular metabolic pathways and marked alterations in substrate utilization in DS cells [37]. These alterations included an overall suppression of glucose utilization, perturbation in the PPP, lower metabolism of glucose-6-phosphate and various other alterations [37].

Control and DS cells (with and without CBS inhibitor treatment) were also subjected to a global untargeted proteomic analysis. This analysis detected many enzymes involved in the interconversion of many of the above analyzed metabolites – as well as a variety of additional proteins, including many enzymes involved in the multitude of metabolic and other cellular processes. The massive differences in the proteome of DS cells vs. control cells are shown in Suppl. Table 1. Similar to prior proteomic analyses of DS cells and tissues [38–40], our analysis demonstrated marked alterations in the proteome of DS cells, affecting, among others, various metabolic pathways, cellular trafficking, DNA structure, stress response, cytoskeleton network, and multiple signaling pathways (Figs. 9–11). In total, 5538 proteins have been reproducibly detected. Approximately 11% of these proteins were significantly different between control and DS group at the significance level of p < 0.05; approximately 56% of these proteins were upregulated and 44% downregulated in DS cells compared to control cells (Fig. 10, Suppl. Table 1). Many of the proteins upregulated in DS were located on other chromosomes, in line with prior observations [40,41].

However, the proteomic analysis did not reveal very significant differences in the expression of enzymes involved in the regulation of central carbon metabolism, yielding the overall conclusion is that the
changes that we observe in terms of bioenergetics and fluxomics are not principally driven by changes in rate limiting enzyme levels, but, rather, by functional biochemical effects, which may be driven by mechanisms that regulate the catalytic activity, rather than the expression level of these proteins. Among many mediators, H$_2$S can induce such effects, for instance through persulfidation of protein cysteines or through its action on metal-containing active centers \[4\]. For instance, in cancer cells, H$_2$S has been shown to drive SQR-dependent respiration causing a reversal of the TCA cycle, glutamine deprivation, increased glycolysis and lipogenesis – effects that are not ultimately linked to altered expression of SQR protein \[42\]. In other cell types – e.g. DS cells – excess H$_2$S can inhibit mitochondrial Complex IV activity \[8\] (see also above) without affecting the expression level of Complex IV (or of other electron transport proteins) \[8\].

Hexokinases are key enzymes which catalyze the conversion of glucose into glucose-6-phosphate. However, hexokinase-1 and hexokinase-2 expression levels were unaffected by DS or AOAA (Table 2). Previous studies \[38\] have demonstrated increases in hexokinase activity in DS; thus, it is likely that this enzyme is activated in DS via some post-transcriptional modification. At the next step of glycolysis, the conversion of glucose-6-phosphate to fructose-6-phosphate is catalyzed by glucose-6-phosphate isomerase. The group of Lubec has previously reported a downregulation of the mRNA for this enzyme in DS brain \[43,44\]; in our proteomic analysis this enzyme was not differentially expressed between control and DS cells (Table 2). Phosphofructokinase-1 (PFK-1) is one of the most important regulatory enzymes of glycolysis. Since this enzyme is encoded on Chromosome 21, in DS, a gene dosage effect was expected and indeed, a statistically significant (approx. 15–25%) upregulation was detected (Table 2). Prior studies have already recognized the functional role of this alteration in DS \[45\]. However, PFK-1 expression was not inhibited by AOAA in DS cells (Table 2). Thus, the effect of AOAA on glycolytic carbon fluxes in DS is unlikely to be due to the regulation of the expression levels of this enzyme. Fructose-bisphosphate aldolase (ALDO) is the next enzyme in glycolysis, which metabolizes fructose 1,6-bisphosphate. The fluxomic analysis revealed higher $^{13}$C fluxes into DHAP in DS cells than in control cells (Fig. 6). However no changes were noted at the expression levels of ALDO (Table 2). Interestingly, ALDO has recently been shown to be sulfhydrated by H$_2$S in endothelial cells, possibly representing an activating modification \[46\]. Thus, a stimulatory effect of H$_2$S on this enzyme in DS is possible (but remains to be studied in the future). In the 6th step of glycolysis, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) catalyzes the conversion of G3P to $\Delta$-glycerate 1,3-bisphosphate, which, is converted to 1,3-bisphospho-$\Delta$-glycerate and, further, by 3-phosphoglycerate kinase (PGK) to 3-phosphoglycerate and subsequently to 2-phosphoglycerate by phosphoglycerate mutate.
Influx of \( ^{13}C \) into 3-phosphoglycerate were found to be stimulated in DS and this effect tended to be attenuated by AOAA (Fig. 7). In our proteomic analysis, GAPDH expression was found to be 18% lower and PGM expression was 9% higher in DS than control cells but these changes were not affected by AOAA (Table 2). Importantly, the activity of GAPDH can be readily affected post-transcriptionally, i.e. without any change in absolute protein levels. In fact, there are several studies implicating the regulation of GAPDH via \( H_2S \)-mediated sulfhydration \[47, 48\]. Thus, the DS-associated stimulation of glycolysis (at least in part) may involve \( H_2S \)-induced GAPDH sulfhydration, but this possibility remains to be directly investigated in the future.

Enolase (ENO, also known as phosphopyruvate hydratase) is a metalloenzyme responsible for the catalysis of the conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP) in the ninth and penultimate step of glycolysis. In our experiments, \( ^{13}C \) flux into pyruvate was significantly higher in DS than in control cells but these changes were not affected by AOAA (Table 2). Importantly, the activity of GAPDH can be readily affected post-transcriptionally, i.e. without any change in absolute protein levels. In fact, there are several studies implicating the regulation of GAPDH via \( H_2S \)-mediated sulfhydration \[47, 48\]. Thus, the DS-associated stimulation of glycolysis (at least in part) may involve \( H_2S \)-induced GAPDH sulfhydration, but this possibility remains to be directly investigated in the future.

Enolase (ENO, also known as phosphopyruvate hydratase) is a metalloenzyme responsible for the catalysis of the conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP) in the ninth and penultimate step of glycolysis. In the final step, in turn, PEP is catalyzed into pyruvate by pyruvate kinase (PK, isoforms M1 and M2). The latter enzyme is subject to multiple levels of regulation, including potential upregulation or activation by \( H_2S \) \[49\]. In the current experimental system, however, neither DS nor AOAA affected the protein level of enolase and pyruvate kinase (PK) M1/M2 (Table 2), which – similar to the case of many other enzymes discussed in earlier paragraphs – does not necessarily mean that the catalytic activity of these enzymes remained unaffected.

The conversion of pyruvate to lactate is catalyzed by lactate dehydrogenase (LDH). In our experiments, \( ^{13}C \) flux into lactate was significantly higher in DS than in control cells but these changes were not affected by AOAA (Table 2). Importantly, the activity of GAPDH can be readily affected post-transcriptionally, i.e. without any change in absolute protein levels. In fact, there are several studies implicating the regulation of GAPDH via \( H_2S \)-mediated sulfhydration \[47, 48\]. Thus, the DS-associated stimulation of glycolysis (at least in part) may involve \( H_2S \)-induced GAPDH sulfhydration, but this possibility remains to be directly investigated in the future.
Fig. 8. DS cells exhibit marked dysregulation of metabolism compared to healthy control cells; effect of AOAA. Various isotopologues (e.g. amino acids, lipids and additional metabolic intermediates) detected by fluxomic analysis are shown in n = 8 human euploid control fibroblasts and n = 8 DS fibroblasts, as summarized in Table 1, with/without AOAA treatment (3 μM). *p ≤ 0.05 and **p ≤ 0.01 indicate significant differences between DSC untreated vs. CC untreated; #p ≤ 0.05 and ##p ≤ 0.01 indicate significant differences between CC + AOAA vs. CC untreated or DSC + AOAA vs. DSC untreated.

Although the fluxomics analysis showed major changes in Krebs cycle metabolites, none of the associated enzymes showed different expression levels between control and DS (Table 2). The only exception was isocitrate dehydrogenase 1 (ICDH1), which exhibited a slight upregulation in DS, which was partially attenuated by CBS inhibition; and malate dehydrogenase 1 (MDH-1), which was slightly elevated in DS cells compared to controls, but this change was unaffected by CBS inhibition. It should be pointed out, however, that changes in the protein level of Krebs cycle enzymes (or enzymes of the mitochondrial electron transport chain, or changes in the expression level of various ATP synthase complex components) cannot translate into functional differences, if the mitochondrial electron transport chain is blocked due to the inhibitory effect of H2S on mitochondrial Complex IV. In this case, the utilization of electron donors by the mitochondria is blocked. Thus, the metabolic processes that serve to produce these donors are functionally suppressed (“backed up”) regardless of the expression level of the proteins involved in the interconversion of the various Krebs cycle metabolites. Nevertheless, it is possible that compensatory changes in the expression of various Krebs cycle or mitochondrial enzymes that would happen in the above scenario, in turn, result in altered metabolic shifts after the reactivation of the electron transport chain – i.e. when pharmacological inhibition of CBS “lifts” the inhibition of mitochondrial Complex IV by H2S.

From the DS-associated alterations in pyrimidine and purine synthesis, one of the most interesting observations of the current results was the change in [13C] flux into de novo NAD+ biosynthesis, because this flux was suppressed by DS and restored by CBS inhibition (Fig. 6). Mammalian NAD+ synthesis occurs through de novo and salvage pathways. In mammalian cells, 90% of free tryptophan is metabolized through the kynurenine pathway, leading to the de novo synthesis of NAD+ [51]. The three different salvage pathways start either from nicotinamide, nicotinic acid, or nicotinamide riboside, with nicotinamide being the major source for the biosynthesis of NAD+ in most mammalian cells [51]. Most of these pathways are not “visible” from our current fluxomic analysis. Nevertheless, the pathway by which NAD+ is synthesized from ribulose 5-phosphate is detectable. This pathway involves multiple steps, including the action of the ATP-consuming enzyme phosphoribosyl pyrophosphate synthetase (PRPS) as a proximal step, as well as a further step that involves the incorporation of nicotinamide via multiple enzymatic steps [51]. Our current fluxomic analysis did not measure PRPP, but prior studies in DS erythrocytes have demonstrated a slight decline in the cellular concentration of this metabolite [52]. On the protein level, neither PRPS1 or PRPS2 levels were affected by DS. Nevertheless – since the activity of these enzymes is ATP dependent – it is conceivable that an overall improvement of cellular bioenergetic status (see Figs. 1–3) after CBS inhibition may also produce an enhancement of PRPP levels and a consequent stimulation of NAD+ biosynthesis.

Taken together, the experiments presented and discussed above have identified significant shifts in central carbon metabolism in DS, and demonstrated that the CBS inhibitor AOAA can revert some of these alterations. However, the fluxomic alterations, for the most part, are not driven through changes in the expression of various central carbon metabolism enzymes – although post-transcriptional regulation of some of the enzymes involved in these processes, via H2S-mediated sulfhydration remains a possibility. The DS-associated fluxomic shifts central carbon metabolism, and the effects of AOAA on some of these alterations are summarized in Fig. 12.

Some additional proteomic changes that are worth mentioning from a bioenergetic/metabolic/mitochondrial standpoint, are the DS-associated upregulation of phosphofructokinase (PFK) and maltate
The current study has several limitations, stemming from a combination of factors. First of all, the study investigates a very complex pathophysiological condition. Second, we attempted to determine the role of another very complex system, the CBS/H2S pathway. Third, we have used a selection of control and DS samples, each from a different human subject (different sex, age). Fourth, the CBS inhibitor used in the current study has some selectivity/specificity issues.
With respect to the complexity of DS, the topic has already been highlighted in many of the prior sections. DS represents with a combination of "gene dosage effects" (producing an upregulation of genes encoded on Chromosome 21) and secondary, compensatory and/or 'domino effects' on other signaling pathways and other proteins and effector pathways (affecting gene products encoded on all other chromosomes). These processes culminate in the dysregulation of thousands of genes and proteins in an age- and cell type-specific fashion. The field has come a long way from the "Lejeune Machine" [69,70], which was an early attempt to integrate some of the pathobiochemical alterations in DS. Nevertheless, it is clear that not only CBS, but also 3-MST is upregulated in DS, and it is likely that both of these species contribute to the biochemical alterations we have observed.

With respect to the complexity of the CBS/H$_2$S pathway, we are facing another level of challenge. CBS, as an essential element of the reverse transsulfuration pathway, is intrinsically linked to the metabolism and interconversion of sulfur-containing amino acids, but also regulates many other metabolites including S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), glutathione (GSH) and others. It is also interlinked with the 3-MST system, which utilizes the 3-mercaptopyruvate generated by cysteine aminotransferase (CAT) by forming a persulfide on its active site ($R–SH$ to $R–SSH$). The persulfide, in turn, releases H$_2$S in the presence of a reductant ($R′–SH$), but 3-MST is also a prominent source of polysulfides, which have biological regulatory roles that are biochemically different from those of H$_2$S [71–73]. Since not only CBS, but also 3-MST is upregulated in DS, it is clear that DS is associated with a combined overproduction of H$_2$S as well as polysulfides, and it is likely that both of these species contribute to the biochemical alterations we have observed.
Table 2
Expression levels of enzymes involved in the interconversion of isopentoliques detected in the fluxomic analysis. Enzyme levels were determined by proteomic analysis and subjected to Log2 normalization. *p ≤ 0.05 and **p ≤ 0.01 indicate significant differences between DSC untreated vs. CC untreated; *p ≤ 0.05 indicates significant differences between CC + AOA vs. CC untreated or DSC + AOA vs. DSC untreated.

| Enzyme          | Pathway     | CC     | CC + AOA | DSC     | DSC + AOA |
|-----------------|-------------|--------|----------|---------|-----------|
| Hexokinase 1    | Glycolysis  | 13.55  | ± 0.03   | 13.53   | ± 0.05    |
|                 |             | ± 13.52| ± 0.05   | ± 13.52| ± 0.05    |
| Hexokinase 2    | Glycolysis  | 11.86  | ± 0.05   | 11.76   | ± 0.05    |
|                 |             | ± 11.73| ± 0.06   | ± 11.73| ± 0.06    |
| glucose-6-phosphate isomerase | Glycolysis | 13.57 | ± 0.16  | 13.70   | ± 0.10 |
|                 |             | ± 13.63| ± 0.12   | ± 13.63| ± 0.12    |
| Phosphofructokinase-1, muscle type | Glycolysis | 11.36 | ± 0.04  | 11.53   | ± 0.02 |
|                 |             | ± 11.54| ± 0.03   | ± 11.54| ± 0.03    |
| Phosphofructokinase-1, liver type | Glycolysis | 12.46 | ± 0.04  | 12.78   | ± 0.02 |
|                 |             | ± 11.87| ± 0.03   | ± 11.87| ± 0.03    |
| Fructose-bisphosphate aldolase | Glycolysis | 10.98 | ± 0.09  | 10.83   | ± 0.10 |
|                 |             | ± 10.81| ± 0.11   | ± 10.81| ± 0.10    |
| Glyceroldehyde 3-phosphate dehydrogenase (GAPDH) | Glycolysis | 12.59 | ± 0.09  | 12.31   | ± 0.07 |
|                 |             | ± 12.05| ± 0.04   | ± 12.05| ± 0.07    |
| 3-phosphoglycerate kinase (3-PGK) | Glycolysis | 14.38 | ± 0.06  | 14.41   | ± 0.07 |
|                 |             | ± 14.39| ± 0.07   | ± 14.39| ± 0.07    |
| Phosphoglycerate mutase (PGM) | Glycolysis | 13.43 | ± 0.04  | 13.56   | ± 0.03 |
|                 |             | ± 13.53| ± 0.03   | ± 13.53| ± 0.03    |
| Enolase 1 (ENO-1) | Glycolysis | 15.73 | ± 0.06  | 15.69   | ± 0.08 |
|                 |             | ± 15.65| ± 0.03   | ± 15.65| ± 0.04    |
| Enolase 2 (ENO-2) | Glycolysis | 11.39 | ± 0.06  | 11.37   | ± 0.07 |
|                 |             | ± 11.40| ± 0.06   | ± 11.40| ± 0.06    |
| Pyruvate kinase M1/M2 | Glycolysis | 15.69 | ± 0.04  | 15.74   | ± 0.04 |
|                 |             | ± 15.75| ± 0.03   | ± 15.75| ± 0.03    |
| Lactate dehydrogenase (LDH) A | Glycolysis | 14.90 | ± 0.04  | 14.96   | ± 0.04 |
|                 |             | ± 14.92| ± 0.03   | ± 14.92| ± 0.03    |
| Lactate dehydrogenase (LDH) B | Glycolysis | 13.48 | ± 0.04  | 13.38   | ± 0.05 |
|                 |             | ± 13.35| ± 0.06   | ± 13.35| ± 0.06    |
| Pyruvate dehydrogenase | Glycolysis | 11.40 | ± 0.04  | 11.34   | ± 0.03 |
|                 |             | ± 11.33| ± 0.03   | ± 11.33| ± 0.03    |
| El subunit alpha | Krebs cycle | ± 0.03 | ± 0.03  | ± 0.03 | ± 0.02 |
| Pyruvate dehydrogenase component X | Glycolysis | 11.82 | ± 0.06  | 11.81   | ± 0.05 |
|                 |             | ± 11.80| ± 0.05   | ± 11.80| ± 0.05    |
| Citrate synthase | Krebs cycle | 12.60 | ± 0.03  | 12.59   | ± 0.05 |
|                 |             | ± 12.57| ± 0.03   | ± 12.57| ± 0.05    |
| Aconitase 1      | Krebs cycle | 12.47 | ± 0.08  | 12.43   | ± 0.06 |
|                 |             | ± 12.45| ± 0.07   | ± 12.45| ± 0.06    |
| Aconitase 2      | Krebs cycle | 12.51 | ± 0.03  | 12.47   | ± 0.05 |
|                 |             | ± 12.42| ± 0.02   | ± 12.42| ± 0.05    |
| Isocitrate dehydrogenase (ICDH) 1 | Krebs cycle | 13.13 | ± 0.06  | 13.33   | ± 0.27 |
|                 |             | ± 13.27| ± 0.06   | ± 13.27| ± 0.05    |
| Isocitrate dehydrogenase (ICDH) 2 | Krebs cycle | 12.53 | ± 0.09  | 12.47   | ± 0.09 |
|                 |             | ± 12.70| ± 0.09   | ± 12.70| ± 0.08    |
| α-ketoglutarate dehydrogenase | Krebs cycle | 12.39 | ± 0.05  | 12.45   | ± 0.06 |
|                 |             | ± 12.44| ± 0.05   | ± 12.44| ± 0.04    |
| Succinyl-CoA synthetase (S-CoAS) GDP-forming subunit beta | Krebs cycle | 11.02 | ± 0.04  | 10.98   | ± 0.03 |
|                 |             | ± 11.01| ± 0.03   | ± 11.01| ± 0.04    |
| Succinyl-CoA synthetase (S-CoAS) | Krebs cycle | 12.42 | ± 0.03  | 12.38   | ± 0.04 |
|                 |             | ± 12.37| ± 0.04   | ± 12.37| ± 0.04    |
| Succinate dehydrogenase (SDH) complex flavoprotein subunit A | Krebs cycle | 11.72 | ± 0.02  | 11.78   | ± 0.04 |
|                 |             | ± 11.77| ± 0.04   | ± 11.77| ± 0.05    |
| Succinate dehydrogenase (SDH) complex subunit B | Krebs cycle | 11.36 | ± 0.02  | 11.34   | ± 0.03 |
|                 |             | ± 11.41| ± 0.03   | ± 11.41| ± 0.03    |

(3) With respect to complexity of DS, age of the cells studied represents an important additional factor. DS is associated with important developmental misalignments, and it is clear that the pathobiocchemistry of DS changes as the individual ages. The age-dependent differences in metabolic processes have recently been highlighted by the recent report by Anderson and co-workers [19]. In the current study, we have studied fibroblasts obtained from 8 healthy controls – which are fairly uniform in terms of their central carbon fluxomic signatures and fibroblasts from 8 DS individuals, mainly newborn or young donors – which are presenting with a larger heterogeneity in their central carbon fluxomic signatures. Combining the cellular responses from these various individuals will, on one hand, increases the “noise” in the system, but on the other hand, improves the biological validity and significance of the experimental findings. In this respect, the DS-associated changes in CBS expression, changes in mitochondrial function and changes in central carbon metabolism appear to be fairly uniform; with the vast majority of samples showing, directionally, the same type of responses (see Figs. 1–8). Nevertheless, individualized analysis can also identify outliers; for example, 1 of 8 subjects in DS did not have higher CBS levels in their fibroblasts than the average expression level of all controls; similarly, 3-MST did not appear to be upregulated in 2 of 8 samples (Fig. 1); 2 DS fibroblasts have not exhibited the suppression of mitochondrial O2 consumption and the reduction of Complex IV activity that was noted in the rest of the samples (Fig. 3). In contrast, the increase in H2S generation was a uniform response in all DS cells (Fig. 1D and E). Thus, all DS cells appear
(4) Finally, we should briefly consider the limitations of the pharmacological agent, AOAA. This molecule has been widely used in the literature to inhibit the catalytic activity of CBS in order to study various processes ranging from cancer metabolism to the neurotransmission, vascular function, hypoxia sensing and others, principally due to the fact that available pharmacological options to inhibit CBS are very limited [3, 4, 34]. However, the effects of AOAA, as far as the modulation of H₂S homeostasis is concerned, go beyond CBS; this compound, as a broad inhibitor of PLP-dependent enzymes, as well as an inhibitor of another H₂S-generating enzyme, CSE [74, 75]. To our knowledge, the relative effect of AOAA on CSE-vs. CBS-derived H₂S production has not yet been determined in cell-based models. However, there is no indication that CSE would be upregulated in DS or that CSE would contribute to the pathogenesis of DS. It should also be pointed out that the concentration of AOAA used in our studies (3 μM) is relatively low, compared to a variety of prior in vitro studies in various models. At this concentration, inhibition of CBS (which is potently inhibited by AOAA) is expected, but significant inhibition of many other PLP-dependent enzymes (on which AOAA is less potent [75]) is less likely. Importantly, the functional and proteomic results presented in the current study both demonstrated that the pharmacological effects of 3 μM AOAA were more pronounced and, in most cases, directionally different in DS cells than in the control cells. It should be also pointed out that in a subset of the cells studied in the current report (Detroit 551 control cells vs. Detroit 539 DS cells; designated as “C5” and “D5”, respectively), the effect of AOAA (3 μM) has already been compared to siRNA-mediated CBS silencing on mitochondrial O₂ consumption and Complex IV activity, and the effects were comparable [9]. Importantly, AOAA has also been shown to exert beneficial effects in a rat model of DS in vivo [8]. All of the above considerations are consistent with the suggestion that in DS in general, and in the current experimental design in particular, CBS is a principal pharmacological target of AOAA. Nevertheless, the current results should be further extended in future studies using genetic or alternative pharmacological approaches.

4. Conclusions

The current report demonstrates fundamental changes in the central carbon metabolism of human DS fibroblasts, that are consistent with the presence of pseudohypoxia, in line with multiple lines of prior observations. There is a blockade of mitochondrial electron transport and aerobic ATP formation in DS cells, which is compensated by a switch to glycolysis. These changes in DS cells are, to a significant part, due to H₂S overproduction in DS, which is generated by CBS in these cells (which is upregulated due to gene dosage effect). The functional consequences of the H₂S-driven pseudohypoxia in DS include a reduction in cellular proliferation rate (Fig. 2) and a reduction in the ability of the cells to process proteins, i.e. a dysregulated autophagy response (Fig. 13); these alterations can be reversed by treatment of the cells with AOAA. In vivo, the consequence of a CBS/H₂S-mediated pseudohypoxia in DS can include impairments in neuronal metabolic activity, neurotransmission, neuronal electrical activity generation, and neurobehavioral function and memory generation; according to recent studies, these alterations...
can also be reversed by genetic correction or pharmacological inhibition of CBS [7–9].

The fluxomic studies presented in the current report indicate that inhibition of H2S overproduction in DS cells improves aerobic, mitochondrial ATP generation and de novo NAD+ biosynthesis; the same intervention also counteracts ROS overproduction and autophagic machinery defects. These effects are expected to fundamentally improve multiple cell functions, which, in turn, would be expected to yield improved cell viability, improved cell differentiation and cellular repair processes, improved processing of proteins (which, in the longer term, may counteract the accumulation of misfolded proteins, a hallmark and key pathophysiological event in various forms of neurodegeneration). All of these findings strengthen the 20-year-old hypothesis (the so-called “Kamoun hypothesis” [5]) that H2S overproduction, via a metabolic inhibitory effect plays a pathogenetic role in DS and support the concept of pharmacological inhibition of CBS as a potential future experimental therapeutic approach in DS.

Ethics approval and consent to participate

The studies are based on publicly available human cell types and did not require additional ethic committee approval.

Consent for publication

The paper does not contain any individual person’s data in any form.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author, C.S. upon reasonable request.

Fig. 13. Pharmacological inhibition of CBS with AOAA attenuates the DS-associated down-regulation of autophagy-related (Atg) components for the formation and maturation of the autophagosome. (A): schematic summary of the pathways involved; (B): representative Western blots; (C): Quantification of the protein expression of ATG7, phosphorylated beclin-1 (pBeclin-1) at Ser15, ATG3, and the lipated, autophagosome-associated LC3-II. β-Actin served as loading control for densitometry. Each bar graph represents the mean ± SEM of n = 8 human euploid control fibroblasts and n = 8 DS fibroblasts. C1–C8 and D1-D8 corresponds to the specific donors listed in Table 1. Dotted connecting lines in the bar graphs indicate the same cell from a specific donor with/without AOAA treatment. *p ≤ 0.05, **p ≤ 0.01 DSC indicates significant differences between DSC untreated vs. CC untreated; #p ≤ 0.05, ##p ≤ 0.01 indicates significant differences between DSC + AOAA vs. DSC untreated.

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Authors’ contributions

Experimental design: TP, LP, EBR, AIN, CS. Experimentation: TP, LP, EBR, AIN. Data analysis: TP, LP, EBR, AIN. Manuscript writing: TP, LP, EBR, AIN, CS.

Declaration of competing interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2022.102416.

List of abbreviations

α-KG α-ketoglutarate
α-KGDH α-ketoglutarate dehydrogenase
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