



# Overproduction of H<sub>2</sub>S, generated by CBS, inhibits mitochondrial Complex IV and suppresses oxidative phosphorylation in Down syndrome

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**Down syndrome (DS) is associated with significant perturbances in mitochondrial function. Here we tested the hypothesis that the suppression of mitochondrial electron transport in DS cells is due to high expression of cystathionine-β-synthase (CBS) and subsequent overproduction of the gaseous transmitter hydrogen sulfide (H<sub>2</sub>S). Fibroblasts from DS individuals showed higher CBS expression than control cells; CBS localization was both cytosolic and mitochondrial. DS cells produced significantly more H<sub>2</sub>S and polysulfide and exhibited a profound suppression of mitochondrial electron transport, oxygen consumption, and ATP generation. DS cells also exhibited slower proliferation rates. In DS cells, pharmacological inhibition of CBS activity with aminooxyacetate or siRNA-mediated silencing of CBS normalized cellular H<sub>2</sub>S levels, restored Complex IV activity, improved mitochondrial electron transport and ATP synthesis, and restored cell proliferation. Thus, CBS-derived H<sub>2</sub>S is responsible for the suppression of mitochondrial function in DS cells. When H<sub>2</sub>S overproduction is corrected, the tonic suppression of Complex IV is lifted, and mitochondrial electron transport is restored. CBS inhibition offers a potential approach for the pharmacological correction of DS-associated mitochondrial dysfunction.**

metabolism | mitochondria | bioenergetics | H<sub>2</sub>S

**D**own syndrome (DS)—trisomy of human chromosome 21 (HSA21)—remains the most common chromosomal disorder (1). It is associated with significant perturbances in many morphological and biochemical features. One of its biochemical hallmarks is mitochondrial dysfunction. Suppression of mitochondrial function—including inhibition of mitochondrial Complex IV—has been documented in cells from DS individuals (DSCs) (2). Complex IV is an essential electron transport component; its best-characterized inhibitors include cyanide (an irreversible inhibitor) and hydrogen sulfide (H<sub>2</sub>S) (a reversible inhibitor) (3).

H<sub>2</sub>S is an endogenous mammalian gaseous mediator, which regulates many cellular processes including mitochondrial function (4). Its effects are often bell shaped: At lower concentrations H<sub>2</sub>S stimulates cellular bioenergetics, while at toxicological concentrations H<sub>2</sub>S suppresses mitochondrial function via inhibition of Complex IV (4). Cystathionine-β-synthase (CBS)—located on HSA21—is a principal enzyme responsible for H<sub>2</sub>S production. Overexpression of CBS (5) and increased levels of the H<sub>2</sub>S metabolite thiosulfate (6) were previously reported in DS. We have now directly tested the hypothesis whether the excess H<sub>2</sub>S produced by CBS is responsible for the suppression of mitochondrial electron transport in DSCs.

DSCs exhibited markedly higher CBS expression than the healthy control cells (CCs). CBS was both cytosolically and mitochondrially localized (Fig. 1A–C). The expression of various H<sub>2</sub>S metabolizing enzymes was unaffected by DS (Fig. 1B). DSCs produced significantly more H<sub>2</sub>S—as well as reactive polysulfides—than CCs (Fig. 1D). The increased CBS expression in DSCs was associated with a profound suppression of cell proliferation, mitochondrial electron transport, oxygen consumption, Complex IV activity, and ATP generation (Fig. 1E–G).

Pharmacological inhibition of CBS with aminooxyacetate (AOAA) (3 μM) (4) normalized H<sub>2</sub>S production (Fig. 1D), restored Complex IV activity (Fig. 1G), improved mitochondrial electron transport (Fig. 1F), and restored DSC proliferation (Fig. 1E). The H<sub>2</sub>S-releasing molecule GYY4137 (4) phenocopied the effect of DS in CCs in terms of bioenergetics and proliferation and reversed the beneficial effects of AOAA in DSCs (Fig. 1E–G). siRNA-mediated silencing of CBS in DSCs recapitulated the effects of AOAA (Fig. 1I–L).

The expression of electron transport chain proteins that are part of Complexes II, III, and IV was increased in DSCs (Fig. 1M), likely representing a compensatory response.

CBS, a key enzyme in the transsulfuration pathway, catalyzes the conversion of homocysteine into cystathionine (7). Lejeune (8) has already hypothesized in the 1990s that the overdosage of CBS may contribute to the metabolic alterations and overall clinical picture in DS. Both the footprint of excess CBS (low homocysteine) and the footprint of mitochondrial inhibition (accumulation of Krebs cycle intermediaries) have been demonstrated by metabolomic analysis of DS subjects (9). Moreover, increased urinary thiosulfate and circulating sulfhemoglobin levels have previously been reported in subjects with DS (6). The current report confirms and extends these findings: DSCs contain higher levels of H<sub>2</sub>S (as well as reactive polysulfides) than CCs.

The hypothesis that CBS-derived H<sub>2</sub>S may be responsible for the metabolic suppression in DS was originally put forward by Kamoun et al. in 2003 (6), but it has not been tested experimentally until now. Our data indicate that CBS-derived H<sub>2</sub>S is, indeed, responsible for the suppression of mitochondrial function in DSCs. When CBS activity or CBS expression is normalized, the H<sub>2</sub>S-mediated tonic suppression of Complex IV is lifted, and the cells regain their ability to perform mitochondrial oxidative phosphorylation (Fig. 1N). In contrast to DSCs, in CCs (which are not under the tonic suppressive effect of high H<sub>2</sub>S), CBS inhibition does not significantly affect proliferation or bioenergetics (Fig. 1N).

H<sub>2</sub>S-mediated suppression of cellular bioenergetics provides a plausible mechanistic explanation for a host of characteristic biochemical and clinical features associated with DS, including the reduced tissue and whole-body O<sub>2</sub> consumption and impaired metabolic fitness—characteristic features of DS (1)—since these processes are directly related to mitochondrial ATP production.

CBS overexpression was recently found to phenocopy the DS-like neurocognitive deficits in mice (10). It is conceivable that

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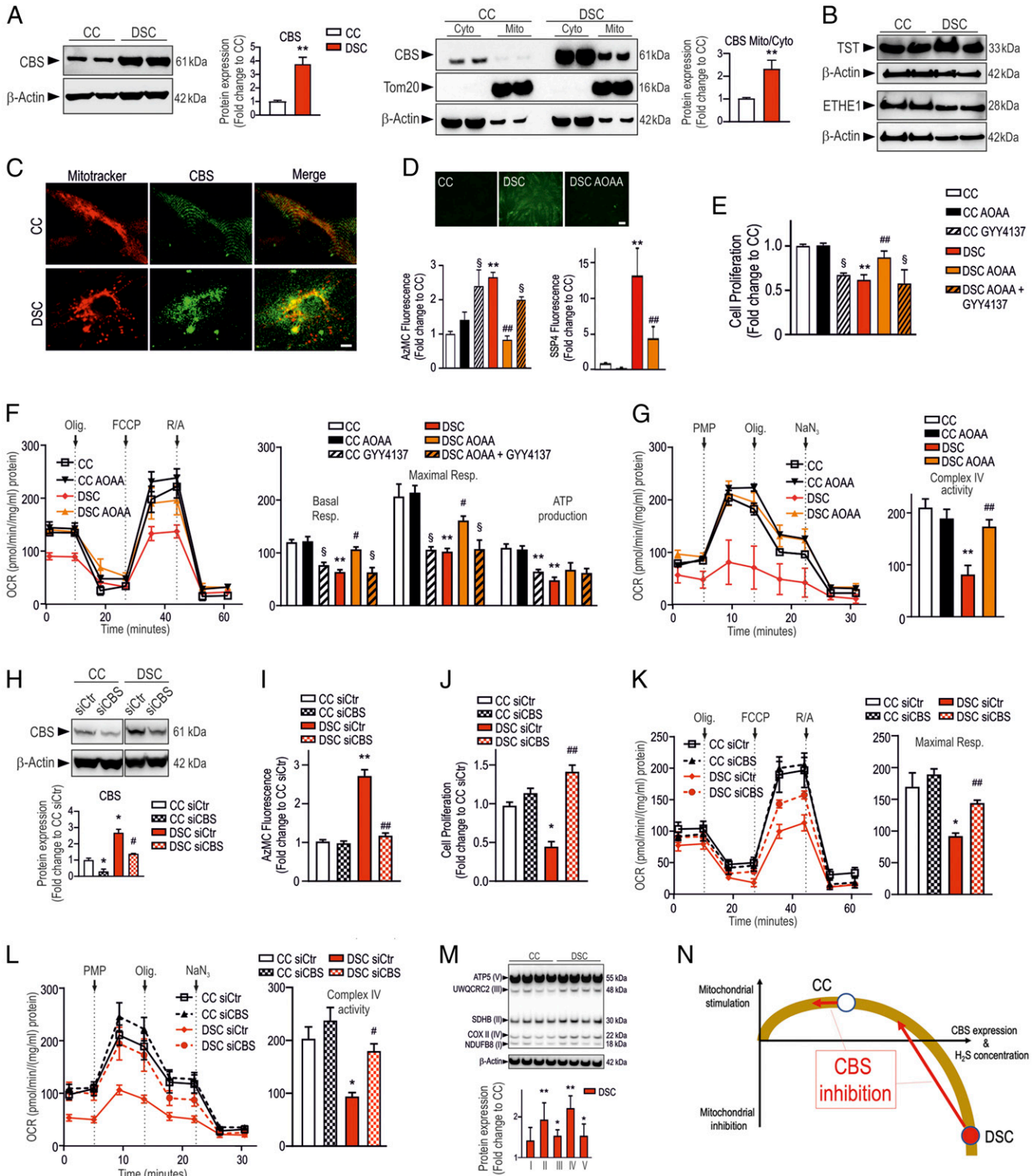
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**Fig. 1.** CBS-derived H<sub>2</sub>S is responsible for the suppression of mitochondrial function in DSCs. DSCs exhibit markedly higher CBS expression—which is, in part, localized to the mitochondria—than healthy CCs, shown by (A) Western blotting and (C) confocal microscopy. (Scale bar: 5  $\mu$ m.) (B) TST and ETHE1 expression was similar in DSCs and CCs. (D) DSCs contain increased amounts of intracellular H<sub>2</sub>S and polysulfide. Top photomicrographs: AzMC-based H<sub>2</sub>S live cell imaging. (Scale bar: 20  $\mu$ m.) (E) DSCs exhibit reduced cell proliferation rate; this is normalized by AOOA (3  $\mu$ M); treatment of CCs with the H<sub>2</sub>S donor GYY4137 (3 mM) phenocopies the inhibition of proliferation seen in DSCs. (F) DSCs exhibit low basal and maximal mitochondrial oxygen consumption rates (OCR); exposure of CCs to GYY4137 phenocopies the metabolic suppression seen in DSCs. (G) Complex IV is blocked in DSCs but it is restored by AOOA. (H–L) siRNA-mediated silencing of CBS in DSCs (H) suppresses H<sub>2</sub>S production (I), normalizes DSC proliferation (J), and enhances OCR (K) and Complex IV activity (L). (M) Increased expression of various mitochondrial proteins belonging to electron chain transport Complexes II, III, and IV in DSCs. (N) Bell-shaped cellular bioenergetic role of H<sub>2</sub>S in DSCs vs. CCs; effect of CBS inhibition. \**P* < 0.05 and \*\**P* < 0.01, significant difference between CCs and DSCs; #*P* < 0.05 and ##*P* < 0.01, significant effect of AOOA in DSCs; \$*P* < 0.05, significant effect of GYY4137 in CCs or significant difference between AOOA and GYY4137+AOOA in DSCs. Data are shown as mean  $\pm$  SEM of at least 3 experiments. One- and 2-way ANOVAs were performed, followed by a post hoc Bonferroni test.

H<sub>2</sub>S overproduction and consequent inhibition of mitochondrial Complex IV explain some of the neurological and neurocognitive deficits associated with DS, because neurons heavily depend on ATP produced by oxidative phosphorylation.

Importantly, H<sub>2</sub>S-mediated inhibition of Complex IV is a reversible biochemical process. Therefore, follow-up studies should be conducted to determine whether pharmacological inhibition of CBS reverses some of the functional defects associated with DS in vivo. Several classes of CBS inhibitors exist that may be useful for such efforts—including AOAA (which has been in clinical trials in the 1970s), various AOAA prodrugs, natural-product CBS inhibitors (apigenin), and benserazide (a clinically approved drug that has a secondary pharmacological effect as a CBS inhibitor) (4).

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## Materials and Methods

Female dermal fibroblasts from control (Detroit 551; ATCC CCL-110) and DS (Detroit 539; ATCC CRL-84) subjects were obtained from LGC Standards and cultured in Advanced DMEM. CBS silencing was achieved by siCBS (SASI\_Hs01\_00214623) (Sigma). Proliferation was quantified by BrdU (11). H<sub>2</sub>S and polysulfide production were measured using 7-azido-4-methylcoumarin (AzMC) (12) and 3',6'-di(O-thiosalicyl)fluorescein (SSP4) (13), respectively. Western blotting was performed using the iBlot 2 Dry Blotting System (11). Mitochondrial localization of CBS was performed by confocal microscopy with an anti-CBS antibody and MitoSOX Red (14) using a Leica TCS SP5 confocal microscope. Bioenergetic measurements were performed by extracellular flux analysis. Complex IV activity was measured in permeabilized cells using ascorbate and TMPD (15).

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