Amino Acid Transport Associated to Cluster of Differentiation 98 Heavy Chain (CD98hc) is at the Crossroad of Oxidative Stress and Amino Acid Availability.

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Running title: CD98 Controls Oxidative Stress and AA Availability

Keywords: SCL3A2/CD98hc/4F2hc, amino acid transport, oxidative stress, cell proliferation, stress response, peptide transport, SLC7 family

ABSTRACT

CD98hc functions as amino acid (AA) transporter (together with another subunit) and integrin signaling enhancer. It is overexpressed in highly proliferative cells, in both physiological and pathological conditions. CD98hc deletion induces strong impairment of cell proliferation in vivo and in vitro. Here, we investigate CD98hc-associated AA transport in cell survival and proliferation. By using chimeric versions of CD98hc, the two functions of the protein can be uncoupled. While recovering the CD98hc AA transport capacity restores the in vivo and in vitro proliferation of CD98hc-null cells, reconstitution of the integrin signaling function of CD98hc is unable to restore in vitro proliferation of those cells. CD98hc-associated transporters (i.e. xCT, LAT1 and y’LAT2 in wild-type cells) are crucial to control reactive oxygen species (ROS) and intracellular AA levels, thus sustaining cell survival and proliferation. Moreover, in CD98hc-null cells the deficiency of CD98hc/xCT cannot be compensated, leading to cell death by ferroptosis. Supplementation of culture media with beta-mercaptoethanol (β-ME) rescues CD98hc-deficient cell survival. In such conditions, null cells show oxidative stress and intracellular AA imbalance and, consequently, limited proliferation. CD98hc-null cells also present reduced intracellular levels of branched-chain AA (BCAA) and aromatic (ARO) AAs and induced expression of peptide transporter 1 (PEPT1). Interestingly, external supply of dipeptides containing BCAAs...
and ARO AAs rescues cell proliferation and compensates for impaired uptake of CD98hc/LAT1 and CD98hc/y+LAT2. Our data establish CD98hc as a master protective gene at the crossroad of redox control and AA availability, making it a relevant therapeutic target in cancer.

Proliferative cells have an increased demand for nutrients such as glucose, AAs, fatty acids, and vitamins. Heteromeric Amino acid Transporters (HATs) are among several families of solute carriers (SLCs) (http://www.bioparadigms.org/slc/intro.htm) that mediate the influx or efflux of solutes (AAs among others) through the plasma membrane of mammalian cells. HATs are composed of a heavy (SLC3 family) and a light (L-type amino acid transporters (LATs) from SLC7 family) subunit, linked by a disulphide bridge (1). The heavy chain carries the complex to the plasma membrane (2), whereas the light chain constitutes the catalytic part of the transporter (3,4). CD98hc (aka SLC3A2, 4F2, FRP1), the only ubiquitously expressed heavy subunit of HATs, can bind to any of six light subunits (LAT1, LAT2, xCT, y+LAT1, y+LAT2 and asc1), which confer substrate specificity to the heterodimer, referred to as CD98 (1). All together these transporters cover a broad substrate spectrum, including all essential amino acids (EAAs). Most HATs are obligatory antiporters, meaning they mediate the simultaneous translocation of two AAs across the membrane in opposite directions (in a 1:1 stoichiometry). As a consequence of this mechanism of transport CD98 heterodimers are unable of net AA import. However, their upregulation can increase the uptake of specific AAs (in exchange with others), rendering the proper AA pool to support cell growth.

Besides its function as transporter, CD98hc behaves as a co-receptor of beta integrins and amplifies their downstream signaling (5,6). A large body of evidence implicates CD98hc in cell proliferation during physiological and pathological conditions (7-9). Furthermore, lack of CD98hc in mouse embryonic stem (ES) cells (in which exon 1, encoding the transmembrane domain of CD98hc, is replaced by a neomycin cassette) blocks cell proliferation in vivo (6). The CD98hc function as integrin signaling enhancer is sufficient to partially rescue the in vivo proliferation defect of CD98hc-null ES cells (6).

In this study we analyzed the role of CD98hc-associated AA transport in cell proliferation and teratoma formation. Interestingly, CD98hc presents specific binding capacity domains: whereas the intracellular domain is necessary and sufficient for interactions with β1 integrins (thus regulating their signaling capacities), the ectodomain (ED) is required for AA catalytic subunit association (10). We show that impaired proliferation of previously generated CD98hc-null ES cells and ES-derived fibroblasts (6) is restored by expression of chimeras that bind the AA transport catalytic subunits. Furthermore, such chimeras are able to specifically promote all AA transport activities observed in WT cells (namely CD98hc/xCT, system xc_; CD98hc/LAT1, system L and CD98hc/y+LAT2, system y+L). Next, we established the biological consequences of deleting CD98hc-mediated AA transport activities and found that ES-derived fibroblasts cannot compensate this deletion. Thus, invalidation of xCT activity results in iron-dependent oxidative (non-apoptotic) cell death called ferroptosis (11,12). Culture medium supplementation with β-ME prevents ferroptosis and restores cell survival. In such conditions, CD98hc-deficient cells present: i) accumulation of reactive oxygen species (ROS) ii) modulation of CD98hc-independent AA transporters and upregulation of peptide transporter PEPT1, iii) intracellular AA imbalance with dramatic increase in cationic AAs (AA⁺) and neutral AAs (AA⁰) but reduced levels of BCAAs and ARO AAs and iv) concomitant limited cell proliferation. An external supply of BCAAs and ARO AAs in the form of dipeptides rescues cell proliferation. Thus, only medium supplementations (with β-ME and BCAA- and ARO AA-containing dipeptides) can compensate for disrupted uptake of EAAs by CD98hc-dependent transport systems xc_, L and y+L. Taken together, our results highlight the critical role of CD98hc-associated AA transport for cell survival and proliferation. We show that CD98hc functions as an integrative and protective hub between oxidative stress and low AA availability.

EXPERIMENTAL PROCEDURES

Cell culture—Wild-type and CD98hc-null mouse ES cells as well as corresponding ES-derived fibroblasts were cultured in complete DMEM high glucose (Gibco) medium supplemented with 10 % v/v FBS (HyClone), 20 mM Hepes pH 7.3, 100 μM non-essential amino
acid (Gibco), 2 mM L-glutamine (Gibco) and, if not stated otherwise, 100 µM β-ME (Sigma Aldrich) at 37°C and 5% v/v CO₂ in an humidified incubator.

**Induction of Teratomas**— A suspension of ES cells (1.5 · 10⁶ cells per site) was injected subcutaneously into athymic BALB/c WEHI nude mice. After 33 days, teratomas were recovered and measured. To ensure similar expression levels in reconstitution experiments, each cell line was supplemented with CD98hc-null ES cells so that a similar number of expressing cells was injected with each clone.

**Cell proliferation assay**— On day 0, wild-type and CD98hc-null ES-derived fibroblasts were seeded in duplicate at 1 · 10⁴ cells per 35-mm diameter dish. After 24h of growth in complete supplemented DMEM medium (detailed above) cells were washed twice with PBS and put to grow in DMEM media with corresponding additional supplemental (1 mM sulfasalazine (SAS), 1 mM N-acetyl-cysteine (NAC), 1 µg/mL vitamin E (Vit E) or BCAA- and ARO AA-containing dipeptides (see below), as indicated).

**Flow citometry with 2’,7’-dichlorodihydro fluorescein diacetate (H2DCFDA)-stained cells**— Briefly, cells were plated and cultured in normal conditions. Forty eight hours after plating cells were washed with Hank’s balanced salt solution (HBSS) once and incubated with 1 µM of H₂DCFDA (Molecular Probes) for 30 min at 37°C. Then cells were harvested, washed in HBSS and levels of intracellular fluorescence (nonfluorescent H₂DCFDA is converted to highly fluorescent 2’,7’-dichlorofluorescein (DCF) upon cleavage of the acetate groups by intracellular esterases and oxidation) were analyzed using a FACS Calibur.

**qPCR/RNA preparation**— RNAs were extracted from cultured ES-derived fibroblasts using TRIzol reagent (Gibco). Reverse transcription was performed using Superscript II reverse transcriptase (Invitrogen). Sets of specific primers (cation transport regulator homolog 1 (CHAC1), sense 5’-CTTGGAAGACCGTAGGGG CTG-3’, antisense 5’-GGTGGGGTGCGCCACATAG G; PEPT1, sense 5’-GCCCGGACCAGATGCAGA CGG-3’, antisense 5’-GGCGGTACACCACACGC GTCC-3’) were used for amplification using 7900 HT Real Time PCR System (Applied Biosystems). Samples were normalized to ribosomal phosphoprotein, large, P0 (RPLP0) (sense 5’- CA CTGGTCTAGGACCCGAGAAG-3’, antisense 5 ’- GGTCCTCTGGAGATTTCG -3’) using the ΔCt method. Statistical significance was determined by means of the Student’s t-test.

**AA uptake measurement**— Transport activities were tested on whole cells as previously described (3) by measuring the transport of corresponding radiolabeled AA (10 µM). Transport activities were confirmed using 1 mM specific inhibitors (and pre-treating cells with 1 mM N-ethylmaleimide (NEM) for 3 min when needed) as indicated in figure legends.

**Western blot**— Whole cell lysates were prepared in radio-immunoprecipitation assay (RIPA) buffer [150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% w/v SDS, 1% w/v Triton X-100, 1% w/v deoxycholate, 5 mM EDTA, 1 mM NaVO₄, 5 mM NaF, 1 mM PMSF and protease inhibitor mixture (Roche)] and centrifuged at 10000 g for 15 min at 4°C. Protein lysates were quantified using the bicinechonic acid (BCA) method (Thermo Scientific Pierce). Proteins were loaded (10 µg of total protein per lane) and resolved in 10% w/v or 12.5% w/v acrylamide gels by SDS-PAGE and transferred to Immobilon membranes (Millipore). They were then immunoblotted (see below) and detected by the ECL method.

**Primary antibodies**— The following primary antibodies were used and validated for protein immunoblotting: i) anti-Nrf2 (rabbit polyclonal antibody against synthetic peptide within human nuclear factor erythroid 2-related factor (Nrf2) C terminal; Abcam #ab62352 lot #GR149891-2; 1:2500; used to recognize mouse Nrf2). This antibody revealed a single band (with same mobility than the one validated in a previous report (13)) that responded to Nrf2 upregulation in CD98hc-null cells treated with 25 µM tert-butylhydroquinone (t-BHQ) for 2 h. ii) anti-SNAT1 (rabbit polyclonal antibody raised against human sodium-coupled amino acid transporter (SNAT) 1; Santa Cruz #sc-67080 lot #G0208; 1:500). It reacts against mouse SNAT1, with several bands appearing in the film. Specificity of the ~50 kDa band was validated by its distinct disappearance after SNAT1 silencing in ES-derived wild-type fibroblasts and in mouse embryonic fibroblasts after lentivirus-mediated...
transduction of SNAT1 shRNA (Sigma Aldrich, #TRCN0000069230) compared to control transduction with scrambled RNA (Sigma Aldrich, #SHC00). iii) anti-p-S6 (rabbit polyclonal antibody against a synthetic phosphopeptide corresponding to residues surrounding Ser235 and Ser236 of human ribosomal protein S6 (S6); Cell Signaling #2211S lot #22 Ref.02/2014; 1:2000). It reacts with S6 only when phosphorylated at serine 235 and 236, as shown in the datasheet. This antibody detected a single band, which decreased after treatment of murine fibroblasts with 2 μM rapamycin for 3 h, proving its specificity. iv) anti-S6 (mouse monoclonal antibody against human S6, Santa Cruz #sc-74459 lot #G0708; 1:8000). This antibody reacts specifically against murine S6 as shown by the presence of a single band in the gels, corresponding to the size of of the band revealed by anti-p-S6, but not responding to treatment with rapamycin. v) anti-p-eIF2α (rabbit polyclonal antibody against a synthetic phosphopeptide corresponding to residues surrounding Ser51 of the alpha subunit of human eukaryotic initiation factor 2 (eIF2); Cell Signaling #9721 lot #9 Ref.04/2012; 1:1000). Specificity against murine eIF2α was validated by upregulation of phosphorylated-eIF2α after murine fibroblasts treatment with 10 nM thapsigargin for 3 h. vi) anti-eIF2α (rabbit polyclonal antibody produced by immunizing animals with a synthetic peptide derived from the carboxy-terminal sequence of eIF2α; Cell Signaling #9722 lot #13 Ref.04/2013; 1:1000) was used to recognize murine total eIF2α. Specificity was validated by the presence of a unique band in the gels, with same size as p-eIF2α, but not responding to thapsigargin treatment. vii) anti-ATF4 (rabbit monoclonal antibody against human activating transcription factor 4 (ATF4); Cell Signaling D4B8 #11815 lot #2; 1:1000) was validated by upregulation of the protein after thapsigargin treatment. viii) anti-PEPT1 (custom made rabbit polyclonal antibody against human PEPT1 kindly provided and validated by Dr. H. Daniel (14,15); 1:5000). ix) anti-tubulin (Sigma Aldrich #T5168 lot #103M4773V; 1:1000) was used as a loading control, correlation between total protein loaded and tubulin signal was checked.

Intracellular AA quantification— Pelleted cells were weighed, and Norleucine was added as internal standard. Samples were deproteinized with 10 % v/v trifluoroacetic acid and lysed by repeated freeze-thaw cycles. After centrifugation at maximum speed, supernatants were ultrafiltered through a 10-kDa spin column (Millipore) and dried using a vacuum chamber. Pellets were resuspended in loading buffer (Biochrom Ltd.), filtered and injected for HPLC analysis. Quantitative analysis of AAs was performed using a Biochrom 30 amino acid analyzer (Biochrom Ltd.). AA peaks were identified on the basis of the retention times of corresponding standards. Quantification was normalized by total protein.

Dipeptide synthesis and supplementation— BCAA- or ARO AA- and L-Ala dipeptides were synthesized following standard procedures by the ICTS “NANBIOSIS”, more specifically by the Synthesis of Peptides Unit (U3) of the CIBER in Bioengineering, Biomaterials & Nanomedicine (CIBER-BBN) at the Barcelona Science Park. Briefly, dipeptides were synthesized in solution by coupling of the corresponding protected N- and C-terminal AAs using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro phosphate (HBTU) in dimethylformamide (DMF) as coupling reagent. Once the reaction was finalized, protected dipeptides were precipitated with cold water, centrifuged and washed again with cold water. Protecting groups were eliminated by addition of trifluoroacetic acid, and after 1 h final dipeptides were precipitated by addition on cold diethyl ether. Dipeptides were redissolved in H2O and lyophilized. Purity was estimated by HPLC and HPLC-MS. Each dipeptide was supplemented at a concentration of 0.25 times of the corresponding BCAA (isoleucine, leucine and valine) or ARO AA (phenylalanine, tyrosine and tryptophan) concentration in complete DMEM medium.

RESULTS

CD98hc-associated AA transport function is sufficient to support cell proliferation in vivo— We previously found that: i) CD98hc-deficient ES cells fail to proliferate and form teratomas in vivo (6) and ii) that the intracellular domain of CD98hc, required for enhanced beta 1 integrin signaling, rescues teratoma growth, although teratomas are as not as large as when CD98hc-null ES cells are reconstituted with wild-type CD98hc (6). Here we asked whether the partial teratoma growth observed could be ascribed to the lack of CD98hc-mediated AA transport function. Rescue
experiments using CD98hc-CD69 chimeras (C69T98E98 and C98T69E98) (depicted in Fig. 1A) only able to bind CD98hc AA transport subunits but not integrins (10) showed restoration of in vivo cell proliferation. Importantly, reconstitution of CD98hc-null ES cells with wild-type CD98hc induced the strongest growth (Fig. 1B-C). As expected, when CD98hc-null cells were reconstituted with CD69, no teratomas were formed (Fig. 1B lower right panel and Fig. 1C). Thus, we show that i) the portion of CD98hc that binds AA transporters promotes cell proliferation in vivo; ii) maximal cell growth rates are only achieved when both CD98hc activities (AA transport and enhanced integrin signaling) are functional. These results suggest that both functions act in synergy to induce teratoma formation.

**CD98hc protects cells against oxidative stress leading to cell survival**— Next, we analyzed the consequences of the lack of CD98hc-mediated AA transport activities. System x_c (CD98hc/xCT) is required for cystine uptake in exchange with glutamate. Hence, it plays a vital role in both redox control and cellular growth by supplying intracellular cysteine, a rate-limiting AA in the synthesis of GSH and necessary for protein synthesis (16). CD98hc-deficient ES-derived fibroblasts generated previously (6) did not survive under standard culture conditions (Fig. 2A, left panel), unless medium was supplemented with β-ME (Fig. 2A, central panel). xCT deficiency leads to ferroptosis. Upregulation of endoplasmic reticulum (ER) stress response gene CHAC1 (cation transport regulator homolog 1) serves as a ferroptosis marker and can be reverted by adding β-ME to culture media (12), as shown in CD98hc-deficient cells (Fig. 2B) and in wild-type cells in which xCT transporter has been inhibited with sulfinylazine (SAS) (Fig. 2B). β-ME supplementation allowed reduction of extracellular cystine into free cysteine, which was subsequently imported by CD98hc-independent transporters (Fig. 2A, right panel), thus guaranteeing cell survival. We tested the effect of N-acetyl-cysteine (NAC) as an alternative source of cysteine and vitamin E (Vit E) as an anti-oxidant (Fig. 2C) on cell survival. Vit E had no positive effect. In contrast, NAC protected CD98hc-null cells from death induced by the withdrawal of β-ME, thus rescuing cell proliferation (Fig. 2C). However, even when supplemented with β-ME, CD98hc-null fibroblasts showed 1.4-fold increase of redox-sensitive probe H2DCFDA labeling compared to wild-type fibroblasts (Fig. 2D). Because of the limitations in the reliability of such probe to accurately measure ROS (17), we also analyzed in cells cultured in β-ME-supplemented medium the expression of the master regulator of intracellular antioxidant response nuclear factor erythroid 2-related factor 2 (Nrf2) (18). It presented indeed an increased expression (~9-fold) in CD98hc-null fibroblasts compared to the wild-type cells (Fig. 2E). In contrast, inhibition of xCT by SAS did not increase Nrf2 expression (Fig. 2E). Thus, CD98hc is required for in vitro cell survival because of its role in cystine uptake via x_c system, protecting cells from ferroptosis. Moreover, CD98hc ablation induces oxidative stress that is independent of xCT activity and non-reverted by β-ME.

**CD98hc-mediated AA transport (via xCT, LAT1 and yLAT2) is sufficient for proper in vitro cell proliferation**— Both wild-type ES-induced teratomas (in vivo) and ES-derived fibroblasts (in vitro) express identical CD98hc-associated transporters as revealed by their mRNA expression (not shown): xCT (more expressed in cultured cells than in vivo, probably due to higher oxygen tension in culture than in vivo conditions (19)), LAT1, y′LAT2, and to a lesser extent Asc1. Thus, the ES-derived fibroblasts provide a suitable in vitro model to establish the role of CD98hc-dependent AA transport in cell proliferation. Wild-type ES-derived fibroblasts presented transport activities corresponding to system x_c, system L and system y′L (Fig. 3A, upper and middle panels). CD98hc drives the trafficking of associated light chains to the plasma membrane (20). Thus, CD98hc-null cells should lack all CD98hc-associated transport subunits in the plasma membrane. Consistently, the system x_c, L and y′L transport activities were absent in CD98hc-deficient cells (Fig. 3A, middle panel). We show that the chimeras previously described to induce leucine transport (C69T98E98 and C98T69E98) (10) (Fig. 3A, lower panel) recovered indeed all CD98hc-mediated transport activities present in wild-type ES-derived fibroblasts (Fig. 3A, middle panel). In sharp contrast, the integrin-signaling chimera (CD98T98E69)(Fig. 3A, lower panel) cannot mediate AA transport (Fig. 3A, middle panel). Next, in order to specifically study the effect of CD98hc-mediated
AA transport on intrinsic cell proliferation capacity, we compared the in vitro cell proliferation rate of CD98hc-null fibroblasts when rescued with chimeras (described above) capable of restoring AA transport or integrin binding (10). First, we show that CD98hc-deficient cells present a major delay in proliferation in vitro compared to wild-type cells. This delay was rescued by reconstitution with full-length CD98hc (Fig. 3B, upper panel). Second, we observed a restoration of in vitro cell proliferation, comparable to wild-type or full-length CD98hc expressing cells, only when null cells were reconstituted with AA transport chimeras (C69T98E98 and C98T69E98)(Fig. 3B, lower panel). Conversely, when only the capacity of integrin signaling (C98T98E69) was reconstituted in CD98hc-null cells, in vitro cell proliferation was still impaired (Fig. 3B, lower panel). Thus, CD98hc AA transport capacity is sufficient to drive proliferation in vitro, whereas the function as integrin signaling enhancer is dispensable. These results highlight the essential requirement of CD98hc-dependent AA transport for cell proliferation regardless of the surrounding environment.

CD98hc supports the balance of intracellular AA content—Deficiency of CD98hc and associated transporters had an impact on the AA intracellular content of CD98hc-null fibroblasts (Fig. 4A). The cell content of both AA⁺ (Arg, Lys and His) and AA₀ (Ala, Ser, Asn, Gln and Met) was increased (2.9- to 4.3-fold and 1.2- to 2.7-fold, respectively) in CD98hc-deficient compared to wild-type fibroblasts. In contrast, BCAAs (Val, Leu and Ile) and ARO AAs (Phe and Tyr) showed an intracellular content decrease of 0.5- to 0.6-fold in CD98hc-null when compared to wild-type cells (Fig. 4A). Analysis of the transport of AAs with altered intracellular content revealed that, in the absence of CD98hc, the expression and activity of CD98hc-independent transporters was modulated. L-Arg uptake was much higher (~240 %) in CD98hc-deficient than in wild-type ES-derived fibroblasts (Fig. 4B). We identified this transport activity as exclusively system y⁺ (since NEM specifically inactivates system y⁺ without affecting system y'L (21)), which presented a ~8-fold increase when compared to wild-type cells (Fig. 4B). Next, we tested whether the expression levels of cationic amino acid transporters (CATs) (system y⁺) could account for such dramatically increased transport activity. In this regard, we detected a 145-fold specific increase in CAT3 (but not CAT1 and CAT2) mRNA levels in CD98hc-null compared to wild-type fibroblasts (data not shown). Using the CAT3 specific inhibitor D-arginine (22) we measured a ~12-fold increase in CAT3 activity in CD98hc-null compared to wild-type cells (Fig. 4C). CAT3 is a CD98hc-independent transporter that mediates the Na⁺-independent transport of cationic amino acids (1). Thus, the upregulation of CAT3 in fibroblasts lacking CD98hc is in accordance with increased AA⁺ intracellular content in such cells (Fig. 4A) as arginine, lysine and histidine are substrates of murine CAT3 (22,23). Regarding the transport of AA₀, by analyzing L-Ala uptake, we identified the presence of system A, a co-transporter of Na⁺ and neutral AAs, mainly excluding BCAAs and ARO AAs (24)(Fig. 4D, grey column), system N, a Na⁺-dependent transporter of neutral AAs (24)(Fig. 4D, white column), and others (part of L-Ala uptake was not ascribed to any specific transporter, but could represent systems ASC, B₀ or B₀,+)(Fig. 4D, black column) in wild-type ES-derived fibroblasts. Depletion of CD98hc resulted in the disappearance of CD98hc-independent system A transport activity (Fig. 4D, grey column) along with the expression of SNAT1 transporter (Fig. 4E) (no differences in mRNA expression of SNAT2, the other system A isoform, were observed)(not shown). In contrast, system N was not changed in CD98hc-ablated cells (Fig. 4C, white column). System N couples AA import with H⁺ efflux (1 AA : 1 Na⁺ [symport] : 1 H⁺ [antiport]), making it less concentrative than system A (24). Despite the depletion of system A, the intracellular content of AA₀ (Ala, Ser, Asn, Gln and Met) was increased in CD98hc-null cells (Fig. 4A). Thus, AA₀ accumulation would be generated by unidentified causes (e.g. reduced consumption rate) not related to AA₀ uptake. Ablation of CD98hc abrogated LAT1 transport activity (system L)(Fig. 3A, middle panel). LAT1 exchanges large AA₀, including BCAAs, ARO AAs and histidine, methionine and glutamine across the plasma membrane (25). System L has four isoforms, two associated with CD98hc (LAT1 and LAT2) and two independent of CD98hc (LAT3 and LAT4) (1,26). All these transporters are Na⁺-independent and can be inhibited by BCH, the leucine analog 2-aminobicyclo [2,2,1] heptane-2-carboxylic acid. Interestingly, in CD98hc-null cells, there was no Na⁺-independent
transport of L-Ile inhibitable by BCH (Fig. 4F). Thus, no CD98hc-independent isoform of system L compensated for LAT1 ablation. Moreover, CD98hc-deficient cells presented only ~10% of wild-type L-Ile uptake in Na\(^+\)-containing transport medium (Fig. 4G), thus arguing in favor of the absence of an efficient compensation for LAT1 by a Na\(^+\)-dependent transporter. Reduced intracellular BCAA and ARO AA content in CD98hc-null cells (Fig. 4A) is consistent with this lack of compensation for LAT1 transport and might underlie the proliferation defect of such cells.

Neither the mechanistic target of rapamycin (mTOR) nor the Integrated Stress Response (ISR) are responsible for CD98hc-null cell proliferation deficiency—The mechanistic target of rapamycin (mTOR) and its downstream effector mTOR complex 1 (mTORC1) play a central role in cell growth and proliferation by responding to amino acid (i.e. leucine, arginine, glutamine) availability (27-30). mTORC1 phosphorylates and activates ribosomal protein S6 (S6) kinase that, in turn, mediates the phosphorylation of S6, an integral component of the 40S subunit of the ribosome (29,31). In protein synthesis no AA can compensate for the absence of another, thus, we thought that the intracellular BCAA and ARO AA shortage of CD98hc-deficient cells could induce protein synthesis repression via mTOR inactivation. Contrary to our hypothesis, we found an increased phosphorylation state of S6 in CD98hc-null compared to wild-type cells (Fig. 5A). In fact, increased activity of mTORC1 in fibroblasts deficient for CD98hc could be attributed to the high content of AA and glutamine in such cells (Fig. 4A), as arginine and glutamine are also regulators of mTORC1 (32-34). Alternatively to mTORC1, eukaryotic cells respond to stress (such as oxidative stress or AA deprivation) by phosphorylating the α subunit of the eukaryotic translation initiation factor 2 (eIF2), which represses global translation coincident with preferential translation of ATF4, a master regulator controlling the transcription of pro-survival target genes (35). This pathway is collectively referred to as integrated stress response (ISR). We analyzed the phosphorylation state of eIF2α in CD98hc-null cells, which was markedly increased (~3-fold) in comparison with wild-type cells (Fig. 5B), thereby suggesting the activation of the eIF2α-mediated stress response pathway. However, despite the strong phosphorylation of eIF2α, ATF4 mRNA (data not shown) and protein levels (Fig. 5C) were substantially repressed in CD98hc-deficient fibroblasts. Dysfunctional ISR (suppressed ATF4 expression in the presence of phosphorylated eIF2α) has been reported in cells affected by chronic stresses (36-40). This could be the case for CD98hc-null ES-derived fibroblasts, which present chronic oxidative stress and imbalanced intracellular AA content. Moreover, CD98hc-deficient cell proliferation was restored without affecting the phosphorylation state of eIF2α (see below), offering serious doubts about the role of phosphorylated eIF2α in blocking general protein synthesis of ES-derived fibroblasts lacking CD98hc. In all, despite our attempts to decipher the molecular mechanisms behind, we can not yet explain the cause for proliferation impairment in CD98hc-null cells.

CD98hc-deficient cell proliferation is restored after BCAA and ARO AA supplementation—As shown above, in the absence of LAT1 activity and presenting only a ~10% of L-Ile uptake (Fig. 3A, middle panel and Fig. 4G), CD98hc-deficient cells maintained a considerable (yet reduced) intracellular content of the LAT1 substrates valine, leucine, isoleucine, phenylalanine, and tyrosine (Fig. 4A). This observation suggests a diminished cell metabolism or alternative sources of these AAs. In this regard, we observed an overexpression of mRNA (Fig. 6A) and protein (Fig. 6B) levels of peptide transporter PEPT1 (while PEPT2 mRNA expression was not affected) (data not shown) in CD98hc-deficient when compared to wild-type fibroblasts. PEPT1 is a proton-dependent transporter responsible for cellular uptake of di- and tri-peptides (41). We took advantage of this overexpression to supplement culture media of CD98hc-KO cells with alanyl dipeptides containing BCAAs and ARO AAs. Intriguingly, the proliferation capacity of CD98hc-deficient fibroblasts was restored to levels of wild-type cells after being grown in dipeptide-supplemented media for 5 days (Fig. 6C), whereas control-supplementation (L-Ala) showed no effect (Fig. 6C, right panel). Next, we performed a time-course analysis and found that upregulation of PEPT1 expression matched the rescue of cell proliferation, with highest mRNA and protein expression being reached at day 5-6 after cell seeding (data not shown). Therefore, cell
proliferation was restored when fibroblasts expressed the transporter that allows extra uptake of dipeptides containing BCAAs and ARO AAs. Strikingly, the intracellular AA content of dipeptide-supplemented fibroblasts showed no recovery of either BCAA or ARO AA content (Fig. 6D), neither phosphorylation of eIF2α nor ATF4 repression was alleviated in the presence of BCAA- and ARO AA-containing dipeptides (data not shown). CD98hc-null ES-derived fibroblasts grown in peptide-supplemented media proliferated normally in the presence of phosphorylated eIF2α, supporting a dysfunctional role of the phosphorylation of the initiation factor on protein synthesis in these cells. Our results reveal the strong dependency of ES-derived fibroblasts on adequate BCAA and ARO AA availability for cell proliferation and the strict requirement of CD98hc-mediated AA transport in teratoma formation and proliferation (45), it has no effect on CD98hc-deficient fibroblasts. This aggravated phenotype might be due to the lack of other transporters (LAT1 and y+LAT2) along with xCT in CD98hc-null cells. Moreover, even when ferroptosis is blocked by β-ME supplementation (consistently with (12)), CD98hc-null fibroblasts still present, with no obvious explanation, accumulation of DCF signal and increased Nrf2 expression. Although it is known as a master regulator of oxidative stress, Nrf2 also participates in intermediary metabolism and mitochondrial physiology (46). Since pharmacological inhibition of xCT does not promote Nrf2 activation, one might question if the observed increased Nrf2 in CD98hc-nulls is directly related to oxidative stress. Further investigation will be needed to clarify this process.

**DISCUSSION**

Proliferating cells must meet specific energetic and biosynthetic demands (42). Their adaptation involves the expression of nutrient transporters, in particular for AAs that cells are unable to store. In this study we unveil the role of CD98hc-mediated AA transport in teratoma formation and proliferation in vivo. We show that CD98hc AA transport function is required for adequate provision of AAs to cells, protecting them against oxidative stress and allowing normal cell proliferation, both in vitro and in vivo.

We show that expression of the CD98hc portion able to bind integrins (C98T998E98) in CD98hc-null cells is not sufficient to restore in vitro cell proliferation, in contrast with in vivo teratoma formation (6), suggesting a specific integrin mediated regulation depending on the properties of the surrounding environment (43). In parallel, expression of any of the chimeras deficient for integrin interaction but containing the CD98hc-ED (C69T98E98, C98T69E98) (6,10) rescues in vivo and in vitro cell proliferation of CD98hc-deficient cells.

Our data suggest that the ED of CD98hc (absent in the chimera exclusively recovering β1 integrin signaling) is crucial for the proper functioning of associated transporters, as CD98hc-null cells expressing ED-containing chimeras catalyze all AA transport activities present in wild-type cells (systems x², L and y¹L).

This observation is in agreement with a recent structural model of CD98hc heterodimers, which shows that CD98hc-ED interacts with the corresponding associated transporter, thus stabilizing it (4).

Distinct AA transporters often share the same substrates, thereby ensuring that the system remains unaffected if one transporter fails. Here we report a general change in intracellular AA content after invalidation of a transporter component, showing that redundancy is not observed with CD98hc-associated transporters. Consequently, CD98hc/xCT and CD98hc/LAT1 (upregulated in highly proliferative cells (44)) and CD98hc/y¹LAT2 are essential for cell survival and proliferation.

In vitro high oxygen tension, cells rely on membrane expression of CD98hc-associated cystine transporter xCT (20), which protects them from cell death by ferroptosis (11). We show that CD98hc-null ES-derived fibroblasts do not survive under routine culture conditions, but grow normally in the presence of β-ME or NAC. Our results are in good agreement with the work by Sato and colleagues on fibroblasts isolated from xCT deficient embryos (45). However, whereas Vit E restores cell survival in xCT-null fibroblasts (45), it has no effect on CD98hc-deficient fibroblasts. This aggravated phenotype might be due to the lack of other transporters (LAT1 and y¹LAT2) along with xCT in CD98hc-null cells. Moreover, even when ferroptosis is blocked by β-ME supplementation (consistently with (12)), CD98hc-null fibroblasts still present, with no obvious explanation, accumulation of DCF signal and increased Nrf2 expression. Although it is known as a master regulator of oxidative stress, Nrf2 also participates in intermediary metabolism and mitochondrial physiology (46). Since pharmacological inhibition of xCT does not promote Nrf2 activation, one might question if the observed increased Nrf2 in CD98hc-nulls is directly related to oxidative stress. Further investigation will be needed to clarify this process.

LAT1 is the other CD98 catalytic subunit overexpressed in highly proliferative cells (44). While inhibition of LAT1 affects cell proliferation (47,48), its genetic or chemical ablation results in mammalian target of rapamycin (mTOR) inhibition and impaired cell growth (27,28,48-50). Our data indicate that >90% of L-isoleucine uptake is lost when CD98hc is absent. Moreover, CD98hc/LAT1 loss leads to cellular depletion of
several essential AAs (BCAAs: leucine, isoleucine and valine, and ARO AAs: phenylalanine and tyrosine), which surprisingly does not impair mTORC1 activity. We hypothesize that the high intracellular content of other AAs such as arginine and glutamine is sufficient to sustain mTORC1 activity (as observed by (32-34)) in CD98hc-null cells. Nonetheless, even with active mTORC1 CD98hc-null cells present a major defect in cell proliferation. In the absence of CD98hc, oxidative stress and/or scarcity of BCAAs and ARO AAs may underlie eIF2α phosphorylation, however p-eIF2α does not appear to play a relevant role in the restriction of general translation of CD98hc-null ES-derived fibroblasts.

Isolated cells (both normal and malignant) depend on 13 AAs for in vitro survival. Over and above the eight AAs required for nitrogen balance (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine), cell cultures require arginine, cyst(e)ine, glutamine, histidine, and tyrosine (51). In spite of the absence of LAT1 activity, CD98hc-deficient cells maintain a considerable intracellular concentration of the BCAA and ARO AA substrates of LAT1. We could explain these somewhat unexpected results by the strong upregulation of di- and tri-peptide transporter PEPT1 (41) expression in CD98hc-deficient cells. Providing dipeptides as an alternative source of BCAAs and ARO AAs is sufficient to recover proper cell proliferation. However, it does neither alleviate stress sensed by eIF2α nor restore the intracellular BCAA and ARO AA content, pointing to the relevance of AA flux rather than AA concentration. PEPT1 has been described to participate in tumor cell growth (52), our data now point to its relevant role in cell proliferation.

By controlling expression at the plasma membrane and coupling transport activities of xCT, LAT1 and y’LAT2, CD98hc confers protection against oxidative and nutritional stresses (ensuring a balanced AA content and a proper supply of EAAs) and thus, provides cells with a proliferative advantage (Fig. 7). Each function of CD98hc, AA transport activity (presented herein) or integrin signaling (6), rescues in vivo cell proliferation. Moreover, our results strongly suggest that both functions can act in synergy providing cells with hyperproliferative capacity in vivo (as can be observed in teratomas formed when full-length CD98hc is expressed in CD98hc-null ES cells). These findings have relevant implications in pathophysiological scenarios. For instance, CD98hc and associated catalytic subunits LAT1 and xCT, as well as integrins, are overexpressed in most tumors (8,44,53), thus promoting cell growth. By expressing such transporters coupled with extracellular matrix receptors, cells can control AA adequacy, ROS impact and anchoring/migration, leading to sustain cell proliferation, critical for tumor cells (54). To date, many studies have focused on the search for either transporter (xCT, LAT1) inhibitors or integrin antagonists as anti-cancer strategies (47,48,55,56). Even though these molecules are appealing targets, encouraging yet limited results have been obtained so far. Targeting CD98hc could represent a novel option widening the therapeutic window of cancer therapy by inducing an immediate blockage of AA transport activities mediated by xCT and LAT1 (with extremely limited compensation capacities) as well as a strong impairment in integrin signaling. CD98hc-targeting drugs could be delivered locally to tumors where this protein is strongly overexpressed. Generating drugs against CD98hc that could inhibit such a pivotal signal integrator would be of potential great use specifically for anti-cancer therapeutic purposes.
ACKNOWLEDGEMENTS
This study was supported by grants from Association pour la Recherche sur le Cancer (ARC R14029AA), from LLCC (R14035A), through the "Investments for the Future" LABEX SIGNALIFE: program reference ANR-11-LABX-0028-01 and by grants from the Spanish Ministerio de Economía y Competitividad (SAF2012-40080-C02-01 and SAF2015-64869-R) and the Generalitat de Catalunya (SGR2009-1355). IRB Barcelona is the recipient of a Severo Ochoa Award of Excellence from the Spanish Ministerio de Economía y Competitividad. SCC was recipient of a Severo Ochoa doctoral fellowship. FT was the recipient of a doctoral fellowship from INSERM Region Provence-Alpes Côte d’Azur/Canceropôle PACA. The authors greatly acknowledge the IRCAN core facilities (supported by le Conseil général 06, FEDER, le Ministère de l’Enseignement Supérieur, la Région Provence Alpes-Côte d’Azur and INSERM) and the Synthesis of Peptides Unit (U3) of the CIBER in Bioengineering, Biomaterials & Nanomedicine (CIBER-BBN) at the Barcelona Science Park. We thank Dr. Juan Pablo Muñoz and Dr. Sónia Rosa Pereira for their technical advice, constructive comments and enlightening discussions.

CONFLICT OF INTEREST
The authors declare no competing financial interests.

AUTHOR CONTRIBUTION
LRB, SCC, EGM, MP and CCF designed research. LRB, SCC, SB, SE, LC, FT and CCF performed research. LRB, MP and CCF analyzed and interpreted data. EGM, HD and MHG contributed with new reagents and analytic tools. LRB, AZ, MP and CCF participated in the conception and design of the article. AZ did a critical revision of the article. LRB, AZ, MHG, MP and CCF gave final approval of the version to be published. LRB, MP and CCF wrote the article.

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FOOTNOTES

* SCC and EGM share second authorship. # MP and CCF share last authorship.

The abbreviations used are: CD98hc, cluster of differentiation 98 heavy chain; AA, amino acid; ROS, reactive oxygen species; β-ME, β-mercaptoethanol; BCAA, branched-chain amino acid; ARO AA, aromatic amino acid; PEPT1, peptide transporter 1; HAT, heteromeric amino acid transporter; SLC, solute carrier; LAT, L-type amino acid transporter; EAA, essential amino acid; ES cell, embryonic stem cell; AA+, cationic amino acid; AA0, neutral amino acid; SAS, sulfasalazine; NAC, N-acetyl-cysteine; VitE, vitamin E; H2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; DCF, 2',7'-dichlorofluorescein; HBSS, Hank’s balanced salt solution; CHAC1, cation transport regulator homolog 1; RPLP0, ribosomal phosphoprotein, large, P0; NEM, N-ethylmaleimide; RIPA, radio-immunoprecipitation assay; BCA, bicinchoninic acid assay; Nrf2, nuclear factor erythroid 2-related factor; t-BHQ, tert-butylhydroquinone; SNAT, sodium-coupled amino acid transporter; S6, ribosomal protein S6; eIF2α, eukaryotic initiation factor 2; ATF4, activating transcription factor 4; HBTU, 2-(1H-benzotriazol-1-yl)1,1,3,tetramethyluroniumhexafluorophosphate; DMF, dimethylformamide; ER, endoplasmic reticulum; CPG, (S)-4-carboxyphenylglycine; BCH, 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid; CAT, cationic amino acid transporter; MeAIB, N-(methylamino)isobutyric acid; mTOR, mechanistic target of rapamycin; ISR, integrated stress response; mTORC1, mTOR complex 1; ED, ectodomain.
**FIGURE LEGENDS**

**FIGURE 1.** CD98hc AA transport capacity restores cell proliferation *in vivo*. A) Depiction of chimeras of CD98hc and CD69 (type II transmembrane protein with functions unrelated to CD98hc) and their interactions with integrins or amino acid transporters. CD98hc protein is depicted in black and CD69 is shown in white. Each chimera is defined by its cytoplasmic (C), transmembrane (T), and extracellular (E) domain derived from either CD98hc (98) or CD69 (69). B) Mice were injected with corresponding ES cells and analyzed after 26 days. C) Depicted is the quantification of tumor volumes (mean values ± SEM) determined at various time points as previously described (6) for wild-type ES cells (WT, ●) and CD98hc-null ES cells reconstituted with CD69 (CD69, ○), full-length CD98hc (CD98hc, ▼) or with chimeras recovering only AA transport, namely C69T98E98 (▲) or C98T69E98 (■). A similar expression level of full-length CD98hc and AA transport chimeras was confirmed by FACS (10).

**FIGURE 2.** β-mercaptoethanol inhibits ferroptosis induced by depletion of CD98hc/xCT transporter and restores CD98hc-null cell survival. A) Wild-type (WT) and CD98hc-null (KO) fibroblast proliferation was compared in the absence (-) (left panel) and presence (+) (middle panel) of β-mercaptoethanol (β-ME) for 5 days. Cells counts at several time points are shown as mean ± SEM. Similar results were obtained at least 3 times. Cystine (Cys-Cys) is transported by system x_c inside the cell where it is reduced to cysteine (CysSH, essential for the formation of reduced GSH). By supplementing culture medium with β-ME, free CysSH is available extracellularly and can be imported inside the cell by CD98hc-independent neutral AA transporters (right panel). B) CHAC1 mRNA expression levels in wild-type (WT, solid bar) and CD98hc-deficient fibroblasts (KO, open bar) grown with no additives (none) and in the presence of 100 µM β-ME (β-ME), 1 mM sulfasalazine (SAS) or the combination of the last two (β-ME + SAS). RPLP0 was used as a housekeeping gene (not depicted). n.d. not determined. Data are presented as mean ± SEM of 8 independent experiments. C) Wild-type (WT, solid bar) and CD98hc-deficient (KO, open bar) fibroblasts were grown with no additives (none) or with 100 µM β-ME (β-ME), 1 mM NAC (NAC), or 1 µg/mL Vit E (Vit E) for 48h. Cell counts are expressed as mean ± SEM of duplicates. A representative experiment is shown, similar results were obtained at least 3 times. D) Percentage of cells positive for the free radical sensor H2DCFDA measured by flow cytometry. Data shows mean ± SEM of 12 measurements. E) Nrf2 protein expression in wild-type (WT, solid bar) and CD98hc-null fibroblasts (KO, open bar) grown in the presence of 100 µM β-ME (β-ME), 1 mM sulfasalazine (SAS) or the combination of the last two (β-ME + SAS). Representative Western blot of 2 independent experiments is shown. Data are normalized by tubulin expression. Quantification corresponds to mean ± SEM of 6 measurements.

**FIGURE 3.** CD98hc AA transport function is required for efficient cell proliferation *in vitro*. A) Depicted are the activities of transporters xCT (exchange of anionic forms of cystine and glutamate), LAT1 (exchange of BCAAs, ARO AAs and other large neutral AAs) and y+LAT2 (exchange of cationic AAs and neutral AAs plus Na+) across the plasma membrane (upper panel). AA transport activities of corresponding systems x_c (black), L (grey) and y+L (white) in wild-type fibroblasts (WT), CD98hc-deficient (KO) fibroblasts and CD98hc-null fibroblasts recovered with full-length CD98hc (CD98hc) or chimeras recovering AA transport (C69T98E98 and C98T69E98) or integrin signaling (C98T98E69). Uptake activity is shown as a percentage of the levels of transport at 2 min (linear conditions) of wild-type cells for each CD98hc-associated transport system. Activities of the transport systems were determined as the uptake of the corresponding radiolabelled amino acid minus its uptake in the presence of specific inhibitor. Thus, system x_c represents the Na+-independent uptake of L-Glu (10 µM) inhabitable by (S)-4-carboxyphenylglycine (S-4-CPG) (1 mM); system L corresponds to the Na+-independent L-Ile (10 µM) uptake that can be inhibited by 2-aminobicyclo [2,2,1] heptane-2-carboxylic acid (BCH) (1 mM) and system y+L corresponds to the L-Arg (10 µM) uptake non-inhibitable by N-ethylmaleimide (NEM) (1 mM) and inhabitable by L-Leu (1 mM) only when Na+ is present. Transport data correspond to mean ± SEM of at least 8 independent measurements (middle panel). Protein domains are depicted (CD98hc in black, CD69 in grey) (lower panel). B) Wild-type (WT, ●) or CD98hc-deficient (KO, ○) fibroblasts recovered with full-length CD98hc (CD98hc, ▼) (upper panel) or CD98hc-null fibroblasts recovered with chimeras restoring AA transport (C69T98E98, and C98T69E98, △) or integrin...
signaling (C98T98E69, ⦿) functions (lower panel, CD98hc is repeated as a reference) were seeded on day 0 and grown in the presence of β-ME for 5 days. Cells were counted every day. Results are expressed as mean ± SEM of duplicates. Experiment was repeated at least 3 times.

**FIGURE 4.** CD98hc depletion leads to imbalanced intracellular AA content and to adaptations of CD98hc-independent transporters. A) Samples from wild-type (WT, solid bar) and CD98hc-null (KO, open bar) fibroblasts were processed as described in Materials and Methods and quantitative analysis of AAs was performed. Results are expressed as mean ± SEM of 3 independent measurements. AA⁺ are presented in red, BCAAs are depicted in yellow, ARO AAs in green and the rest of AA₀ in black. AAs are grouped and underlined to indicate that they are substrates of the corresponding transport system (below). B) Na⁺-dependent L-Arg (10 µM) uptake at 2 min inhibitable by NEM (1 mM) alone (system y⁺, white column) or in combination with L-Leu (1 mM) (system y'L, black column). Transport data correspond to mean ± SEM of at least 12 independent measurements. C) CAT3 transport activity measured as Na⁺-independent uptake of L-Arg (10 µM) by wild-type (WT, solid bar) and CD98hc-null (KO, open bar) fibroblasts inhibitable by D-Arg (5 mM) in the presence of L-Leu (1 mM). Transport data correspond to mean ± SEM of 3 independent experiments. D) Na⁺-dependent L-Ala (100 µM) uptake at 2 min inhibitable by N-(methylamino)isobutyric acid (MeAIB) (10 mM) alone (system A, grey column) or in combination with L-Asn (10 mM) (system N, white column). Residual transport is depicted as others (black column). Transport data correspond to mean ± SEM of at least 8 independent measurements. E) Protein expression of SNAT1 in wild-type (WT) and CD98hc-null (KO) fibroblasts. Representative Western blot is shown. Data are normalized by tubulin expression. Three independent experiments showed similar results. F) Na⁺-independent L-Ile (10 µM) uptake at 2 min inhibitable by BCH (1 mM) in wild-type (WT, solid bar) and CD98hc-deficient (KO, open bar) fibroblasts. Transport data correspond to mean ± SEM of at least 8 independent measurements. G) L-Ile (10 µM) uptake levels at 2 min in the presence of Na⁺ in wild-type (WT, solid bar) and CD98hc-null fibroblasts (KO, open bar). Transport data correspond to mean ± SEM of at least 12 independent measurements. (* p<0.05; ** p<0.01; *** p<0.001; Student's t-test)

**FIGURE 5.** Stress responses triggered in CD98hc-depleted fibroblasts. A) mTORC1 is activated in CD98hc-deficient fibroblasts. Phosphorylation of S6 is compared between wild-type (WT, solid bar) and CD98hc-null (KO, open bar) fibroblasts. B) eIF2α phosphorylation is compared between wild-type (WT, solid bar) and CD98hc-null (KO, open bar) fibroblasts. C) ATF4 protein expression in wild-type (WT, solid bar) and CD98hc-null (KO, open bar) cells. A-C) Representative Western blots of 2 independent experiments are shown. Data are normalized by tubulin expression. Quantification corresponds to mean ± SEM of at least 3 independent experiments run in duplicates. (* p<0.05; *** p<0.001; Student's t-test).

**FIGURE 6.** Supplementation with BCAA- and ARO AA- containing dipeptides restores the proliferation of CD98hc-null fibroblasts. A) PEPT1 mRNA expression levels in wild-type (WT) and CD98hc-deficient (KO) fibroblasts. RPLP0 was used as a housekeeping gene (not depicted). Data are presented as mean ± SEM of 3 independent experiments. B) PEPT1 protein expression in wild-type and CD98hc-null fibroblasts. Representative Western blot of 2 independent experiments is shown. Data are normalized by tubulin expression. Quantification corresponds to mean ± SEM of 8 measurements (right panel). C) Proliferation of wild-type (WT) and CD98hc-null (KO) fibroblasts grown with no additives or in the presence of BCAA- and ARO AA- containing dipeptides (WT + dipeptides and KO + dipeptides) was measured over 6 days. Cell counts at several time points are shown as mean ± SEM of 3 independent experiments (left panel). Cell count at day 6 including KO + Ala as control (right panel)(*** p<0.001; Student's t-test). D) Samples from CD98hc-null (KO, open bar) and CD98hc-null + dipeptides (KO + dipeptides, dotted open bar) fibroblasts were processed as described in Materials and Methods and quantitative analysis of BCAAs and ARO AAs was performed. Results are expressed as mean ± SEM of 3 independent measurements. (* p<0.05; ** p<0.01; *** p<0.001; Student's t-test)
FIGURE 7. CD98hc serves as a hub of the stress response network. In wild-type fibroblasts (upper panel) CD98hc-associated transporters (LAT1, xCT and y’LAT2) ensure that cells have a balanced AA content, which allows them to counterbalance oxidative stress (via CD98hc/xCT) and to fuel protein synthesis and concomitant cell proliferation. When CD98hc is not present (lower panel), all associated transporters fail to reach the plasma membrane, and therefore there is no effective AA transport activity of LAT1, xCT and y’LAT2 transporters. β-ME supplementation is then required to inhibit cell death by ferroptosis. In supplemented culture media, CD98hc-null cells present ROS accumulation (triggering Nrf2) and imbalanced AA flux (increased concentration of AA\(^\text{+}\) caused by upregulation of CAT3 CD98hc-independent AA transporter, accumulation of AA\(^\text{0}\) and shortage of BCAAs and ARO AAs), which lead to a defect in cell proliferation. External supply of BCAAs and ARO AAs in form of dipeptides (which can enter the cell via PEPT1 di- and tri-peptide transporter and compensate for the lack of CD98hc/LAT1 transport activity) restores cell proliferation.
A. 

| CD98hc | C69T98E98 | C98T69E98 | C98T98E98 | CD69 |
|--------|-----------|-----------|-----------|------|
|       |           |           |           |      |
|       | +         | +         | +         | -    |
|       | -         | -         | -         | -    |

B. 

WT ES cells

C69T98E98

C98T69E98

CD69

C. 

Tumor size (mm²)

Days following injection

Ballina et al. Fig. 1
A. Graphs showing cell growth under -β-ME and +β-ME conditions. The graph on the left represents WT and KO cells, while the graph on the right shows the same for Cys and Cys precursors.

B. Bar graph showing CHAC1 mRNA levels under different conditions. The graph compares WT and KO cells with treatments including β-ME, SAS, and β-ME + SAS.

C. Bar graph showing number of cells (x1000) under different conditions. The graph compares WT and KO cells with treatments including none, β-ME, NaC, and Vit E.

D. Bar graph showing percentage of DCF positive cells under WT and KO conditions.

E. Western blot analysis of α Nrf2 and α tubulin under WT, KO, and (+β-ME) conditions.
Ballina et al. Fig. 3
Arg  Lys  His  Pro  Thr  Gly  Ala  Ser  Asn  Gin  Met  Val  Leu  Ile  Phe  Tyr  Trp

AA content (nmol AA / µg prot)

B.  

WT  KO

0  200  400  600  800  1000 pmols L-Arg / mg prot · min

0  50  100  150 pmols L-Ile / mg protein · min

C.  

CAT3 activity (pmols L-Arg / mg protein · min)

WT  KO

D.  

WT  KO

0  1000  2000  3000  4000  5000 pmols L-Ala / mg prot · min

y+  y+L

E.  

0  7  14  21 pmols L-Lys / mg prot · min

F.  

none  BCH

0  100  200 pmols L-Ile / mg protein · min

G.  

WT  KO

0  200  400  600  800 pmols L-Ile / mg prot · min

Ballina et al. Fig. 4
A. 

![Graph showing P-S6 / total (a.u.) for WT and KO with a comparison of α P-S6 (S235/236), α S6, and α tubulin.]

B. 

![Graph showing P-eIF2α / total (a.u.) for WT and KO with a comparison of α P-eIF2α (S51), α eIF2α, and α tubulin.]

C. 

![Graph showing ATF4 (a.u.) for WT and KO with a comparison of α ATF4 and α tubulin.]

Ballina et al. Fig. 5
WILD TYPE CELL

CD98hc/xCT \[ \text{CSSC-} \text{Glu-} \] BCAA

ARO AA

GSH

BALANCED AA CONTENT

RESISTANCE TO OXIDATIVE STRESS

PROTEIN SYNTHESIS

CD98hc/LAT1

CD98hc/LAT2

IMBALANCED AA FLUX

AA+

AA0

ARO AA

BCAA

Ballina et al. Fig. 7
Amino Acid Transport Associated to Cluster of Differentiation 98 Heavy Chain (CD98hc) is at the Crossroad of Oxidative Stress and Amino Acid Availability.
Laura R. de la Ballina, Sara Cano-Crespo, Elena González-Muñoz, Susanna Bial, Soline Estrach, Laurence Cailleteau, Floriane Tissot, Hannelore Daniel, Antonio Zorzano, Mark H. Ginsberg, Manuel Palacin and Chloé C. Féral

*J. Biol. Chem.* published online March 5, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M115.704254

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