

## Molecular and cellular characterization of the Down syndrome critical region protein 2

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### Abstract

Down syndrome (DS) is caused by trisomy for human chromosome 21 and is the most common genetic cause of mental retardation. The distal 10 Mb region of the long arm of the chromosome has been proposed to be associated with many of the abnormalities seen in DS. This region is often referred to as the Down syndrome critical region (DSCR). We report here the results of our analyses of the DSCR protein 2 (DSCR2). Results from transiently transfected COS-1 and HEK293 cells suggest that DSCR2 is synthesized as a 43 kDa precursor protein, from which the N-terminus is cleaved resulting in a polypeptide of 41 kDa. The polypeptide is modified by still uncharacterized co- or post-translational modifications increasing the predicted molecular weight of 32.8 kDa by about 10 kDa. Analyses of the only putative N-glycosylation site by *in vitro* mutagenesis excluded the possibility of the contribution of N-glycosylation to this increase in molecular weight. Further, the results of intracellular localization studies and membrane fractionation assays indicate that DSCR2 is targeted to a cytoplasmic compartment as a soluble form.

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Down syndrome (DS) is the most prevalent genetic cause of mental retardation affecting approximately one in 700 newborns. DS is characterized by a combination of several clinical abnormalities, in addition to mental retardation. These phenotypic features include congenital heart disease, short stature, hypotonia, significant hearing loss, and early onset Alzheimer disease with dementia [1–3]. The incidence of leukemia is significantly increased in DS patients, the relative risk being 10–20 times higher than that of the normal population, and acute megakaryocytic leukemia occurs 200–400 times more frequently than in the normal population [4].

Cytogenetic and clinical studies of DS patients have implied that the syndrome is caused by complete or partial triplication of chromosome 21. Our earlier studies

have demonstrated that DS is a contiguous gene syndrome, and make it clear that a single DS chromosomal region is not responsible for all of the DS phenotypic features and other gene products interact with the genes located on chromosome 21 [3]. However, studies of patients' karyotypes with partial triplication of chromosome 21 have shown that a 5–10 Mb chromosomal region at 21q22.2–21q22.3 is associated with many of the phenotypic features of DS patients. This region is often called the Down syndrome critical region (DSCR) in that it contains genes which contribute a large part of the variance of these features. Several genes have been identified in this region including DSCR1–5 [5–10].

Here we have begun to assess the biochemical and cellular properties of the novel DSCR2 protein, which is of unknown function and which has no homology to any known protein. The human DSCR2 gene encodes a protein of 288 amino acids, resulting in a predicted polypeptide of 32.8 kDa, which was originally called

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chromosome 21 leucine rich protein (c21-LRP) due to a high content (10%) of leucine residues [6]. Computational prediction programs suggest two hydrophobic regions for DSCR2, which possibly represent transmembrane domains, located at amino acid positions 72–91 and 217–243 (Fig. 1). Due to the lack of any organelle targeting signals, DSCR2 is predicted to be a cytoplasmic protein. The observed 85.4% homology between human and mouse proteins suggests a high conservation in evolution and consequently an important role for development or survival of mammalian cells [10]. The mouse polypeptide has an extra glutamine residue in the N-terminal low complexity region resulting in a predicted protein of 289 amino acids. This low complexity region encompasses amino acids 19–36 and contains 10 glutamic acid residues.

Expression analysis by Northern blotting has revealed that DSCR2 is expressed at low levels in human brain and leukocytes, with highest expression in testis and Jurkat cells [6]. Lower DSCR2 mRNA levels were detected by RT-PCR in colon and breast, and DSCR2 expression was detected in all fetal tissues tested. The expression pattern in adult mouse tissues was also determined by Northern blotting. These analyses revealed that the highest expression levels of DSCR2 were detected in mouse testis and that expression continues throughout embryogenesis starting as early as E11. Moderate DSCR2 expression levels were detected in adult mouse brain, liver, and kidney. Expression analyses of cultured T98G and Jurkat cell lines indicated that DSCR2 expression is regulated at the rate of proliferation, with higher expression in dividing cells and lower at the quiescent stage of cells [11].

To obtain more information about the DSCR2 polypeptide, we analyzed its biochemical properties and intracellular targeting in transiently transfected HEK293 and COS-1 cells. Our results imply that DSCR2 is synthesized as a 43 kDa precursor protein, which is processed to a 41 kDa polypeptide by N-terminal trimming. The 10 kDa difference between the predicted and observed molecular weights of the protein is not due to

the usage of the only putative N-glycosylation site at position 129 but it is more likely due to other co- or post-translational modifications. Additionally, the results obtained from membrane fractionation and intracellular localization analyses by immunofluorescence microscopy suggest that DSCR2 is a soluble cytoplasmic protein in HEK293 and COS-1 cells.

**Materials and methods**

*Construction of expression plasmids and in vitro mutagenesis.* The human DSCR2 cDNA was cloned into a pBluescript vector (Stratagene, La Jolla, CA) as described previously by direct cDNA selection using human chromosome 21 specific BAC and cDNA libraries generated from a 14-week-old trisomy 21 fetal brain [11]. The coding region of the wild-type human DSCR2 gene was PCR cloned into a mammalian expression vector pCMV-Script (Stratagene) in the presence or absence of an N- (hereafter called N-FLAG-DSCR2/pCMV-Script) or C-terminal (hereafter called C-FLAG-DSCR2/pCMV-Script) FLAG epitope sequence using linker primers and the pBluescript vector containing the DSCR2 cDNA. To remove the putative N-glycosylation site residing at position 129 from the C-FLAG-DSCR2/pCMV-Script construct, the asparagine residue was changed to glutamine by QuickChange site-directed in vitro mutagenesis kit (Stratagene) according to manufacturer’s protocols. The correct nucleotide sequences of all expression constructs were confirmed by DNA sequencing.

*Cell culture and transfections.* HEK293 and COS-1 cells were purchased from the American Type Culture Collection (Manassas, VA). HEK293 were maintained in MEM (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (Cellgro), non-essential amino acids (Cellgro), and antibiotics (Invitrogen, Carlsbad, CA) in 5% CO<sub>2</sub> at 37 °C. COS-1 cells were maintained in DMEM (Cellgro) supplemented with 10% fetal bovine serum and antibiotics in 5% CO<sub>2</sub> at 37 °C. Cells were plated 1 day prior to transfections, which were carried out using the LipofectAMINE PLUS reagent (Invitrogen) following the manufacturer’s guidelines.

*DSCR2 specific antibody production.* To obtain a DSCR2 specific antibody (hereafter called 1a), rabbits were immunized by subcutaneous injection with 500 µg of a synthetic peptide, corresponding to amino acids 46–60 of the human DSCR2 polypeptide, coupled to keyhole limpet hemocyanin conjugate emulsified in complete Freund’s adjuvant. The immunizations were repeated four times over a span of 3 weeks, and the blood was collected 1 week after the last immunization. To isolate the antiserum, blood was centrifuged for 10 min at 13,000g and the serum fractions were used in experiments.

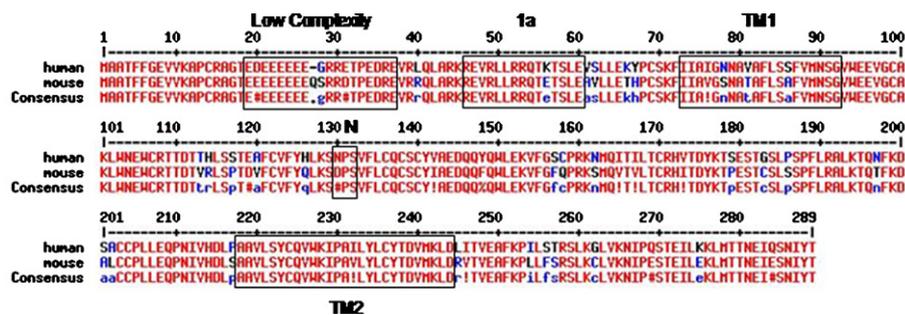


Fig. 1. Comparison of human and mouse DSCR2 polypeptides. Predicted transmembrane regions (TM1 and TM2), putative N-glycosylation site (N), the low complexity region located in the N-terminus of the polypeptide, and the peptide sequence used to raise the 1a antibody (1a) are indicated by boxes.

**Immunoprecipitation and Western blotting.** For immunoprecipitation assays, HEK293 cells were transfected with N-FLAG-DSCR2/pCMV-Script, C-FLAG-DSCR2/pCMV-Script or pCMV-Script. Two days post-transfection, cells were harvested using RIPA lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Igepal, 0.5% deoxycholic acid, and 0.1% SDS) supplemented with protease inhibitors (Complete; Roche Applied Science, Indianapolis, IN). Cell lysates were subjected to immunoprecipitation using either the 1a (immunoprecipitation/Western blotting assays and membrane fractionation) or FLAG (metabolic labeling) (Stratagene) antibodies and protein A/G PLUS–Sephrose (Santa Cruz Biotechnology, Santa Cruz, CA). After an overnight incubation at 4 °C, immunocomplexes were washed three times with ice-cold RIPA buffer and the pellets were resuspended in 2× Laemmli buffer. The protein samples were separated on 10% SDS–PAGE gels and the results were obtained either by Western blotting using the FLAG antibody (immunoprecipitation/Western blotting assays and membrane fractionation) or by fluorography (metabolic labeling, see below).

**Metabolic labeling.** For metabolic labeling, HEK293 cells were plated a day before transfections, which were performed as described above using the N-FLAG-DSCR2/pCMV-Script, C-FLAG-DSCR2/pCMV-Script or the pCMV-Script constructs. Cells were metabolically labeled 2 days post-transfection by starving them in methionine- and cysteine-free medium (Invitrogen) for 1 h and then labeled with 50 µCi/ml of both [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine (Amersham Biosciences UK, Little Chalfont, Buckinghamshire, UK) for 1 h. After the labeling, cells were harvested and lysed with RIPA buffer supplemented with protease inhibitors. Lysed cells were immunoprecipitated with FLAG antibody and protein A/G–Sephrose. Immunocomplexes were separated on 10% SDS–PAGE and visualized by fluorography (Amplify, Amersham Biosciences, UK).

**Membrane fractionation.** HEK293 cells were transiently transfected with the CLN5 expression construct [13] N-FLAG-DSCR2/pCMV-Script or C-FLAG-DSCR2/pCMV-Script constructs. Actin was detected as an endogenously expressed protein. The fractionation was performed as described before [14] using membrane fractionation buffer A (10 mM Hepes, pH 7.0, 0.15 M KCl, 1 mM EGTA, 0.5 mM MgCl<sub>2</sub>, and 1 mM dithiothreitol) supplemented with protease inhibitors. Briefly, cells were mechanically lysed with a syringe and a 26-gauge needle. To remove any unbroken cells, aggregates, and potential inclusion bodies, lysates were centrifuged at 10,000g for 5 min at 4 °C. Supernatants were then subjected to ultracentrifugation at 120,000g for 90 min at 4 °C. The fractions were analyzed by Western blotting using antibodies to FLAG (for DSCR2), CLN5 (to confirm the membrane fraction), and actin (to confirm the soluble fraction).

**Immunofluorescence microscopy.** To determine the subcellular localization of the DSCR2 protein, HEK293, and COS-1 cells were plated on coverslips 1 day prior to transfections, which were performed with N-FLAG-DSCR2/pCMV-Script or C-FLAG-DSCR2/pCMV-Script constructs. To determine the final destination of the polypeptides, the protein synthesis was halted for 2 h two days after transfections by adding 50 µg/ml cycloheximide (Sigma–Aldrich, St. Louis, MO) to the cells. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature and blocked with 0.5% bovine serum albumin (Fraction V, Sigma–Aldrich) or blocked and permeabilized with 0.5% bovine serum albumin (Fraction V)/0.2% saponin (Sigma–Aldrich) for 15 min at room temperature. Alternatively, cells were fixed with cold methanol, using the blocking procedure described above. Thereafter, cells were labeled with the FLAG antibody, washed with the blocking solution, and incubated with fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody. After washing with phosphate-buffered saline, the cells were mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA) and viewed with a fluorescence microscope (Carl Zeiss MicroImaging, Thornwood, NY) using a fluorescence in situ hybridization image capture system (MetaSystems Isis, Altusheim, Germany).

## Results

### *Expression of wild-type, FLAG-tagged, and N129Q DSCR2 polypeptides*

The human DSCR2 gene encodes a polypeptide of 288 amino acids, resulting in a protein with a predicted molecular weight of 32.8 kDa [6] (Fig. 1). To facilitate the detection of DSCR2 expression, we first cloned the coding region of the gene into a mammalian expression vector with and without the N- or C-terminal FLAG epitope. At the same time, we raised antibodies in rabbits using a peptide homologous to amino acids 45–59 of the human DSCR2 protein (hereafter called 1a). To analyze the specificity of the newly produced 1a antibody in immunoprecipitation assays and Western blotting, we transiently transfected HEK293 cells with the C-FLAG-DSCR2/pCMV-Script expression construct or the pCMV-Script vector. The immunoprecipitation was performed using either the 1a antiserum or the preimmune serum. The results of these experiments revealed that the 1a antiserum specifically detects the DSCR2 polypeptide in immunoprecipitation assays (Fig. 2A) but does not recognize the denatured form of the protein in Western blotting (data not shown). Therefore, the 1a antibody was used only in immunoprecipitation, whereas the FLAG antibody was used in both Western blotting and immunoprecipitation assays. These experiments also indicated that DSCR2 has two different polypeptide forms of 44 and 42 kDa. Since human DSCR2 has one potential N-glycosylation site (Asp-X-Ser) at position 129, we sought to clarify if these two DSCR2 forms are due to a differential N-glycosylation status of the polypeptide. To facilitate these analyses, we first mutagenized the only potential N-glycosylation site from asparagine to glutamine (N129Q) using C-FLAG-DSCR2/pCMV-Script as a template. After transfections of HEK293 cells with the C-FLAG-DSCR2/pCMV-Script and N129Q expression constructs, the cell lysates were immunoprecipitated with the 1a antibody and the immunocomplexes were analyzed by Western blotting using the FLAG antibody. The analyses of cell lysates overexpressing the glycosylation mutant N129Q protein resulted in two bands with similar molecular weights to those of wild-type proteins (Fig. 2B). This result suggests that the two observed DSCR2 forms are not generated by differential N-glycosylation status of the proteins and the asparagine residue at position 129 is not modified by N-glycans, but rather that size difference is due to other cellular processes.

Next, we analyzed whether the observed two forms are caused by N-terminal proteolytic trimming of the newly synthesized polypeptides. For these analyses, we transfected the HEK293 cells with the DSCR2 construct with either N- or C-terminal FLAG epitope, immunoprecipitated the cell lysates with the 1a antibody, and stained the Western blots with the FLAG antibody.

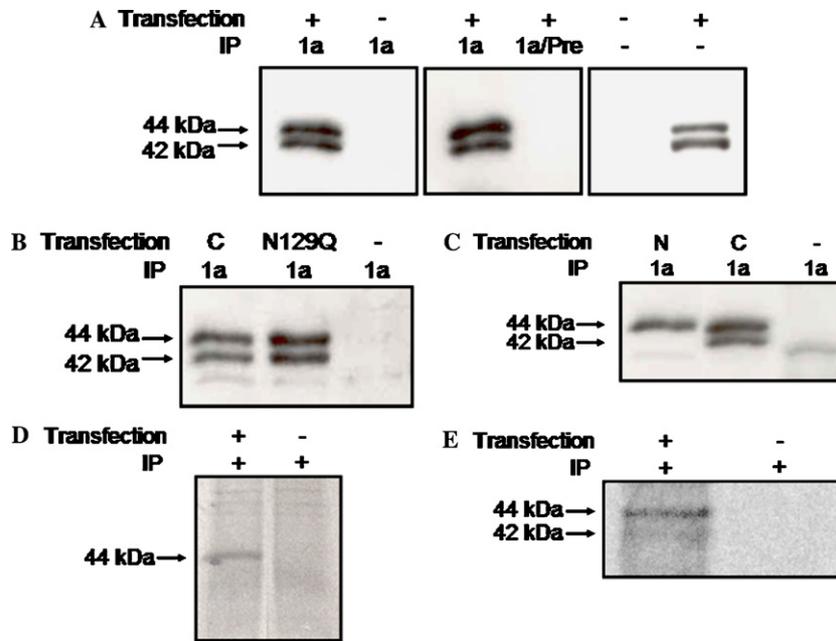


Fig. 2. Expression of DSCR2 in HEK293 cells. (A) Cells were transfected with C-FLAG-DSCR2/pCMV-Script expression construct (+) or pCMV-Script vector (-) and cell lysates were immunoprecipitated with a DSCR2 specific antibody (1a) or pre-serum from the same rabbit before immunization (1a/Pre), followed by Western blotting using the FLAG antibody. The right-hand panel shows a Western blot of transfected cells (non-immunoprecipitated) using the FLAG antibody. (B) Cells were transfected with C-FLAG-DSCR2/pCMV-Script (C), C-FLAG-DSCR2/pCMV-Script lacking the only potential N-glycosylation site at position 129 (N129Q) or pCMV-Script (-) and cell lysates were immunoprecipitated with the 1a antibody. Western blotting was performed with the FLAG antibody. (C) Cells were transfected with N-FLAG-DSCR2/pCMV-Script (N), C-FLAG-DSCR2/pCMV-Script (C) or pCMV-Script (-) and cell lysates were either immunoprecipitated with the 1a antibody or remained non-immunoprecipitated (-). Western blotting was performed with the FLAG antibody. (D) Cells were transfected with the N-FLAG-DSCR2/pCMV-Script expression construct (+) or the pCMV-Script vector (-) and metabolically labeled with [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine. Thereafter, cell lysates were immunoprecipitated with the FLAG antibody (+). Proteins were separated on SDS-PAGE and the results were obtained by fluorography. (E) Cells were transfected with the C-FLAG-DSCR2/pCMV-Script expression construct (+) or the pCMV-Script vector (-) and metabolically labeled with [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine, which was followed by cell lysis and immunoprecipitation with the FLAG antibody (+). Proteins were separated on SDS-PAGE and the results were obtained by fluorography. Transfections and immunoprecipitations are shown above each panel and molecular weights of the proteins bands are indicated on the left.

The expression of N-FLAG-DSCR2/pCMV-Script construct resulted in a single protein band of 44 kDa and the lower molecular weight band was never detected when the N-FLAG construct was used (Fig. 2C). These results suggest that the N-terminus of the DSCR2 polypeptide is cleaved after synthesis, rendering a protein of 42 kDa. In this case, the N-terminal trimming also removes the FLAG epitope located in the N-terminus of the polypeptide, making the lower molecular weight protein undetectable with the FLAG antibody.

To further confirm the molecular weights of the DSCR2 polypeptides, we metabolically labeled HEK293 cells transiently transfected by either N- or C-terminally tagged constructs with [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine. The cell lysates were analyzed by fluorography after immunoprecipitation with the FLAG antibody. The results of these analyses were consistent with those observed by Western blotting, showing one band of 44 kDa after transfection with the N-FLAG-DSCR2/pCMV-Script construct and two bands of 44 and 42 kDa following transfection using the DNA construct with a C-terminal FLAG tag (Figs. 2D and E).

#### *DSCR2 is a soluble protein*

Computational analyses of the primary amino acid sequence of the DSCR2 polypeptide suggest that it has two hydrophobic regions at positions 72–91 and 217–243, predicting two transmembrane domains for the polypeptide [6]. To test if these predictions hold true, we performed a membrane fractionation assay of HEK293 cells transiently transfected with either the N-FLAG-DSCR2/pCMV-Script or the C-FLAG-DSCR2/pCMV-Script constructs. Before membrane fractionation, any unbroken cells, aggregates, or inclusion bodies were removed by 10,000g centrifugation. After centrifugation at 120,000g, cell lysates were immunoprecipitated with the 1a antibody and analyzed by Western blotting using the FLAG antibody. The results of these experiments indicated that DSCR2 is located in the supernatant after centrifugation of postnuclear supernatants at 120,000g, suggesting that DSCR2 is a soluble protein (Figs. 3A and B). As found in our Western blotting experiments, the N-terminally tagged expression construct resulted in only one protein band of 44 kDa, whereas the C-termi-

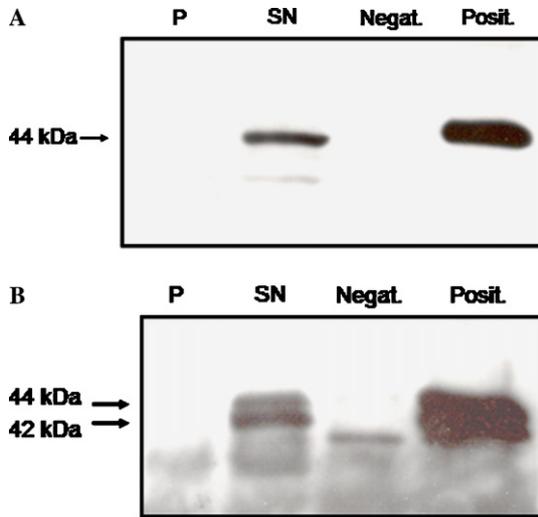


Fig. 3. Membrane fractionation of DSCR2. HEK293 cells were transiently transfected with N-FLAG-DSCR2/pCMV-Script (A) or C-FLAG-DSCR2/pCMV-Script (B), mechanically lysed, and subjected to ultracentrifugation at 120,000g. Fractions were immunoprecipitated with the 1a antibody and Western blotting was performed with the FLAG antibody. Molecular weights of the protein bands are indicated on the left. P, pellet; SN, supernatant; Negat., negative control for Western blotting with pCMV-Script transfection and without immunoprecipitation; and Posit., positive control for Western blotting with N-FLAG-DSCR2/pCMV-Script (A) or C-FLAG-DSCR2/pCMV-Script (B) without immunoprecipitation.

nally tagged construct resulted in two bands of 44 and 42 kDa. These observations imply that both observed DSCR2 forms are soluble and that the predicted hydrophobic regions of the polypeptide are not associated with membranes in HEK293 cells. As a control, we performed similar membrane fractionation assays for two other proteins, CLN5 and actin, of which the long form of CLN5 is known to be a membrane protein [12] and actin is a sol-

uble protein. As expected, CLN5 was found associated with the membrane fraction and actin with the soluble fraction (data not shown).

*DSCR2 is targeted to cytoplasm in COS-1 and HEK293 cells*

Analyses of the primary amino acid sequence of DSCR2 suggest a cytoplasmic location with a likelihood of 69.6% for the DSCR2 polypeptide [6]. To determine if DSCR2 is targeted to a cytoplasmic compartment, we transiently transfected COS-1 and HEK293 cells with DSCR2 expression constructs tagged with the FLAG epitope in either the N- or C-terminus. To determine the final destination of the polypeptides, protein synthesis was halted with cycloheximide for 2 h before staining the cells with the FLAG antibody. Intracellular localization images were captured by immunofluorescence microscopy. The staining pattern of both cell lines showed distribution throughout the cells, in a pattern resembling that of the cytoplasm (Figs. 4A–D). This observation held true for both N- and C-terminally tagged constructs. These staining results suggest that in transiently transfected COS-1 and HEK293 cell lines, DSCR2 is targeted to a cytoplasmic compartment. Identical results were observed with two different fixation methods using either methanol or 4% PFA followed by saponin permeabilization. When protein synthesis in cell lines transfected with the N-terminal FLAG epitope was blocked by cycloheximide, fluorescent staining of the proteins became significantly fainter, whereas staining of cells expressing C-FLAG-DSCR2/pCMV-Script construct remained intact (data not shown). These observations further support the possibility that the N-terminus of the polypeptide is proteolytically trimmed after synthesis, as suggested by the immunoprecipitation/Western blotting assays.

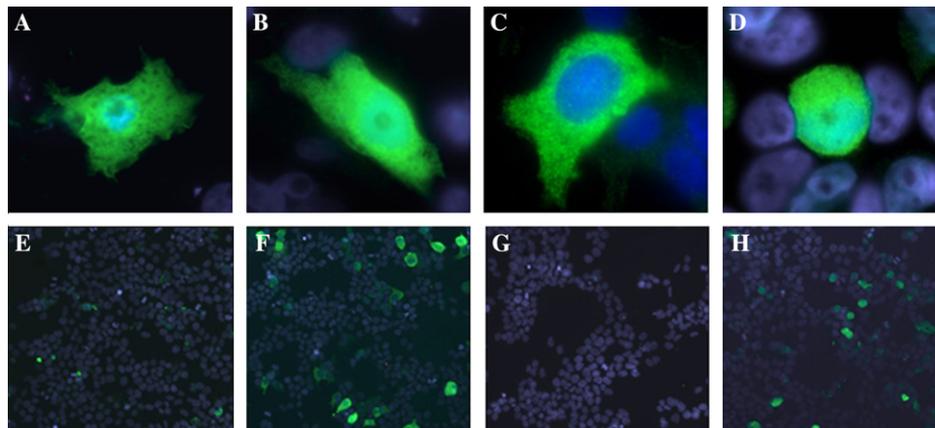


Fig. 4. Subcellular localization of DSCR2. COS-1 (A,B) and HEK293 (C–H) cells were transiently transfected with N-FLAG-DSCR2/pCMV-Script (A,C,E,F) or C-FLAG-DSCR2/pCMV-Script (B,D,G,H) and fixed with paraformaldehyde. Cells were either blocked with bovine serum albumin and permeabilized with saponin (A,B,C,D,F,H) or just blocked with bovine serum albumin (E,G) before staining with the FLAG antibody. The secondary antibody was conjugated with fluorescein isothiocyanate. Cells were viewed with an immunofluorescence microscope. Magnifications: A–D, 630 $\times$ ; E–H, 100 $\times$ .

It has previously been suggested that both N- and C-termini of DSCR2 may be located intracellularly, with the loop between two potential transmembrane domains being extracellular [6]. To confirm the intracellular/extracellular topology of both termini, HEK293 cells were transfected with the constructs with the FLAG epitope in either the N- or C-terminus. Then, transfected cells were stained with the FLAG antibody with or without saponin permeabilization after PFA fixation. Both protein forms showed significantly higher degree of positive cells when saponin permeabilization of the cell membrane was performed before staining. These results further confirm that both termini of the DSCR2 polypeptide are located intracellularly.

## Discussion

The clinical phenotypes of DS are caused by an overexpression of a subset of genes located on human chromosome 21. Analyses of trisomic mouse models for DS have revealed that despite segmental trisomy of murine chromosome 16, which is orthologous to human chromosome 21, about 9% of these genes are not overexpressed, and the expression of some genes is even decreased [15]. However, the expression of most trisomic genes is increased and these genes, as well as the genes with decreased expression, are assumed to contribute to the clinical manifestations of DS. Cytogenetic studies of patients with partial triplication have shown that the distal part of chromosome 21 is sufficient to cause many of the phenotypic abnormalities found in DS patients. One of the genes located in this chromosomal region is the novel gene DSCR2, whose function or contribution to the manifestations of DS is currently unknown. To facilitate the evaluation of the cellular consequences of overexpression of DSCR2, we studied the biosynthesis, maturation, and targeting of this polypeptide in cellular systems using transiently transfected COS-1 and HEK293 cells. Our expression analyses reveal that the protein encoded by the DSCR2 gene is synthesized as a 44 kDa precursor, when a FLAG epitope tag is added either to the N- or C-terminus. This epitope increases the molecular weight of the polypeptide by approximately 1 kDa, suggesting that the DSCR2 polypeptide itself is approximately 43 kDa. Despite several attempts, we were not able to raise DSCR2 specific antibodies that recognize the denatured form of the protein in Western blotting, and therefore we used FLAG antibodies to detect the DSCR2 polypeptide in immunostaining. However, the newly produced 1a antibody recognized DSCR2 polypeptides in immunoprecipitation assays, and was therefore used to concentrate expressed polypeptides prior to immunostainings. Moreover, when DSCR2 constructs with a C-terminal FLAG epitope were used, a lower molecular weight band of 42 kDa

was also observed. Subtraction of the molecular weight increased by the FLAG tag suggests that the smaller DSCR2 form is 41 kDa. This kind of phenomenon is often due to differential glycosylation status of the isoforms, usage of alternative methionine in translation of the polypeptide, or proteolytic trimming of the polypeptide. Since DSCR2 has one putative N-glycosylation site at position 129 and the N-glycosylation of one asparagine residue is known to increase the molecular weight of the polypeptide by about 2 kDa, we first analyzed whether these protein bands represent differentially N-glycosylated isoforms of the polypeptide. Elimination of the only putative N-glycosylation site by mutagenizing the asparagine residue to glutamine resulted in identical protein bands to wild type proteins on Western blots. Based on these observations, we concluded that DSCR2 is not modified by N-glycans and the observed size difference of 2 kDa is not due to differential N-glycosylation of the polypeptide. Because the second methionine on the DSCR2 polypeptide is located at position 89, it was very unlikely that the size difference was due to the usage of this alternative methionine in the initiation of translation. Therefore, we next analyzed if the observed size difference is due to proteolytic trimming of the polypeptide, using a DSCR2 cDNA construct with an N-terminal FLAG epitope, and compared these results to those obtained when the C-FLAG-DSCR2 construct was used. Comparison of these stainings revealed that the N-terminal FLAG is detectable only in the higher molecular weight form, suggesting that the observed lower molecular weight band is the result of maturation of the polypeptide by N-terminal cleavage. Support for this hypothesis was apparent from immunofluorescent stainings of transiently transfected COS-1 and HEK293 cells. When protein synthesis was halted for two hours, the staining intensities of N-terminally tagged proteins decreased significantly, whereas stainings of the C-terminally tagged construct remained intact.

The precise cleavage site of the polypeptide is not currently known but based on the prediction of the primary amino acid sequence, cleavage may occur at the region surrounding the amino acid residue located at position 20. This potential cleavage site resides in the low complexity region encompassing the amino acids 19–36 (Fig. 1), which is assumed to be involved in interactions with other proteins. Therefore, it is possible that this glutamic acid rich region is bound by another protein that is involved in cleavage of the newly synthesized polypeptide resulting in a mature protein with a lower molecular weight. Alternatively, the putative binding protein may assist in targeting the DSCR2 polypeptide to the correct cellular compartment.

Earlier predictions have suggested a molecular weight of 32.8 kDa for the full-length DSCR2 [6], which differs

by about 10 kDa from the molecular weight observed by our immunoprecipitation/Western blotting assays and metabolic labeling analyses. The difference between the predicted and observed molecular weights may partially be explained by the inaccuracy of the prediction programs used. Additionally, it is possible that DSCR2 is co-translationally or post-translationally modified by other modifications, rather than N-glycosylation increasing the molecular weight.

Prediction programs have suggested that DSCR2 is a membrane protein due to the presence of two hydrophobic regions [6]. To elucidate if one or both of these amino acid regions are associated with membranes, we performed membrane fractionation experiments using transiently transfected HEK293 cells. Both DSCR2 forms were observed in the supernatant, suggesting that none of the predicted hydrophobic regions represent a transmembrane domain, and that DSCR2 is a soluble protein.

A cytoplasmic location has been proposed for the DSCR2 protein, since analyses of the primary amino acid sequence have not been able to detect any organelle targeting signals. Therefore, our next experiments aimed to clarify if these predictions for intracellular location held true. For this purpose, transiently transfected COS-1 and HEK293 cells were analyzed by immunofluorescence microscopy. Both N- and C-terminally tagged DSCR2 polypeptides showed a staining pattern typical of cytoplasm, suggesting that both newly synthesized and mature proteins are trafficked to the cytoplasmic compartment. Confirmation of this cytoplasmic location was performed in the experimental procedures by omitting the permeabilizing reagent, saponin. Both expression constructs used resulted in almost completely negative staining patterns in the absence of saponin, whereas several positively stained cells were observed when cells were permeabilized. These results suggest that both the N- and C-termini of the DSCR2 polypeptide are located intracellularly. Consequently, the observed cytoplasmic location is in accordance with the predicted location. The results of these intracellular localization and membrane fractionation experiments may direct future experiments to characterize interacting molecules located in the soluble fraction of the cytoplasmic compartment.

In conclusion, our described experiments indicate that the wild-type DSCR2 polypeptide is synthesized as a 43 kDa precursor protein, which is proteolytically trimmed at the N-terminus to result in a 41 kDa polypeptide. This polypeptide is co-translationally or post-translationally modified by events other than N-glycosylation, and is targeted to the cytoplasmic compartment as a soluble form. These results are important first steps toward the characterization of the cellular function of the DSCR2 protein and eventually, the contribution of its overexpression to the molecular pathogenesis and clinical manifestation of DS.

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