European Journal of Human Genetics (2012) 20, 870–877

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ARTICLE

C4ST-1/CHST11-controlled chondroitin sulfation interferes with oncogenic HRAS signaling in Costello syndrome

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Costello syndrome is a pediatric genetic disorder linked to oncogenic germline mutations in the *HRAS* gene. The disease is characterized by multiple developmental abnormalities, as well as predisposition to malignancies. Our recent observation that heart tissue from patients with Costello syndrome showed a loss of the glycosaminoglycan chondroitin-4-sulfate (C4S) inspired our present study aimed to explore a functional involvement of the chondroitin sulfate (CS) biosynthesis gene *Carbohydrate sulfotransferase 11/Chondroitin-4-sulfotransferase-1* (*CHST11/C4ST-1*), as well as an impaired chondroitin sulfation balance, as a downstream mediator of oncogenic HRAS in Costello syndrome. Here we demonstrate a loss of C4S, as well as a reduction in *C4ST-1* mRNA and protein expression, in primary fibroblasts from Costello syndrome patients. We go on to show that expression of oncogenic HRAS in normal fibroblasts can repress *C4ST-1* expression, whereas interference with oncogenic HRAS signaling in Costello syndrome fibroblasts elevated *C4ST-1* expression, thus identifying *C4ST-1* as a negatively regulated target gene of HRAS signaling. Importantly, we show that forced expression of *C4ST-1* in Costello fibroblasts could rescue the proliferation and elastogenesis defects associated with oncogenic HRAS signaling in these cells. Our results indicate reduced *C4ST-1* expression and chondroitin sulfation imbalance mediating the effects of oncogenic HRAS signaling in the pathogenesis of Costello syndrome. Thus, our work identifies *C4ST-1*-dependent chondroitin sulfation as a downstream vulnerability in oncogenic RAS signaling, which might be pharmacologically exploited in future treatments of not only Costello syndrome and other RASopathies, but also human cancers associated with activating RAS mutations.
European Journal of Human Genetics (2012) **20,** 870–877; doi:10.1038/ejhg.2012.12; published online 8 February 2012

Keywords: Costello syndrome; HRAS; C4ST-1; CHST11; chondroitin sulfate

INTRODUCTION

Costello syndrome (MIM 218040) is a pediatric disease associated with poor postnatal growth, mental retardation, craniofacial abnormalities, loose skin on limbs and neck, cardiac and elastogenesis defects, increased cellular proliferation and development of tumors.[1–9]

Recently, Costello syndrome has been identified as the first example of a disorder associated with activating germ-line mutations in the HRAS proto-oncogene.[10–17] In addition to Costello syndrome, a group of multiple congenital anomaly syndromes collectively named RASopathies have been shown to be associated with germline mutations in various genes of the RAS/MAPK pathway.[8,18,19] Indeed, inhibitors of a number of components of the RAS/MAPK pathway are currently being evaluated as pharmacological interventions in RASopathies.[18]

Glycosaminoglycans (GAGs), including chondroitin sulfate (CS), are involved in a wide variety of biological processes.[20–23] Part of the CS biosynthesis in the Golgi entails the transfer of sulfate groups to specific carbon positions of the disaccharide units by sulfotransferase enzymes.[20–23] The balance of chondroitin sulfation is tightly controlled through spatial and temporal expression of distinct chondroitin sulfotransferase genes.[21,24,25] Moreover, this tightly controlled chondroitin sulfation balance has been shown to be disturbed in human disease and malignancies.[26–31] Carbohydrate sulfotransferase 11/Chondroitin-4-sulfotransferase-1 (CHST11/C4ST-1; subsequently named C4ST-1) catalyzes the transfer of sulfate to the C-4 position of CS disaccharides, thus creating chondroitin-4-sulfate (C4S) as one of its products.[21] We have recently generated a loss-of-function mutation in *C4ST-1* and demonstrated severely decreased C4S levels and severe cartilage defects in homozygous mutant mouse embryos.[24,32]

Although other laboratories have shown that RAS signaling can affect the sulfation status and expression of CS proteoglycans *in vitro*,[33,34] we have established previously that cellular defects of fibroblasts derived from Costello syndrome patients can be reversed by degrading CSs in these cultures.[35] We also demonstrated an altered chondroitin sulfation balance with decreased C4S levels in the heart tissue of Costello syndrome patients.[36] These data suggest a possible link between oncogenic HRAS signaling and the balance of chondroitin sulfation in Costello syndrome.

Here we provide evidence that HRAS-mediated loss of *C4ST-1* expression and C4S levels are critical steps in the pathogenesis of Costello syndrome. We report that C4S levels, and *C4ST-1* mRNA and protein expression, are significantly decreased in fibroblasts from Costello syndrome patients. Forced expression of

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Received 26 August 2011; revised 9 January 2012; accepted 10 January 2012; published online 8 February 2012

oncogenic HRAS in normal fibroblasts could repress *C4ST-1* expression, whereas pharmacological interference with oncogenic HRAS signaling in CS fibroblasts elevated *C4ST-1* expression. These results therefore identified *C4ST-1* as a negatively regulated target gene of the HRAS signaling. Importantly, we show that forced expression of *C4ST-1* in Costello fibroblasts could rescue the proliferation and elastogenesis defects associated with oncogenic HRAS signaling in these cells. Thus, our results indicate that reduced *C4ST-1* expression and a subsequent imbalance in chondroitin sulfation have a central role in mediating the effects of oncogenic HRAS signaling in the pathogenesis of Costello syndrome. Moreover, these results identify *C4ST-1* and associated C4S levels as a downstream vulnerability in oncogenic RAS signaling, which might be an avenue to be pharmacologically exploited in future treatments not only of Costello syndrome, but also of RAS-associated human cancers.

METHODS

Fibroblast cultures

Primary fibroblasts were derived from forearm skin biopsies of five children, ranging in age from 1 month to 16 years (C7669, C12195, C12196, C-TC and C563), diagnosed and previously described[35] as Costello syndrome, and fibroblasts from two normal children of matching ages (4184 and 4185), as well as human foreskin fibroblasts (ATCC, Manassas, VA, USA). All biopsies were obtained with parental consent and Institutional Ethics Committee approval, and fibroblasts were isolated and cultured as previously described,[35] then stored as multiple samples of passage two in the cell repository of The Hospital for Sick Children in Toronto. For rescue of Costello syndrome defects, Costello syndrome fibroblast lines C7669 and C563 were stably transfected with either the CAGIP vector alone or with the CAGIP-Flag-hC4ST-1 expression plasmid, using the Amaxa Nucleofection system (Lonza, Walkersville, MD, USA) according to the manufacturer's recommendations.

Biochemical and cell biological analysis

Immunofluorescence was performed using the following antibodies: mouse α-chondroitin-4-sulfate (Seikagaku, ACCI, East Falmouth, MA, USA, 1:100), mouse α-chondroitin-sulfate (Sigma, Oakville, ON, Canada, 1:100), rabbit α-tropoelastin (EPC, Owensville, MO, USA, 1:300), mouse α-C4ST-1 (Abcam, Cambridge, MA, USA, 1:100), mouse α-BrdU (clone BU-33, Sigma, 1:500). Secondary antibodies were Cy2-conjugated and propidium iodide was used as a nuclear counterstain. The analysis of proliferation rates by BrdU incorporation was performed as previously described.[24] Western blot analysis was performed by separating cell lysates in TNTE-based lysis buffer on 10% SDS-PAGE gels, followed by transfer to nitrocellulose membranes and incubation with mouse α-C4ST-1 (Abcam, 1:100) and mouse α-beta-tubulin (Abcam, 1:1000) antibodies.

Absolute quantification of C4ST-1, C4ST-2 and C4ST-3 expression in fibroblasts

The cDNAs of C4ST-1, C4ST-2 and C4ST-3 were amplified with primers that provided an additional sequence for the bacterial T7 promoter at the 5′-end. *In vitro* transcription, using T7-RNA-polymerase was performed to obtain C4ST-1, C4ST-2 and C4ST-3 RNA, followed by RT reactions to obtain cDNA. Serial dilutions of 10, 0.5, 0.05 and 0.005 ng/μl were utilized in qRT-PCR reactions to obtain standard curves. Subsequently, we performed RT reactions on RNA from normal fibroblast cultures as previously described,[32] and quantified C4ST-1, C4ST-2 and C4ST-3 absolute expression levels by real-time PCR (qRT-PCR) using the ABI HT7600 system (ABI, Foster City, CA, USA), according to the manufacturer's instruction using an annealing temperature of 60 °C. The following primer pair sequences (5′–3′) were used for these amplifications:

hC4ST-1: 5′-AGTATGTTGCACCCAGTCATGCG-3′; 5′-GCAGGACAGCAGT GTTTGAGAGC-3′. hC4ST-2: 5′-GGGCGCGAGGTTCCCAGC-3′; 5′-AACAGC CGGGCCTTGGTCATC-3′. hC4ST-3: 5′-GCCCGGCATTTGGAAACAGAGC-3′; 5′-GAGCGCGGGTCCTGATCCAG-3′. hHPRT: 5′-AAACAATGCAGACTTTGC TTTCC-3′; 5′-GGTCCTTTTCACCAGCAAGCT-3′.

Sequencing of *HRAS* and *C4ST-1* genes in Costello syndrome

Genomic DNA from Costello syndrome patients was amplified using the following primer pairs (5′–3′) at an annealing temperature of 65 °C and 30 PCR cycles: HRAS exon 2 (first coding exon): HRAS-pr-F 5′-GGACCGCTG TGGGTTTGCCC-3′; HRAS-in1-R 5′-AGCCCTATCCTGGCTGTGTCCTG-3′. HRAS exons 3–5: HRAS-in1-F 5′-ATGAGGGGCATGAGAGGTACCAGG-3′; HRAS-3′-UTR-R GGCGTGAGCCCAGACCCCGG-3′. C4ST-1 exon 2 (first coding exon): hC4ST-1-x1 5′-TCCCCGCAGCCAGGACAAAGCCAT-3′; hC4ST-1-x2 5′-CAGAAATCAGGCGGAGCGAGCTAGAG-3′. C4ST-1 exon 3: hC4ST-x3 5′-TCCCCTGCTAGATTTTTCAGAGATGCTACATAG-3′; hC4ST-1-x4 5′-GCCTGTTTCTTCTTCCCTACTCTGTGC-3′. C4ST-1 exon 4: hC4ST-1-x5 5′-GTGGATTTATGATCTACTTGTAGCCAAGTGG-3′; hC4ST-1-x6 5′-AGATG TTGTACTTCTGGGTGAACTTGTTGCG-3′. hC4ST-1-x7 5′-TCCTGTTTGTC CGGGAGCCCTTCG-3′; hC4ST-1-x8 5′-AATCGAGTTTGTAGACTTCGTACA GCTGCG-3′. hC4ST-1-x9 5′-CTGAAGTTCCCCACCTATGCAAAGTCTACG-3′; hC4ST-1-x10 5′-CTGCAAAGACAGCTGTCCTTCCCTACG-3′. The same primers were used for sequencing reactions.

RESULTS

Costello syndrome is associated with severely reduced C4ST-1 expression and chondroitin sulfation imbalance

In a previous publication, we described reduced levels of C4S GAG chains in heart tissue of Costello syndrome patients.[36] Here we first aimed to extend this analysis to primary fibroblasts from Costello syndrome patients. Confluent cultures of fibroblasts from unaffected individuals (normal) and Costello syndrome patients (Costello) were stained with an antibody against C4S, using immunofluorescence techniques (Figures 1a and b). Although a C4S signal was easily detected in normal fibroblasts (Figure 1a), C4S was almost completely absent in Costello fibroblasts (Figure 1b).

Biosynthesis of C4S can be mediated by three closely related, but distinct enzymes: C4ST-1, C4ST-2 and C4ST-3. To evaluate which of these enzymes might have a predominant role in C4S synthesis in normal fibroblasts, we compared the expression of these genes (Figure 1c). We prepared mRNA from normal fibroblasts, and performed absolute quantification of mRNA levels of the three *C4ST* genes using real-time PCR (qRT-PCR). This analysis showed that *C4ST-1* is by far the predominant *C4ST* gene expressed in normal human fibroblasts, whereas expression of *C4ST-2* and *C4ST-3* was barely detectable (Figure 1c). Indeed, *C4ST-1* mRNA levels were approximately 30- and 500-fold higher when compared with *C4ST-2* and *C4ST-3*, respectively (Figure 1c). Thus, in subsequent experiments, we concentrated on the role of the predominant C4ST-1 in Costello syndrome.

Next, we tested whether the reduction of immuno-detectable C4S GAG chains in Costello fibroblasts is associated with a reduction in *C4ST-1* expression. For these experiments, we used primary fibroblasts from five patients clinically diagnosed with CS (C12195, C12196, C7669, C-TC, C563). To ensure that these cells are *bona fide* Costello syndrome samples, we first analyzed the mutational status of the *HRAS* gene by sequencing of the coding region. The genetic basis of Costello syndrome has been identified as heterozygous gain-of-function mutations in distinct regions of the *HRAS* coding sequence. Indeed, all five samples carried heterozygous mutations in amino acid 12 (G12A, G12S; Table 1), which have previously been described as HRAS gain-of-function mutations in Costello syndrome, as well as various cancers.[8,10,12,13,15,16,37] We next prepared mRNA from fibroblasts from the five Costello syndrome cell lines, as well as two different unaffected individuals (normal 1 and 2), and determined *C4ST-1* mRNA levels by qRT-PCR (Figure 1d). Although both normal samples showed high levels of *C4ST-1* expression, *C4ST-1* mRNA levels were severely reduced in all Costello syndrome samples, with lowest levels observed in C7669 and C563 cells (Figure 1d). In the

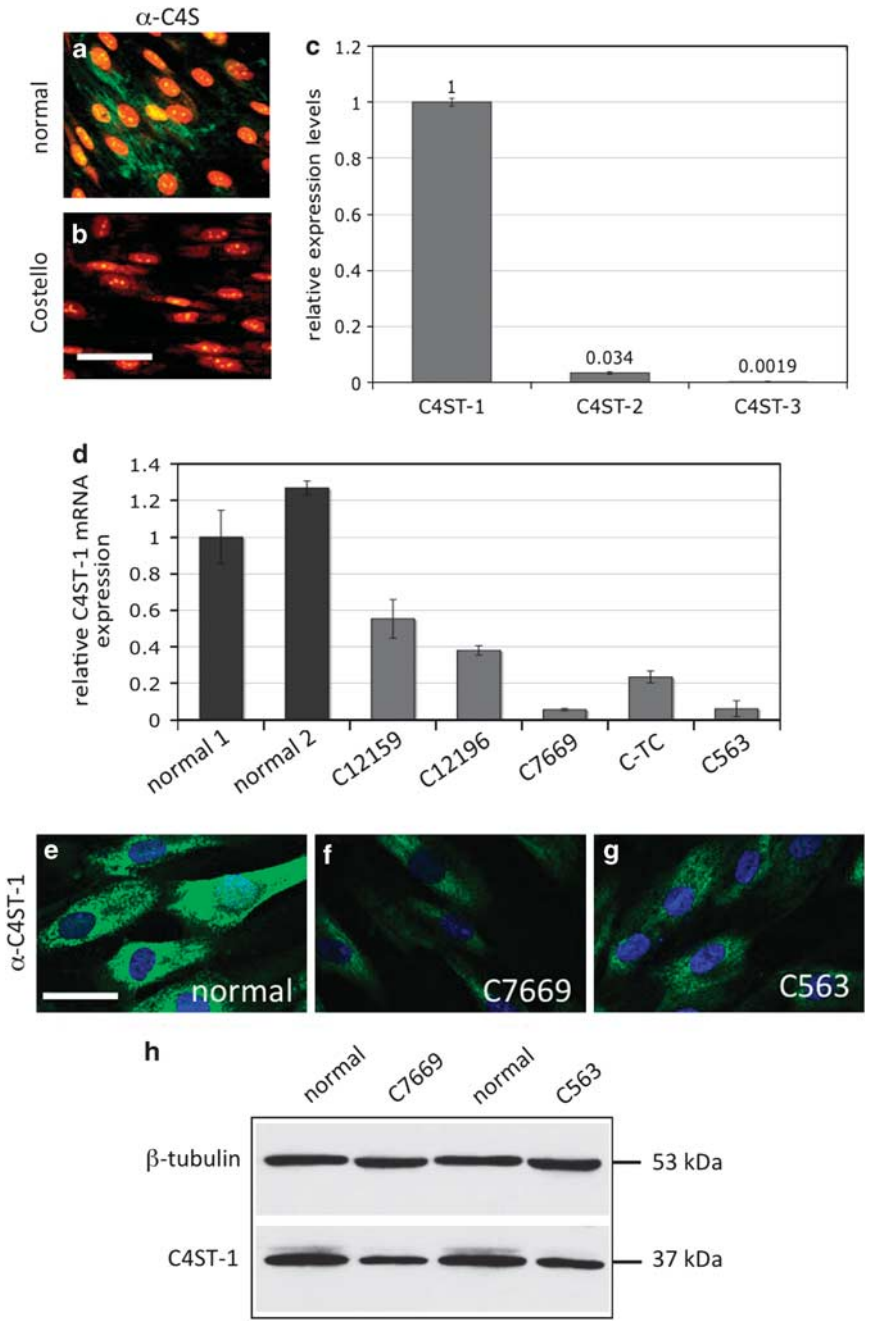

Figure 1 *C4ST-1* expression and C4S levels are reduced in Costello syndrome. (**a**, **b**) Immunofluorescent detection of C4S in normal and Costello syndrome using an α-C4S antibody. (**b**) Reduced levels of C4S (green signal) in Costello fibroblasts compared with normal cells (**a**; nuclei are counterstained orange-red with propidium iodine; scale bar represents 10 μm). (**c**) Absolute quantification of mRNA expression levels of *C4ST-1*, *C4ST-2* and *C4ST-3* in normal human primary fibroblasts by qRT-PCR. *C4ST-1* is expressed at drastically higher levels compared with *C4ST-2* and *C4ST-3*. (**d**) Expression of *C4ST-1* in normal and Costello syndrome samples. qRT-PCR revealed a reduction in *C4ST-1* mRNA expression in primary fibroblasts from all five Costello syndrome patients, when compared with two normal individuals. The average value from three measurements ± SD is shown. (**e**–**g**) Immunofluorescent detection of C4ST-1 in normal (**e**) and Costello syndrome C7669 (**f**), and C563 (**g**) fibroblasts using an α-C4ST-1 antibody. Both Costello syndrome samples show reduced levels of C4ST-1 protein (green signal; nuclei are counterstained blue with DAPI; scale bar represents 5 μm). (**h**) Western blot analysis of C4ST-1 protein expression using α-C4ST-1 and α-beta-tubulin antibodies demonstrates a reduction in C4ST-1 protein levels in C7669 and C563, when compared with normal samples.

following experiments, we utilized these Costello lines C7669 and C563 to establish a proof of principle of an involvement of C4ST-1 in the mediation of HRAS signaling. To extend our analysis to C4ST-1 protein expression, we performed both immunofluorescence and western blot detection of C4ST-1 protein on normal, C7669 and C563 fibroblasts. In immunofluorescence experiments, normal fibroblasts showed strong expression of C4ST-1 protein (Figure 1e), whereas expression was severely reduced in C7669 (Figure 1f) and C563 (Figure 1g) cells. Western blot experiments showed reduced levels of a normal-sized C4ST-1 protein in C7669 and C563 cells (Figure 1h). Accordingly, sequence analysis of the C4ST-1 coding sequence did not reveal any alterations in the Costello syndrome

Table 1 Mutational analysis of the *HRAS* coding sequence in Costello syndrome patients

Patient	*HRAS mutation*
C12195	G12A het
C12196	G12S het
C7669	G12S het
C-TC	G12S het
C563	G12S het

samples when compared with normal samples (data not shown). Together, these data demonstrated that Costello syndrome is associated with reduced levels of *C4ST-1* mRNA, C4ST-1 protein and C4S GAG chains.

Negative regulation of C4ST-1 expression by HRAS signaling

The results presented above let us to hypothesize that oncogenic HRAS signaling in Costello syndrome could negatively regulate C4ST-1 expression levels. To test this hypothesis, we wanted to determine whether experimental modulation of HRAS signaling in fibroblasts could affect C4ST-1 expression. Our hypothesis would predict that activation of HRAS signaling in normal fibroblasts could interfere with C4ST-1 expression; conversely, pharmacological inhibition of HRAS signaling in Costello syndrome fibroblasts should lead to increased expression of *C4ST-1*.

To test the first hypothesis that oncogenic HRAS could repress C4ST-1 expression, we transiently transfected normal fibroblasts with an oncogenic CAGIP–HRAS–G12V expression plasmid, in combination with a GFP expression plasmid to identify transfected cells (CAGIP–HRAS–V12/GFP), and tested by immunofluorescence detection whether C4ST-1 expression was reduced in tranfected cells when compared with non-transfected cells. As a control, the empty CAGIP vector along with the GFP expression plasmid was transfected. GFP-positive cells transfected with CAGIP/GFP (Figure 2b) showed no elevated HRAS levels, as expected (Figure 2c). Moreover, in these samples, levels of C4ST-1 expression were similar in transfected and non-transfected cells (Figure 2d). In contrast, GFP-positive cells tranfected with CAGIP–HRAS–V12/GFP (two fields shown; Figures 2f and j) showed elevated levels of HRAS (Figures 2g and k), indicating that our HRAS-V12 expression cassette is functional. These GFP-positive cells expressing oncogenic HRAS-V12 showed a clear reduction in C4ST-1 expression levels when compared with neighboring non-transfected cells (Figures 2h and l). To quantify this effect, we measured the luminosity of the C4ST-1 signal (Figure 2m). Although there was no difference in luminosity between the control CAGIP-transfected and non-transfected cells, expression of HRAS-V12 caused a drastic decrease in C4ST-1 luminosity (Figure 2m) when compared with neighboring non-transfected cells, thus confirming that oncogenic HRAS signaling could indeed repress C4ST-1 expression.

We next tested our second hypothesis that pharmacological inhibition of HRAS signaling in Costello syndrome fibroblasts could lead to increased expression of *C4ST-1*. The MAPK pathways are critical downstream mediators of RAS signal transduction, and have been shown to be upregulated in response to oncogenic HRAS mutations in Costello syndrome.[8,18,38,39] Thus, C7669 fibroblasts were treated with inhibitors of the MAP-kinases Erk (PD98059), p38 (SB203580) and SAPK/JNK (SP600125) for 48 h, and subsequently analyzed for *C4ST-1* mRNA expression by qRT-PCR (Figure 2n). Blocking all three MAPK cascades was able to significantly elevate the *C4ST-1* expression by approximately 2- to 3.5-fold when compared with a DMSO-treated control. These results show that inhibition of RAS signaling in C7669 cells could re-establish higher levels of *C4ST-1* expression. Combined, these experiments demonstrated that C4ST-1 is a negatively regulated target of oncogenic HRAS signaling in Costello syndrome fibroblasts.

Re-establishing expression of C4ST-1 rescues impaired elastogenesis and increased proliferation in Costello syndrome fibroblasts

Our results, thus far, showed that activated HRAS signaling in Costello syndrome could negatively regulate C4ST-1 expression and leads to a significant reduction in C4ST-1 and C4S levels in fibroblasts from Costello syndrome patients. Fibroblasts from Costello syndrome patients have previously been shown to be unable to deposit elastin fibers in the extracellular matrix.[35,40–42] At the same time, Costello syndrome fibroblasts have drastically increased proliferation rates when compared with normal fibroblasts.[35] Therefore, we investigated a potential functional involvement of C4ST-1 in the mediation of these biological effects of oncogenic HRAS signaling in Costello syndrome. We hypothesized that re-establishment of higher C4ST-1 levels through forced expression of *C4ST-1* might be able to rescue the elastogenesis defects and normalize the increased proliferation associated with oncogenic HRAS signaling in Costello syndrome fibroblasts. To test this hypothesis, C7669 and C563 fibroblasts were stably transfected with either a control plasmid (CAGIP) or a C4ST-1-expression plasmid (C4ST-1). Quantitative RT-PCR analysis showed increased *C4ST-1* expression in C7669 and C563 cells when transfected with the C4ST-1 expression plasmid, but not with the empty vector control (Figure 3a). Pools of transfected cells, as well as individual clones (Figures 3b–i; and data not shown) were analyzed for elastin fiber deposition, using an anti-elastin antibody in immunofluorescence experiments. We found that in contrast to normal fibroblasts that produced abundant elastic fibers (Figures 3b and f), Costello syndrome fibroblasts C7669 (Figure 3c) and C563 (Figure 3g) displayed a complete absence of extracellular elastic fibers, with most of the elastin protein localized intracellularly, as previously shown.[35,41,42] Stable transfection of the CAGIP empty vector did not rescue the elastin fibers deficiency in either C7669 (Figure 3d) or C563 (Figure 3h) cells. However, upon forced expression of *C4ST-1* in these cells, extracellular elastic fiber formation was re-established in both C7669 (Figure 3e) and C563 (Figure 3i) fibroblasts.

Oncogenic HRAS signaling in Costello syndrome fibroblasts has been shown to lead to increased proliferation.[35] We sought to analyze the effect of forced *C4ST-1* expression by immunofluorescence detection of BrdU incorporation into proliferating cells (Figures 4a–h). We analyzed pools of stably transfected C7669 and C563 cells, as well as two individual clones for both control vector (CAGIP) and C4ST-1 in the case of C7669 cells. Cultures of fibroblasts derived from two normal donors showed BrdU incorporation in approximately 18–21% of the cells (Figures 4a, e and i), whereas Costello fibroblasts showed 62 (C7669; Figures 4b and i) and 38% (C563; Figures 4f and i) BrdU-positive cells. Stable transfection of the control CAGIP vector alone in C7669 (Figures 4c and i) or C563 (Figures 4g and i) cells did not significantly alter proliferation rates of either pools or individual clones. However, forced expression of C4ST-1 was able to significantly reduce proliferation rates of both C7669 (Figures 4d and i) and C563 cells (Figures 4h and i).

These results showed that re-establishing *C4ST-1* expression in Costello syndrome fibroblasts could rescue the defects in elastogenesis and normalize increased proliferation induced by activated HRAS signaling in these cells. These results indicate a functional involvement

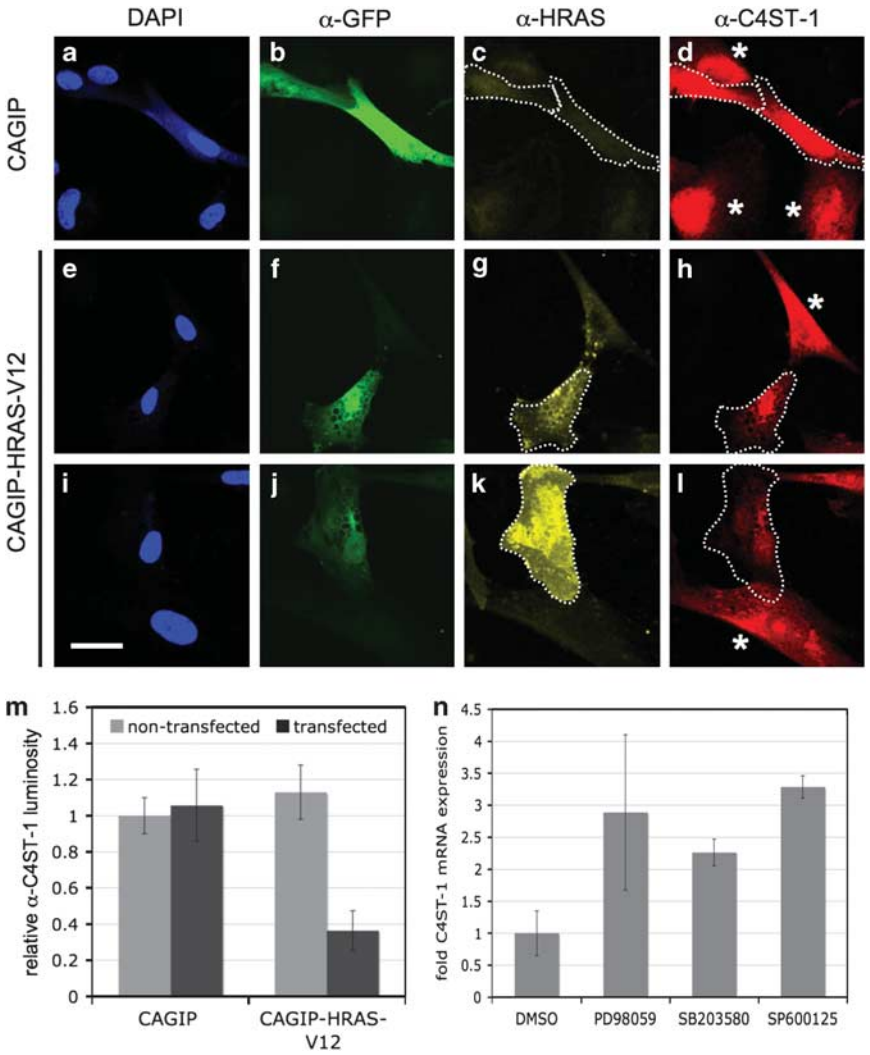

Figure 2 Oncogenic HRAS signaling represses C4ST-1 expression. (**a**–**l**) Expression of HRAS-V12 leads to reduced C4ST-1 expression in normal fibroblasts. Immunofluorescent detection of GFP (green), HRAS (yellow) and C4ST-1 (red; nuclei are counterstained blue with DAPI), in the absence and presence of HRAS-V12. (**a**–**d**) Expression of the control CAGIP plasmid together with a GFP expression plasmid as transfection control shows a GFP-positive transfected cell (**b**), which does not overexpress HRAS (**c**). Consequently, no difference in C4ST-1 expression (**d**) was observed in transfected cells (outlined by white line) and non-transfected cells (marked by star). (**e**–**h**; **i**–**l**) Two fields of fibroblasts transfected with HRAS-V12. GFP-positive transfected cells (**f**, **j**) showed overexpression of HRAS-V12 (**g**, **k**), and a concomitant reduction in C4ST-1 expression (**h**, **l**). Transfected cells are outlined by white line; non-transfected cells are marked by a star. Scale bar in (**i**) represents 5 μm. (**m**) Quantification of C4ST-1 luminosity from immunofluorescence experiments shown in (**d**, **h**, **l**). A minimum of 30 cells were analyzed per condition. Transfection of the CAGIP control vector did not lead to a significant change in C4ST-1 luminosity. Transfection of an HRAS-V12 expression constructs caused a significant decrease of C4ST-1 luminosity, when compared with untransfected cells. (**n**) Inhibition of RAS/MAPK signaling in Costello syndrome fibroblasts can increase *C4ST-1* expression. C7669 cells were treated for 48 h with DMSO, the MAP-kinase inhibitors PD98059 (Erk inhibitor), SB203580 (p38 inhibitor) or SP600125 (SAPK/JNK inhibitor). Subsequently, *C4ST-1* mRNA levels were quantified by qRT-PCR. All MAP-kinase inhibitors were able to elevate *C4ST-1* expression levels.

of *C4ST-1* expression and C4S levels in the mediation of oncogenic HRAS signaling during the pathogenesis of Costello syndrome, and might be suggestive of C4ST-1/C4S as a potential pharmacological target to interfere with oncogenic HRAS signaling.

DISCUSSION

The results presented in this study establish a crucial role for *C4ST-1* expression and the balance of chondroitin sulfation in the mediation of HRAS signaling in the pathogenesis of Costello syndrome. Together with our previous findings describing a functional involvement of pericellular CS in elastogenesis,[35] and a loss of C4S in cardiac tissue of Costello patients,[36] these data support our notion of a functional involvement of an altered chondroitin sulfation balance in the development of Costello syndrome. Specifically, our data suggest that HRAS-mediated reduction of C4ST-1 expression is necessary for the phenotypic manifestation of Costello skin abnormalities, which include elastogenesis defects. Moreover, as proliferation abnormalities are associated with multiple aspects of the Costello syndrome, including cardiac defects and neoplasias, our data suggest that reduced C4ST-1 expression is a prerequisite for a range of abnormalities observed in Costello syndrome.

Recently, cellular senescence was reported in fibroblasts with forced expression of HRAS-activating mutations.[39] Strikingly, we previously demonstrated that mice homozygous for a C4st-1 loss-of-function

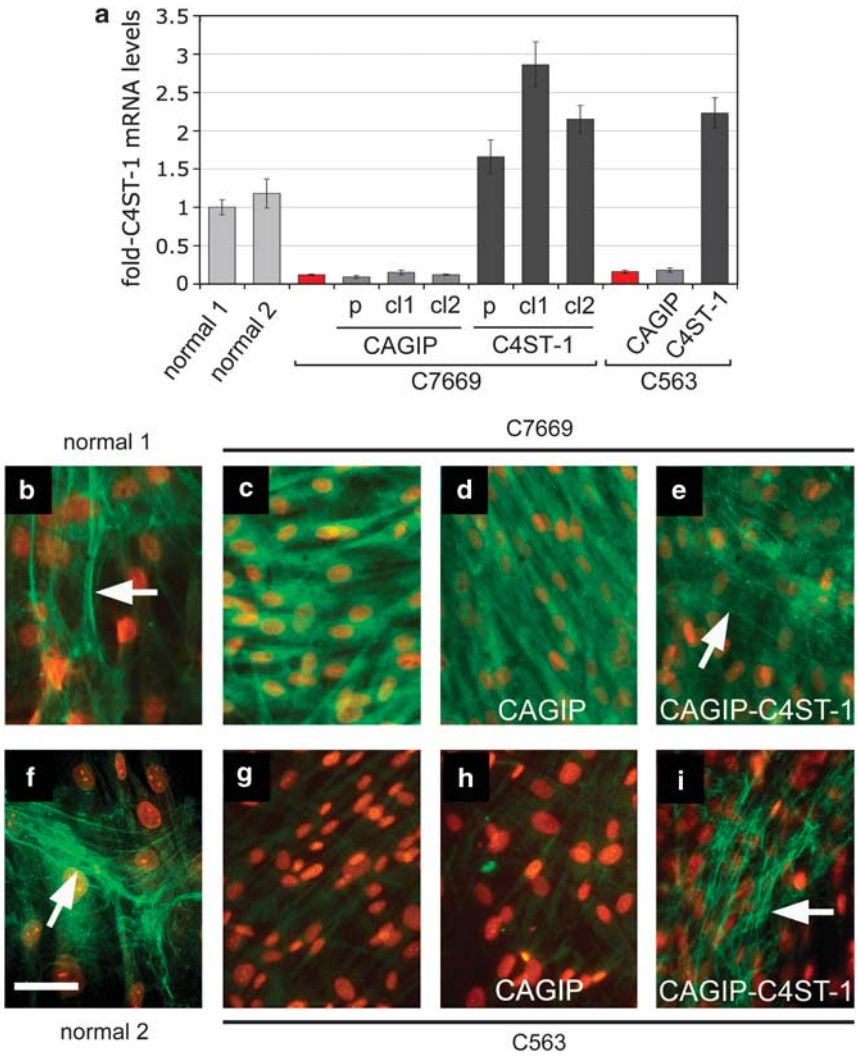

Figure 3 Forced expression of C4ST-1 can rescue the elastogenesis defects in Costello syndrome fibroblasts. (**a**) qRT-PCR analysis of *C4ST-1* mRNA expression levels. C7669 and C563 display severely reduced *C4ST-1* expression levels (red bars), when compared with fibroblasts samples from two unaffected individuals. *C4ST-1* expression is not altered in cell stably expressing the CAGIP control vector, but stable transfection of the CAGIP–C4ST–1 vector (C4ST-1) lead to increased expression of *C4ST-1* in both pools (p) and clones (cl1, cl2) of C7669 cells, as well as pools of C563 cells. (**b**, **i**) Immunofluorescence using an α-tropoelastin antibody (green signal), showing the presence of extracellular elastic fibers (arrows) in two normal fibroblast samples (**b**, **f**). Extracellular elastin fibers are absent in C7669 (**c**) and C563 (**g**) fibroblasts. Instead, C7669 cells show only intracellular tropoelastin staining (**c**). Stable transfection of the parental control plasmid CAGIP into C7669 (**d**) or C563 (**h**) fibroblasts did not alter the deficiency in elastic fiber formation. Stable transfection of CAGIP–Flag-C4ST-1 re-established extracellular elastic fiber formation (arrows) in C7669 (**e**) and C563 (**i**) fibroblasts. Nuclei are counterstained red with propidium iodide. Scale bar in (**e**) represents 10 μm.

gene-trap mutation showed signs of senescence in the developing cartilage growth plate, including loss-of-matrix molecules and an osteoarthritis-like phenotype.[24] Thus, senescence appears to be associated with both gain-of-function of HRAS and loss-of-function of C4ST-1, consistent with our model that loss of C4ST-1 function is important for phenotypic manifestation of oncogenic HRAS signaling.

In addition to Costello syndrome, a group of multiple congenital anomaly syndromes collectively named RASopathies have been shown to be associated with germline mutations in various genes of the RAS/MAPK pathway.[8,18,19] For example, mutations in *KRAS*, *BRAF*, *MEK1* and *MEK2* have been identified in cardio-facio-cutaneous syndrome, and *PTPN11*, *SOS1*, *NRAS*, *KRAS*, *RAF1* and *SHOC2* mutations are associated with Noonan syndrome. Although each syndrome exhibits unique phenotypic features, there are numerous overlapping features between the syndromes, including characteristic facial features, cardiac defects, cutaneous abnormalities, neurocognitive delay and a predisposition to malignancies. It is tempting to speculate that misregulation of common sets of downstream targets, including *C4ST-1*, might be responsible for the overlapping phenotypic features of RASopathies; however, we currently do not know whether C4ST-1 expression is altered in other RASopathy syndromes.

Although both the Costello cell lines tested here showed an absence of extracellular elastin fibers, we observed obvious differences in intracellular tropoelastin staining. There are conflicting reports regarding the levels of elastin expression in Costello syndrome: several studies on samples from single patients described a reduction in elastin mRNA expression,[40,42,43] whereas we previously reported normal levels of tropoelastin in three Costello samples, and identified decreased extracellular elastin deposition

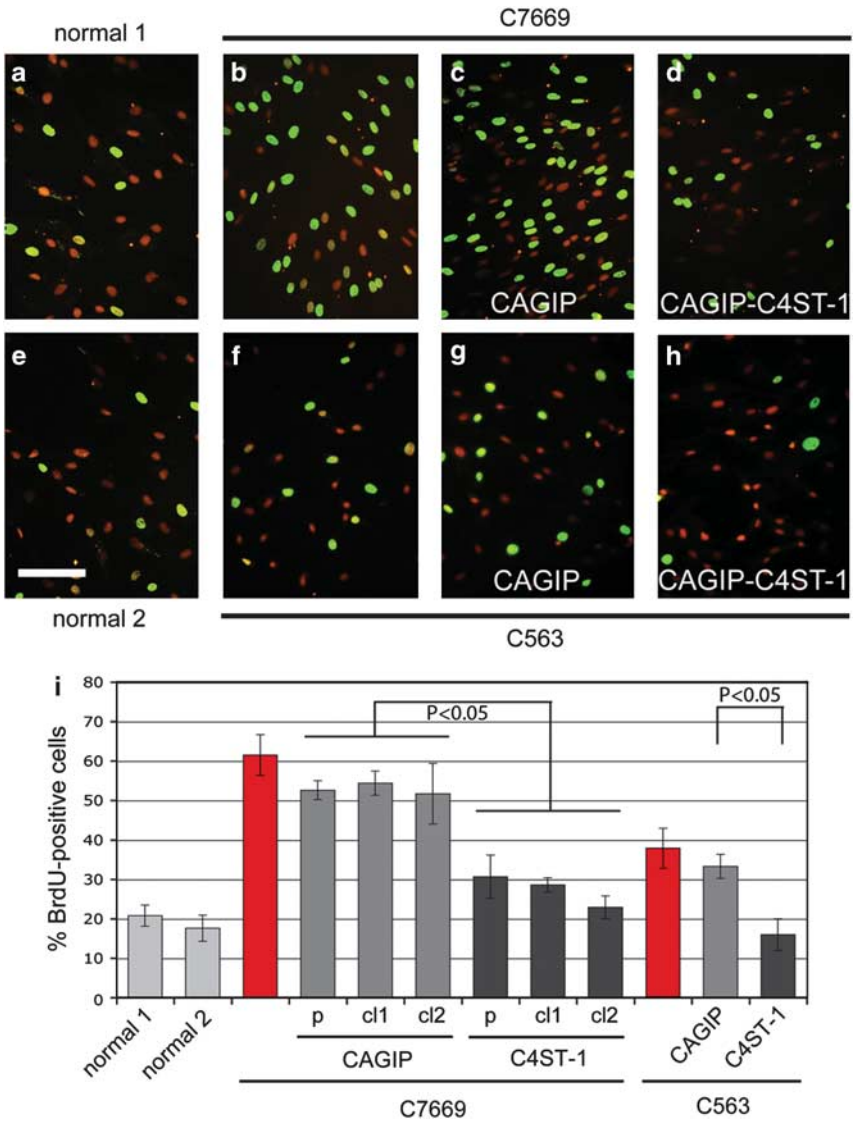

Figure 4 Forced expression of C4ST-1 can interfere with proliferation in Costello syndrome fibroblasts. (**a**–**h**) Labeling and visualization of proliferating fibroblasts using an α-BrdU antibody. When compared with two normal fibroblasts samples (**a**, **e**), C7669 (**b**) and C563 (**f**) cells showed increased BrdU staining (green signal). Stable expression of the pCAGIP control plasmid in C7669 (**c**) and C563 (**g**) fibroblasts did not significantly alter BrdU staining. Stable expression of pCAGIP–C4ST-1 in C7669 (**d**) and C563 (**h**) fibroblasts reduced BrdU staining to levels observed in normal cells. Nuclei were counterstained red by propidium iodide. Scale bar in (**e**) represents 50 μm. (**i**) Quantification of BrdU-positive cells staining showed that C7669 fibroblasts displayed an approximately three-fold increase in proliferation, and C563 an approximately two-fold increase in proliferation, when compared with two normal fibroblast lines. A pool (p) or two individual clones (cl1, cl2) of C7669 cells stably expressing CAGIP–C4ST-1 showed normalized proliferation rates; this was not achieved by stable transfection of the CAGIP control plasmid alone, in either pools or two individual clones. Similarily, a pool of C563 cells stably expressing CAGIP–C4ST-1 showed normalized proliferation rates, which again was not observed by stable expression of the CAGIP control plasmid alone. The average value of five measurements ± SD is shown.

in these Costello samples.[35] Taken together, these data might suggest that although the absence of extracellular elastin fibers is a common theme in Costello syndrome, expression levels of intracellular tropoelasin could vary significantly in different patients, as we observed in the two Costello samples analyzed here.

More than 30% of human cancers contain activating mutations in RAS genes,[44] which makes our identification of the C4ST-1/C4S axis as a downstream vulnerability of the RAS-signaling pathway a potential valuable tool not only in the better understanding and treatment of Costello syndrome, but also in the development of new therapeutic approaches for the treatment of RAS-associated cancers. From a clinical point of view, CS is registered as an oral drug on the European market for the treatment of osteoarthritis symptoms, has been extensively studied and shown to be safe, well tolerated and effective.[45] Thus, CS-based therapeutic approaches have the potential to be a promising avenue to interfere with oncogenic RAS signaling in human disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was funded by the Canadian Institutes of Health Research (CIHR) to AH and JLW and by the Heart and Stroke Foundation of Ontario to AH. MK was supported by a CIHR postdoctoral fellowship. JLW is a CIHR investigator and an International Research Scholar of the Howard Hughes Medical Institute.

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