SUMF1 enhances sulfatase activities *in vivo* in five sulfatase deficiencies

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Sulfatases are enzymes that hydrolyse a diverse range of sulfate esters. Deficiency of lysosomal sulfatases leads to human diseases characterized by the accumulation of either GAGs (glycosaminoglycans) or sulfolipids. The catalytic activity of sulfatases resides in a unique formylglycine residue in their active site generated by the post-translational modification of a highly conserved cysteine residue. This modification is performed by SUMF1 (sulfatase-modifying factor 1), which is an essential factor for sulfatase activities. Mutations in the *SUMF1* gene cause MSD (multiple sulfatase deficiency), an autosomal recessive disease in which the activities of all sulfatases are profoundly reduced. In previous studies, we have shown that SUMF1 has an enhancing effect on sulfatase activity when co-expressed with sulfatase genes in COS-7 cells. In the present study, we demonstrate that SUMF1 displays an enhancing effect on sulfatases activity when co-delivered with a sulfatase cDNA via AAV (adeno-associated virus) and LV (lentivirus) vectors in cells from individuals affected by five different diseases owing to sulfatase deficiencies or from murine models of the same diseases [i.e. MLD (metachromatic leukodystrophy), CDPX (X-linked dominant chondrodysplasia punctata) and MPS (mucopolysaccharidosis) II, IIIA and VI]. The SUMF1-enhancing effect on sulfatase activity resulted in an improved clearance of the intracellular GAG or sulfolipid accumulation. Moreover, we demonstrate that the SUMF1-enhancing effect is also present *in vivo* after AAV-mediated delivery of the sulfamidase gene to the muscle of MPSIIIA mice, resulting in a more efficient rescue of the phenotype. These results indicate that co-delivery of SUMF1 may enhance the efficacy of gene therapy in several sulfatase deficiencies.

Key words: adeno-associated virus (AAV), formylglycine-generating enzyme (FGE), lentivirus, lysosomal storage disorder, sulfatase, sulfatase-modifying factor 1 (SUMF1).

INTRODUCTION

Sulfatases are a large family of enzymes that are involved in the degradation of sulfated substrates such as GAGs (glycosaminoglycans) and sulfolipids. It has been shown that all sulfatases are strictly related, sharing both sequence and structural features [1,2]. Current analysis of the human genome sequence reveals the presence of 17 distinct sulfatase genes [3].

Eight inherited metabolic disorders in humans are caused by sulfatase deficiencies, resulting in impaired desulfation of sulfatase natural substrates. In the case of deficiencies of lysosomal sulfatases, sulfated substrates accumulate in the cells and tissues of patients, causing a lysosomal storage disorder. Deficiencies of lysosomal sulfatases include MLD (metachromatic leukodystrophy), which is characterized by the storage of sulfolipids in the central and peripheral nervous systems, and five different types of MPS (mucopolysaccharidosis) (types II, IIIA, IIID, IVA and VI), which are due to the accumulation of GAGs in several tissues and organs [4,5]. Two additional genetic diseases are associated with non-lysosomal sulfatase deficiencies, XLI (X-linked ichthyosis) and CDPX (X-linked dominant chondrodysplasia punctata) [6,7].

Active sulfatases contain a unique FGly (formylglycine) catalytic residue, which is located in the N-terminal region and is generated in the endoplasmic reticulum from a cysteine precursor through a multistep process [8]. Such post-translational modification is required for sulfatase catalytic activity. In a severe human inherited disease, called MSD (multiple sulfatase deficiency), the activity of all sulfatases is profoundly impaired due to a defective post-translational modification. The phenotype of MSD patients combines all of the clinical symptoms observed in individual sulfatase deficiencies [1]. The gene encoding the FGE (formylglycine-generating enzyme), named *SUMF1* (sulfatase-modifying factor 1), has been identified and found to be mutated in patients with MSD [9,10]. SUMF1 is able to recover sulfatase activity in MSD fibroblasts. Moreover, SUMF1 strongly enhances sulfatase activity when it is co-transfected with sulfatase cDNAs in wild-type cultured cells [9]. These data demonstrate that SUMF1 is an essential factor for sulfatase activity and suggest that SUMF1 overexpression can be exploited to improve the production of recombinant active sulfatases for enzyme-replacement therapy.

Takakusaki et al. [11] have recently shown that intravenous injection of both ARS (arylsulfatase) A- and SUMF1-expressing plasmids by a hydrodynamics-based gene transfer procedure resulted in a significant increase in ARSA activity in both liver and serum of injected MLD mice, when compared with animals receiving ARSA plasmids alone. Whereas hydrodynamic plasmid delivery is unlikely to be feasible in a clinical setting and is intrinsically limited by transient expression, this study indicates

Abbreviations used: AAV, adeno-associated virus; ARS, arylsulfatase; BM, bone marrow; CDPX, X-linked dominant chondrodysplasia punctata; CMV, cytomegalovirus; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; GAG, glycosaminoglycan; GFP, green fluorescent protein; HA, haemagglutinin; HEK-293T, human embryonic kidney; HSPC, haemopoietic stem/progenitor cells; IDS, iduronate sulfatase; LV, lentivirus; MLD, metachromatic leukodystrophy; MPS, mucopolysaccharidosis; MSD, multiple sulfatase deficiency; PBMC, peripheral blood mononuclear cell; PGK, phosphoglycerate kinase; p-NC, *p*-nitrocatechol sulfate; SGSH, sulfamidase; SUMF1, sulfatase-modifying factor 1.

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that co-expression of SUMF1 may enhance the activity of an exogenous sulfatase delivered to sulfatase-deficient cells *in vivo*.

In the present work, using stable AAV (adeno-associated virus)- and LV (lentivirus)-mediated delivery, we performed an extensive analysis of the capability of SUMF1 to increase the specific activity of sulfatases in ARSA-, ARSE-, ARSB-, IDS (iduronate sulfatase)- and SGSH (sulfamidase)-deficient cell lines and in MPSIIIA mice. Our results identify SUMF1 as a valuable tool for further improving protein and gene-based therapies for sulfatase deficiencies.

MATERIALS AND METHODS

Vector cloning and production of LV and AAV vectors

VSV (vesicular-stomatitis virus)-pseudotyped LV stocks were produced by transient co-transfection of the transfer constructs pCCLsin.cPPT.hPGK.eGFP.Wpre, pCCLsin.cPPT.hPGK.ARSA-HA.Wpre, pCCLsin.cPPT.hPGK.ARSE.Wpre, pCCLsin.cPPT.hPGK.SUMF1.Wpre, pCCLsin.cPPT.hPGK.SUMF1-3Xflag.Wpre or pCCLsin.cPPT.hPGK.SUMF1(C336R)-3Xflag.Wpre, the third-generation packaging constructs pMD2.Lg/p.RRE and pRSV.Rev, and the pMD2.G envelope construct in HEK-293T (human embryonic kidney) cells, followed by ultracentrifugation of conditioned medium as described in [12]. Stocks were titred by end-point expression titre in HeLa cells, when possible [using anti-GFP (green fluorescent protein) and HA (haemagglutinin) antibodies] and/or quantified for particle content by HIV-1 group-specific antigen p24 immunocapture assay.

The human *SGSH*, *ARSB* and *SUMF1* genes were amplified by PCR and cloned into NotI/ClaI sites of pAAV2.1-CMV-GFP plasmid [13] to obtain pAAV2.1-CMV-SGSH, pAAV2.1-CMV-ARSB and pAAV2.1-CMV-SUMF1 vectors respectively (GFP was replaced by *SGSH*, *ARSB* or *SUMF1* cDNAs). The *IDS* cDNA [14] was cloned into NotI/ClaI sites of pAAV2.1-CMV-GFP plasmid. The pAAV2.1-CMV-expressing plasmids were used to triple transfect HEK-293T cells to produce AAV serotype 1 and serotype 5 vectors according to protocols described previously [15]. AAV vectors were produced by the AAV TIGEM (Telethon Institute of Genetics and Medicine) Vector Core.

Cell cultures and infections

Six-week-old male MLD mice were killed with CO_2, and BM (bone marrow) was harvested by flushing the femurs and the tibias. HSPCs (haemopoietic stem/progenitor cells) were purified using the Enrichment of Murine Hematopoietic Progenitors kit (StemCell Technologies). For transduction, 10^6 cells/ml were exposed to PGK (phosphoglycerate kinase)–ARSA–HA/PGK–GFP LVs (0.5–2 μg of p24 equivalent/ml) for 12 h and, after 12 h of wash out, to PGK–GFP–SUMF1 LVs (2 μg of p24 equivalent/ml), in StemSpan® SFEM (serum-free expansion medium) (StemCell Technologies), in the absence of serum and with cytokines [100 ng/ml mSCF (mouse stem cell factor), 100 ng/ml hFlt3L (Fms-like kinase-3), 100 ng/ml mIL3 (mouse interleukin 3) and 200 ng/ml hIL-6 (human interleukin 6)]. After transduction, cells were kept in liquid culture in the presence of serum and of the same cytokine cocktail for 10–15 days for further testing. MLD patients' PBMCs (peripheral blood mononuclear cells) were isolated from peripheral blood by Ficoll separation, after informed consent obtainment from patients or their legal tutors (Protocollo TIGET 03). After isolation, cells were plated at the concentration of 5×10^5 cells/ml in 5 % human serum and stimulated with 5 μg/ml OKT3 (anti-CD3 monoclonal antibody) and 5 μg/ml anti-CD28 for 36 h. Transduction of stimulated cells was performed by two cycles of 12-h exposure to the viral supernatant (25–100 ng of p24/ml) at 37 °C and 5 % CO_2 in the presence of 0.5 μg/ml OKT3 and 0.5 μl/ml anti-CD28. After transduction, cells were then washed and plated at a density of 10^6 cells/ml in the presence of 6 units/ml IL-2 (interleukin 2). HeLa cells and CDPX fibroblasts were transduced with 25–100 ng of p24/ml of the abovementioned vectors. Human MPSII, MPSIIIA and MPSVI fibroblast cell lines were obtained from Cell Line Bank of the Giannina Gaslini Institute in Genova, Italy. Human CDPX cells were present in the TIGEM database. They were maintained in α-MEM (minimal essential medium) containing 10 % (v/v) fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin G and 100 μg/ml streptomycin. MEFs (mouse embryonic fibroblasts) were isolated from 14.5-day MPSIIIA, MLD and normal mouse embryos following standard protocols [16]. Cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin G and 100 μg/ml streptomycin. For infection, the appropriate AAV 2/1 vectors (10^5 viral particles/cell in DMEM without serum) were added to subconfluent fibroblasts for 2 h. The infecting medium was then replaced with fresh complete medium, and the cells were collected after 6 days for sulfatase enzymatic assays.

Western blotting

Cell were lysed in RIPA buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 1 % Triton X-100, 1 % sodium deoxycholate and 0.1 % SDS) supplemented with protease inhibitor cocktail (Sigma). Western blots were performed using anti-FLAG M2 monoclonal antibody (Sigma).

Sulfatase enzymatic assays

For detecting ARSA specific activity, the p-NC (*p*-nitrocatechol sulfate) assay was adapted for ARSA–HA specific activity detection. ELISA plates were coated with anti-HA antibody by overnight incubation at 4 °C, diluted 1:50 in 0.1M $NaHCO_3$, pH 8.9. After blocking with 10 % (v/v) rat serum in PBS, 100 μl of serum was plated on coated wells and incubated at room temperature (25 °C) for 2 h. After incubation, wells were washed three times with PBS containing 0.05 % (v/v) Tween 20 and 100 μl of p-NC was added. After 90 min of incubation at 37 °C, p-NC was collected, the reaction stopped by adding 1 ml of 1 M NaOH, and the final product was read at 515 nm using a spectrophotometer. ARSB and ARSE enzymatic activities were tested as described in [17]. Sulfamidase and IDS were assayed with fluorogenic substrates as described previously [18,19]. All sulfatase activities were normalized against the total amount of protein quantified using the Bradford protein assay (Bio-Rad).

Metabolic labelling of GAGs

Fibroblasts infected with AAV vectors were pulsed with $^{35}SO_4$ for 72 h (8 μCi/ml $Na_2{}^{35}SO_4$ in F12/10 % fetal bovine serum medium without antibiotics). The radiolabelled medium was then replaced with normal growth medium, and, after 72 h, the cells were collected and pellets were assayed for the amount of labelled material.

Animals and injection

MPSIIIA mice (strain *Sgsh*^{mps3a}/PstJ) were obtained from the Jackson Laboratory and were derived from the MPSIIIA mouse strain described in [20]. For muscle injection, right gastrocnemius of newborn MPS-IIIA mice was injected with 10 μl of PBS containing the appropriate virus mixture. We used a total of

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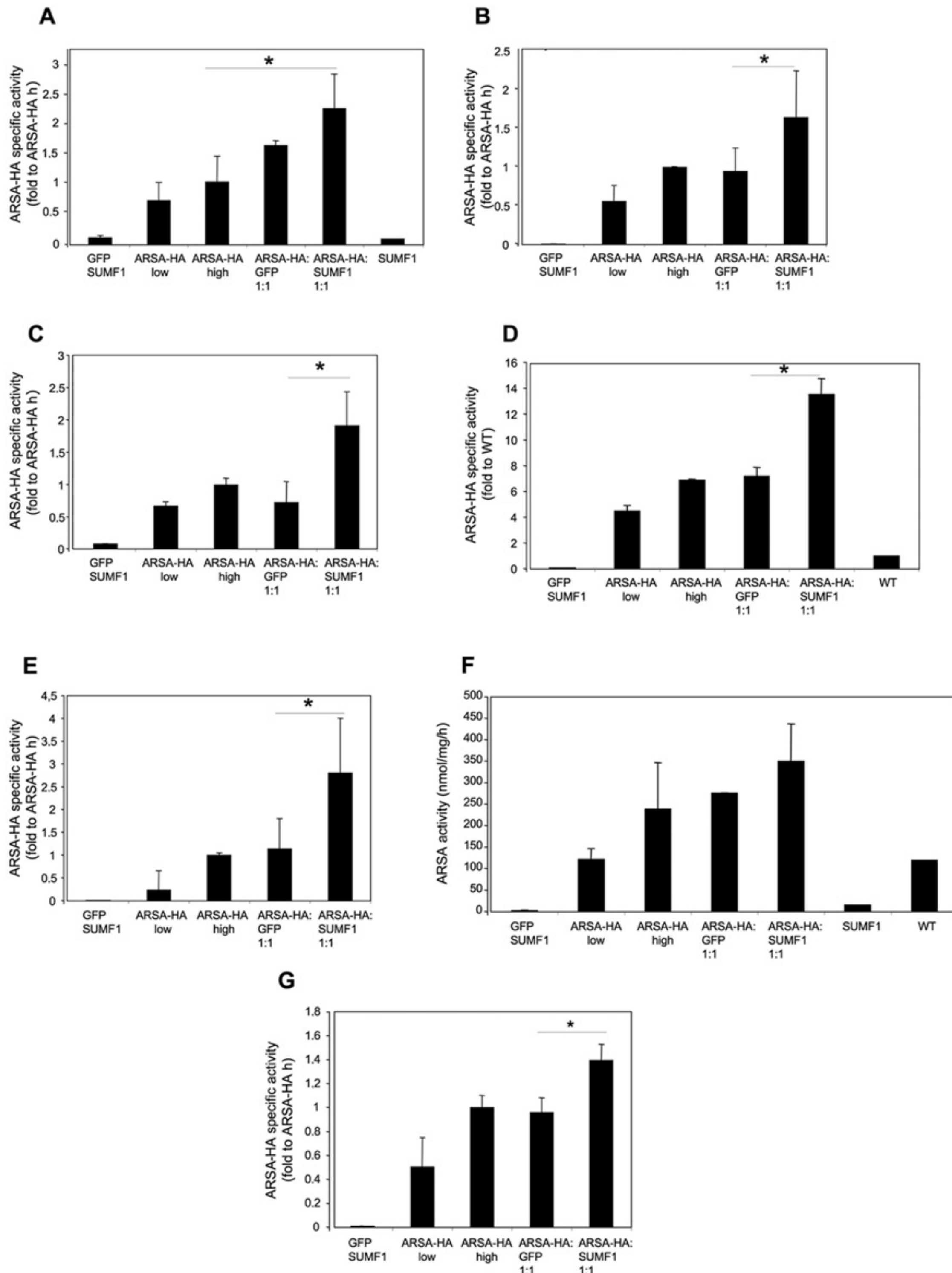

Figure 1 Effects of SUMF1 on ARSA activity upon LV transduction of HeLa and ARSA-deficient cells

HeLa cells (**A**–**C**), murine ARSA$^{-/-}$ HSPCs (**D**–**E**) and MLD patients' stimulated T-cells (**F**–**G**) were transduced with either ARSA–HA LV, at low or high (h) vector input (see the Materials and methods sections for details), or with ARSA–HA (high vector concentration) and either SUMF1 or GFP LVs at a 1:1 ratio. ARSA–HA specific activity was measured either by conventional p-NC assay (**A**, **D**, **F**) or by the immunocapture assay (**B**, **C**, **E**, **G**) on cell extracts (**A**, **B**, **D**–**G**) and supernatant (**C**). ARSA activity in (**A**–**C**, **E**, **G**) is expressed as fold difference from the levels detected in samples transduced with ARSA at high vector input (ARSAh), in (**D**) as fold of the levels detected in wild-type (WT) samples, and in (**F**) in nmol/mg per h. In all tested cell types, an increase of ARSA–HA activity was detected upon ARSA and SUMF1 co-expression as compared with controls (either ARSA–HA- or ARSA–HA/GFP-transduced cells). $^*P < 0.05$ ($n = 6$).

10^{12} viral particles for each injection. After 6 weeks, the injected, control uninjected and unaffected mice were killed, and the right gastrocnemius was collected. Tissues were homogenized and ruptured by freeze–thaw three to five times. After clarification at 13 000 g for 5 min at 4 °C, the supernatants were tested for sulfamidase activity.

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All animal experimentations was conducted in accordance to the guidelines of The Animal Care and Use Committee of Cardarelli Hospital (Naples, Italy), or according to protocols approved by the Animal Care and Use Committee of the Fondazione San Raffaele del Monte Tabor (IACUC #255) and communicated to the Ministry of Health and local authorities according to the Italian law.

Immunofluorescence and GFP staining

Immunofluorescence analysis was performed on paraffin sections of muscle tissues fixed in methacarn. After rehydration, the specimens were incubated with 10 % (v/v) normal serum and 0.1 % (w/v) BSA in TBS (Tris-buffered saline) for 30 min, and then incubated for 2 h with a primary sheep polyclonal antibody against human sulfamidase (a gift from John Hopwood, Lysosomal Diseases Research Unit, Women's and Children's Hospital, North Adelaide, SA, Australia). After washing, sections were incubated with secondary TRITC (tetramethylrhodamine β-isothiocyanate)-conjugated anti-goat antibody and mounted with 1:1000 DAPI (4′,6-diamidino-2-phenylindole)/Vectashield (Vector Laboratories). The specimens were then visualized under fluorescence microscope and pictures were taken with a 63× magnification objective. For GFP staining, the muscle paraffin sections were rehydrated, mounted with 1:1000 DAPI/Vectashield and visualized under fluorescence microscope. Pictures were taken under a 63× magnification objective.

RESULTS AND DISCUSSION

To study the effect of SUMF1 on ARSA activity, two LV vectors were constructed, one carrying the *ARSA* cDNA, and one carrying *SUMF1* cDNA, both under the control of the human PGK promoter. To permit *in situ* immunodetection of ARSA, in all the experiments, we used a C-terminally tagged transgene, in which the gene is fused in-frame with the sequence encoding the HA peptide from the human influenza virus. The HA-tagged enzyme had a specific activity comparable with that of the unmodified enzyme, and was properly sorted to the lysosomal compartment [21,22]. Either ARSA–HA and SUMF1 or ARSA–HA and GFP were co-expressed in HeLa cells, HSPCs from ARSA-knockout mice and stimulated T-cells from MLD patients upon LV transduction, using previously optimized protocols [12,23]. After transduction, cells were maintained in culture for 2–4 weeks to monitor steady-state effects of ARSA–SUMF1 co-expression. ARSA specific activity was tested on cell extracts and conditioned medium by using a conventional p-NC assay and by an HA-specific immunocapture assay. Co-expression of ARSA–HA and SUMF1 resulted in a significant increase of ARSA specific activity in both cell extracts and conditioned medium from co-transduced HeLa cells, as compared with cells transduced either with ARSA–HA at high and low vector input or with ARSA–HA and GFP (Figures 1A–1C). HSPC purified from the BM of ARSA-knockout mice were transduced at high and low vector concentration (see the Materials and methods section for details) with the ARSA–HA LV, and subsequently with either GFP or SUMF1 LV. Co-expression of SUMF1 led to a 30–60 % increase of ARSA–HA specific activity as compared withs ARSA–GFP co-expressing cells or to ARSA-transduced cells (Figures 1D and 1E). As shown previously [23], transduction of MLD patients' stimulated T-lymphocytes with ARSA-encoding LV resulted in reconstitution of ARSA activity up to normal donors' levels (Figures 1F and 1G). Interestingly, when both ARSA and SUMF1 were co-expressed, also in these cells a significant increase of ARSA specific activity above the levels detected in single-transduced or ARSA- and GFP-co-transduced cells was observed (Figures 1F and 1G). These experiments demonstrate that SUMF1 co-delivery with ARSA can significantly enhance its specific activity in three different cell types, suggesting the potential use of SUMF1 to further improve the efficacy of gene therapy strategies for MLD.

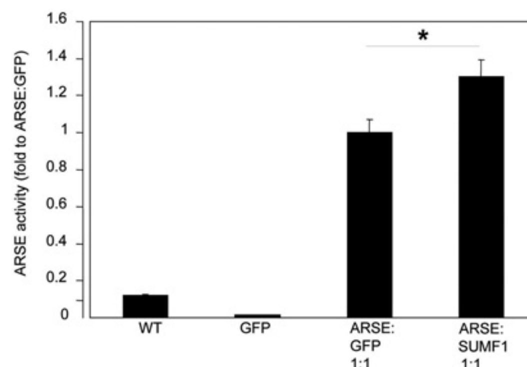

Figure 2 Effects of SUMF1 on ARSE activity upon LV transduction of CDPX cells

CDPX fibroblasts were single-transduced with GFP-encoding LV, or were double-transduced with both ARSE and either SUMF1 or GFP LVs at a 1:1 ratio. ARSE activity was measured in cell extracts of transduced CDPX cells and in wild-type (WT) fibroblasts. Co-infection of SUMF1 together with ARSE (1:1 ratio) resulted in an increase of ARSE activity as compared with control (ARSE/GFP, 1:1). $^{*}P < 0.05$ ($n = 3$).

A similar approach was used to transduce fibroblasts derived from CDPX patients. *ARSE* cDNA was cloned in the LV vector under the human PGK promoter. A fibroblast cell line from a CDPX patient was co-transduced with LV vectors carrying either ARSE and SUMF1 or ARSE and GFP (Figure 2). ARSE activity was 20–25 % higher in cells transduced with the ARSE–SUMF1 LVs as compared with cells transduced with the ARSE–GFP LVs, demonstrating an enhancing effect of SUMF1 on ARSE activity.

AAV vectors were used to transduce fibroblasts derived from MPSII, MPSIIIA and MPSVI patients. Different sulfatase cDNAs were inserted into AAV2/1 vectors under the control of the CMV (cytomegalovirus) promoter (*IDS*, *SGSH* and *ARSB* cDNAs for the infection of MPSII, MPSIIIA and MPSVI fibroblasts respectively). MPSII, MPSIIIA and MPSVI fibroblasts were either infected with the AAV2/1 vectors containing sulfatase cDNAs or co-infected with an AAV2/1 vector containing *SUMF1* cDNA, at different sulfatase/SUMF1 virus ratios. For each infection, we used a total of 10^5 viral particles/cell. At 6 days post-infection, we assayed the cell pellets for sulfatase activities. In MPSII fibroblasts co-infected with AAV2/1–CMV–IDS and AAV2/1–CMV–SUMF1 in equal doses (IDS/SUMF1, 1:1) IDS activity was approx. 2-fold greater than the activity in fibroblasts infected with AAV2/1–CMV–IDS (Figure 3A). The enhancing effect of SUMF1 was dependent upon the ratio between AAV2/1–CMV–IDS and AAV2/1–CMV–SUMF1 virus doses (Figure 3A). Importantly, the increase in IDS activity due to the co-expression of SUMF1 correlated with a functional reduction in GAG storage in infected MPSII fibroblasts. Indeed, $^{35}SO_4$ labelling experiments demonstrated that the accumulation of $^{35}SO_4$-labelled material in fibroblasts infected with both IDS and SUMF1 was quantitatively reduced compared with $^{35}SO_4$ incorporation detected in fibroblasts infected with IDS alone (Figure 3A).

Similar results were obtained in MPSIIIA and MPSVI fibroblasts after infection with AAV2/1–CMV–SGSH and AAV2/1–CMV–ARSB respectively. Sulfatase activities were significantly higher in cells co-infected with the AAV2/1–CMV–SUMF1

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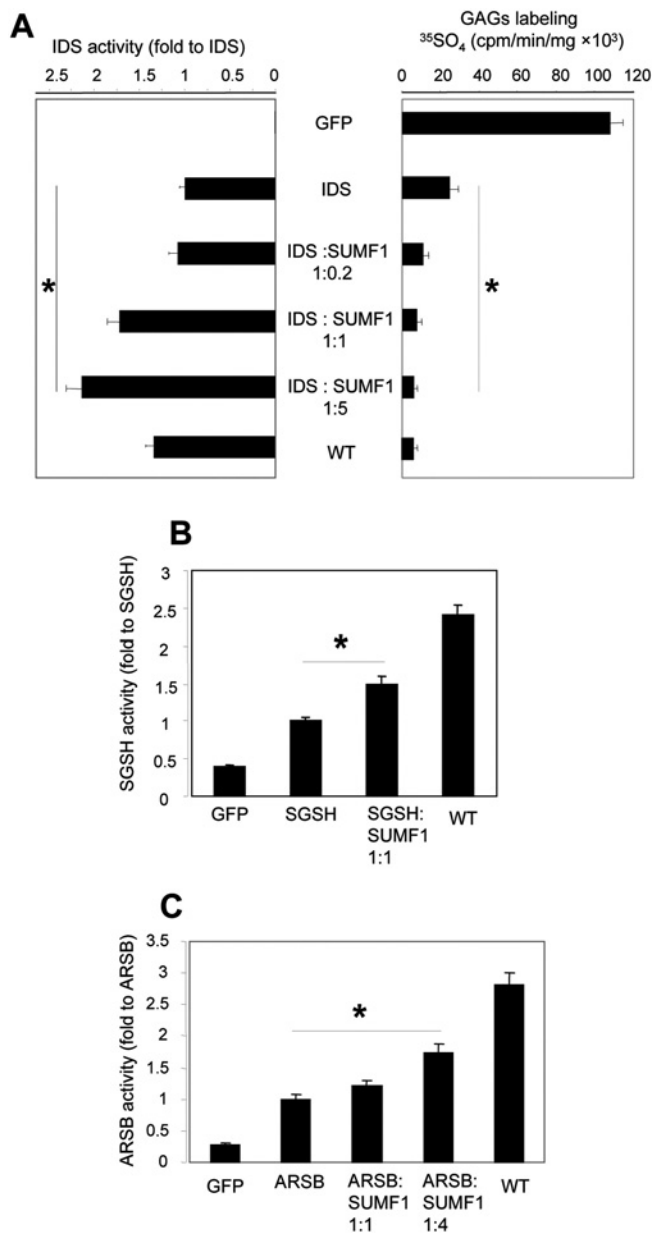

Figure 3 AAV-mediated co-expression of SUMF1 with IDS, SGSH or ARSB in MPSII, MPSIIIA and MPSVI human fibroblasts

MPSII (**A**), MPSIIIA (**B**) and MPSVI (**C**) human fibroblasts were infected with AAV2/1–CMV–GFP or AAV2/1–CMV–sulfatase (IDS for MPSII, SGSH for MPSIIIA and ARSB for MPSVI fibroblasts) or with both AAV2/1–CMV–sulfatase (IDS, SGSH or ARSB) and AAV2/1–CMV–SUMF1 at different sulfatase/SUMF1 ratios. The dose of AAV–sulfatase was the same in all infections, and the total amount of infecting viral particles (10^5 viral particles/cell) was kept constant by using AAV2/1–CMV–GFP in the infecting viral mixture. The IDS, SGSH and ARSB activities are shown as fold increase over sulfatase-infected fibroblast activity. The amount of GAG storage in (**A**) was measured by labelling the cell medium with $^{35}SO_4$ as described in the Materials and methods section. Co-expression of both IDS and SUMF1 at a ratio of either 1:1 or 1:5 resulted in both an increase of IDS activity and a decrease of GAG accumulation as compared with controls (either IDS or IDS/SUMF1 1:0.2). The infection of either SGSH/SUMF1 (1:1 ratio) or ARSB/SUMF1 (1:4 ratio) resulted in an increase of sulfatase activities as compared with controls (SGSH, ARSB or ARSB/SUMF1, 1:1). $*P < 0.05$ ($n = 3$). WT, wild-type.

virus (Figures 3B and 3C). Taken together, these results demonstrate that, in sulfatase-deficient fibroblasts, co-delivery of SUMF1 with sulfatase cDNAs results in a significant enhancement of sulfatase activity, and such an effect correlates with a reduction of GAG storage in the cells.

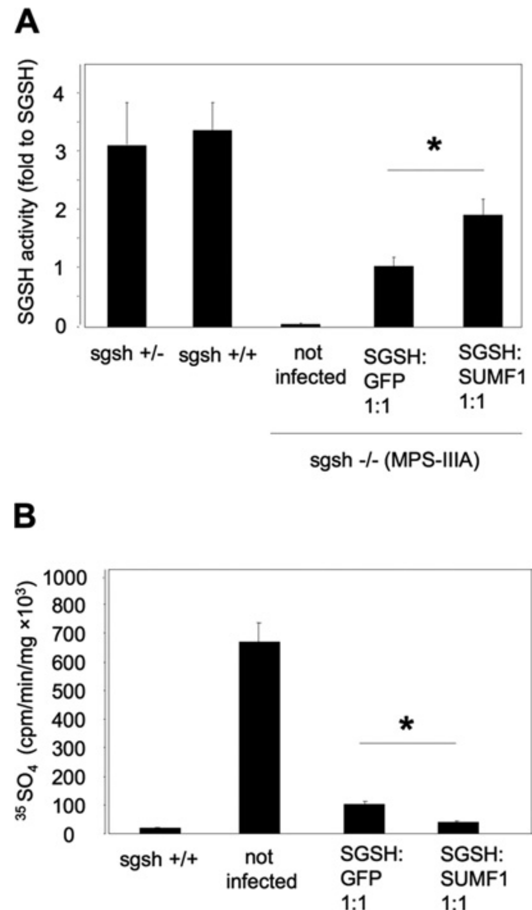

Figure 4 AAV-mediated transfer of SGSH with or without SUMF1 in MPSIIIA MEFs

MEFs were obtained from wild-type (Sgsh +/+), heterozygotes (Sgsh +/−) and MPSIIIA (Sgsh −/−) mice. (**A**) MPSIIIA MEFs were double-infected with both AAV2/1–CMV–SGSH and either AAV2/1–CMV–GFP or AAV2/1–CMV–SUMF1 at a 1:1 ratio. Sulfamidase activity is shown as fold increase over the activity of MPSIIIA MEFs infected with SGSH/GFP. (**B**) WT and MPSIIIA MEFs (both infected and uninfected) were metabolically labelled with $^{35}SO_4$ to quantify the amount of GAG storage. Co-expression of both SGSH and SUMF1 (1:1 ratio) resulted in both an increase of SGSH activity and a decrease of GAG accumulation as compared with controls (SGSH/GFP, 1:1). $*P < 0.05$ ($n = 3$).

To test the ability of SUMF1 to enhance sulfatase activity *in vivo*, we used an MPSIIIA mouse model which carries a spontaneous missense mutation (D31N) in the catalytic site of SGSH enzyme that reduces its activity to approx. 3 % of normal levels [20,24]. The disease progression in MPSIIIA mice is similar to that observed in MPSIIIA patients, making the mice an excellent model for evaluating pathogenic mechanisms of the disease and for the development and testing of treatment strategies [25,26].

We first tested the capability of SUMF1 to enhance the activity of SGSH in MEFs obtained from MPSIIIA mice. SGSH activity in MPSIIIA MEFs was undetectable, as expected (Figure 4A). By using AAV2/1 vectors, we co-infected MPSIIIA MEFs with both SGSH and either GFP or SUMF1 at a 1:1 ratio. At 6 days after infection, we tested the activity of SGSH and analysed GAG storage in cell pellets. Delivery of both SGSH and SUMF1 AAVs to MEFs yielded enzyme activity 2-fold higher than the activity in MEFs infected with both SGSH and GFP (Figure 4A). This enhancing effect also correlated with a decrease in GAG storage in MEFs co-infected with both SGSH and SUMF1 (Figure 4B).

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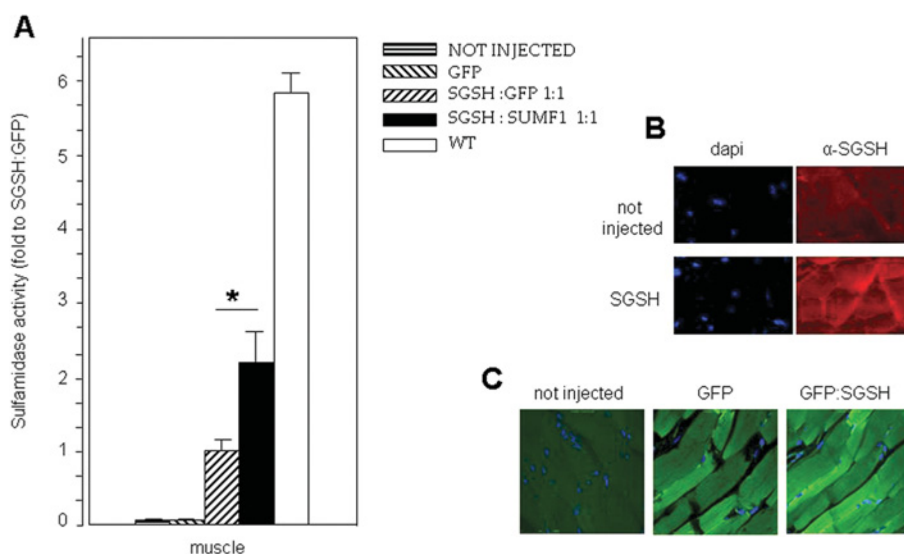

Figure 5 Sulfamidase activity in the muscle of MPS IIIA mice after AAV-mediated transfer of SGSH with or without SUMF1

(**A**) Newborn MPSIIIA mice were divided into four experimental groups. One group was not injected ($n = 3$), while the other three groups received an injection into the right gastrocnemius of AAV2/5–CMV–GFP ($n = 3$), a 1:1 mixture of AAV2/5–CMV–SGSH and AAV2/5–CMV–GFP ($n = 6$) or a 1:1 mixture of AAV2/5–CMV–SGSH and AAV2/5–CMV–SUMF1 vectors ($n = 7$). The total number of viral particles used for each injection (10^{12}) was kept constant. After 6 weeks, the muscle tissues (right gastrocnemius) were collected from either injected or uninjected MPSIIIA mice, and from wild-type (WT) ($n = 4$) 6-week-old mice. Sulfamidase activity was measured in total tissue homogenates and expressed as fold activation over SGSH/GFP-injected muscle. Co-delivery of both SGSH and SUMF1 to mouse muscles resulted in an increase of SGSH activity in injected tissues as compared with controls (SGSH/GFP). $^{*}P = 0.096$. (**B**) Immunofluorescence of muscle sections from AAV2/5–CMV–SGSH-injected MPSIIIA mice revealed a strong transduction of myocells after delivery of AAV–SGSH vector. In muscle sections of uninjected MPSIIIA mice, a low background signal was detected. This is due to the cross-reaction of the anti-(human sulfamidase) antibody with murine sulfamidase. (**C**) GFP staining of both GFP- and SGSH/GFP-injected muscles indicates a widespread expression of the GFP in the myocells. DAPI staining of nuclei is shown in blue.

To test the ability of SUMF1 to enhance SGSH activity *in vivo*, we delivered both *SGSH* and *SUMF1* genes into the right gastrocnemius of newborn MPSIIIA mice. Mice were injected with AAV2/5–CMV–GFP or were co-injected with either a 1:1 mixture of AAV2/5–CMV–SGSH and AAV2/5–CMV–GFP or a 1:1 mixture of AAV2/5–CMV–SGSH and AAV2/5–CMV–SUMF1 vectors, keeping constant the total amount of viral particles (10^{12} for each injection). After 6 weeks, we killed the mice and collected the muscles from injected and control uninjected or unaffected animals. Imunofluorescence staining demonstrated that human SGSH was strongly expressed in the injected muscles (Figure 5B). The level of SGSH activity measured in the injected muscles for which AAV2/5–CMV–SGSH and the AAV2/5–CMV–GFP mixture was used reached 17 % of normal activity and was approx. 8-fold greater with respect to activity measured in muscle of uninjected mice (Figure 5A). Most importantly, muscle injected with AAV2/5–CMV–SGSH and AAV2/5–CMV–SUMF1 showed a further increment of SGSH activity to levels 2-fold higher than that detected in muscle injected with the AAV2/5–CMV–SGSH and AAV2/5–CMV–GFP mixture (Figure 5A). To exclude the possibility that the lower sulfamidase activity detected in the muscles injected with both SGSH and GFP could be due to an immune-cell-mediated clearance of GFP-expressing cells, we checked for GFP expression in both GFP- and GFP + SGSH-injected muscles. The results showed that both tissues display a broad GFP signal (Figure 5C), suggesting stable transduction in all conditions.

We demonstrated previously that at least 11 SUMF1 mutations found in MSD human patients resulted in severely impaired sulfatase-enhancing activity when the corresponding mutated *SUMF1* cDNAs were co-expressed with sulfatases in COS-7 cells [17]. In order to check that the observed enhancement of activities was due to the specific action of SUMF1, we co-infected three cell lines (MPSII and CDPX human fibroblasts, and MPSIIIA MEFs) with LV carrying the specific sulfatase and the SUMF1 C336R mutant. This mutation has been shown to completely abolish the enhancing activity of SUMF1 in both COS-7 cells [17] and $SUMF1^{-/-}$ MEFs (I. Annunziata, personal communication). In all three sulfatase-deficient cell lines, the SUMF1 C336R mutant was unable to produce significant increases of activity of the co-delivered sulfatase (Figure 6). In contrast, co-infection of vectors carrying the wild-type *SUMF1* and sulfatase cDNAs produced a clear synergistic effect on sulfatase activity, as expected (Figure 6). Similar levels of SUMF1 proteins (wild-type and mutant) were detected in infected fibroblasts (Figure 6B, and results not shown).

Overall, our data demonstrate an enhancing effect of SUMF1, using two different viral approaches, LV and AAV, on the activity of five different sulfatases both *in vitro* and *in vivo*. Importantly, these data were obtained under conditions in which the activity of each individual sulfatase was deficient. Furthermore, our results suggest that the optimal ratio between SUMF1 and each sulfatase may vary among different sulfatases. Phenotype rescue by sulfatase gene delivery in a mouse model of sulfatase deficiency has already been proven in the case of the MLD mouse model [12,22,27]. In this case, delivery of the *ARSA* gene alone in either the brain parenchyma or in HSPCs, without co-delivery of SUMF1, resulted in an efficient reconstitution of ARSA activity and correction of the phenotype of treated animals. Furthermore, a complete rescue of the visceral phenotype was also observed after AAV-mediated gene transfer of IDS in a mouse model of MPSII [14]. However, the amount of endogenous SUMF1, which is responsible for the activation of exogenous sulfatases in treated sulfatase-deficient mice, may become a limiting factor in certain cells and tissues and/or in certain conditions throughout life. Also, each sulfatase may behave differently (e.g. because of a different

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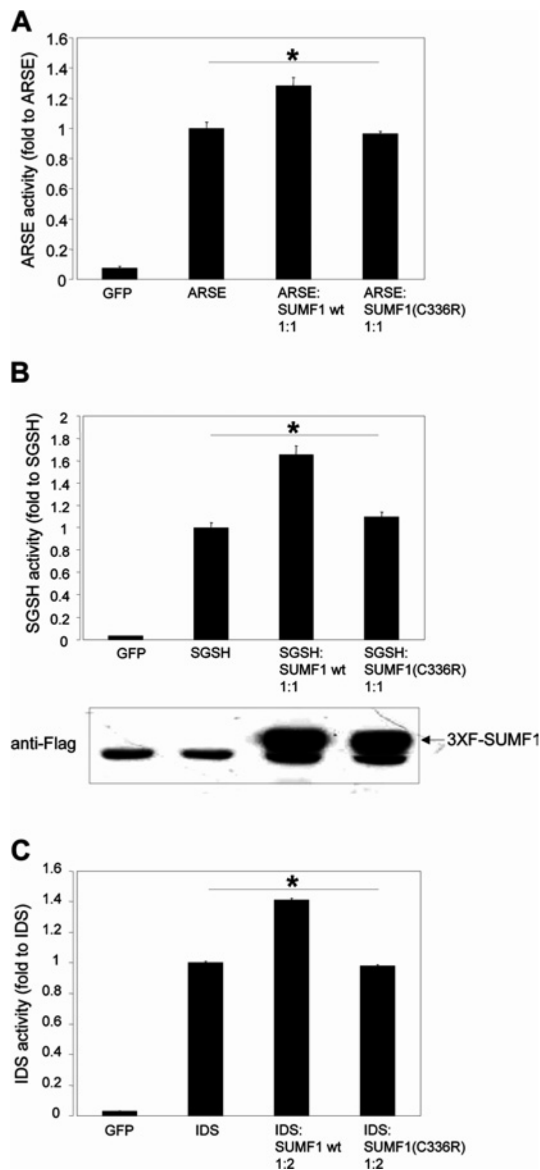

Figure 6 SUMF1 C336R mutant impairs the sulfatase-enhancing activity

CDPX human fibroblasts (**A**), MPSIIIA MEFs (**B**) and MPSII human fibroblasts (**C**) were single-transduced with GFP-encoding LV, or were double-transduced with both sulfatase (ARSE, SGSH or IDS for CDPX, MPSIIIA and MPSII fibroblasts respectively) and 3Xflag-SUMF1 wt, 3xFlag-SUMF1 C336R mutant or GFP LVs. The ARSE, SGSH and IDS activities are shown as fold increase over sulfatase–GFP-infected fibroblasts activity. Co-infection of SUMF1 C336R mutant with sulfatase did not result in any significant increase of sulfatase activity as compared with sulfatase–SUMF1 wt co-infection. $^{*}P < 0.05$ ($n = 3$). A Western blot of the FLAG-tagged SUMF1 proteins is shown (**B**, lower panel).

affinity for SUMF1) in the amounts of SUMF1 required for activation. Finally, as most gene therapy approaches for lysosomal storage disorders aim at the generation of ‘protein factory’ organs, producing the highest possible levels of circulating enzymes, SUMF1 should be considered to be a viable and useful tool to achieve this goal in those lysosomal diseases that are caused by sulfatase deficiencies.

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