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► **To cite this version:**

Alessandro Fraldi, Alessandra Biffi, Alessia Lombardi, Ilaria Visigalli, Stefano Pepe, et al.. SUMF1 enhances sulfatase activities in vivo in five sulfatase deficiencies. *Biochemical Journal*, 2007, 403 (2), pp.305-312. 10.1042/BJ20061783 . hal-00478708

HAL Id: hal-00478708

<https://hal.science/hal-00478708>

Submitted on 30 Apr 2010

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

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Key words: SUMF1, FGE, sulfatase, Lysosomal Storage Disorder, AAV, Lentivirus

Short page heading title: *in vivo* SUMF1 enhancing effect

Abstract

Sulfatases are enzymes that hydrolyze a diverse range of sulfate esters. Deficiency of lysosomal sulfatases leads to human diseases characterized by the accumulation of either glycosaminoglycans (GAGs) 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 Multiple Sulfatase Deficiency (MSD), an autosomal recessive disease in which the activities of all sulfatases are profoundly reduced. In previous studies we showed that SUMF1 has an enhancing effect on sulfatase activity when co-expressed with sulfatase genes in Cos7 cells. Here we demonstrate that SUMF1 displays an enhancing effect on sulfatases activity when co-delivered with a sulfatase cDNA via adeno-associated (AAV)- and lenti (LV)-viral vectors in cells from individuals affected by five different diseases due to sulfatase deficiencies or from murine models of the same diseases (i.e. MLD, CDPX, MPSII, MPSIIIA and MPSVI). 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.

Introduction

Sulfatases are a large family of enzymes involved in the degradation of sulfated substrates such as glycosaminoglycans (GAGs) 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 (LSD). Deficiencies of lysosomal sulfatases include metachromatic leukodystrophy (MLD), which is characterized by the storage of sulfolipids in the central and peripheral nervous systems, and five different types of mucopolysaccharidoses (MPS types II, IIIA, IIID, IVA, and VI), which are due to the accumulation of glycosaminoglycans (GAGs) in several tissues and organs [4,5]. Two additional genetic diseases are associated with non-lysosomal sulfatase deficiencies, X-linked ichthyosis (XLI) and chondrodysplasia punctata (CDPX) [6,7].

Active sulfatases contain a unique formylglycine (FGly) catalytic residue, which is located in the N-terminal region and is generated in the endoplasmatic reticulum from a cysteine precursor through a multistep process [8]. Such post-translation modification is required for sulfatase catalytic activity. In a severe human inherited disease, called Multiple Sulfatase Deficiency (MSD), the activity of all sulfatases is profoundly impaired due to a defective post-translational modification. The phenotype of MSD patients combines all the clinical symptoms observed in individual sulfatase deficiencies [1]. The gene encoding the formylglycine-generating enzyme (FGE), 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 sulfatases cDNAs in wild type cultured cells [9]. These data demonstrate that *SUMF1* is an essential factor for sulfatase activity and suggest that *SUMF1* over-expression can be exploited to improve production of recombinant active sulfatases for enzyme replacement therapy (ERT).

Takakusaki and co-workers have recently shown that intravenous injection of both ARSA and *SUMF1* expressing plasmids by a hydrodynamics-based gene transfer procedure, resulted in a significant increase in ARSA activity both in liver and serum of injected MLD mice, when compared to animals receiving ARSA plasmids alone [11]. Whereas hydrodynamic plasmid delivery is unlikely to be feasible in a clinical setting and intrinsically limited by transient

expression, this study indicates 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- and LV-mediated delivery we performed an extensive analysis of the capability of SUMF1 to increase the specific activity of sulfatases in Arylsulfatase A (ARSA), Arylsulfatase E (ARSE), Arylsulfatase B (ARSB), Iduronate Sulfatase (IDS) and Sulfamidase (SGSH) deficient cell lines and in MPSIIIA mice. Our results identify SUMF1 as a valuable tool for further improving protein and gene based therapies for sulfatases deficiencies.

Methods

Vector cloning and production of LV and AAV vectors. VSV-pseudotyped LV stocks were produced by transient co-transfection of the transfer constructs pCCLsin.cPPT.hPGK.eGFP.Wpre or pCCLsin.cPPT.hPGK.ARSA-HA.Wpre or pCCLsin.cPPT.hPGK.AARSE.Wpre or pCCLsin.cPPT.hPGK.SUMF1.Wpre or 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 293T cells, followed by ultra-centrifugation of conditioned medium, as described [12]. Stocks were titered by end-point expression titer in HeLa cells, when possible (using GFP and HA antibodies) and/or quantified for particle content by HIV-1 Gag p24 immunocapture assay. LV

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 respectively pAAV2.1-CMV-SGSH, pAAV2.1-CMV-ARSB and pAAV2.1-CMV-SUMF1 vectors (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 293T cells to produce AAV serotype 1 and serotype 5 vectors according protocols previously described [15]. AAV vectors were produced by the AAV TIGEM Vector Core.

Cell cultures and infections. Six-week-old male MLD mice were killed with CO₂ and BM was harvested by flushing the femurs and the tibias. Hematopoietic stem/progenitor cells (HSPC) were purified using the Enrichment of Murine Hematopoietic Progenitors kit (StemCell Technologies, Vancouver, BC). For transduction, 1×10^6 cells/ml were exposed to PGK-ARSA-HA/PGK-GFP LVs (0,5-2 μ g p24 equivalent/ml) for 12 hours and, after 12 hours of wash out, to PGK-GFP/SUMF1 LVs (2 μ g p24 equivalent/ml), in Stem Span SFEM expansion medium (StemCell Technologies), in

the absence of serum and with cytokines (mSCF 100ng/ml, hFlt3L 100ng/ml, mIL3 100ng/ml, hIL6 200ng/ml). 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' PBMC were isolated from peripheral blood by Ficoll separation, upon 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 OKT3 5 μ g/ml and anti-CD28 5 μ g/ml for 36 hours. Transduction of stimulated cells was performed by two cycles of 12-hour exposure to the viral supernatant (25-100ng of p24/ml) at 37°C and 5% CO₂ in the presence of OKT3 0.5 μ g/ml and anti-CD28 0.5 μ l/ml. After transduction, cells were then washed and plated at the density of 1×10^6 /mL in the presence of IL2 6U/ml. HeLa cells and CDPX fibroblasts were transduced with 25-100ng of p24/ml of the already mentioned vectors. Human MPSII, MPSIIIA and MPSVI fibroblast cell lines were obtained from Cell line Bank of "Giannina Gaslini" Institute in Genova. Human CDPX cells were present in TIGEM database. They were maintained in α -MEM medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin G and 100 μ g/ml streptomycin. Mouse Embryo Fibroblasts (MEFs) were isolated from 14.5 day-old MPSIIIA, MLD and normal mouse embryos following standard protocols [16]. Cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin G and 100 μ g/ml streptomycin. For infection, the appropriate AAV 2/1 vectors (10^5 viral particles/cell in DMEM without serum) were added to sub-confluent fibroblasts for 2 hours. The infecting media was then replaced with fresh complete medium and the cells collected after 6 days for sulfatase enzymatic assays.

Western blotting. Cells were lysed in RIPA buffer supplemented with protease inhibitor cocktail (Sigma). Western blottings were performed using anti-FLAG M2 monoclonal antibody (Sigma).

Sulfatase enzymatic assays. For detecting ARSA specific activity, p-Nitrocatecholsulfate assay (p-NC) assay was adapted for ARSA-HA specific activity detection. Elisa plates were coated with anti-HA antibody by O.N. incubation at 4°C, 1:50 in 0.1M NaHCO₃ pH=8.9. After blocking with PBS 10% rat serum, 100 μ l of serum were plated on coated wells and incubated at room temperature for 2 hours. After incubation, wells were washed 3 times with PBS 0.05% Tween-20, and 100 μ l p-NC were added. After 90 minutes of incubation at 37°C, p-NC was collected, the reaction stopped by adding 1ml NaOH 1M, and the final product read at spectrophotometer (515nm wavelength). ARSB and ARSE enzymatic activities were tested as described in [17]. Sulfamidase and Iduronate 2-sulfatase were assayed with fluorogenic substrates as previously described [18,19]. All sulfatase

activities were normalized against the total amount of protein quantified by a Bradford protein assay (Bio-Rad).

Metabolic labeling of GAGs. Fibroblasts infected with AAV vectors were pulsed with $^{35}\text{SO}_4$ for 72 hours (8 $\mu\text{Ci/ml}$ $\text{Na}_2^{35}\text{SO}_4$ in F12/10% FCS medium without antibiotics). The radio-labeled medium was then replaced with normal growth medium and after 72h the cells were collected and pellets assayed for the amount of labeled material.

Animals and injection. MPSIIIA mice were obtained from Jackson Laboratory (strain name: *Sgsh^{mps3a}/PstJ*) and were derived strain from MPSIIIA mice described in [20]. For muscle injection, right gastrocnemius of newborn MPS-IIIa mice was injected with 10 μL of PBS containing the appropriate virus mix. We used a total of 10^{12} viral particles for each injection. After six weeks, the injected, control not-injected and unaffected mice were sacrificed and the right gastrocnemius collected. Tissues were homogenized and ruptured by freezing/thawing 3-5 times. After clarification (13.000 x g for 5 min at 4°C), the supernatants were tested for sulfamidase activity.

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% normal serum and 0,1% bovine serum albumin in TBS for 30 min, and then incubated for 2 hours with a primary sheep polyclonal antibody against human sulfamidase (a gift of John Hopwood). After washing, sections were incubated with secondary anti-goat TRITC-conjugated antibody and mounted with 1:1000 Dapi/Vectashield. The specimens were then visualized under fluorescence microscope and pictures were taken with a 63 X 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 with a 63 X magnification objective.

Results and Discussion

To study the effect of SUMF1 on ARSA activity two lentiviral (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 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 hemagglutinin protein of the human influenza virus. The HA-tagged enzyme had a specific activity comparable with that of the un-modified 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, HSPC from ARSA knock-out 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 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 to cells transduced either with ARSA-HA at high and low vector input or with ARSA-HA and GFP (Fig. 1A,B,C). HSPC purified from the bone marrow (BM) of ARSA knock-out mice were transduced at high and low vector concentration (see methods 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 to ARSA-GFP co-expressing cells or to ARSA transduced cells (Fig. 1D,E). As already shown [23], transduction of MLD patients' stimulated T lymphocytes with ARSA encoding LV resulted in reconstitution of ARSA activity up to normal donors' levels (Fig. 1F,G). 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 (Fig. 1F,G). These experiments demonstrate that SUMF1 co-delivery together with ARSA can significantly enhance its specific activity in 3 different cell types, suggesting the potential use of SUMF1 to further improve the efficacy of gene therapy strategies for MLD.

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 (Fig. 2). ARSE activity was 20-25 % higher in cells transduced with the ARSE/SUMF1 LVs

as compared to 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 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 the SUMF1 cDNA, at different sulfatase:SUMF1 virus ratios. For each infection we used a total of 10^5 viral particles/cell. Six 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) iduronate sulfatase activity was approximately 2-fold compared to the activity in fibroblasts infected with AAV2/1-CMV-IDS (Fig. 3A). The enhancing effect of SUMF1 was dependent upon the ratio between AAV2/1-CMV-IDS and AAV2/1-CMV-SUMF1 virus doses (Fig. 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, $^{35}\text{SO}_4$ labeling experiments demonstrated that the accumulation of $^{35}\text{SO}_4$ -labeled material in fibroblasts infected with both IDS and SUMF1 was quantitatively reduced compared to $^{35}\text{SO}_4$ incorporation detected in fibroblasts infected with IDS alone (Fig. 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 virus (Fig. 3B,C). 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 effect correlates with a reduction of GAG storage in the cells.

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 approximately 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 (Mouse Embryo Fibroblasts) obtained from MPSIIIA mice. SGSH activity in MPSIIIA MEFs was undetectable as expected (Fig. 4A). By using AAV2/1 vectors, we co-infected MPSIIIA MEFs with

both SGSH and either GFP or SUMF1 at 1:1 ratio. Six days after infection we tested the activity of SGSH and analyzed GAG storage in cell pellets. Delivery of both SGSH and SUMF1 AAVs to MEFs yielded enzyme activity two fold higher than the activity in MEFs infected with both SGSH and GFP (Fig. 4A). This enhancing effect also correlated with a decrease in GAG storage in MEFs co-infected with both SGSH and SUMF1 (Fig. 4B).

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 mix of AAV2/5-CMV-SGSH and AAV2/5-CMV-GFP or a 1:1 mix of AAV2/5-CMV-SGSH and AAV2/5-CMV-SUMF1 vectors, keeping constant the total amount of viral particles (10^{12} pts for each injection). Six weeks later, we sacrificed the mice and collected the muscles, from injected and control not-injected or unaffected animals. Immunofluorescence staining demonstrated that human SGSH was strongly expressed in the injected muscles (Fig. 5B). The level of SGSH activity measured in the injected muscles for which AAV2/5-CMV-SGSH and AAV2/5-CMV-GFP mix was used reached 17% of normal level activity and was about 8-fold greater with respect to activity measured in muscle of not-injected mice (Fig. 5A). Most importantly, muscle injected with AAV2/5-CMV-SGSH and AAV2/5-CMV-SUMF1 showed further increment of SGSH activity to levels 2 fold higher than that detected in muscle injected with AAV2/5-CMV-SGSH and AAV2/5-CMV-GFP mix (Fig. 5A). To exclude 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 (Fig. 5C), suggesting stable transduction in all conditions.

We previously demonstrated that at least eleven SUMF1 mutations found in MSD human patients resulted in severely impaired sulfatase-enhancing activity when the correspondent 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 showed to completely abolish the enhancing activity of SUMF1 both in Cos-7 cells [17] and SUMF1 *-/-* MEFs [Annunziata I. personal communications]. In all three sulfatase deficient cell lines the SUMF1 C336R mutant was unable to produce significant increases of activity of the co-delivered sulfatase (Fig. 6A,B,C). In contrast, co-infection of vectors carrying the wt SUMF1 and sulfatase cDNAs produced a clear synergistic effect on sulfatases activity, as expected (Fig. 6A,B,C). Similar levels of SUMF1 proteins (wt and mutant) were detected in infected fibroblasts (Fig. 6B and data 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 in conditions in which the activity of each individual sulfatase was deficient. Further, 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 parenchima or in HSPC, 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 Iduronate sulfatase 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. due to a different 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 a viable and useful tool to achieve this goal in those lysosomal diseases that are caused by sulfatase deficiencies.

ACKNOWLEDGEMENTS

We thank Ida Annunziata for providing LV expressing Flag-SUMF1 proteins, Marco Sardiello and Anthony Fedele for critical reading of the manuscript, and Lucia Sergi Sergi for experimental help in LV production. This work was supported by grants from Italian Telethon (TIGEM) to M.P.C, the “Istituto Superiore di Sanità” (project n. 526/A18) to A.B. and from Italian Telethon (TIGET), the European Community (CONSERT), and the Italian Ministry of Scientific Research (FIRB) and of Health to L.N.

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Abbreviations

Iduronate Sulfatase (IDS), Sulfamidase (SGSH), Glucosamine 6-Sulfate (G6S), Galactose 6-Sulfate (Gal 6S), Arylsulfatase B (ARSB), Arylsulfatase A (ARSA), Arylsulfatase C (ARSC) and Arylsulfatase E (ARSE).

Figure legends

Figure 1. Effect of SUMF1 on ARSA activity upon lentiviral transduction of HeLa and ARSA deficient cells

HeLa cells (**A-C**), murine ARSA $-/-$ HSPC (**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 methods 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, E, F, G**) and supernatant (**C**). ARSA activity in A, B, C, E, G is expressed as fold of the levels detected in samples transduced with ARSA at high vector input (ARSA_h), in D as fold of the levels detected in wild type (WT) samples,

and in F in nmol/mg/hour. In all tested cell types an increase of ARSA-HA activity was detected upon ARSA and SUMF1 co-expression as compared to controls (either ARSA-HA or ARSA-HA/GFP transduced cells)(* p<0.05) (n=6).

Figure 2. Effect of SUMF1 on ARSE activity upon lentiviral 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 normal (WT) fibroblasts. Co-infection of SUMF1 together with ARSE (1:1 ratio) resulted in an increase of ARSE activity as compared to control (ARSE:GFP 1:1) (* p<0.05) (n=3)

Figure 3. AAV-mediated co-expression of SUMF1 with either 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 (either 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 (1×10^5 viral particles/cell) was kept constant by using AAV2/1-CMV-GFP into the infecting viral mix. The IDS, SGSH and ARSB activities are shown as fold increase to sulfatase-infected fibroblasts activity. The amount of GAG storage in A was measured by labeling the cell medium with $^{35}\text{SO}_4$ as described in methods. Co-expression of both IDS and SUMF1 at either 1:1 or 1:5 ratios resulted in both an increase of IDS activity and in a decrease of GAGs accumulation as compared to 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 sulfatases activities as compared to controls (SGSH, ARSB or ARSB:SUMF1 1:1) (* p<0.05) (n=3).

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

MEFs were obtained from WT (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 1:1 ratio. Sulfamidase activity is shown as fold increase to the activity of MPSIIIA MEFs infected with SGSH:GFP. (B) WT and MPSIIIA MEFs (both infected

and not-infected) were metabolically labeled with $^{35}\text{SO}_4$ 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 in a decrease of GAGs accumulation as compared to controls (SGSH:GFP 1:1) (* $p < 0.05$) ($n=3$).

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 4 experimental groups. One group was not injected ($n=3$), while the other three groups were injected into the right gastrocnemius with either AAV2/5-CMV-GFP ($n=3$), or a 1:1 mix of AAV2/5-CMV-SGSH and AAV2/5-CMV-GFP ($n=6$), or a 1:1 mix 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} pts) was kept constant. After six weeks the muscle tissues (right gastrocnemius) were collected from either injected or not-injected MPSIIIA mice, and from unaffected (WT, $n=4$) six weeks old mice. Sulfamidase activity was measured in total tissue homogenates and expressed as fold activation to SGSH:GFP-injected muscle. Co-delivering of both SGSH and SUMF1 to mouse muscles resulted in an increase of SGSH activity in injected tissues as compared to 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 delivering of AAV-SGSH vector. In muscle sections of not-injected MPSIIIA mice a low background signal is 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 indicate a widespread expression of the GFP in the myocells. Dapi staining of nuclei is shown in blue.

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, respectively for CDPX, MPSIIIA and MPSII fibroblasts) and either 3XFlag-SUMF1 wt, 3XFlag-SUMF1 C336R mutant or GFP LVs. The ARSE, SGSH and IDS activities are shown as fold increase to 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 to sulfatase:SUMF1 wt co-infection (* $p < 0.05$) ($n=3$). A western blot of the flag tagged SUMF1 proteins is shown (B, bottom).

Fig. 1

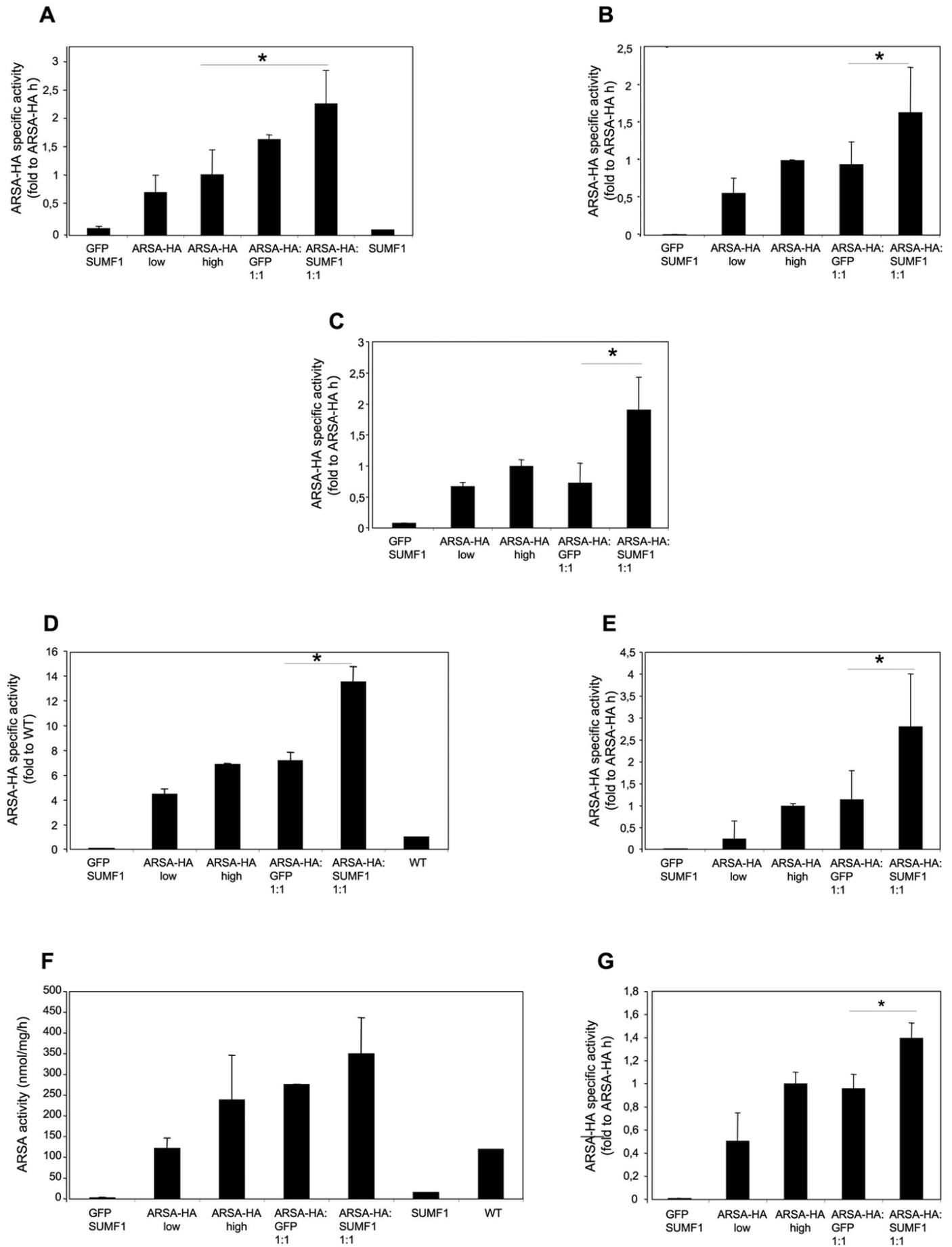


Fig. 2

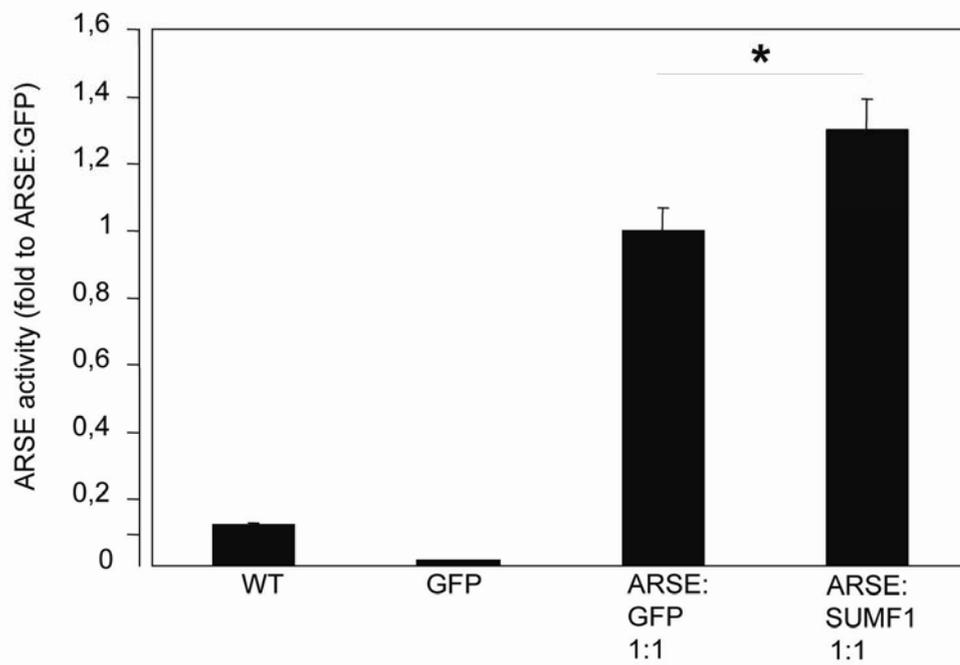


Fig. 3

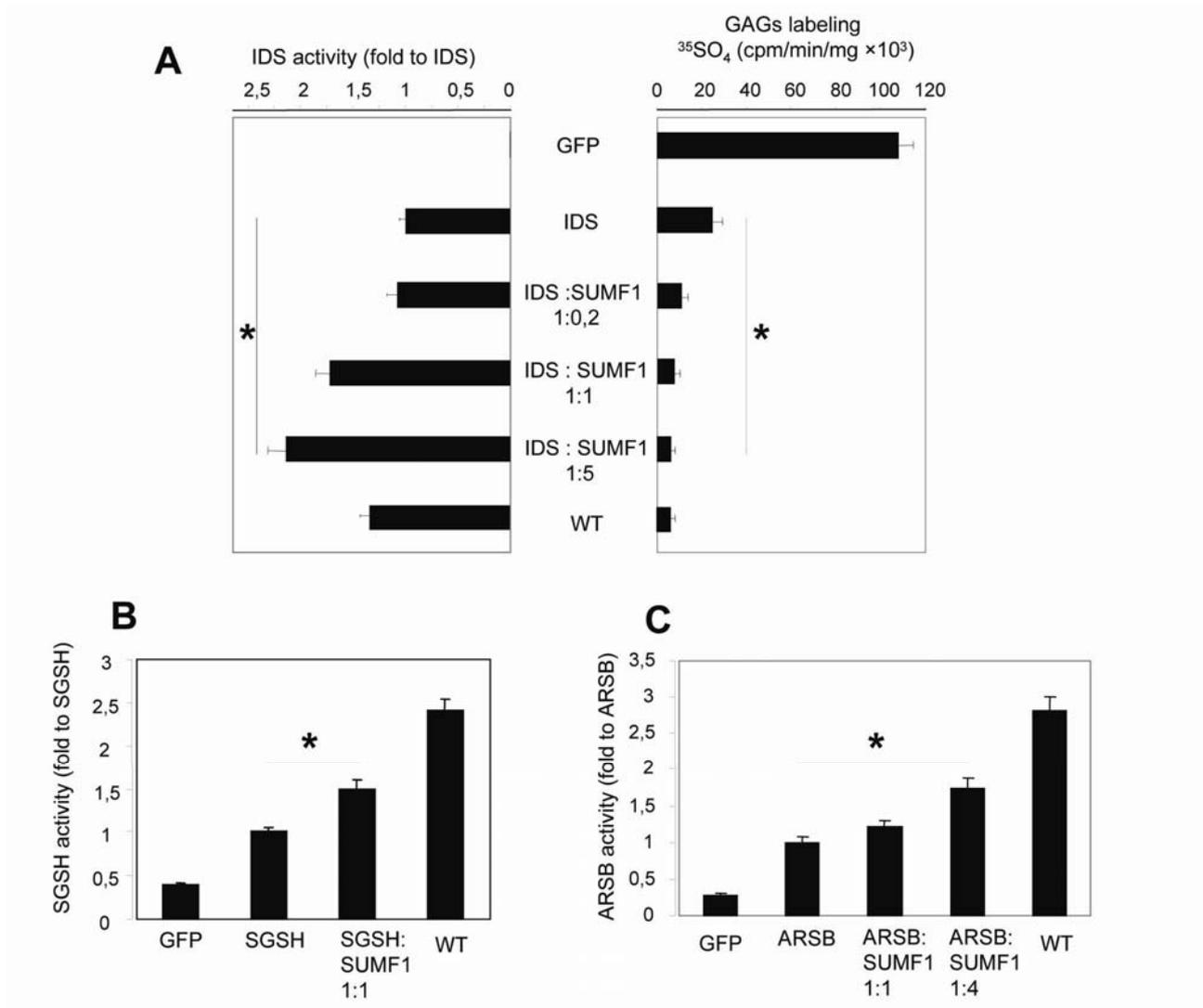


Fig. 4

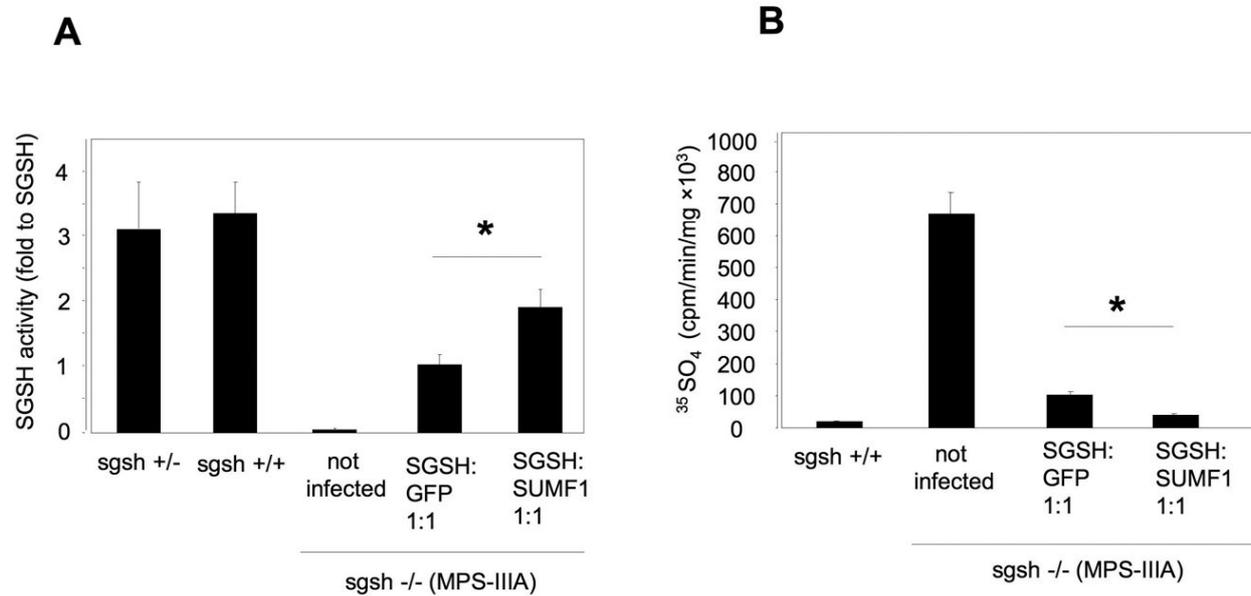


Fig. 5

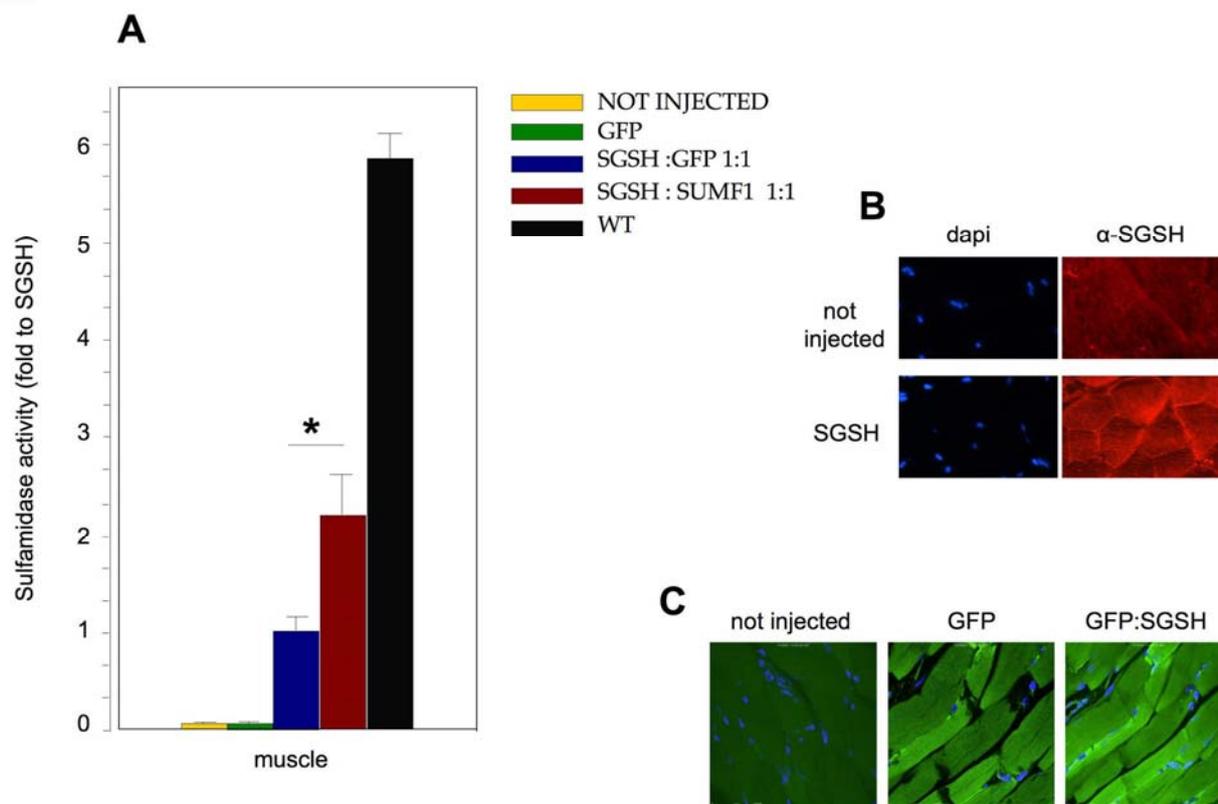


Fig. 6

