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1 **Dermatan Sulfate Epimerase 1-Deficient Mice have Reduced**
2 **Content and Changed Distribution of Iduronic acids in Dermatan**
3 **Sulfate and an Altered Collagen Structure in Skin**

4
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13 Running title: FUNCTION OF DERMATAN SULFATE

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Dermatan sulfate epimerase 1 (DS-epi1) and 2 convert glucuronic acid to iduronic acid in chondroitin/dermatan sulfate biosynthesis. Here we report on the generation of DS-epi1-null mice and the resulting alterations in the chondroitin/dermatan polysaccharide chains. The numbers of long blocks of adjacent iduronic acids are greatly decreased in skin decorin and biglycan chondroitin/dermatan sulfate, along with a parallel decrease of iduronic-2-O-sulfated-galactosamine-4-O-sulfated structures. Both iduronic acid blocks and iduronic acids surrounded by glucuronic acids are also decreased in versican-derived chains. DS-epi1-deficient mice are smaller than wild-type littermates, but otherwise have no gross macroscopic alterations. The lack of DS-epi1 affects the chondroitin/dermatan sulfate in many proteoglycans and the consequences for skin collagen structure were initially analyzed. We found that the skin collagen architecture was altered, and electron microscopy showed that the DS-epi1-null fibrils have a larger diameter than the wild-type fibrils. The altered chondroitin/dermatan sulfate chains carried by decorin in skin are likely to affect the collagen fibril formation and reduce the tensile strength of DS-epi1-null skin.

1 Chondroitin sulfate (CS) is an unbranched polymer chain composed of alternating
2 glucuronic acid (GlcA) and N-acetyl galactosamine (GalNAc) (36,49). In dermatan
3 sulfate (DS) D-glucuronic acid is converted to its epimer L-iduronic acid (IdoA)
4 (25). The extent of this modification, which varies from a few percent of the
5 glucuronic acid being epimerized to a predominant presence of iduronic acid,
6 depends on the variable epimerase activity in tissues and on the core protein
7 attached to the chain in CS/DS-proteoglycans (PG) (41,47). The same CS/DS-
8 proteoglycan has different content of iduronic acid depending on the cell type and
9 tissue of origin (4,5). The name CS/DS denotes the hybrid GlcA-IdoA nature of the
10 chain. It has long been known that the distribution of iduronic acids within the chain
11 is not random but follows two patterns: either they are clustered together, forming
12 long iduronic acid blocks, or they are isolated, i.e. interspersed among surrounding
13 glucuronic acid (11). Dermatan sulfate epimerase 1 and 2, in short DS-epi1 and DS-
14 epi2, encoded in mouse by *Dse* and *Dsel(like)* genes, respectively, are present from
15 *Xenopus tropicalis* to humans but not in worms and fly (23,34). During DS
16 biosynthesis, epimerization is followed by the action of eight C-specific O-
17 sulfotransferases, which transfer a sulfate group to C-2 of both IdoA and GlcA, and
18 to C-4, C-6, C-4/C-6 of GalNAc (18). These modification reactions, individually
19 affecting only part of the available substrate, produce structural variability in the
20 CS/DS chain. Considerable efforts have been made to characterize specific
21 sequences in the CS/DS chains responsible for binding to protein, and the
22 subsequent mediation of a biological effect (28). For instance (IdoA-2OS-GalNAc-
23 4OS)₃ and GalNAc-4/6-diOS-containing structures bind and activate heparin

1 cofactor II, which is the major antithrombotic system in the subendothelial layer
2 (48). IdoA/GlcA-2OS-GalNAc-6OS-containing structures bind to pleiotrophin,
3 mediating neuritogenic activity (3,44). IdoA-GalNAc-4OS-containing structures
4 bind to basic fibroblast growth factor and the complex has been shown to be active
5 in wound healing (46).

6 CS/DS-PGs are mainly found in the extracellular matrix (ECM). They
7 belong to four families: lecticans; e. g. versican, aggrecan, brevican and neurocan;
8 collagens: e. g. collagen IX; basement membrane proteoglycans: e. g. SMC3,
9 collagen XV, perlecan, containing both heparan sulfate and CS/DS; Small Leucine-
10 rich Repeat Proteoglycans (SLRP). Some proteoglycans of the first three groups are
11 referred to as CS-PGs. The actual presence of iduronic acid, depending on the tissue
12 examined and on the developmental stage, has been overlooked in many cases
13 (37,44). The archetypical SLRP family members, decorin, biglycan, fibromodulin
14 and lumican, bind fibrillar collagens and affect the collagen fibril and scaffold
15 formation in connective tissues (15). Decorin and biglycan are substituted with one
16 and two CS/DS chains, respectively. Decorin is involved in collagen type I fibril
17 formation and matrix assembly in a wide range of connective tissues and binds near
18 the C-terminus of collagen monomers delaying their accretion to the growing fibrils.
19 We have identified an SYIRIADTNIT sequence in decorin as essential for binding
20 to collagen (16). The role of the decorin CS/DS chain *in vivo* has not been explored,
21 although *in vitro* studies suggest that IdoA promotes the binding of CS/DS to
22 collagen (31) and is required for self-association of CS/DS chains (6,10,22).

1 Here the function of DS-epi1 in mice was disrupted. DS-epi1-deficient mice
2 show CS/DS with a marked deficiency in iduronic acid-containing structures. The
3 deletion of DS-epi1 is likely to affect many types of proteoglycans and to result in a
4 complex phenotype. We focus on skin alterations presumably caused by altered
5 decorin/biglycan CS/DS chains.

7 MATERIALS AND METHODS

8 **Materials** - Cell culture reagents were from Invitrogen. Red-Sepharose gel,
9 Superose 6 HR 10/30, Superdex Peptide 10/300 GL, and PD-10 columns, ECL-Plus
10 reagent were from GEHealthcare. DE52 anion exchange gel was from Whatman.
11 $^{35}\text{SO}_4$ (1500 Ci/mmol) was from PerkinElmer. Chondroitinase ABC, B, and AC-I
12 were from Seigakaku. Brilliant blue G colloidal gel-staining solution was from
13 Sigma.

14 **Construction of the targeting vector and generation of chimeric mice** - A
15 genomic *Dse/Sart2* clone was isolated from a mouse genomic PAC library
16 (RPC121; Geneservice, Cambridge, UK) (33). A 6.3 kb SphI-StuI fragment was
17 ligated in pBluescript II KS (Stratagene) and a phosphoglycerate kinase-neomycin
18 resistance cassette (pGKNeo) (26) was inserted in the reverse transcriptional
19 orientation in a unique XhoI site located in the second exon of *Dse/Sart2* (Fig. 1A).
20 The targeting vector was linearized at a NotI site and used to transfect R1 mouse
21 embryonic stem cells at Lund Transgenic Core Facility as described (45). Clones
22 were picked and analyzed by southern blot after NcoI digestion using a 420 bp
23 external probe. This probe hybridizes with a 6.3 kb wild type and a 4.8 kb Nco I

1 fragment (Fig. 1B). Three individually targeted clones were injected into C57BL/6
2 blastocysts to generate chimeric mice. Chimeric males were obtained from two
3 clones and were mated with C57BL/6 females. F1 mice with germ line transmission
4 were intercrossed to produce all genotypes in a mixed C57BL/6//129/SvJ genetic
5 background. All experiments in this study were conducted on littermates of this
6 mixed genetic background. Mice derived from two ES clones were characterized
7 for: *i*) *Dse* allelic distribution at weaning, *ii*) *Dse* expression by qRT-PCR and by
8 western blot, *iii*) shape at birth of the tail, and *iv*) weight measured between birth
9 and weaning. Given the complete identity of the *Dse*^{-/-} mice from the two ES
10 clones, according to the above four criteria, only mice derived from one ES clone
11 were further characterized. Mice were genotyped by PCR from tail DNA using the
12 following primers: forward common for both alleles 5'-
13 AGCACATTGCAGCTCGGCTTAC-3', reverse for wild-type allele 5'-
14 GCTGCCATCCTCTCCATGTAGTC-3', reverse for neo-cassette mutated allele 5'-
15 TGGATGTGGAATGTGTGCGAGG-3'. The use of animals for research complied
16 with national guidelines, and permission was given by the regional ethical board.

17 **Quantitative RT-PCR** – Total RNA was extracted from tissues using RNAeasy
18 kit (Qiagen), DNase-treated with DNA-free™ Kit (Applied Biosystems), and
19 cDNA synthesis was performed with SuperScript® VILO™ cDNA synthesis kit
20 (Invitrogen). The samples were mixed with primers and SYBR Green Master Mix
21 (Applied Biosystems) and amplified in an ABI Prism instrument (Applied
22 Biosystem, Foster City, CA) starting with an initial 2-min heating at 50°C and 10-
23 min heating at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. The

1 data were analyzed using SDS 2.1 software (Applied Biosystems). The calculated
2 threshold cycle (Ct) values were normalized to the Ct-value for glyceraldehyde-3-
3 phosphate dehydrogenase (GAPDH). The primers used were *Dse*: 5'-
4 TGTGTGCTGTATCCTGAGAACA -3' and 5'-CAAGGCGCATCTTTCACCAAC
5 -3'; *GAPDH*: 5'-AGGTCGGTGTGAACGGATTTG-3' and 5'-
6 TGTAGACCATGTAGTTGAGGTCA-3'.

7 **Establishment of primary fibroblasts cell culture** - Lung and skin fibroblasts
8 were derived from 2-month-old animals. Small pieces of the organs were adhered to
9 plastic and cultivated in MEM, 10%FBS. Outcoming cells were grown for 10 days
10 and split (passage 1). Cells were utilized for the experiments between passage 4 and
11 passage 7.

12 **Western blot and immunohistochemistry** - Cells were lysed in 20 mM MES pH
13 6.5, 150 mM NaCl, 10% glycerol, 2 mM DTT, 1 mM EDTA, 1% Triton X-100,
14 protease inhibitors, i.e. PMSF 1mM, aprotinin leupeptin and pepstatin each at 1
15 µg/ml (lysis buffer). Spleen, lung, kidney, skin, and brain organs were homogenized
16 in ~3-fold excess (v:w) of lysis buffer with the use of a Potter. Protein content was
17 determined in cleared lysates by Bradford method (BioRad), using BSA as
18 standard. Alternatively, 5-mg organ lysates were bound in batch to 50 µl of Red-
19 Sepharose gel, incubated 60 min, washed 5 times with lysis buffer, and finally
20 eluted by reducing Laemli sample buffer.

21 DS-epi1 was detected using 1µg/ml of an immunopurified anti-DS-epi1 rabbit
22 polyclonal antibody obtained after antigen peptide immunization:
23 (KWSKYKHDLAAS corresponding to aminoacid 509-520 of the human/murine

1 sequence), Innovagen, Sweden. ECL-Plus was used as HRP substrate.

2 Immunohistochemistry was done as described (45) using antisera against decorin
3 and biglycan.

4 **DS-epimerase activity** - 200 µl of organ or cell lysates were desalted using as
5 dialysis buffer 20 mM MES (pH 5.5 at 37°C), 10% glycerol, 0.5 mM EDTA, 0.1%
6 Triton X-100, 1 mM DTT, protease inhibitors. Protein content was determined after
7 dialysis and equal amount of proteins of samples from the different genotypes were
8 assayed for epimerase activity. The final assay volume was 100 µl of 0.8 X dialysis
9 buffer, 2 mM MnCl₂, 0.5% NP40, and 30,000 dpm of the labeled chondroitin
10 substrate ([5-³H]defructosylated K4, prepared according to (14)). Incubations were
11 carried out at 37°C for 20 h and the released tritium was quantified as described in
12 (23).

13 **Preparation of *in vivo* labeled CS/DS** – Ten-day-old mice were i.p. injected with
14 50µl PBS, containing 200µCi ³⁵SO₄, and kept alive for two h before sacrifice. To
15 assess the structure of the CS/DS chains from the whole body, the entire mice were
16 cut in pieces and grinded in 30 ml of 100mM Tris pH 8.5 (at 55°C), 200 mM NaCl,
17 5 mM EDTA buffer. The mixture was made 0.1% SDS, proteinase K was added till
18 final 100µg/ml, and incubated at 55°C overnight. Further proteinase was added to
19 cleared material and the incubation was continued for additional 6 h. The material
20 was diluted 1:4 with 200 mM acetate pH 5.5, bound in batch with 1 ml DE-52 anion
21 exchange, washed with 50mM acetate pH 5.5, 200 mM NaCl, eluted with 50 mM
22 acetate pH 5.5, 1.5 M NaCl, added 10 µg CS-6-sulfated as carrier, and desalted
23 using PD-10 column run in water. GAG chains were cleaved by β-elimination in

1 50mM KOH, 100 mM NaBH₄ at 45°C for 16-18 h and re-isolated again on DE-52
2 operated in principle as above. Heparan sulfate chains were depolymerised by
3 deamination reaction at pH 1.5 (43), followed by re-isolation of CS/DS chains on
4 Superose 6 run in 0.2 M NH₄HCO₃.

5 **Preparation of labeled skin decorin/biglycan- and versican-CS/DS chains.**

6 Skin from 10-day-old mice, labeled as above, was extracted by Potter
7 homogenization in 10 volumes (v/w) of guanidine 4M, 50 mM acetate pH 5.8,
8 10mM EDTA, 5 mM NEM, protease inhibitors. Cleared extracted material was
9 dialyzed versus excess of 6M urea, 50 mM acetate pH 5.5, 0.2M NaCl, 1 mM
10 EDTA, protease inhibitors. After dialysis, Triton was added till 0.1% and PG were
11 bound to DE-52 gel, washed with dialysis buffer, 0.1% Triton, and eluted by 4M
12 guanidine, 50 mM acetate pH 5.8, 10mM EDTA. 100µg dextran T500 was added as
13 carrier, and PGs were size-separated on a Superose 6 column, run in 4M guanidine,
14 50 mM acetate pH 5.8, 10m M EDTA, 0.1% Triton. 10 µg CS-6-sulfated was added
15 as carrier, and the isolated PGs were desalted by PD-10 columns run in water, and
16 the GAG chains were cleaved from the core protein by β-elimination , re-purified
17 on DE-52, freed from HS, and finally recovered, as described above.

18 **Preparation of unlabeled skin decorin/biglycan** – Skin from 3- to 4-month-old
19 mice was extracted in guanidine-containing buffer, dialyzed versus urea-containing
20 buffer and DE-52 purified as above. DE-52 purified material, without addition of
21 any carrier, was ethanol-precipitated and run on Superose 6 as above, without
22 addition of any carrier. Elution fractions were analyzed by western blot with anti-
23 biglycan and anti-decorin antibodies. Pooled material was dialyzed stepwise versus

1 Hepes 50 mM pH 7.4, NaCl 0.15 M solutions containing decreasing concentration
2 of urea (6 M, 3 M, no urea), and finally extensively versus water. Samples were
3 lyophilized and resuspended in PBS. Most of the material was soluble, as it was
4 recovered in the supernatant after 20,000 x g centrifugation. Purity was verified by
5 SDS-PAGE (see Fig. 9).

6 **Chondroitinases treatment of CS/DS and analysis of the split products –**
7 Cleavage of CS/DS chains by lyases (Seikagaku), as well as their analyses, were
8 according to (34).

9 **Collagen staining –** Tissues were fixed in 95% ethanol, 1% acetic acid and
10 decalcified in 6% EDTA in phosphate-buffered saline for 1 week prior to
11 dehydration and embedding. Sections were stained with Chromotrope 2R and Sirius
12 Red (Sigma).

13 **Electron microscopy –** Tissues from 3-month-old wild-type and *Dse*^{-/-} mice were
14 analyzed by transmission electron microscopy. After fixation in 0.15 M sodium
15 cacodylate-buffered 2.5% glutaraldehyde, and post-fixation in 0.1 M *s*-collidine-
16 buffered 2% osmium tetroxide, samples were embedded in epoxy resin. Ultrathin
17 sections were analyzed in a Philips CM-10 electron microscope, and collagen fibril
18 diameters were quantified with ImageJ software (NIH). To calculate fibril density
19 the area occupied by collagen fibrils was measured by converting the image into
20 black (collagen fibrils) and white (empty area) pixels, and their concurrent
21 quantification was performed using ImageJ software (NIH). The Mann–Whitney U
22 test was used to calculate significance, with $P < 0.05$ considered statistically
23 significant (32).

1 **Differential scanning calorimetry** - Differential scanning calorimetry
2 measurements were performed with either dissected skin, or with acid-solubilized
3 collagen (Vitrogen) preincubated for 4 h in 37°C in PBS in presence of extracted
4 decorins from wild-type or *Dse*^{-/-} mice. The thermograms were recorded in VP-
5 DSC (MicroCal), scan rate 0.25°C/min, and medium feedback. Each thermogram
6 was corrected by subtraction of a linear baseline based on a blank buffer sample,
7 and normalized for collagen concentration.

8 **Collagen fibrillogenesis assay** – Acid-solubilized collagen (Vitrogen) was
9 neutralized and diluted (100 µg/mL) in 150 mM NaCl, 20 mM HEPES, pH 7.4,
10 with decorin extracted from wild-type or *Dse*^{-/-} mice (2 µg/ml). Solutions were
11 degassed and then incubated at 37°C in a spectrophotometer where the absorbance
12 was continuously recorded at 400 nm.

13 **Tensile strength determination.** Dorsal skin samples from 5-month-old *Dse*-deficient
14 (n=5) and wild type (n=5) mice were harvested immediately after sacrifice. Skin samples
15 were cut using a template 4 mm wide by 20 mm long. Two skin samples, of identical
16 width and length, were harvested from each mouse. The long axis of all samples
17 coincided with the anterior-posterior direction of the mice. The thickness of each sample
18 was determined using a micrometer at two consecutive locations along the middle 5-mm
19 part of the skin specimens, and an average of the two measures was used for analysis.
20 Approximately 7 mm of each end of the skin samples were subsequently allowed to air
21 dry at room temperature, while keeping the middle 5-mm part moist by PBS-soaked
22 gauze. Thereafter, the dried sample ends were glued to the uncoated aluminium endplates
23 of the mechanical rig with cyanoacrylate glue. When the glue was hardened, the skin

1 samples and the endplates were attached to the mechanical test frame and submerged in a
2 petri dish with PBS. Subsequently, the mechanical test frame was placed under a
3 stereoscopic microscope (SMZ1000, Nikon, Tokyo, Japan) and the failure test was
4 initiated. All failure tests were conducted with a constant plate-plate elongation velocity
5 of 2.0 mm/min corresponding to 40% strain/min.

6 The microtensile mechanical rig was equipped with a 50-Newton load cell with 1%
7 accuracy (Deben UK Ltd., Suffolk, UK.), a specimen chamber, and two specimen clamps
8 driven by Maxon motor 118516 with 241062 encoder fitted that achieved and measured
9 changes in grip displacement.

10 The cross-sectional area of the skin samples were calculated based on the measured
11 width and thickness of the individual samples (width x thickness) on the assumption
12 that the samples had a square cross-section. Sample stress was calculated as the
13 tensile force (N) divided by the cross-sectional area (m²) of the samples and
14 reported in megapascal units (MPa). Peak stress was defined as the greatest
15 magnitude of stress prior to tissue failure. All samples failed between the endplates
16 of the mechanical rig, approximately in the middle of the specimen.

18 RESULTS

19
20 **Generation of *Dse*-knockout mice.** In order to functionally disrupt the *Dse* gene,
21 a neomycin-resistance cassette was inserted after the ATG translational start in exon
22 2 (Fig. 1A). The resulting 6.3-kb targeting vector was electroporated into 129/Sv
23 embryonic stem cells. Out of 62 ES clones screened for homologous recombination

1 by Southern blot, ten clones contained the mutated allele (Fig. 1B). Three positive
2 ES clones were injected into blastocysts, of which two yielded chimeric mice.
3 *Dse*^{+/-} mice were generated by mating chimeric male with C57BL/6 female.
4 Breeding of the resulting heterozygous 129/Sv//C57BL/6 mice, i.e. with a mixed
5 genetic background, resulted in *Dse*-deficient and wild-type littermates, which were
6 analyzed in this report (Fig. 1C). The mice (n=198) were 18% *Dse*^{-/-}, 33% *Dse*^{+/+}
7 and 49% *Dse*^{+/-}.

8 ***Dse*^{-/-} mice are smaller and have a kinked tail.** At birth *Dse*^{-/-} mice were
9 smaller with a 20-30% reduced body weight as compared to wild-type littermates
10 (Fig. 2A and 2B). All *Dse*^{-/-} pups had a tail with a kink that was not present after
11 four weeks of age. At 80 days of age the *Dse*-deficient mice remained 5-10%
12 shorter (crown-rump length) and 10% lighter (Fig. 2B). Adrenal gland, brain,
13 intestines, kidney, lungs, and other major organs of 6-month-old *Dse*-deficient mice
14 were necroscopically and histologically examined, and no gross changes were
15 observed. Skin (see below) showed histological alterations. *Dse*^{-/-} mice had
16 reduced fertility; intercrosses of *Dse*^{-/-} mice gave litters of 2-4 pups (9 litters),
17 compared to 8-12 pups obtained from heterozygous or wild-type parents. Six *Dse*^{-/-}
18 mice were followed till 16 months of age and don't have increased mortality.

19 **In *Dse*^{-/-} mice DS-epimerase 1 expression is abolished and epimerase activity**
20 **is reduced.** DS-epi1 is highly expressed in spleen. A qRT-PCR product of the
21 expected size was obtained from wild-type spleen ($C_t = 27$, or ΔC_t of 6.5 compared
22 with the reference 18S rRNA gene) but was absent, even after 40 PCR cycles, in
23 *Dse*^{-/-} organ. Spleen was also analyzed for DS-epi1 protein expression by western

1 blot. Analysis of 100 μ g of spleen lysates revealed a \sim 100 kDa band present in
2 wild-type samples and absent in *Dse*^{-/-} samples (Fig. 3A). This band co-migrated
3 with the band derived from DS-epi1 overexpressed in 293HEK cells. To increase
4 the sensitivity of the analysis, DS-epi1 was enriched from 5 mg of lysates by Red-
5 Sepharose gel and western blotted. The \sim 100 kDa DS-epi1 band was reduced to
6 about 50% in *Dse*^{+/-} compared to wild-type, and was missing in *Dse*^{-/-} spleens
7 derived from two mice, each originating from two different ES clones. A less
8 predominant band of \sim 78kDa was present only in the enriched sample from spleen
9 and might represent a degradation product. Identical results were generated from
10 lung tissue (data not shown). DS-epi 1 expression was similarly analyzed in skin
11 fibroblasts derived from adult wild-type and *Dse*^{-/-} mice (Fig. 3A). A single \sim 100
12 kDa band was detected in wild-type but not in the *Dse*^{-/-} cells. Similar results were
13 obtained from adult lung fibroblasts and mouse embryonic fibroblasts derived from
14 wild-type and *Dse*^{-/-} mice (data not shown). Epimerase activity was assayed in five
15 organ lysates from 4-week-old mice, which showed a greatly diversified specific
16 epimerase activity: skin released 1,170 dpm of tritium from the substrate/h/mg of
17 protein, spleen 753, lung 466, kidney 127, and brain 41. The *Dse*^{+/-} lysates
18 contained activity that spanned 47 till 107% of the corresponding wild-type organs
19 (Fig. 3B). *Dse*^{-/-} spleen and kidney contained 4% of the activity compared to the
20 wild-type samples, lung 18%, skin 35%, brain 86% (Fig. 3B). The remaining
21 activity is most likely catalyzed by DS-epi2. Specific epimerase activity was 2,074
22 dpm/h/mg in wild-type adult skin fibroblasts, and decreased 35% in the
23 corresponding *Dse*^{-/-} cells.

1 **The whole body-derived *Dse*^{-/-} dermatan sulfate lacks iduronic acid blocks**
2 **and has reduced amount of iduronic acid-2-sulfated-N-acetylgalactosamine-4-**
3 **sulfated structures.** To obtain a general picture of the alterations in CS/DS, GAG
4 chains were ³⁵S-labeled *in vivo* and extracted from the entire mouse. Similar
5 amounts of ³⁵S radioactivity were recovered in the CS/DS chains from mice of all
6 three genotypes. Moreover, the size distribution of the ³⁵S-labeled CS/DS chains, as
7 seen on gel permeation, was similar in the three genotypes (data not shown).
8 Purified CS/DS chains were analyzed for the amount and distribution of iduronic
9 acid by chondroitinase B and AC-I, which cleave GalNAc~IdoA and
10 GalNAc~GlcA linkages, respectively. Digestion of wild-type chains by
11 chondroitinase B yielded a disaccharide peak, derived from at least three contiguous
12 IdoA-GalNAc sequences in the native chain, amounting to 7.3% of the total
13 radioactivity of the split products (Fig. 4A). Wild-type tetrasaccharides, derived
14 from ~IdoA-GalNAc-GlcA-GalNAc~IdoA sequences in the native chains,
15 amounted to 11.5%. The corresponding sample from the *Dse*^{-/-} mouse showed 1%
16 of the disaccharides (~ 7-fold reduction compared to wild-type), and 2.3% of the
17 tetrasaccharides (~5-fold reduction). The sum of hexa-, octa-, and decasaccharide
18 fractions, coming from ~IdoA-(GalNAc-GlcA)_{n 2-4}-GalNAc~IdoA sequences in the
19 native chains, were a minor component both in the wild-type and *Dse*^{-/-} samples
20 and were very similar, amounting to 7.2% and 6.9%, respectively. The split product
21 size distribution coming from wild-type and *Dse*^{+/-} samples was almost
22 superimposable. Disregarding the possible occurrence of nonsulfated disaccharide
23 units, lacking radiolabel, calculations from the disaccharide till decasaccharide

1 fractions resulted in total IdoA content of 14.9% in wild-type, 16.8% in *Dse+/-*
2 chains, and 3.4% in *Dse-/-* chains, which therefore have 23% remaining IdoA
3 compared to wild-type (Fig 4B). Digestion by chondroitinase AC-I leaves
4 unaffected structures constituted by adjacent (IdoA-GalNAc)_n units. Wild-type mice
5 produced such structures with more than 4 adjacent units, eluting the vast majority
6 of them in the void volume of the column (Fig. 4C). These structures, called
7 iduronic acid blocks, amounted to 19% of the total radioactivity in *Dse+/+* chains,
8 decreased to 15.1% in *Dse+/-*, and were missing in *Dse-/-* chains.

9 *Dse+/+* and *Dse-/-* CS/DS chains were also cleaved by chondroitinase ABC, and
10 the resulting disaccharides were separated by HPLC. The iduronic-2-sulfated-N-
11 acetylgalactosamine-4-sulfated structures, recovered as ΔB after the analysis, were
12 reduced by 85% in *Dse-/-* chains (Table I).

13 **Skin decorin/biglycan- and versican-CS/DS *Dse-/-* chains contain less**
14 **iduronic acid blocks and iduronic acid-2-sulfated-N-acetylgalactosamine-4-**
15 **sulfated structures.** ³⁵S-sulfate *in vivo* labeled versican and a mixture of decorin
16 and biglycan were purified, and their CS/DS chains recovered. Either versican-
17 derived chains or decorin/biglycan-derived chains were of comparable size, as
18 shown by gel filtration, irrespective of the different genotypes (Fig. 5A).

19 Chondroitinase B or AC-I digestion made it possible to determine the amount and
20 distribution of iduronic acid along the chain, as outlined above. In skin
21 decorin/biglycan-CS/DS iduronic acid content decreased from 67.1% in wild-type
22 to 17.9% in *Dse-/-*, as calculated from the chondroitinase B split product
23 distribution (Fig. 5B). The entire decrease of iduronic acid content could be

1 attributed to the 90% decrease of the iduronic acid blocks, which accounted for
2 58.2% of the total chain in wild-type and 5.6% in *Dse*^{-/-} (Fig. 5C). Skin versican
3 CS/DS wild-type chains contained 13.3% of iduronic acid, which decreased to 1.4%
4 in *Dse*^{-/-} (Fig. 5D). Iduronic acid blocks were 7.5% of the total wild-type chain and
5 0.6% of the *Dse*^{-/-} chains (Fig. 5E).

6 Size distribution of decorin/biglycan-derived *Dse*^{+/+} iduronic acid blocks,
7 obtained after chondroitinase AC-I digestion, was analyzed and the modal length
8 value was 15 kDa, corresponding to approximately 30 disaccharide residues (Fig.
9 5F), as long as the intact, i.e. uncleaved, chain (Fig. 5A).

10 Furthermore, the iduronic-2-sulfated-N-acetylgalactosamine-4-sulfated structures
11 were reduced by 70% in *Dse*^{-/-} decorin/biglycan chains (Table I).

12 **DS-epi1 deficiency alters skin morphology, collagen fibril ultrastructure, and**
13 **skin tensile strength.** Skin samples obtained from five *Dse*^{-/-} mice contained
14 sparser loose connective tissue in the hypodermal layer (Fig. 6 A-D). These
15 structural changes did not alter the morphology or the numbers of hair follicles
16 (wild-type = 33±10 hair follicles/tail cross-section, n=7; *Dse*^{-/-} 29.5±4.5, n=8). The
17 collagen content, as determined by hydroxyproline, was not significantly different
18 in the skin of wild-type (20.8 ng hydroxyproline/mg wet weight, n=4, range=16.8-
19 25.3) and *Dse*-deficient mice (18.6 ng hydroxyproline/mg wet weight, n=4,
20 range=17.3-20.8). Immunostaining of decorin showed a similar staining pattern and
21 indicate that the distribution and amount of decorin is similar in wild-type and *Dse*-
22 deficient skin (Fig.6 E-G). Similarly, staining with an anti-biglycan antiserum
23 showed no change in distribution or amount of biglycan (not shown).

1 Transmission electron microscopy (TEM) pictures were taken at different levels
2 of dermis and hypodermis (Fig. 7A). Analysis of the skin collagen fibril
3 ultrastructure by TEM showed a shift towards thicker fibrils in *Dse*^{-/-} skin. The
4 mean fibril diameter shifted from 60 nm in wild-type to 85 nm in *Dse*^{-/-} skin. The
5 differences in fibril diameter remain in dermal and hypodermal areas. In some *Dse*^{-/-}
6 /- hypodermal areas collagen fibrils were absent. This agrees with the observations
7 by light microscopy (Fig. 6). On the other hand, *Dse*^{-/-} tail tendons showed a minor
8 shift towards thicker fibrils compared to the wild-type tendon, and most of the
9 fibrils had the same diameter in both genotypes (Fig. 7B). There were no
10 differences in collagen fibril diameter or morphology in the Achilles tendon (Fig.
11 7C). Fibril density did not change between the two genotypes (Table II).

12 We compared the relative amount of *Dse* mRNA in wild-type tissues by qRT-
13 PCR. *Dse* mRNA was more abundant in skin than in the tendons, with spleen and
14 lung having the highest amounts (Fig. 7D).

15 The altered collagen fibrils in the *Dse*^{-/-} skin were accompanied by a decrease in
16 mechanical strength (Fig. 8). The stress at failure was 5.86 ± 1.88 MPa (n=5) in
17 wild-type skin and 3.48 ± 1.12 MPa (n=5) in *Dse*^{-/-} skin, representing a 41%
18 reduction.

19 **Skin decorin/biglycan from wild-type and *Dse*^{-/-} mice have differential effects**
20 **on collagen fibril formation *in vitro*.** To compare the biophysical properties of
21 skin collagen from wild-type and *Dse*^{-/-} mice, the collagen denaturation was
22 analyzed in a differential scanning calorimeter. Skin from the *Dse*^{-/-} mice denatures

1 more heterogeneously having two denaturation peaks at 60 and 62°C, as compared
2 to wild-type skin with only the 60°C peak (Fig. 9A).

3 Decorin and biglycan are prominent CS/DS-proteoglycans in skin and have been
4 shown to participate in collagen fibril formation (1). Decorin and biglycan were
5 extracted from the skin of wild-type or *Dse*^{-/-} adult mice with similar yields and
6 obtained as a mixture containing 90-95% decorin. Both PGs migrated in a similar
7 manner in SDS-PAGE (Fig. 9B), irrespective of the genotype. Similar amounts of
8 decorin (Fig. 9C) and biglycan (data not shown) were present in the skin of the two
9 genotypes. Acid-solubilized collagen was incubated in the presence of extracted
10 decorin/biglycan preparations from the different genotypes. Differential scanning
11 calorimeter analysis of the resulting collagen fibrils, which melted at around 50 °C,
12 showed a two-peak fibril-denaturation pattern in the presence of *Dse*^{-/-}
13 decorin/biglycan and a single peak after addition of wild-type decorin/biglycan
14 (Fig. 9D), similar to the results obtained with intact skin. The denaturation of
15 collagen monomers, occurring at 42°C, was unaffected. We also investigated the
16 kinetics of *in vitro* collagen fibril formation after addition of the decorin/biglycan
17 preparations, using turbidometry. In this assay, decorin isolated from the skin of
18 three wild-type mice all inhibited the formation of collagen fibrils (Fig. 9E). In
19 contrast, decorin/biglycan isolated from the skin of three *Dse*^{-/-} mice stimulated
20 fibril formation. After removal of the CS/DS chains by chondroitinase ABC, all
21 decorin/biglycan preparations inhibited fibril formation, regardless of genotype.
22 These results demonstrate that the iduronic acid blocks in CS/DS chains carried by
23 decorin/biglycan have a direct effect on collagen fibril formation.

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DISCUSSION

Biosynthesis of CS/DS is a multistep process, requiring at least 22 enzymes directly involved in the assembly and modification of the chain, starting from the activated monosaccharides and the sulfate donor PAPS. Multi-enzyme complexes are thought to accomplish these coordinated and fast reactions. The final structure of CS/DS is subjected to different levels of regulation *in vivo*, which is still poorly understood despite all the enzymes having been cloned and studied *in vitro*. DS-epi1 is one of the two DS-epimerases converting glucuronic acid to iduronic acid (23,34). An *in vivo* model with ablated DS-epi1 can provide new insights into the biological roles of specific CS/DS structures and the regulation of the biosynthetic machinery.

DS-epi1 mRNA and protein were not detectable in *Dse*^{-/-} mice. In adult animals DS-epi1 is the enzyme responsible for the majority of the epimerase activity in spleen, kidney, lung, and skin. The remaining activity in these tissues can most likely be attributed to DS-epi2. Adult brain, where the activity of DS-epi2 is predominant, is an exception. This is in agreement with mRNA expression data in brain, showing the highest expression of DS-epi2 and the lowest of DS-epi1 (13,27). *Ds*-epi1 deficiency results in a 77% reduction of iduronic acid in newly synthesized CS/DS by *Dse*^{-/-} mice. Skin decorin/biglycan and versican contain 67% and 13% of iduronic acid in their wild-type chains, respectively; they were similarly

1 affected by ablation of Ds-epi1, which led to 73% and 89% reduction of iduronic
2 acid, respectively. Clearly DS-epi1 plays a role in the modification of both
3 proteoglycans, at least in skin. In wild-type skin decorin/biglycan the iduronic acid
4 blocks, with a modal length value of 15 kDa, can comprise almost the entire chain.
5 In *Dse*^{-/-} mice skin only 10% of iduronic acid blocks remain in decorin/biglycan.
6 On the other hand, isolated iduronic acid residues, i.e. surrounded by glucuronic
7 acid, are not affected in skin decorin/biglycan. On the contrary, in skin versican and
8 in the whole body CS/DS, probably being the latter sample predominantly
9 composed of versican-type chains, both isolated iduronic acid and iduronic acid
10 blocks are downregulated. Our report is the first showing that versican contains
11 iduronic acid blocks, at least in skin.

12 Interestingly, *Dse*^{+/-} mice have approximately half the amount of the enzyme, but
13 still produce chains which are almost identical to the wild-type chains. Our data also
14 show that decreased epimerization does not affect the length of the CS/DS chains,
15 differently to the effect of CS4ST1 ablation (50). Disaccharide fingerprint of CS/DS
16 chains showed that, as a consequence of the reduction of the iduronic acid blocks,
17 also the disulfated structures in the native chains (IdoA-2OS-GalNAC-4OS)_n are
18 greatly reduced, both in the whole body CS/DS and in skin decorin/biglycan. Our
19 results support previous findings that locate the position of these disulfated
20 structures within the iduronic acid blocks (17,24). In summary, DS-epi1 is the main
21 but not the only enzyme synthesizing iduronic acid blocks *in vivo*. The presented
22 data confirm the results obtained in lung fibroblasts (34), and further suggest that
23 differential level of expression of the two epimerases in the different tissues,

1 together with the expression of the 4-O-sulfotransferase D4ST (35), is a primary
2 determinant of the amount and distribution of iduronic acid.

3 *Dse*^{-/-} mice in a mixed 129/Sv//C57BL/6 genetic background were vital, without
4 any gross organ alteration. This is different from the perinatal mortality of heparan
5 sulfate (HS) epimerase-deficient mice, which lack kidneys and have altered lungs
6 (21). This probably reflects a different role for iduronic acid in HS and CS/DS in
7 embryo development and the fact that only one epimerase is present in HS
8 biosynthesis. *Dse*^{-/-} mice were smaller at birth, but grew normally. We can
9 speculate that the maternal/fetal-derived placenta - an organ rich in DS and decorin
10 (12) - might have heparin cofactor II-mediated thrombotic imbalance and/or altered
11 ECM, and therefore could play a role in the retardation of intrauterine growth. An
12 indication of a combined maternal and fetal contribution to the severity of the
13 intrauterine phenotype is the reduced litter size generated by knock-out parents.

14 Many organs might be affected under close examination by the deficiency of DS-
15 epi1, which is ubiquitously expressed, with the possible exception of brain where
16 DS-epi2 is the predominant epimerase. Therefore it will be interesting to study if
17 organs rich in DS-epi1 like spleen and kidney, despite a normal morphological
18 appearance, present some functional defects. We initially analyzed the skin of *Dse*-
19 deficient mice since this tissue showed histological changes under light microscope
20 and has high content of dermatan sulfate. *Dse*^{-/-} skin has a different histological
21 appearance than wild-type skin and the hypodermal region contains a sparse
22 connective tissue. This resembles the skin from decorin-deficient mice [Fig. 3C and
23 3D in (7)]. The skin morphology did not lead to any alterations in the amount of

1 collagen, as determined by hydroxyproline content. Light microscopic investigation
2 of *Dse*-deficient tail tendon and Achilles tendons revealed no morphological
3 abnormalities. Collagen fibrils in the skin of *Dse*^{-/-} mice are thicker than those in
4 the wild-type skin. Decorin is an abundant CS/DS proteoglycan in skin, binds to
5 collagen and affects collagen fibril formation *in vitro* (38). The decorin (*Dcn*)⁻ and
6 *Dse*-deficient mice both have changes in skin collagen fibril structure (7). In *Dcn*-
7 deficient skin the fibrils show irregular outlines and a larger variation in diameters
8 as compared to wild-type. The mean fibril diameter in *Dcn*-deficient and wild-type
9 skin is similar, whereas the *Dse*-deficient skin has collagen fibrils with an increased
10 mean diameter of 85 nm, as compared to 60 nm in the wild-type. *Dcn*-deficient tail
11 tendons have abnormal collagen fibrils, some larger than 600 nm, with multiple
12 lateral fusions. In contrast, *Dse*-deficient tail and Achilles tendons were largely
13 unaffected. The lack of tendon phenotype is likely due to the measured lower level
14 of DS-epi1 mRNA in tendons compared to skin, and to a lower content of iduronic
15 acid in tendons (4). The skin from *Dcn*⁻ and *Dse*-deficient mice both have reduced
16 tensile strength as a possible result of altered collagen fibril assembly. The stress at
17 failure was reduced by 40% and 70% as compared to wild-type *in Dse*⁻ and *Dcn*-
18 deficient skin (7), respectively. In summary, *Dcn*^{-/-} and *Dse*^{-/-} skins have
19 overlapping but not identical alterations in collagen structures. This can be
20 explained considering the total ablation of the proteoglycan in *Dcn*^{-/-}, as opposed to
21 ablation of some of its functional components in *Dse*^{-/-}. The *Dse*-deficient skin
22 phenotype was not the result of an altered expression level of decorin or biglycan.
23 In fact, the amount of decorin and biglycan in skin, as determined by western blot,

1 immunohistochemistry, and chemical quantification after extraction, indicated no
2 change in the amount due to *Dse* ablation. This is different from the functionally
3 defective galactosyl transferase I, responsible for a variant of the Ehlers-Danlos
4 syndrome, which leads to altered amount of decorin, both as proteoglycan and as
5 non-glycanated core protein (42). Our results therefore suggest that the iduronic
6 acid blocks carried by decorin chain have a role in determining the structure and
7 mechanical properties of collagen. We obtained further support by using
8 preparations containing predominantly decorin and minor amount of biglycan,
9 isolated from wild-type and *Dse*-deficient mouse skin. The denaturation of collagen
10 fibrils pre-formed in the presence of *Dse*^{-/-} PGs shows two closely spaced melting
11 temperatures in differential scanning calorimetry, while wild-type PGs resulted in a
12 single melting temperature. These patterns of denaturation were reproduced with
13 intact skin from wild-type and *Dse*^{-/-} mice and indicate that the iduronic acid blocks
14 of dermatan sulfate affect the biophysical property of collagen fibrils. We also used
15 decorin/biglycan from the two genotypes in turbidometric measurements of
16 collagen fibril formation. PGs from wild-type skin inhibited the fibril formation,
17 whereas *Dse*^{-/-} PGs promoted fibrillogenesis. These results suggest that IdoA
18 blocks have a role in the fibril formation process, and agree with previously
19 reported roles of the dermatan sulfate chain (39).

20 The interaction between antiparallel CS/DS chains of two decorin molecules has
21 been proposed to contribute to the lateral growth of collagen fibrils (40). In a skin
22 healing model and in skin development from embryonic stage till adulthood, longer
23 decorin chains correlate with wider interfibrillar spacing (19,30). In the mentioned

1 skin healing model the longer chains contained fewer (IdoA-2OS-GalNAC-4OS)_n
2 structures and therefore presumably fewer iduronic acid blocks (20), and thinner
3 fibrils were present. Therefore, the structural changes described in our study in skin
4 decorin/biglycan CS/DS chains, which contain less iduronic acid but have similar
5 length, are different from the changes described during development and healing.

6 Understanding the role of the CD/DS chains is complicated by their multiple
7 potential interactions. In *Dse*^{-/-} mice altered amount and/or distribution of iduronic
8 acid-containing structures, thought to be important for CS/DS:CS/DS interaction
9 (10), might lead to modified affinity of these CS/DS self-interactions. This in turn
10 could explain the changes in the fibril structures. CS/DS chains have been shown to
11 contribute to the binding of decorin to collagen I (29). Iduronic acid affects CS/DS
12 chain flexibility, which often favors protein binding (8). *Dse*^{-/-} chains might have
13 altered conformation affecting collagen I/decorin binding and ultimately the growth
14 of the fibrils. Alternatively, fibril surface-associated proteins like the FACIT-
15 collagen XIV (2,9) might bind not optimally via decorin CS/DS to the growing
16 fibril, affecting its structure. Finally, behaviour of cells in skin, for instance
17 fibroblasts could be modified by altered DS chains.

18 In conclusion, our results indicate that decorin/biglycan iduronic acid
19 blocks have a role in skin collagen fibril formation and in the biophysical
20 characteristics and mechanical properties of the skin.

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19

1 TABLE I.

2 **Disaccharide composition of *Dse*^{+/+} and *Dse*^{-/-} CS/DS chains.**

3 The CS/DS preparations were digested with chondroitinase ABC and the resulting
4 disaccharides analyzed by anion-exchange HPLC.

	Whole body		Skin		
	Total CS/DS ^a		Decorin/biglycan-derived ^b		
	<i>Dse</i> ^{+/+}	<i>Dse</i> ^{-/-}	<i>Dse</i> ^{+/+}	<i>Dse</i> ^{-/-}	
	<i>mol%</i>				
10	ΔO unit, ΔHexA-GalNAc	n.a. ^c	n.a.	1.0	1.3
11	ΔA unit, ΔHexA-GalNAc4S	86.6	86.6	89.5	95.1
12	ΔC unit, ΔHexA-GalNAc6S	7.6	10.1	0.6	0.8
13	ΔB unit, ΔHexA2S-GalNAc4S	3.4	0.5	9.0	2.7
14	ΔE unit, ΔHexA-GalNAc4S6S	1.7	1.7	n.d. ^d	n.d.
15	ΔD unit, ΔHexA2S-GalNAc6S	0.7	1.1	n.d.	n.d.

16 ^a *In vivo* labeled CS/DS chains were prepared from the whole mouse, as described
17 under “Experimental procedures”.

18 ^b Unlabeled CS/DS chains were prepared from skin decorin/biglycan from 4-month-
19 old mice. The disaccharides obtained after chondroitinase ABC digestion were
20 labeled with the reducing reagent NaB³H₄.

21 ^c n.a., not applicable

22 ^d n.d., none detected (less than 0.1%)

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TABLE II.

Fibril density calculated from TEM images.

	Fibril density
	<i>(% area occupied by fibrils in the TEM images)±SE</i>
Wild-type dermis	72 ± 5
Dse-/- dermis	68 ± 7
Wild-type hypodermis	62 ± 5
Dse-/- hypodermis	60 ± 8
Wild-type tail tendon	75 ± 7
Dse-/- tail tendon	88 ± 8
Wild-type Achilles	74 ± 8
Dse-/- Achilles	77 ± 10

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FOOTNOTES

1) The abbreviations used are: CS, chondroitin sulfate; Δ units, 4,5-unsaturated hexuronic acid residues, originating from the degradative process catalyzed by chondroitinase ABC; DS, dermatan sulfate; DS-epi1, DS-epimerase 1; DS-epi2, DS-epimerase 2; GlcA, glucuronic acid; GalNAc, N-acetyl-galactosamine; HS, heparan sulfate; IdoA, iduronic acid; MES, 4-morpholineethanesulfonic acid;

2) This work was supported by grants from the Swedish Science Research Council, the Medical Faculty of Lund University, the Albert Österlund Foundation, the Greta and Johan Kock Foundation, Polysackaridforskning AB, and the Tissue in Motion Medical Faculty Program.

3) The authors are grateful to Ragnar Mattsson - Lund Transgenic Core Facility - for the help in generating the knock-out mice and for precious advices, and to Ricardo Feinstein – National Veterinary Institute, Uppsala, Sweden – for the help in histological examinations.

FIGURE LEGENDS

FIG.1. Targeted disruption of the *Dse* gene.

(A) Schematic view of the *Dse* genomic structure (*first line*). Restriction enzyme map of the targeted *Dse* locus in exon 2 (*second line*); the location of the PCR product obtained from the wild-type allele is indicated. Targeting vector (*third line*); the location of the PCR product obtained from the mutated allele is indicated. The origin of the 6.3 and 4.8 kb *NcoI* fragments and the location of the common probe is shown in the *last two lanes*. (B) Southern blot of DNA isolated from ES cells. (C) PCR genotyping of the mice.

FIG. 2. Macroscopic appearance of the *Dse*^{-/-} mice.

(A) *Dse*^{-/-} newborn. *Arrow* points to the kinked tail. (B) Eight wild-type and *Dse*^{-/-} males were weighed (± 2 S.D.). Similarly, female *Dse*^{-/-} mice were smaller than wild-type mice (data not shown).

FIG. 3. DS-epi1 expression and epimerase activity in *Dse*^{-/-} mice.

(A) *Left gel*: spleen homogenates were directly western blotted (100 μ g protein applied) or DS-epi1 was enriched by Red-Sepharose, starting from 5 mg of initial lysates. *Central gel*: 20 μ g of lysates from control or DS-epi1-overexpressing 293HEK cells. *Right gel*: 20 μ g of lysates from adult skin primary fibroblast from *Dse*^{+/+} and *Dse*^{-/-} mice. Membranes were probed with anti-Ds-epi1 antibody. (B) Equal amount of protein for each organ, irrespective to the genotype of origin, was

1 assayed for epimerase activity. Values (\pm 2 S.D. of triplicates) are reported as
2 percent of specific epimerase activity referred to the wild-type organ. *Empty bars*,
3 *Dse+/-*; *black bars*, *Dse-/-*. As example, the wild-type skin homogenates released
4 3,067 dpm of tritium from the labeled substrate during 20 h incubation.

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6 FIG. 4. Analysis of *Dse-/-* CS/DS present in the whole body.

7 Ten-day-old pups were *in vivo* ^{35}S labeled and CS/DS was purified from the whole
8 body. Chains were cleaved with (A) chondroitinase B or (C) chondroitinase AC-I.
9 Split products were separated on the size-permeation Superdex Peptide column.
10 Elution positions of di-, tetra-, hexa-, octa- and deca-saccharides are indicated by
11 *arrows*. AC-I-resistant iduronic acid blocks are shown. *Triangles*, *Dse+/+*; *squares*,
12 *Dse+/-*; *circles*, *Dse-/-*. (B) Proportion of iduronic acid residues in the CS/DS
13 chains, as calculated from (A).

14
15 FIG. 5. Analysis of *Dse-/-* skin decorin/biglycan- and versican-CS/DS chains.

16 Ten-day-old pups were *in vivo* ^{35}S labeled. Skin decorin/biglycan and versican were
17 isolated and (A) their CS/DS chains analyzed by size-permeation on a Superose 6
18 column, run in 0.2 M NH_4HCO_3 . Heparins of different molecular weight were used
19 as markers. *Filled triangles*, wild-type decorin/biglycan chains; *empty triangles*,
20 *Dse-/-* decorin/biglycan chains; *filled squares*, wild-type versican chains; *empty*
21 *squares*, *Dse-/-* versican chains. (B-E) CS/DS chains were cleaved by
22 chondroitinase B and AC-I, as indicated. Split products were separated as in Fig. 4.
23 *Squares*, *Dse+/+*; *triangles*, *Dse+/-*; *circles*, *Dse-/-*. (F) Wild-type decorin/biglycan-

1 derived iduronic acid blocks, as obtained after chondroitinase AC-I and pooled as
2 indicated in (C), were analyzed by size-permeation on a Superose 6 column, run in
3 1 M NaCl.

4
5 FIG. 6. Skin morphology and immunohistochemistry.

6 Tail skin (A-B) stained with Chromotrope 2R and visualized with light microscope
7 or (C-D) stained with Sirius Red and visualized with polarized light. Sparser loose
8 connective tissue appears in the hypodermis of *Dse*^{-/-} (*arrow*). Bar = 100 μ m. D =
9 dermis; E = epidermis; H = hair follicles; T = tendons. (E-F) Histoimmunological
10 detection of decorin. (G) Pre-immune serum control.

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12 FIG. 7. Ultrastructure analysis of collagen fibrils in skin, tail tendon, and Achilles
13 tendon.

14 *Right panels*, skin (A), tail tendon (B), Achilles tendon (C) were examined by
15 electron microscopy. *Left panels (A-C)*, diameters of 1,000 fibrils from each source
16 were measured and presented in histograms. *Solid bars*, wild-type; *open bars*,
17 *Dse*^{-/-}. (D) Quantitative RT-PCR of *Dse* mRNA from different wild-type tissues.

18 Values (\pm 2 S.D. of triplicates) are normalized for spleen mRNA.

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20 FIG. 8. Biomechanics of skin.

21 Stress at failure was measured by applying force to skin samples and measuring the
22 stress at which the skin ruptured. Duplicate samples from five mice of each
23 genotype were measured. Bars indicate mean values; P = 0.018.

1

2 FIG. 9. Biophysical properties of *Dse*^{-/-} skin and effect of skin decorin/biglycan on
3 collagen fibril formation *in vitro*.

4 (A) Differential scanning calorimetry of intact skin. Skin samples from 3-month-old

5 mice were immersed in PBS in a calorimetric cell and heated over a temperature
6 range of 30-90 °C. Thermograms (no peaks after 70°C) were normalized for buffer

7 blank run without skin. (B) 500 ng of extracted decorin/biglycan from adult skin
8 were applied to SDS-PAGE, and stained with Blue G Colloidal solution. (C)

9 Proteoglycans were extracted from identical amount (fresh weight) of tail skin of 4-
10 month-old mice. PGs were purified on anion exchange DE52 column and identical
11 volumes of the eluates were western blotted and stained with anti-decorin antibody.

12 (D) Differential scanning calorimetry of *in vitro* formed collagen fibrils. Prior to
13 calorimetry, 500 µg acid-solubilized collagen was incubated for 4 h at 37°C with
14 addition of 10 µg wild-type or *Dse*^{-/-} decorin/biglycan extracts from skin (one
15 preparation is shown in (B)). Collagen fibrils were then heated in the calorimeter
16 over a temperature range of 30-60°C. Thermograms are normalized for buffer blank

17 run without collagen. (E) Turbidometric measurements of *in vitro* collagen
18 fibrillization. 50 µg acid-solubilized collagen was mixed with 1 µg decorin/biglycan
19 extracts, either pre-treated or non-treated with chondroitinase ABC. The samples
20 were then incubated at 37°C in a spectrophotometer and readings of turbidity were
21 taken every 4 min and plotted.

Fig. 1

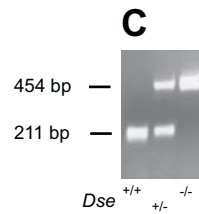
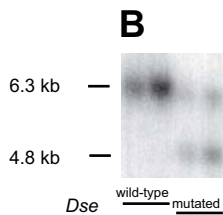
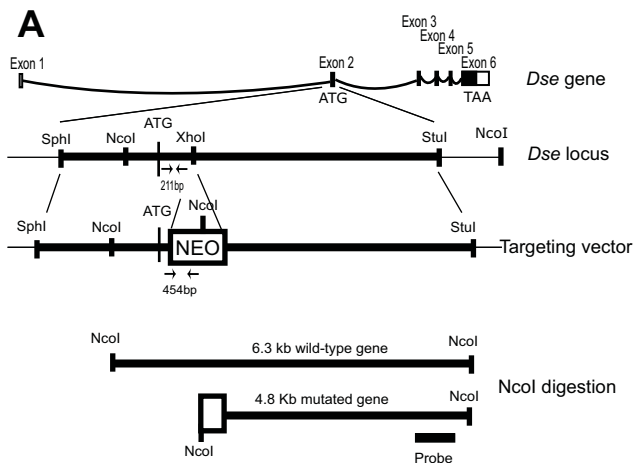


Fig. 2

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B

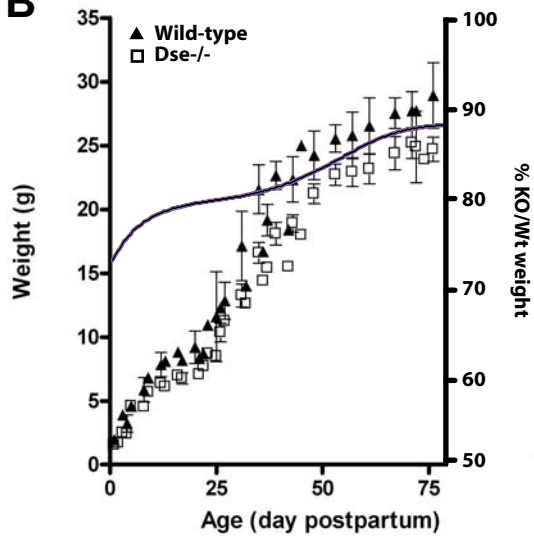


Fig. 3

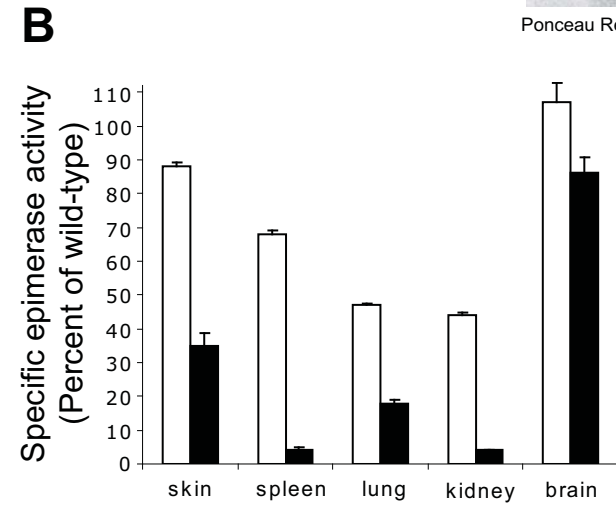
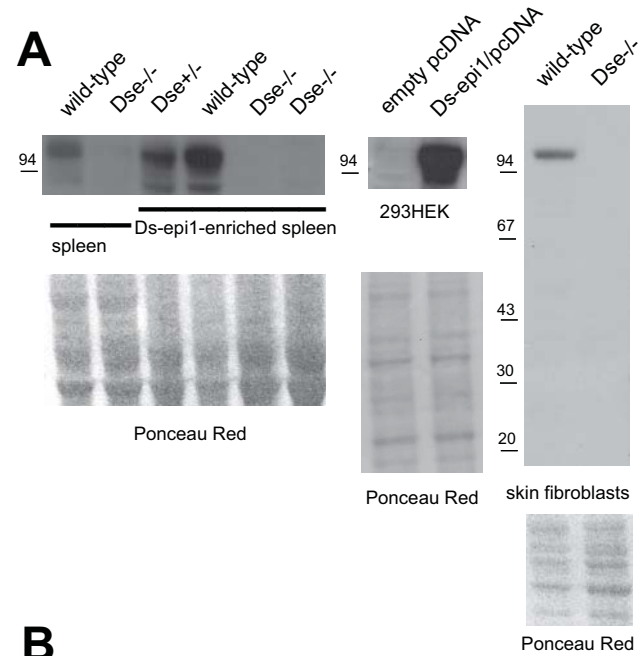


Fig. 4

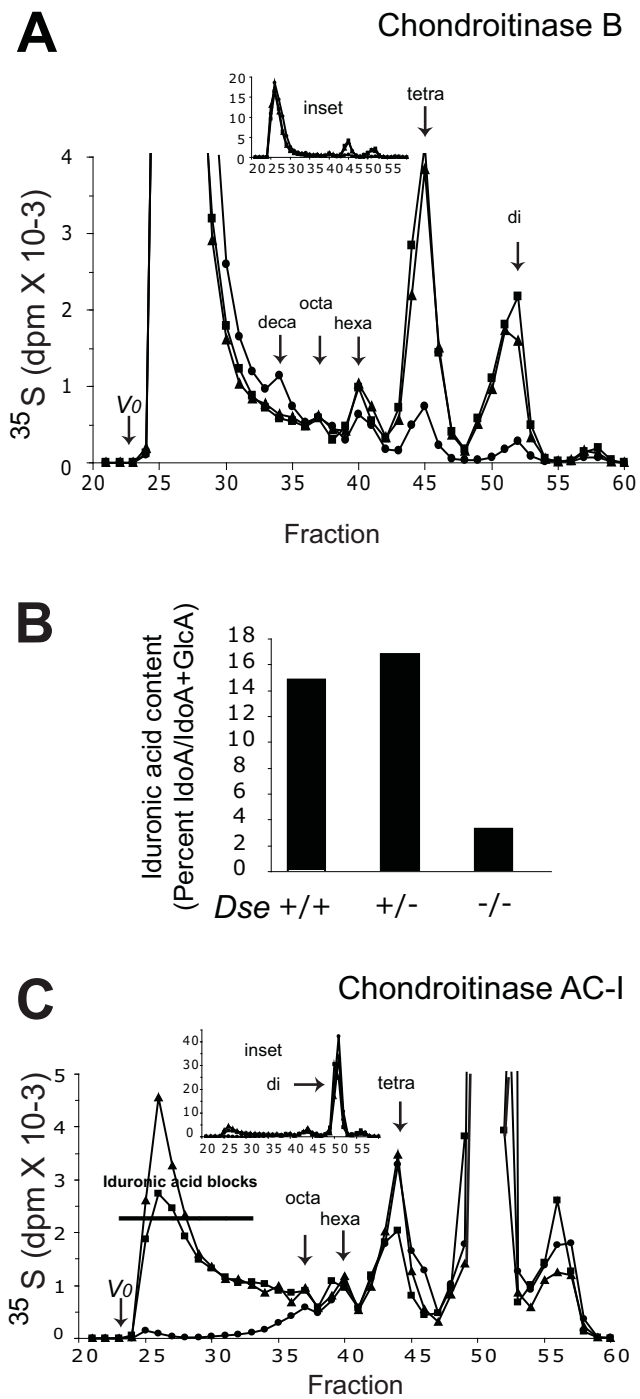


Fig. 5

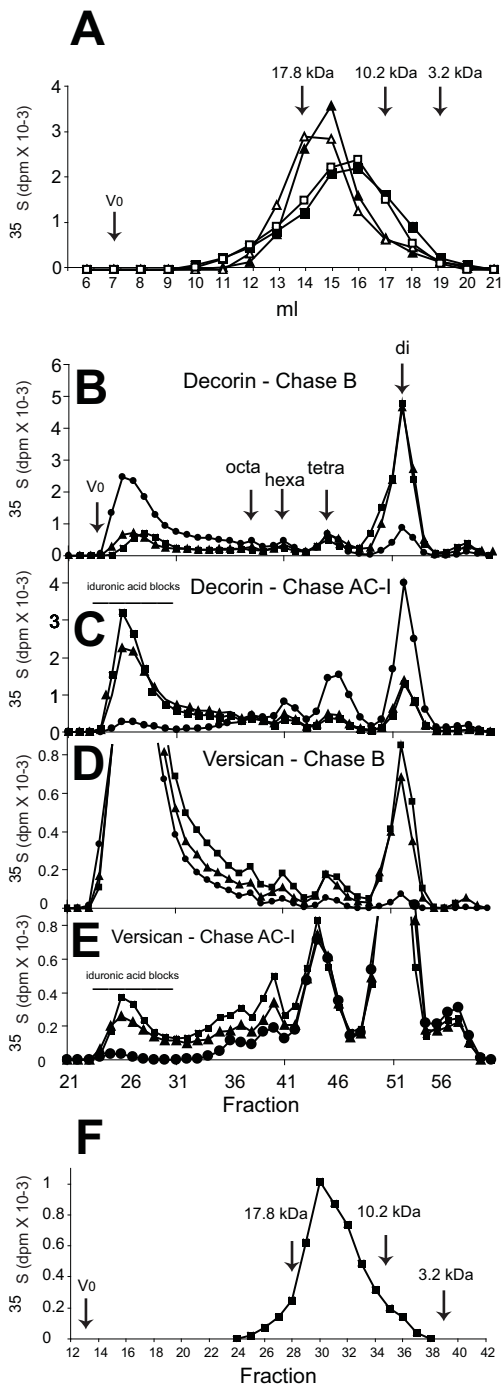


Figure 6

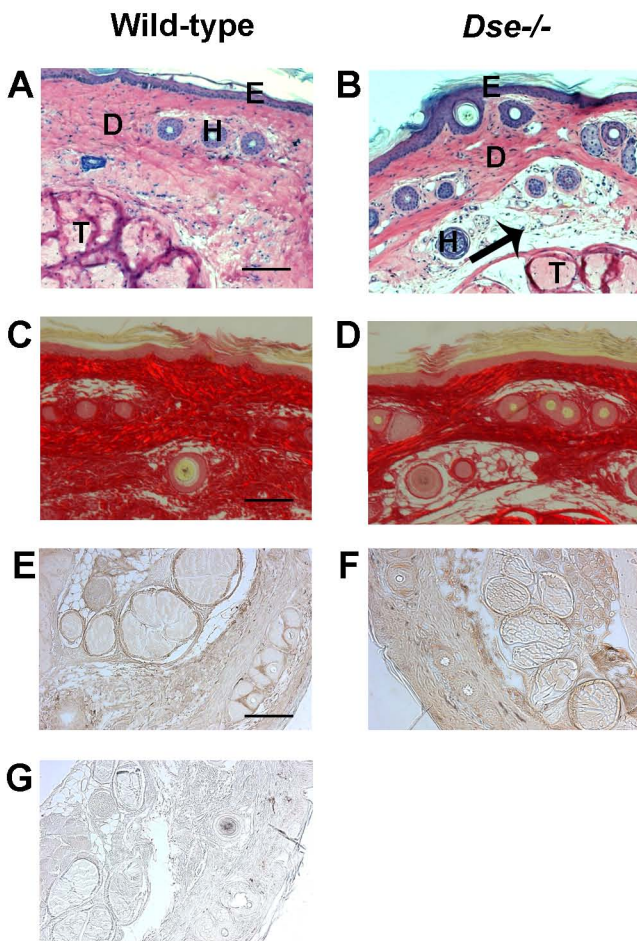


Figure 7

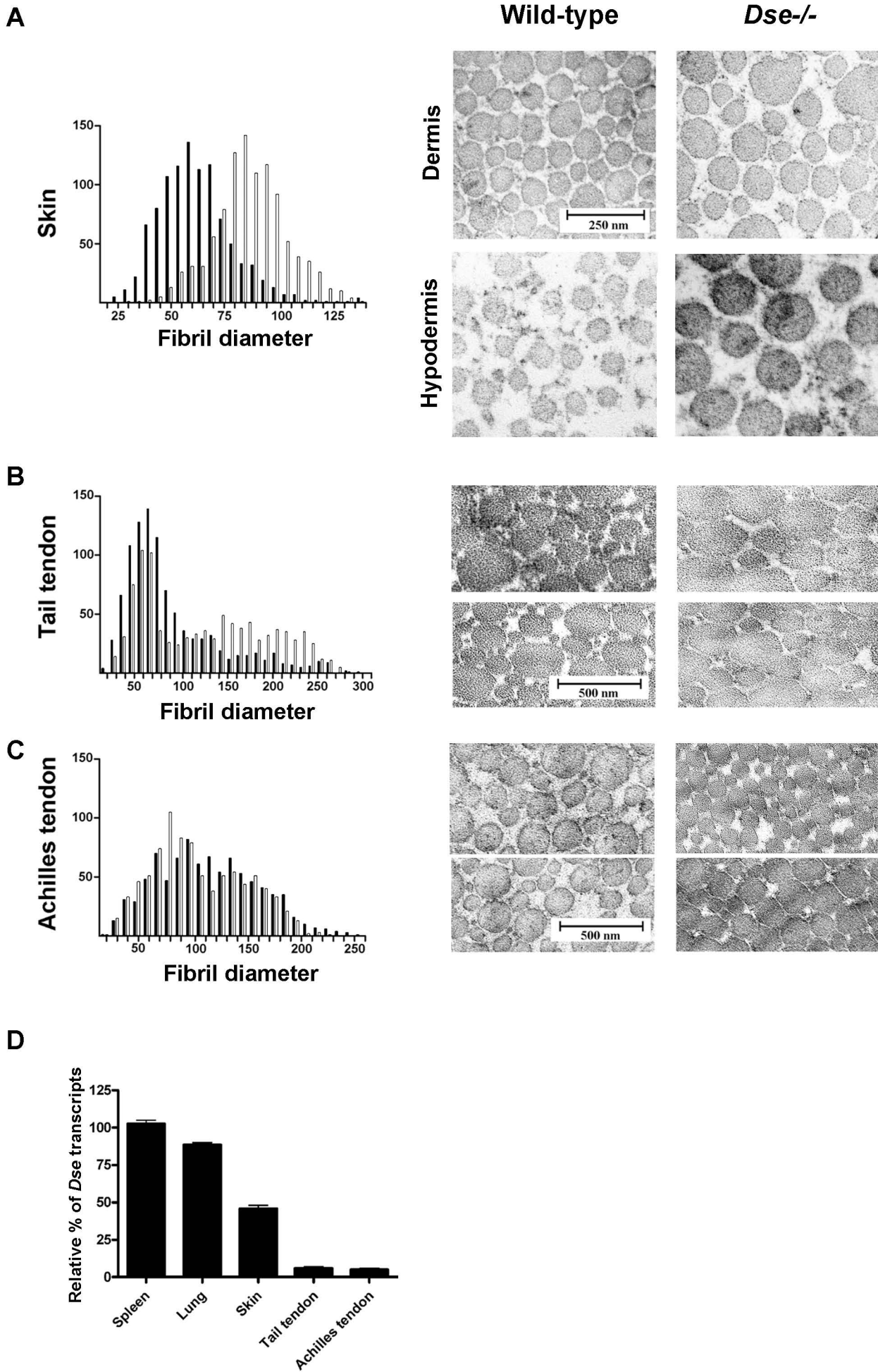


Figure 8

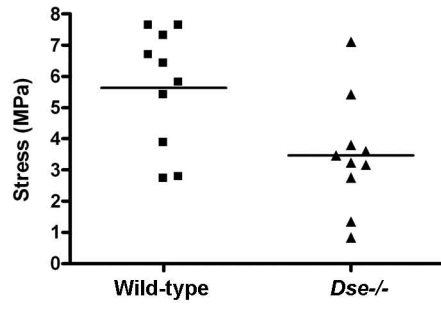
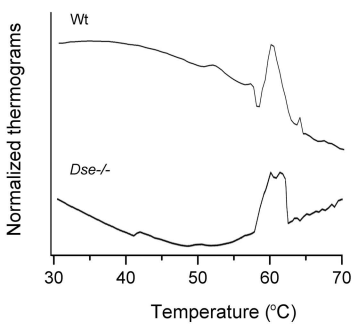
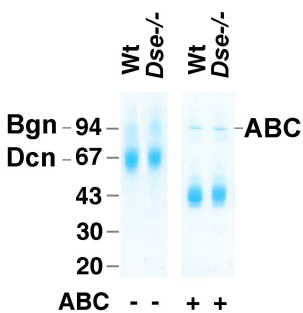


Figure 9

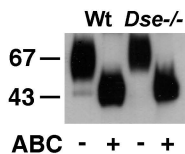
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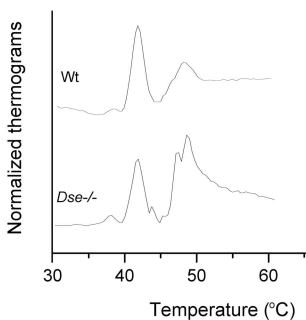
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