Differences in corneal phenotypes between destrin mutants are due to allelic difference and modified by genetic background

Sharon V. Kawakami-Schulz  
*University of Wisconsin - Madison*

Angela M. Verdoni  
*Washington University School of Medicine in St. Louis*

Shannon G. Sattler  
*University of Wisconsin - Madison*

Akihiro Ikeda  
*University of Wisconsin - Madison*

Sakae Ikeda  
*University of Wisconsin - Madison*

Follow this and additional works at: [https://digitalcommons.wustl.edu/open_access_pubs](https://digitalcommons.wustl.edu/open_access_pubs)

**Recommended Citation**  
Kawakami-Schulz, Sharon V.; Verdoni, Angela M.; Sattler, Shannon G.; Ikeda, Akihiro; and Ikeda, Sakae, "Differences in corneal phenotypes between destrin mutants are due to allelic difference and modified by genetic background." Molecular Vision. 18, 606-616. (2012).  
https://digitalcommons.wustl.edu/open_access_pubs/1799

This Open Access Publication is brought to you for free and open access by Digital Commons@Becker. It has been accepted for inclusion in Open Access Publications by an authorized administrator of Digital Commons@Becker. For more information, please contact vanam@wustl.edu.
Differences in corneal phenotypes between destrin mutants are due to allelic difference and modified by genetic background

Sharolyn V. Kawakami-Schulz, Angela M. Verdoni, Shannon G. Sattler, Akihiro Ikeda, Sakae Ikeda

Department of Medical Genetics, University of Wisconsin, Madison, WI

Purpose: Mutations in destrin (Dstn) cause corneal abnormalities in mice. A null mutation, Dstn\(^{corn1}\), results in corneal epithelial hyperproliferation, inflammation, and neovascularization in the A.BY background (A.BY Dstn\(^{corn1}\)). Homozygosity for a point mutation, Dstn\(^{corn1-2J}\), results in mild thickening of the corneal epithelium but no corneal neovascularization in a C57BL/6 (B6) background (B6 Dstn\(^{corn1-2J}\)). The goal of this study was to determine whether phenotypic differences are due to allelic differences between Dstn\(^{corn1}\) and Dstn\(^{corn1-2J}\), or are the result of genetic background effects.

Methods: We generated two congenic (Cg) mouse lines, B6.Cg-Dstn\(^{corn1}\) and A.BY.Cg-Dstn\(^{corn1-2J}\), to compare to the original A.BY Dstn\(^{corn1}\) and B6 Dstn\(^{corn1-2J}\) lines. We performed immunohistochemistry to assay F-actin accumulation, neovascularization, proliferation, and inflammation. By Western blot analysis we tested the expression of serum response factor (SRF), a known regulator of the Dstn\(^{corn1}\) phenotype.

Results: The Dstn\(^{corn1}\) mutation leads to neovascularization, hyperproliferation, and inflammation in the cornea of A.BY Dstn\(^{corn1}\) as well as B6.Cg-Dstn\(^{corn1}\) mice. We did not observe significant corneal neovascularization or hyperproliferation in either A.BY.Cg-Dstn\(^{corn1-2J}\) or B6 Dstn\(^{corn1-2J}\) mice. Actin accumulation, neovascularization, epithelial proliferation and inflammation in B6.Cg-Dstn\(^{corn1}\) cornea are significantly reduced when compared to A.BY Dstn\(^{corn1}\) cornea. SRF changes are consistent in Dstn\(^{corn1}\) mutants, regardless of genetic background.

Conclusions: Differences in the abnormal phenotypes of Dstn mutants result from allelic differences between Dstn\(^{corn1}\) and Dstn\(^{corn1-2J}\). Moreover, phenotypes of Dstn\(^{corn1}\) mice are modified by genetic background, suggesting the existence of genetic modifiers. Protein analysis suggests that a genetic modifier affects phenotypic severity functionally downstream from or in a pathway independent from SRF. These data demonstrate that natural genetic variation affects phenotypic severity in Dstn\(^{corn1}\) mice.

Mutations in the gene for the actin depolymerizing factor destrin (DSTN), lead to abnormalities in the structure and function of the cornea and subsequent blindness in mice [1-3]. Since actin depolymerizing factors including DSTN function to maintain a homeostatic ratio of actin in two forms, filamentous actin (F-actin) and globular actin (G-actin) [4], Dstn mutant mice demonstrate the importance of actin dynamics regulation to the proper structure and functions of the cornea. We have previously reported both phenotypic and gene expression differences between two mutations of the Dstn gene, Dstn\(^{corn1}\) and Dstn\(^{corn1-2J}\) [2,3]. The Dstn\(^{corn1}\) mutation is characterized by deletion of a ~35 Kb region containing the entire coding sequence for Dstn, while the Dstn\(^{corn1-2J}\) allele has been identified as a point mutation in exon 3, resulting in a Proline to Serine amino acid change in the actin binding domain [3]. Dstn\(^{corn1}\) was isolated and has been maintained in the A.BY genetic background, while Dstn\(^{corn1-2J}\) has been propagated in the C57BL/6 (B6) background.

There are four major classes of phenotypes that distinguish the two Dstn mutations. First, while both mutants display abnormal levels of F-actin accumulation in the corneal epithelium, consistent with the role of DSTN to depolymerize F-actin, A.BY Dstn\(^{corn1}\) mice display extreme misregulation of actin dynamics, leading to structural breakdown of the corneal epithelium. B6 Dstn\(^{corn1-2J}\) mice display mild F-actin accumulation but maintain overall structural integrity of the corneal epithelium [3]. Second, while A.BY Dstn\(^{corn1}\) mice develop neovascularization into the cornea, B6 Dstn\(^{corn1-2J}\) cornea remain free of any abnormal vasculature. Third, the A.BY Dstn\(^{corn1}\) mutants demonstrate increased epithelial thickening due to proliferating cells throughout the corneal epithelium when compared to B6 Dstn\(^{corn1-2J}\) [1,3]. Fourth, A.BY Dstn\(^{corn1}\) mutants displayed significant recruitment of inflammatory cells to the corneal epithelium when compared to B6 Dstn\(^{corn1-2J}\) [5]. Since these mutations are on different genetic backgrounds, it was impossible to deduce whether phenotypic differences between Dstn\(^{corn1}\) and Dstn\(^{corn1-2J}\) were solely due to differences in mutant alleles, or if the mutants are differentially affected because of genetic backgrounds.
To observe each mutation independently of background effects, we used the original lines, A.BY Dstn\(^{onl}\) and B6 Dstn\(^{onl-2J}\) to create a pair of congenic (Cg) lines, B6.Cg-Dstn\(^{onl}\) and A.BY.Cg-Dstn\(^{onl-2J}\). By comparing A.BY Dstn\(^{onl}\) to A.BY.Cg-Dstn\(^{onl-2J}\) and B6 Dstn\(^{onl-2J}\) to B6.Cg-Dstn\(^{onl}\), we are able to assess the allelic differences between the two mutants independent of genetic background effects. Moreover, by comparing A.BY Dstn\(^{onl}\) to B6.Cg-Dstn\(^{onl}\) and A.BY.Cg-Dstn\(^{onl-2J}\) to B6 Dstn\(^{onl-2J}\), we can observe the effects of genetic background on the disease phenotypes attributed to different types of mutations. These analyses demonstrated that both allelic difference and genetic background affect the severity of corneal abnormalities caused by the Dstn mutation.

**METHODS**

**Mouse husbandry:** A.BY-H2\(^b\) H2-T18\(^b\)/SnJ (A.BY WT), C57BL/6J WT (B6 WT), A. BY-H2\(^b\) H2T18\(^b\)/Snl-Dstn\(^{onl}\) (A.BY Dstn\(^{onl}\)), and C57BL/6Jsmn-Dstn\(^{onl-2J}\) (B6 Dstn\(^{onl-2J}\)) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred in an animal facility at the University of Wisconsin-Madison. All mouse procedures were performed in accordance with the protocols approved by the Animal Care and Use Committee at the University of Wisconsin-Madison and conform to the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research.

**Generation of congenic mouse lines:** B6.Cg-Dstn\(^{onl}\) mice were generated by crossing A.BY Dstn\(^{onl}\) mice to B6 WT mice, selecting for mice carrying a Dstn\(^{onl}\) allele, and backcrossing to B6 WT for 8 generations. Heterozygous mice were intercrossed and progeny genotyped as homozygous for the Dstn\(^{onl}\) mutation were used for analysis. A.BY.Cg-Dstn\(^{onl-2J}\) mice were generated by crossing B6 Dstn\(^{onl-2J}\) mice to A.BY WT mice, selecting for mice carrying a Dstn\(^{onl-2J}\) allele, and backcrossing to A.BY WT for 10 generations. Heterozygous mice were intercrossed and progeny genotyped as homozygous for the Dstn\(^{onl-2J}\) mutation were used for analysis.

**Genotyping:** All mice were genotyped using polymerase chain reaction (PCR). To genotype for Dstn, primers mADF-F31 (5′-GTC CCA TGA ATG TGA ATT GC-3′) and mADF-R28 (5′-CCC TGG TGA CCT TTC ATC-3′) were used for amplification. To genotype for Dstn\(^{onl}\), primers mADF-F32 (5′-GCC ACA TCA TTA GCT TTT GAA G3′) and mADF-R30 (5′-TGG CAC TCC TGC TGT CAC-3′) were used for amplification. To genotype for Dstn\(^{onl-2J}\), primers mADF-F35 (5′TGG AGG GTG TGC TTT CTC TAC-3′) and mADF-R32 (5′-CTA CGA AGA TAA TAA GGT GGG C-3′) were used for amplification, followed by digestion of the PCR product with the restriction enzyme BanI.

**Immunohistochemistry:** All mice were analyzed at postnatal day 58 (P58), unless otherwise indicated. Immunohistochemistry on frozen corneas was performed as described previously [2]. Briefly, after removal of the eye, corneas were removed and fixed in parafomaldehyde and gradients of sucrose before sectioning. Sections were blocked with phosphate-buffered saline (PBS) with 0.5% Triton X and 2% normal donkey serum before overnight incubation with primary antibodies. Sections were then rinsed with PBS, incubated with a secondary antibody, and stained with DAPI. The primary antibodies and dilutions used for the analysis were: Ki67 (1:100; Thermo Scientific, Fremont, CA), myeloperoxidase (1:200; R&D Systems, Minneapolis, MN), and cluster of differentiation 45 (CD45; 1:100; BD Pharmingen, San Diego, CA). Sections were incubated with Alexa Fluor 488 or 568 conjugated secondary antibody (1:400; Invitrogen, Carlsbad, CA) or Alexa Fluor 568 conjugated phalloidin (1:50; Invitrogen) and were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1:200; Sigma-Aldrich, St. Louis, MO).

Immunohistochemistry on whole cornea was performed as described previously [2]. Briefly, after removal of the eye, corneas were removed and fixed in parafomaldehyde overnight. Corneas were then rinsed in PBS and post-fixed in 100% acetone, followed by additional PBS rinses. Corneas were blocked overnight in PBS with 0.8% Triton and 2% normal donkey serum, followed with an overnight incubation with the primary antibody in block solution. The primary antibody for cluster of differentiation 31 (CD31; BD Pharmingen) was used at a dilution of 1:50. Corneas were transferred to block solution containing phalloidin (1:50; Invitrogen) and an Alexa Fluor 488 conjugated secondary antibody (1:200; Invitrogen). Corneas were counterstained with DAPI (1:200; Sigma-Aldrich).

**Imaging:** Images acquired on sections and whole mount cornea were captured on an Eclipse E600 microscope (Nikon, Tokyo, Japan) using a SPOT camera (Spot Diagnostics, Sterling Hills, MI) or a Zeiss 510 confocal laser scanning system and Axio Imager microscope using LSM 510 software (release 4.2; Carl Zeiss Microlsaging, Thornwood, NY). For images obtained of the surface of eye, mice were sacrificed and immediately placed under the objective of a Zeiss Stemi SV11 dissecting scope attached to a Nikon COOLPIX 995 digital camera (Nikon).

**Western blotting:** For each line, six corneas pooled from three mice were homogenized in RIPA buffer (1× PBS with 1% NP-40 and 0.1%SDS) containing a protease inhibitor cocktail. Protein concentrations were determined using the Precision Red protein assay reagent (Cytoskeleton, Denver, CO) according to the manufacturer’s instructions. Equal amount of protein were subjected to SDS–PAGE using 4%−12% Bis-tris gels and antibodies against SRF (1:1,000; Santa Cruz Bionotechnology, Santa Cruz, CA), and FMR1 (1:4,000; Millipore, Billerica, MA). Blots were treated with horseradish peroxidase conjugated secondary antibodies (1:2,000; Millipore, Billerica, MA). Blots were treated with horseradish peroxidase conjugated secondary antibodies (1:2,000; Millipore, Billerica, MA). Blots were treated with horseradish peroxidase conjugated secondary antibodies (1:2,000; Millipore, Billerica, MA). Blots were treated with horseradish peroxidase conjugated secondary antibodies (1:2,000; Millipore, Billerica, MA). Blots were treated with horseradish peroxidase conjugated secondary antibodies (1:2,000; Millipore, Billerica, MA).
Jackson Immunoresearch, West Grove, PA) before detection using a chemiluminescent reagent (Amersham ECL Plus western blotting detection system; General Electric, Buckinghamshire, UK). Blots were then exposed to X-ray film (Thermo Scientific, Rockford, IL). Western blot analyses were performed in both biologic and technical triplicate. FMRI1 (fragile X mental retardation syndrome 1 homolog) was used as a loading control, as previously described [6].

**Analysis of Dstn<sup>corn1</sup>/Dstn<sup>+</sup> relative density ratio:** The Dstn<sup>corn1</sup> relative density/Dstn<sup>+</sup> relative density measurements were performed via scanning densitometry using ImageJ software. Bands identified as SRF were normalized against their respective FMRI1 control bands. Normalized values, obtained in biologic triplicate, were then pooled for analysis.

**Histological quantification of proliferating cells and inflammatory cells:** Corneal frozen sections were stained for the proliferation marker Ki67, the pan-leukocyte marker CD45, the neutrophil marker myeloperoxidase (MP), and the nuclear marker DAPI. Cells were counted using ImageJ software on digital images taken using the SPOT Image Analysis system. Two separate, nonconsecutive sections were analyzed for each eye for each phenotype.

**Quantification of the vascularized area of the cornea:** Digital images of all corneal flat mounts were collected using the Spot Image Analysis system. Vascularized area and total corneal area were measured using ImageJ software with a method similar to that of Bock et al. [7]. Briefly, filters were applied to subtract background, reduce noise, and enhance contrast. The total corneal area was outlined using the innermost vessel of the limbal arcade as the border, and the area of CD31-positive vessels within the cornea was then calculated and normalized to the total corneal area (expressed as the percentage of vascularized cornea) using thresh holding analysis. Filters were applied to subtract background, reduce noise, and enhance contrast. The total corneal area was outlined using the innermost vessel of the limbal arcade as the border, and the area of CD31-positive vessels within the cornea was then calculated and normalized to the total corneal area (expressed as the percentage of vascularized cornea) using thresh holding analysis.

**Statistical analysis:** For comparison of three groups, a one-way ANOVA was performed, followed by pairwise t-tests with a Bonferroni correction if the ANOVA resulted in a p<0.05. For comparison of two groups, a two-tailed, unpaired t-test was used. GraphPad Prism software (GraphPad, San Diego, CA) was used for statistical analysis and to create all graphs reporting numerical values. *p<0.05, **p<0.01, ***p<0.001.

**RESULTS**

Characterization of genetic mutations in the gene for DSTN and phenotypic differences in the cornea of A.BY Dstn<sup>corn1</sup> and B6 Dstn<sup>corn1–2J</sup> had been reported previously [1,3,5]. In this study, we created the congenic lines B6.Cg-Dstn<sup>corn1</sup> and A.BY.Cg-Dstn<sup>corn1–2J</sup> to compare to the original lines. Dstn<sup>corn1</sup> mice lack the coding region for DSTN and represent a null mutant of the Dstn protein, while Dstn<sup>corn1–2J</sup> mice are characterized by a non-synonymous mutation in the actin binding domain of DSTN that most likely results in hypomorphic activity.

Even upon gross visual analysis, these two Dstn mutations exhibit phenotypic differences at P58 (Figure 1). The corneal surface of A.BY Dstn<sup>corn1</sup> and B6.Cg-Dstn<sup>corn1</sup> mice is roughened and blood vessels are clearly visible. Cataracts in the lens are another characteristic feature of Dstn<sup>corn1</sup> eyes in both backgrounds at P58. The corneal surface of A.BY.Cg-Dstn<sup>corn1–2J</sup> and B6 Dstn<sup>corn1–2J</sup> also appears rough when compared to WT, but to a lesser degree. In mice with the Dstn<sup>corn1–2J</sup> mutation, a cataract has not fully formed by P58 but develops in all mice as they age (data not shown). Additionally, neither Dstn<sup>corn1–2J</sup> mutant displays corneal neovascularization (Figure 1).

By staining the whole mount cornea and frozen sections of the cornea with phalloidin, which specifically binds to F-actin, we compared F-actin accumulation among the four lines of mice to WT. We observed that, even when comparing the mutations in the same background (A.BY Dstn<sup>corn1</sup> vs A.BY.Cg-Dstn<sup>corn1–2J</sup> and B6.Cg-Dstn<sup>corn1–2J</sup> versus B6 Dstn<sup>corn1–2J</sup>), Dstn<sup>corn1–2J</sup> displays milder F-actin accumulation compared to Dstn<sup>corn1</sup>. The level of F-actin accumulation in the B6.Cg-Dstn<sup>corn1</sup> cornea is less severe compared to the A.BY Dstn<sup>corn1</sup> cornea (Figure 2).

To examine the amount of corneal neovascularization, we performed immunofluorescence using an anti-CD31 antibody on whole cornea. Quantification of the vascularized area in A.BY Dstn<sup>corn1</sup> to A.BY.Cg-Dstn<sup>corn1–2J</sup> and B6.Cg-Dstn<sup>corn1–2J</sup> to B6 Dstn<sup>corn1–2J</sup> and WT mice revealed that significant corneal neovascularization occurs only in mice with the Dstn<sup>corn1–2J</sup> mutation, in both the A.BY and B6 backgrounds. There is no significant difference in the amount of vasculature in Dstn<sup>corn1–2J</sup> compared to WT (Figure 3A,B). Neovascularization is significantly reduced in the cornea of B6.Cg-Dstn<sup>corn1</sup> mice compared to A.BY Dstn<sup>corn1</sup> mice. While this difference is apparent and significant by P28, the difference increases in significance with age (Figure 3C).

To detect proliferating cells in the corneal epithelium, we performed immunohistochemistry using an anti-Ki67 antibody on whole cornea. Quantification of the vascularized area in A.BY Dstn<sup>corn1</sup> to A.BY.Cg-Dstn<sup>corn1–2J</sup> and B6.Cg-Dstn<sup>corn1–2J</sup> to B6 Dstn<sup>corn1–2J</sup> and WT mice revealed that significant corneal neovascularization occurs only in mice with the Dstn<sup>corn1–2J</sup> mutation, in both the A.BY and B6 backgrounds. There is no significant difference in the amount of vasculature in Dstn<sup>corn1–2J</sup> compared to WT (Figure 3A,B). Neovascularization is significantly reduced in the cornea of B6.Cg-Dstn<sup>corn1</sup> mice compared to A.BY Dstn<sup>corn1</sup> mice. While this difference is apparent and significant by P28, the difference increases in significance with age (Figure 3C).

To detect proliferating cells in the corneal epithelium, we performed immunohistochemistry using an anti-Ki67 antibody. We compared the amount of proliferation in each of the four lines and found that the number of proliferating cells in the corneal epithelia of Dstn<sup>corn1</sup> mice is significantly more than that in the corneal epithelia of Dstn<sup>corn1–2J</sup> mice in both the A.BY and B6 backgrounds (Figure 4A,B). Hyperproliferation was significantly increased in the corneal epithelia of A.BY Dstn<sup>corn1</sup> mice compared to B6.Cg-Dstn<sup>corn1–2J</sup> mice (Figure 4C). The trend of increased hyperproliferation in the corneal epithelium of A.BY
Dstr<sup>corn1</sup> compared to B6.Cg-Dstr<sup>corn1</sup> is present by P28 and becomes significant by P58 (Figure 4C). In contrast, when comparing these phenotypes between A.BY.Cg-Dstr<sup>corn1–2J</sup> and B6 Dstr<sup>corn1–2J</sup> mice, we did not observe any significant difference.

To detect inflammatory cells in the corneal epithelium, we performed immunohistochemistry using anti-CD45 and anti-myeloperoxidase antibodies. We compared the amount of inflammation in the cornea of mice from each of the four lines. We found that there is significantly increased recruitment of inflammatory cells to the cornea of Dstr<sup>corn1</sup> mice when compared to Dstr<sup>corn1–2J</sup> or WT mice, an observation common to both backgrounds. There is also a significant increase in inflammatory cells in Dstr<sup>corn1–2J</sup> mutant corneas compared to WT (Figure 5A,B). The level of inflammation was significantly increased in the cornea of A.BY Dstr<sup>corn1</sup> mice compared to B6.Cg-Dstr<sup>corn1</sup> mice (Figure 5C). In the corneal epithelium of Dstr<sup>corn1</sup> mice, the trend of increased inflammation in the A.BY compared to the B6 background is seen at P28 and becomes significant by P58 (Figure 5C). In contrast, when comparing these phenotypes between A.BY.Cg-Dstr<sup>corn1–2J</sup> and B6 Dstr<sup>corn1–2J</sup> mice, we did not observe any significant difference.

We had previously identified the transcription factor serum response factor (SRF) as a major contributor to Dstr<sup>corn1</sup> phenotypes [2,6]. To assess the quantity of SRF in the Dstr<sup>corn1</sup> mutants in both genetic backgrounds, we performed western blot analysis. We found that the level of SRF is higher in Dstr<sup>corn1</sup> when compared WT in both backgrounds (Figure 6A). Additionally, statistical analysis reveals that the level of induction of SRF expression in Dstr<sup>corn1</sup> mutants, that is, the increase relative to WT, is not significantly different between genetic backgrounds. (Figure 6B).

Our results suggest that phenotypic differences between Dstr mutants are due to allelic differences. Actin accumulation, neovascularization, proliferation and inflammatory response differences between Dstr<sup>corn1</sup> and Dstr<sup>corn1–2J</sup> remain consistent when compared in two genetic backgrounds. Notably, phenotypes caused by the Dstr<sup>corn1</sup> mutation are also modified by genetic background. In every case, the abnormal phenotype is dampened in the cornea.
Figure 2. F-actin in the cornea of Dstn mutants and WT mice in both the A.BY and B6 background at P58. A: Phalloidin staining of whole mount cornea at P58 shows that F-actin (red) accumulation more severe in Dstn\textsuperscript{com1} cornea compared to Dstn\textsuperscript{com1-2J} cornea in both the A.BY and B6 background. F-actin accumulation is less severe in the cornea of B6.Cg-Dstn\textsuperscript{com1} when compared to A.BY Dstn\textsuperscript{com1}. Bar, 200 μm. B: Phalloidin staining of frozen sections illustrates the degree of F-actin (gray in upper panels, red in lower panels) accumulation in each Dstn mutant compared to WT. Two portions of an A.BY Dstn\textsuperscript{com1} cornea are shown to represent its irregular surface. Bar, 20 μm. All slides were counterstained with DAPI to mark cell nuclei (blue).
of B6.Cg-\textit{Dsn}^{com1} mice compared to A.BY \textit{Dsn}^{com1} mice. We crossed the \textit{Dsn}^{com1} mutants and found that F1 mice demonstrate a level of neovascularization that is intermediate to and significantly different from A.BY \textit{Dsn}^{com1} and B6.Cg-
**DISCUSSION**

*Dstr*\(^{corn1}\) and *Dstr*\(^{corn1-2J}\) contribute to observed phenotypic differences: By observing corneal phenotypes on the same genetic background, this study shows that the differences in

---

*Dstr*\(^{corn1}\) (Figure 7A,B). These results suggest the presence of a genetic factor that is variable between B6 and A.BY and affects the phenotype in a dosage-dependent manner.
abnormal phenotypes that we observe in Dstn mutant mice are due to the allelic difference between Dstn<sup>com1</sup> and Dstn<sup>com1–2J</sup>. These observations are consistent with our previous finding that Dstn<sup>com1</sup> is a null mutation, while Dstn<sup>com1–2J</sup> is a point mutation [3]. Since the point mutation in Dstn<sup>com1–2J</sup> leads to an amino acid change in the actin binding region of DSTN, the protein resulting from the Dstn<sup>com1–2J</sup> mutation likely has residual functions leading to the milder phenotypes in Dstn<sup>com1–2J</sup> cornea compared to Dstn<sup>com1</sup> cornea where no DSTN protein is produced.

**Genetic modification is specific to the Dstn<sup>com1</sup> mutation.** The cornea of B6.Cg-Dstn<sup>com1</sup> mice display milder forms of each phenotype when compared to A.BY Dstn<sup>com1</sup>. This modification is not seen when comparing the cornea of B6 Dstn<sup>com1–2J</sup> to A.BY.Cg-Dstn<sup>com1–2J</sup>, suggesting the existence of genetic modifiers that specifically interact with molecules or pathways affected by the Dstn<sup>com1</sup> mutation. In fact, our previous microarray study to compare A.BY Dstn<sup>com1</sup> and B6 Dstn<sup>com1–2J</sup> to their WT counterparts revealed that the Dstn<sup>com1</sup> mutation led to differential expression of 1,226 annotated genes, whereas the Dstn<sup>com1–2J</sup> mutation led to changes in only 202 annotated genes [2]. Based on these observations, we speculate that the modifier or modifiers may belong to or interact with the set of ~1000 genes whose differential expression is observed in Dstn<sup>com1</sup> and not in Dstn<sup>com1–2J</sup>. Another possible reason which could explain genetic modification specific to the Dstn<sup>com1</sup> mutation is the involvement of a compensating mechanism that may be activated by a complete loss of destrin (Dstn<sup>com1</sup>) but not by a point mutation (Dstn<sup>com1–2J</sup>). There are 2 other members of the

---

**Figure 5. Recruitment of inflammatory cells to the cornea of Dstn mutants.**

- **A:** Immunofluorescence using CD45 (red) to mark inflammatory cells and myeloperoxidase to mark neutrophils specifically in Dstn mutant and WT cornea on A.BY and B6 backgrounds at P58. All slides were counterstained with DAPI to mark cell nuclei (blue). Bar, 20 μm.
- **B:** Quantification of CD45 positive cells revealed significantly increased inflammation in both Dstn<sup>com1</sup> and Dstn<sup>com1–2J</sup> mutant cornea compared to WT at P58 (p<0.001 for both backgrounds).
- **C:** The trend for increased inflammation in A.BY Dstn<sup>com1</sup> compared to B6.Cg-Dstn<sup>com1</sup> is present by P28 and increases to significance by P58. Sample sizes: A.BY.Cg-Dstn<sup>com1–2J</sup> P58 n=4, A.BY Dstn<sup>com1</sup> P58 n=8, A.BY WT P58 n=4, B6 Dstn<sup>com1–2J</sup> P58 n=4, B6.Cg-Dstn<sup>com1</sup> P58 n=6, B6 WT P58 n=4, A.BY Dstn<sup>com1</sup> P28 n=6, B6.Cg-Dstn<sup>com1</sup> P28 n=3. Unit length=300 μm. Error bars, SEM * denotes statistical significance resulting from t-tests, with omitted bars representing non-significance. *p<0.05, **p<0.01, ***p<0.001.
actin depolymerizing factor family in mice, cofilin 1 which is ubiquitously expressed and cofilin 2 which is muscle specific [8]. We previously observed upregulation of cofilins in the A.BY Dstncorn1 cornea, indicating the existence of the compensating mechanism by cofilins [3]. It is possible that compensation by cofilins is more effective in the B6 background compared to the A.BY background, leading to milder phenotypes in the B6.Cg-Dstncorn1 cornea compared to the A.BY Dstncorn1 cornea.

**Effects of genetic variation on the mouse cornea:** Genetic background effects have been observed for various phenotypes in the mouse cornea. The rate of wound healing, along with epithelial migration, was found to be greater in the cornea of B6 mice compared to BALB/c mice [9]. Induced corneal neovascularization [10,11] as well as lymphangiogenesis [12,13] has also been shown to be greatly affected by the genetic background. Central corneal thickness, which is known to be associated with the risk for glaucoma, differs among inbred strains of mice [14] and quantitative trait locus (QTL) analysis identified a genetic locus that is significantly associated with this phenotype [15]. Our data demonstrates that genetic background has a significant effect on the severity of Dstncorn1 phenotypes, indicating the presence of a QTL that interacts with this mutation. We subsequently found that the F1 neovascularization phenotype is intermediate to both Dstncorn1 mutants, suggesting that the locus or loci may act in a semi-dominant manner. Future identification of genetic factors responsible for these phenotypic differences in the cornea of Dstncorn1 mice should provide valuable information regarding the molecular networks that regulate homeostasis and pathogenesis in the cornea.

The genetic modifier of Dstncorn1 acts in a pathway independent of SRF: Notably, all known phenotypes caused by the Dstncorn1 mutation are similarly affected by genetic background. This observation suggests that genetic factors responsible for these phenotypic differences act on the part of a molecular pathway that affects all of these phenotypes. We previously identified one transcription factor that appears to be responsible for all phenotypes caused by the Dstncorn1 mutation. Gene expression analysis revealed that multiple molecules that are downstream of the transcription factor SRF, as well as SRF itself, are abnormally upregulated in the cornea of Dstncorn1 mice [2]. Inactivation of SRF specifically in the corneal epithelium of the Dstncorn1 mice rescued all phenotypes caused by this mutation [6]. Data from this study suggest that the level of induction of SRF in Dstncorn1 mutants does not appear to be directly responsible for the variation in severity of the phenotypes. That is, since the levels of induction of SRF in A.BY Dstncorn1 and B6.Cg-Dstncorn1 are not significantly different, we conclude that there exists an additional regulator responsible for the dampening of phenotypes in the B6 background. Since genetic factors responsible for phenotypic differences between A.BY Dstncorn1 and B6.Cg-Dstncorn1 are not significantly different, we conclude that there exists an additional regulator responsible for the dampening of phenotypes in the B6 background. Since genetic factors responsible for phenotypic differences between A.BY Dstncorn1 and B6.Cg-Dstncorn1 mice affect all phenotypes in a similar manner, we hypothesize that these genetic factors may act downstream from SRF in a molecular pathway, or else function in an independent manner. Further investigation of the SRF pathway along with identification of these genetic factors will test this possibility.

**Conclusions:** This study has shown that phenotypic differences between the Dstn mutants Dstnoral and Dstnoral–2 are due to allelic differences. Additionally, further analysis revealed that phenotypes caused by the Dstnoral mutation are
modified by genetic background. Our work indicates that there are additional factors that regulate the \textit{Dstr}\textsubscript{corn1} phenotypes other than SRF, which was previously determined as a major contributor. Further identification of factors that play a role in the formation of \textit{Dstr}\textsubscript{corn1} phenotypes will provide valuable insights into the regulation of actin dynamics, absence of vasculature, controlled epithelial proliferation and inflammatory response in the cornea.

ACKNOWLEDGMENTS
The authors would like to thank Satoshi Kinoshita for generating frozen sections, Zhen Zhang for development and technical support of E-mouse Lab (e-mouselab.com), Paul Schulz for statistics assistance, as well as the University of Wisconsin-Madison Genetics Confocal Facility for the use of the confocal microscope. This work was supported by a grant from the National Institutes of Health (NIH R01 EY016108) and a core grant to Waisman Center (NIH P30 HD03352). Support for S.V.K-S was partially provided by the NIH pre-doctoral training program in Genetics (T32 GM07133).

REFERENCES
1. Smith RS, Hawes NL, Kuhlmann SD, Heckenlively JR, Chang B, Roderick TH, Sundberg JP. Corn1: a mouse model for corneal surface disease and neovascularization. Invest Ophthalmol Vis Sci 1996; 37:397-404. [PMID: 8603845]
2. Verdoni AM, Aoyama N, Ikeda A, Ikeda S. Effect of destrin mutations on the gene expression profile in vivo. Physiol Genomics 2008; 34:9-21. [PMID: 18381839]
3. Ikeda S, Cunningham LA, Boggess D, Hawes N, Hobson CD, Sundberg JP, Naggett JK, Smith RS, Nishina PM. Aberrant actin cytoskeleton leads to accelerated proliferation of corneal epithelial cells in mice deficient for destrin (actin...
4. Bamburg JR. Proteins of the ADF/cofilin family: essential regulators of actin dynamics. Annu Rev Cell Dev Biol 1999; 15:185-230. [PMID: 10611961]

5. Verdoni AM, Smith RS, Ikeda A, Ikeda S. Defects in actin dynamics lead to an autoinflammatory condition through the upregulation of CXCL5. PLoS ONE 2008; 3:e2701. [PMID: 18628996]

6. Verdoni AM, Schuster KJ, Cole BS, Ikeda A, Kao WW, Ikeda S. A pathogenic relationship between a regulator of the actin cytoskeleton and serum response factor. Genetics 2010; 186:147-57. [PMID: 20610412]

7. Bock F, Onderka J, Hos D, Horn F, Martus P, Cursiefen C. Improved semiautomatic method for morphometry of angiogenesis and lymphangiogenesis in corneal flatmounts. Exp Eye Res 2008; 87:462-70. [PMID: 18789928]

8. Vartiainen MK, Mustonen T, Mattila PK, Ojala PJ, Thesleff I, Partanen J, Lappalainen P. The three mouse actin-depolymerizing factor/cofilins evolved to fulfill celltype-specific requirements for actin dynamics. Mol Biol Cell 2002; 13:183-94. [PMID: 11809832]

9. Pal-Ghosh S, Tadvalkar G, Jurjus RA, Zieske JD, Stepp MA. BALB/c and C57BL6 mouse strains vary in their ability to heal corneal epithelial debridement wounds. Exp Eye Res 2008; 87:478-86. [PMID: 18809399]

10. Rohan RM, Fernandez A, Udagawa T, Yuan J, D'Amato RJ. Genetic heterogeneity of angiogenesis in mice. FASEB J 2000; 14:871-6. [PMID: 10783140]

11. Rogers MS, D'Amato RJ. The effect of genetic diversity on angiogenesis. Exp Cell Res 2006; 312:561-74. [PMID: 16321383]

12. Regenfuss B, Onderka J, Bock F, Hos D, Maruyama K, Cursiefen C. Genetic heterogeneity of lymphangiogenesis in different mouse strains. Am J Pathol 2010; 177:501-10. [PMID: 20489140]

13. Nakao S, Maruyama K, Zandi S, Melhorn MI, Taher M, Noda K, Nusayr E, Doetschman T, Hafezi-Moghadam A. Lymphangiogenesis and angiogenesis: concurrence and/or dependence? Studies in inbred mouse strains. FASEB J 2010; 24:504-13. [PMID: 19858096]

14. Lively GD, Jiang B, Hedberg-Buenz A, Chang B, Petersen GE, Wang K, Kuehn MH, Anderson MG. Genetic dependence of central corneal thickness among inbred strains of mice. Invest Ophthalmo Vis Sci 2010; 51:160-71. [PMID: 19710407]

15. Lively GD, Koehn D, Hedberg-Buenz A, Wang K, Anderson MG. Quantitative trait loci associated with murine central corneal thickness. Physiol Genomics 2010; 42:281-6. [PMID: 20423963]