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The Role of Heparan Sulfate Proteoglycans in Optic Disc and Stalk Morphogenesis

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Abstract

Background—Heparan sulfate proteoglycans (HSPG) are important for embryonic development via the regulation of gradient formation and signaling of multiple growth factors and morphogens. Previous studies have shown that Bmp/Shh/Fgf signaling are required for the regionalization of the optic vesicle (OV) and for the closure of the optic fissure (OF), the disturbance of which underlie ocular anomalies such as microphthalmia, coloboma and optic nerve hypoplasia.

Results—To study HSPG-dependent coordination of these signaling pathways during mammalian visual system development, we have generated a series of OV-specific mutations in the heparan sulfate (HS) *N*-sulfotransferase genes (*Ndst1* and *Ndst2*) and HS *O*-sulfotransferase genes (*Hs2st*, *Hs6st1* and *Hs6st2*) in mice. Interestingly, the resulting HS undersulfation still allowed for normal retinal neurogenesis and optic fissure closure, but led to defective optic disc and stalk development. The adult mutant animals further developed optic nerve aplasia/hypoplasia and displayed retinal degeneration. We observed that MAPK/ERK signaling was down-regulated in *Ndst* mutants, and consistent with this, HS-related optic nerve morphogenesis defects in mutant mice could partially be rescued by constitutive Kras activation.

Conclusions—These results suggest that HSPGs, depending on their HS sulfation pattern, regulate multiple signaling pathways in optic disc and stalk morphogenesis.

Keywords

HSPG; Ndst; sulfotransferase; Fgf; Optic Disc; Optic Stalk

INTRODUCTION

Optic nerve hypoplasia and coloboma are important causes of childhood visual disorders and blindness. Although it is clear that these congenital ocular deformations result from failures of optic fissure closure and optic nerve development, the genetic bases of these

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developmental defects are only poorly understood (Chang et al., 2006; Ghiasvand et al., 2011).

Multiple morphogenetic pathways, including Sonic hedgehog (Shh), bone morphogenetic protein (Bmp), retinoic acid (RA), fibroblast growth factor (Fgf) and Wnt signaling, are required for mammalian retinal development. During early retinal development, the proximal-distal patterning of optic vesicle is controlled by midline-derived Shh, while the dorsal-ventral patterning is regulated by Bmp and RA signaling (Chiang et al., 1996; Furuta and Hogan, 1998; Matt et al., 2008; Lupo et al., 2011). Homozygous *Shh* null mutant mice exhibit cyclopia and optic nerve aplasia (Chiang et al., 1996), and *Bmp7* knockout mice display coloboma and optic nerve and optic disc aplasia (Morcillo et al., 2006). Once the optic cup is formed, Fgf and Wnt signaling are required for the regionalization of the presumptive neural retina (NR) and the retinal pigmented epithelium (RPE), respectively. Specific depletion of the Wnt signaling component β-catenin in the dorsal optic cups leads to the failure of RPE specification (Westenskow et al., 2009). In recent studies on Fgf signaling, we and others have shown that Fgf signaling is required not only for neural retina determination but also for optic disc and optic fissure development (Cai et al., 2010; Cai et al., 2013; Chen et al., 2013).

To study the coordination of the various signaling pathways during embryonic development, we have previously used mammalian lens and lacrimal gland as models to investigate the role and mechanisms of heparan sulfate proteoglycans (HSPGs) (Pan et al., 2006; Pan et al., 2008; Qu et al., 2011a; Qu et al., 2011b; Qu et al., 2012). As a conserved and crucial part of the extracellular matrix, HSPGs are functionally involved in the regulation of multiple intercellular signaling molecules, including but not restricted to Shh, Wnt and Fgf, during organ morphogenesis. HSPGs consist of glycoprotein cores covalently linked to heparan sulfate (HS) glycosaminoglycan chains. Nascent HS consisting of alternating residues of Glucuronic acid and N-acetyglucosamine is initially modified by N-deacetylation of N-acetylglucosamine residues, followed by their subsequent N-sulfation. Both reactions are catalyzed by one or more of the four *N*-deacetylase-*N*-sulfotransferase (Ndst) family members. Importantly, HS N-sulfation serves as an essential prerequisite for subsequent HS modifications by heparan sulfate 2-*O*, *3-O* and 6-*O* sulfotransferases (Hs2st/Hs6st) and one epimerase, that together create ligand-specific sulfated binding sites on the proteoglycan-linked HS chain (Bishop et al., 2007). Consistent with this, we previously found that FGF-regulated early lens and lacrimal development was disrupted in *Ndst* and *Hs2st/Hs6st* mutants, whereas BMP and Wnt signaling appeared unaffected (Pan et al., 2006; Pan et al., 2008; Qu et al., 2011a; Qu et al., 2011b; Qu et al., 2012).

In the present study, we examined the role of HSPGs in retinal development by specifically disrupting *Ndst1/Ndst2* and *Hs2st/Hs6st* in early optic vesicle development. The resulting HS sulfation mutants exhibited normal retinal neurogenesis and optic fissure closure, but defective optic disc and stalk development, resulting in optic nerve hypoplasia and aplasia. Whereas we have previously shown that constitutive Kras activity fully rescued optic nerve dysgenesis caused by the loss of Fgf-Frs2-Shp2 signaling (Cai et al., 2013), similar optic nerve defects in the HS sulfation mutants were only partially ameliorated by activated Kras

signaling. Therefore, our results show that HS is required for other signaling pathways in addition to the established FGF-MAPK signaling in optic disc and stalk morphogenesis.

RESULTS

Combined ablation of *Ndst1/Ndst2* or *Hs2st/Hs6st1/Hs6st2* HS biosynthetic genes in the optic vesicle caused optic nerve dysgenesis and retinal degeneration

We have previously shown that two of the four *Ndst* genes, *Ndst1* and *Ndst2*, are expressed in E12.5 mouse retinae (Pan et al., 2006). The systemic deletion of *Ndst1* function in mice is lethal at birth, however, *Ndst2* knockout animals are grossly normal except for a mast cell specific defect (Forsberg et al., 1999; Grobe et al., 2005). *Ndst1* systemic knockout embryos exhibit ocular phenotypes ranging from small eyes to a complete lack of eyes. However, the remaining retinae in either *Ndst1* or *Ndst2* deficient animals are normal, suggesting that *Ndst* genes play redundant roles in retina development (Pan et al., 2006). To test this idea, we first generated *Ndst1* conditional mutants by employing *Six3*-Cre, which is an optic vesicle-specific deleter active as early as at embryonic day (E)9.5 (Cai et al., 2010; Cai et al., 2013). *Six3-Cre;Ndst1*$^{flox/flox}$ were viable and fertile without any obvious ocular phenotypes (data not shown). The compound *Six3-Cre;Ndst1*$^{flox/flox}$*;Ndst2*$^{KO/KO}$ mutants also showed normal eye size and a fully fused ventral ocular structure (Fig. 1A–D). Closer examination of adult eyes, however, revealed hypoplasia or aplasia of optic nerves (100% penetrant) (Fig. 1E–H and Table 1). The thickness of retinae and the number of retinal ganglion cells at 4 weeks were reduced by 39% and 50%, respectively ($P< 0.001$, $n=10$). These results indicate that the two HSPG *N*-deacetylase/N-sulfotransferase enzymes are required for optic nerve development and retinal homeostasis.

To investigate the function of HSPG *O*-sulfation enzymes in retinal development, we also generated *Hs2st/Hs6st* compound mutants, lacking the single 2-O sulfotransferase gene and up to two of the three 6-O sulfotransferase genes. Although single or double ablation of *Hs2st*, *Hs6st1* and *Hs6st2* by *Six3-Cre* did not affect ocular development, possibly due to compensatory upregulation of 6-O sulfation in response to abolished 2-O sulfation (data not shown) (Merry et al., 2001; Merry and Wilson, 2002), we observed significant optic nerve defects and retinal degeneration in *Six3-Cre;Hs6st1*$^{flox/flox}$*;Hs6st2*$^{KO/KO}$*;Hs2st* $^{flox/flox}$ animal (also 100% penetrant) (Fig. 1I–P and Table 1). These results support that HS *O*-sulfation is a critical determinant for retinal development. Since *Ndst1/Ndst2* and *Hs2st/Hs6st1/Hs6st2* mutants share similar ocular defects, we chose *Six3-Cre;Ndst1*$^{flox/flox}$*;Ndst2*$^{KO/KO}$ for further analysis.

Normal neurogenesis in *Ndst1/Ndst2* mutants

Optic nerve development is dependent on proper differentiation of retinal ganglion cells, which project axons through the optic disc to connect to the brain. To determine the molecular mechanisms of the observed optic nerve defects in HS-sulfation mutants, we analyzed the expression of key transcriptional factors required for retinal development. At E11.5, the RPE marker *Mitf* is properly restricted to the outer layer of the optic vesicle and the ventral retina, while the NR marker *Chx10* was expressed correctly in the inner layer of the optic vesicle (Fig. 2A–D). Similarly, normal expressions of *Otx1* was detected in the

distal retinae and the RPE differentiation marker *Dct1* was confined to the RPE (Fig. 2E–J). These results suggested that the regionalization of optic vesicles was unaffected in *Ndst1/Ndst2* mutants. Sagittal sections of E13.5 embryos showed that mutant eyes were fused at the Pax2-positive ventral retina, consistent with a lack of coloboma defects in these mice (Fig 2. K–N, arrows). In transverse section, however, Pax2 was already down regulated in the presumptive optic disc region (Fig 2. O–P, arrows). Sox2 is a transcriptional factor for neural retinal progenitors, while *Math5* and *Brn3b* are specifically required for ganglion cell development. We found that these transcriptional factors are expressed in expected spatial patterns in *Ndst1/Ndst2* mutants (Fig. 2Q–T and data not shown). Additionally, the cell proliferation markers Cyclin D1 and Ki-67 were also expressed indistinguishably between control and mutant retinae (Fig. 2U–X). These results suggest that the optic nerve dysgenesis in *Ndst1/Ndst2* mutants is not due to defects in the optic vesicle patterning or ganglion cell differentiation.

Failure of optic disc and stalk morphogenesis in HS-sulfation mutants

Since *Ndst1/Ndst2* mutants showed normal neural retina determination and ganglion cell genesis, we next analyzed the growth of ganglion cell axons and the development of the optic discs. The optic discs are composed of Pax2-positive astrocytes, which express Netrin-1 to guide the projection of retinal ganglion cell axons. Double staining of *Netrin-1* by RNA *in situ* hybridization and Pax2 by immuno-fluorescence demonstrated that *Ndst1/Ndst2* mutants had reduced numbers of optic disc cells in central retinae, which were also grossly disorganized (Fig. 3A–D″ and Table 2).

We examined the pathway-finding of axons within retinae and the optic stalks using NF-165, an neurofilament protein marker that traces each optic nerve fiber. We found the retinal ganglion cell axons in *Ndst1/Ndst2* mutants were misrouted to the sub-retinal space, apparently because they were unable to properly project into the optic disc and stalk (Fig. 3E–H″). Similar defects were observed in *Hs2st/Hs6st1/Hs6st2* mutants (Fig. 3I–J″), suggesting that the specification of the optic disc and guidance of retinal ganglion axons both require HS *O*-sulfations, consistent with the described strong reduction of HS 2-*O*- and 6-*O*-sulfation in *Ndst1*/*Ndst2* compound mutant cells and tissues (Holmborn et al., 2004; Grobe et al., 2005; Raman et al., 2011; Sheng et al., 2011). Although analysis of Pax2 and NF-165 expression on the sagittal sections demonstrated that optic fissures were closed properly in *Ndst1/Ndst2* mutants, only a few axons were detected in optic stalks in these mice (Fig. K–N″). These results suggest that optic disc and stalk morphogenesis were disrupted during embryogenesis of *Ndst1/Ndst2* mutants.

Disrupted Fgf-Fgfr association in HS sulfation mutants

The ocular phenotypes in *Ndst1/Ndst2* mutants are highly similar to what we have reported in mouse mutants that have lost either *Fgfr1* and *Fgfr2* or their downstream mediators *Frs2a* and *Shp2* (Cai et al., 2013). To determine whether *Ndst1/Ndst2* mutations disrupted FGF signaling, we next analyzed the Fgf-Fgfr binding activity by the ligand and carbohydrate engagement (LACE) assay. In this assay, recombinant Fgf10 was incubated with an Fgfr2-Ig chimera protein on brain cryosections, which presented the endogenous HS as the required co-receptor for functional trimeric Fgf10/Fgfr2/HS assembly (Pan et al., 2006). The Fgfr2-Ig

chimera protein is unable to bind HS in the absence of Fgf ligand under the experimental conditions used. In situ formation of Fgf10/Fgfr2/HS complex was detected using a secondary antibody that recognizes the Ig domain fused to Fgfr2. Consistent with the timing of the *Six3-Cre* deleter, we observed that the Fgf-Fgfr binding activity in *Ndst1/Ndst2* mutants gradually diminished from E10.5 to E12.5 (Fig. 4A–F). At E13.5, LACE signals were mostly lost in the central retina and the optic stalk, where the *Six3-Cre* deleter is known be the most active (Fig. 4G–J). Therefore, HS-dependent Fgf-Fgfr assembly into signaling-competent trimeric complexes was impaired in *Ndst1/Ndst2* mutants, indicating defective FGF signaling in the affected tissues.

Fgf-MAPK signaling was disrupted in *Ndst* mutants, and constitutive Kras activation partially rescued the ocular defects

We have previously shown that genetic ablation of *Fgfr1/Fgfr2* or *Frs2a/Shp2* abrogated MAPK/ERK signaling in the central retina and the optic stalk (Cai et al., 2013). To further determine if Fgf signaling was impaired in *Ndst1/Ndst2* mutants, we developed two approaches: one is based on phospho-ERK immunostaining; another is to generate compound mutants with a gain-of-function of Ras signaling. We detected that ERK phosphorylation was downregulated in both the central retina region and the optic stalk in *Ndst1/Ndst2* mutants (Fig. 5A–H). In contrast, when *Six3-Cre;Ndst1*$^{flox/flox}$*; Ndst2*$^{KO/KO}$ mutants were crossed with *LSL-Kras*^{G12D}, an oncogenic allele of *Kras* that can be conditionally activated by Cre deletion, the resulting activation of Ras signaling restored ERK phosphorylation in mutant retinae (Fig. 5I and Table 2). This led to a partial recovery of optic disc development, as indicated by Pax2 expression (Fig. 5J and Table 2). However, NF-165 staining showed that many axons of retinal ganglion cells were still misrouted to the subretinal space (Fig. 5J, arrow). As a result, optic nerves in *Six3-Cre;Ndst1*$^{flox/flox}$*; Ndst2*$^{KO/KO}$*;LSL-Kras*^{G12D} mutants remained thinner than those of wild type controls (Fig. 5K, L and Table 1). Therefore, restoration of FGF-Ras signaling did not fully compensate for the loss of HS sulfation during the optic disc and stalk development, suggesting that other factors required for these processes depend on the specifically regulated HS sulfation.

DISCUSSION

In this study, we have generated a series of mouse mutants with deficient HS sulfation in the optic vesicle and observed resulting hypoplasia or aplasia of the optic disc, stalk and nerve. Mice and cells lacking Ndst1 and Ndst2 isoenzyme function are known to lack HS 2-O and 6-O sulfates in addition to their reduced relative levels of N-sulfation, consistent with the established Ndst gateway function during HS biosynthesis (Holmborn et al., 2004; Grobe et al., 2005; Raman et al., 2011; Sheng et al., 2011). In contrast, HS from cells and mice with targeted deletions in Hs2st and Hs6st function completely lacks 2-O sulfates and is expected to show strongly reduced 6-O sulfation with increased N-sulfation levels (Merry et al., 2001; Habuchi and Kimata, 2010; Qu et al., 2011a). The phenotypic similarities between *Ndst1/Ndst2* and *Hs2st/Hs6st* mutants may thus be best explained by a predominant role of HS O-sulfation in optic disc, stalk and nerve development. These findings are consistent with impaired Fgf function and retinal axon guidance abnormalities observed at the optic chiasm in Hs2st, Hs6st1 and Hs2st/Hs6st1 compound mutant mice (Pratt et al., 2006; Habuchi and

Kimata, 2010). By genetic manipulation of the endogenous Ras signaling activity, we demonstrated that retinal development in *Ndst1/Ndst2* mutants could indeed be partially rescued by constitutive MAPK/ERK signaling, supporting our previous study in lens and lacrimal gland development, where activated Ras-ERK signaling also subverted HS sulfation deficiency to reverse FGF signaling defects (Qu et al., 2011b). Together, these results strongly suggest that FGF-Ras-ERK signaling is one of the key downstream pathways regulated by the extracellular heparan sulfate proteoglycans in retinal development, consistent with the established role of HS 6-O sulfation and Ndst activity in specific Fgf functions in other developmental processes (Habuchi and Kimata, 2010; Qu et al., 2011a; Qu et al., 2011b; Qu et al., 2012).

One of our recent studies on *Six3-Cre* induced FGFR/Frs2/Shp2 conditional mutants provided direct evidence that FGF/MAPK signaling is also required for optic disc and nerve development (Cai et al., 2013). However, whereas both *Fgfr1/Fgfr2* and *Frs2/Shp2* mutant eyes displayed coloboma as their optic fissures failed to close, we failed to observe similar defects in either *Ndst1/Ndst2* or *Hs2st/Hs6st* mutants presented in this work. This lack of coloboma defects in HS mutants can be explained by residual FGF binding and Fgf/Fgfr formation by undersulfated HS, or by the slow turnover rate of HS in retinal cells, resulting in the persistence of extracellular HS after the Cre-mediated deletion of HS-sulfation genes. Consistent with the latter scenario, we observed that the Fgf/Fgfr binding as indicated by LACE signal in *Ndst1/Ndst2* mutation slowly diminished from E10.5 to E12.5 until the optic fissure was already closed, thus precluding any coloboma defects. Indeed, when we deleted *Ndst* genes used *Rx-Cre*, another optic vesicle specific Cre line that acts earlier than *Six3-Cre* (Cai et al., 2010), we observed optic coloboma (Table 1). Therefore, the timing of HS depletion plays an important role in the severity of ocular phenotype.

We have presented evidence that depletion of HS disrupted the assembly of FGF/FGFR and phosphorylation of ERK in developing retina, which can be rescued by constitutively active Kras. It is notable, however, that Kras signaling only partially restored the formation of the optic disc, suggesting that there are additional HSPG-dependent signaling pathways not rescued by Kras signaling. This is in agreement with previous studies showing that HSPGs regulated multiple signaling pathways, including Wnt, Shh, BMP, all of which have been implicated in optic disc development (Kirkpatrick and Selleck, 2007). Moreover, Kras signaling also failed to rescue the misrouting of retinal ganglion cell axons in *Ndst1/Ndst2* mutants, consistent with reports that retinal-specific ablation of HS co-polymerase gene *Ext1* or systemic knockouts of *Hs2st* and *Hs6st1* genes resulted in defective axon guidance due to impaired Netrin-1 and Slit-1 signaling (Pratt et al., 2006; Ogata-Iwao et al., 2011). Therefore, in line with these previous studies, our work demonstrates that HSPGs also play essential roles in regulating multiple signaling pathways in the optic disc and nerve development.

EXPERIMENTAL PROCEDURES

Mice

Ndst1^{flox} mice have been previously reported (Grobe et al., 2005). *Hs6st1*^{flox} is a kind gift from Dr. Wellington V. Cardoso (Columbia University, New York, NY) (Izvolsky et al.,

2008). *Hs2st*^{flox} is a kind gift from Dr. Jeffrey D. Esko (University of California San Diego, La Jolla, CA) (Stanford et al., 2010). *Ndst2*^{KO} is a kind gift from Dr. Lena Kjellén (University of Uppsala, Uppsala, Sweden) (Forsberg et al., 1999). *Six3-Cre* mice were kindly provided by Dr. Yasuhide Furuta (M.D. Anderson Cancer Center, Houston, TX) (Furuta et al., 2000). *Hs6st2*^{KO} and *LSL-Kras*^{G12D} mice were obtained from Mutant Mouse Regional Resource Centers (MMRRC) and the Mouse Models of Human Cancers Consortium (MMHCC) Repository at National Cancer Institute, respectively (Tuveson et al., 2004). All mice were maintained in mixed genetic background. The floxed animals which do not carry *Six3-Cre* transgene were used as controls. All experiments were performed in accordance with institutional guidelines.

Immunohistochemistry

Immunohistochemistry was performed as previously described with the following antibodies were used: anti-Pax2 (PRB-276P) (from Covance, Berkeley, CA), anti-Sox2 (#Ab5603, Chemicon, Temecula, CA), anti-phospho-ERK1/2 (#9101) and anti-Cyclin D1 (#2926) (from Cell Signaling Technology, Beverly, MA), anti-Ki67 (#550609, BD Pharmingen San Diego, CA) anti-NF165 (#2H3, from Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), anti-Mitf (#MS-771-P0, Thermo Scientific, Fremont, CA) (Pan et al., 2006; Pan et al., 2008). At least 3 embryos of each genotype were fully sectioned and stained for each marker. The central retina sections across the optic disc region were used in cell counting. The thickness of the adult retina was measured from the ganglion cell layer to the outer nuclear layer based on the DAPI nuclear staining.

RNA *in situ* hybridization

RNA *in situ* hybridization on cryo-sections were carried out as previously described (Pan et al., 2006; Cai et al., 2010). The following probes were used: *Netrin-1* (from Valerie Wallace, Ottawa Health Research Institute, Ottawa, Ontario, Canada), *Math5* (from Dr. Tom Glaser, University of Michigan, Ann Arbor, MI), *Brn3b* (from Dr. Lin Gan, University of Rochester, Rochester, NY), *Chx10*, *Mitf* and *Dct1* (from Dr. Roderick R. McInnes, Hospital for Sick Children, Toronto, Ontario, Canada), *Otx1* (from Naoki Takahashi, Nara Institute of Science and Technology, Nara, Japan). At least 3 independent and fully sectioned eye balls of each genotype were analyzed for each RNA probe.

Ligand and carbohydrate engagement (LACE) assay

The LACE assay was used to probe the in situ binding affinity of Fgf–Fgfr complexes to heparan sulfate on retina sections as previously described (Pan et al., 2006). Recombinant Fgf10 and Fgfr2b were obtained from R&D Systems, Minneapolis, MN.

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Key findings

- Heparan sulfate *N*- and *O*-sulfotransferases are both required for optic nerve development.
- FGF signaling is disrupted by impaired Heparan sulfate sulfation in the optic disc.
- Constitutive Kras activation partially rescues optic disc development but not retinal axon projection in tissue-restricted HS mutant mice.

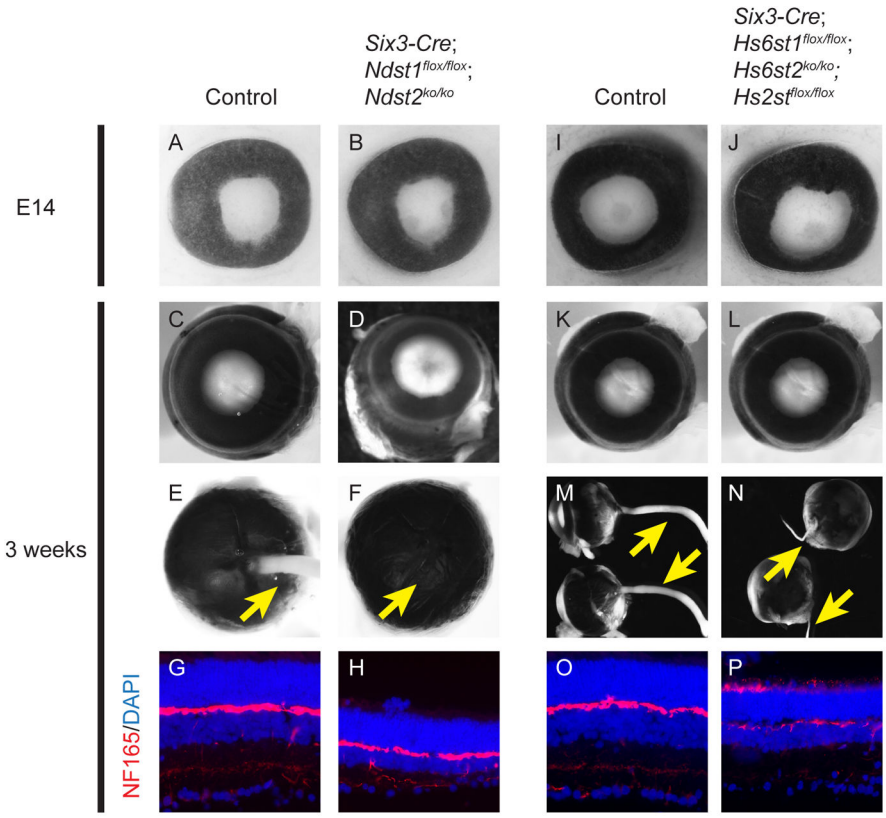

Figure 1. Comparable ocular defects in *Six3-Cre; Ndst1$^{flox/flox}$; Ndst2$^{KO/KO}$* and *Six3-Cre; Hs6st1$^{flox/flox}$; Hs6st2$^{KO/KO}$; Hs2st$^{flox/flox}$* mice

(**A**–**H**) Conditional *Ndst1/Ndst2* mutants displayed normal closure of the optic cup in the front (**A**–**D**), but optic nerve hypoplasia or aplasia at the back side of the eye (**E** and **F, arrow**). Retinal thinning was evident at adult stages (**G** and **H**).

(**I**–**P**) *Hs2st/Hs6st1/Hs6st2* conditional mutants also showed hypoplastic optic nerves and degenerating retinae.

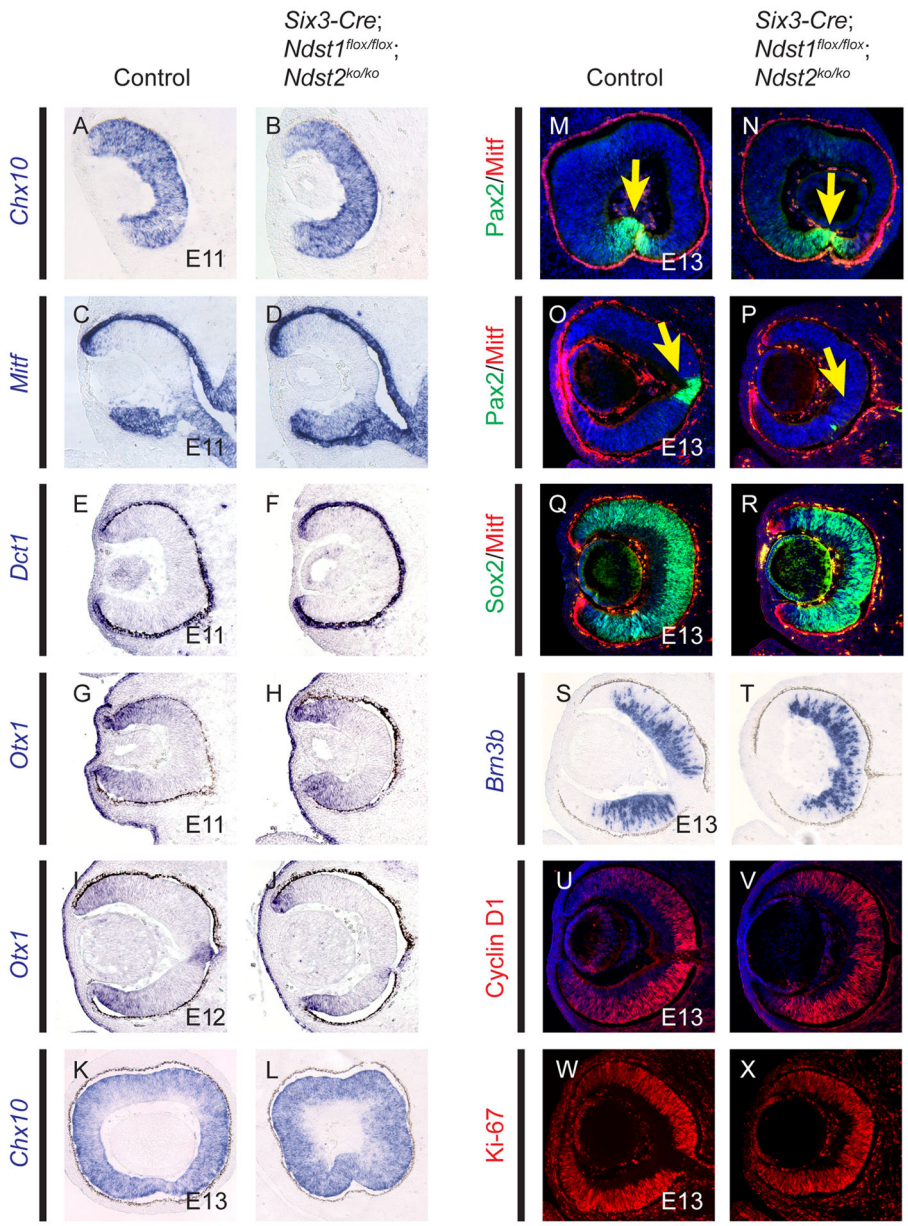

Figure 2. Molecular markers for earlier neural retina development in *Ndst* mutants

(**A**–**J**) The NR marker *Chx10*, RPE markers *Mitf* and *Dct1*, and ciliary margin marker *Otx1* are expressed normally in *Ndst1/Ndst2* conditional mutants.

(**K**–**N**) Sagittal sections of *Ndst1/Ndst2* mutants showed that the optic cup is fused at the ventral side (arrows).

(**O**–**P**) Transvers sections showed that Pax2 were reduced in the optic disc region (arrows).

(**Q**–**T**) Sox2 and *Brn3b* expression indicates unaffected differentiation of retinal ganglion cells in conditional *Ndst1/Ndst2* mutants.

(**U**–**X**) No significant differences in the expression of cell proliferation markers Cyclin D1 and Ki-67 were observed in eyes of E13 *Ndst1/Ndst2* conditional mutants.

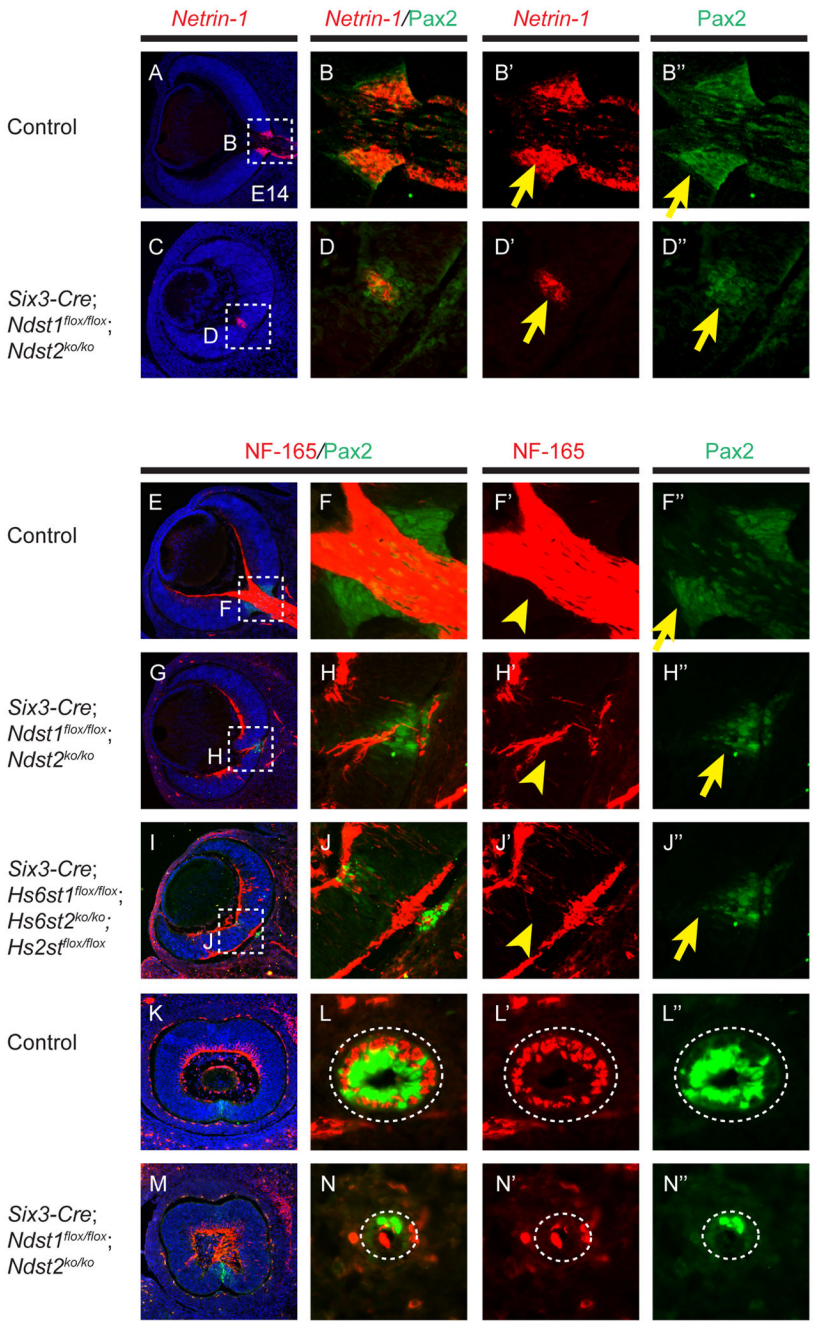

Figure 3. Disrupted optic disc and stalk development in *Ndst* and *Hs6st/2st* conditional mutant mice

(**A–D″**) The optic disc markers *Netrin-1* and Pax2 were down regulated in E14 *Ndst1/Ndst2* mutants. Arrows indicate *Netrin-1* and Pax2-expressing optic discs.

(**E–N**) Abnormal development of optic stalks and nerves was observed in both E14 *Ndst* and *Hs6st/2st* mutants, as indicated by altered expression of NF-165 and Pax2. Arrowheads denote the misrouting of retinal ganglion cell axons. Optic stalks were marked by circling the cells surrounding the NF-165-expressing neural fibers in dashed white lines (L and N).

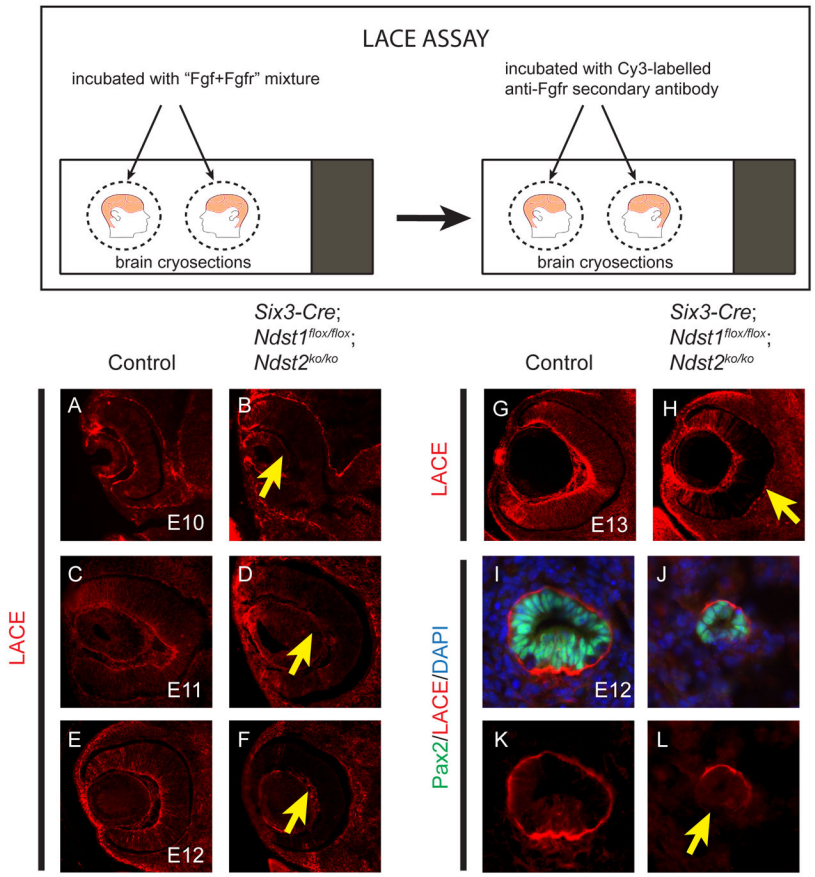

Figure 4. Ligand and carbohydrate engagement (LACE) analysis of disrupted HS-dependent Fgf-Fgfr assembly in *Ndst* mutants

(A–H) LACE assay on the frontal sections of *Ndst* mutants showed a gradual loss of HS-dependent Fgf-Fgfr association in central retinae isolated from *Ndst* mutant E10 to E13 mice (arrows).

(**I–L**) LACE signals were also diminished in Pax2-positive *Ndst* mutant optic stalks on sagittal sections.

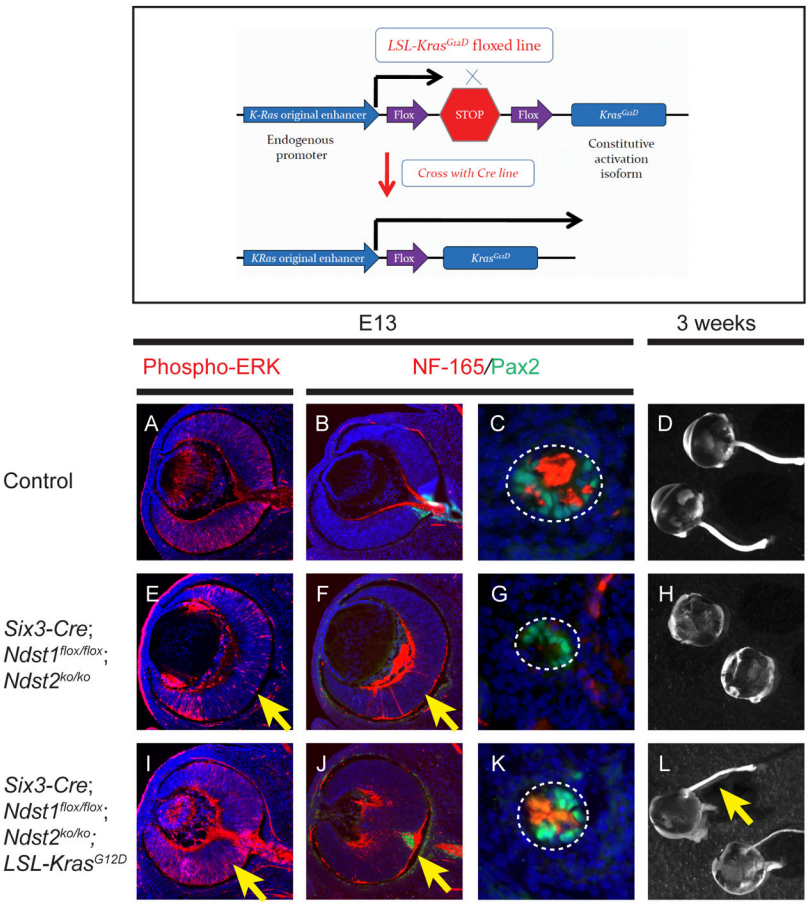

Figure 5. MAPK signaling defects in *Ndst* mutant are partially rescued by Kras

(A–H) In *Ndst1/Ndst2* mutants, phospho-ERK was downregulated in central retina, and Pax2 expression was also found to be strongly reduced in the putative optic disc region (E and F, arrows indicate the missing optic discs). As a result, the NF-165-expressing axons projected to the sub-retinal space, failing to reach the optic stalk (F and G), and the optic nerve was missing in adult animal (H).

(**I–L**) Partial rescue of developmental defects by constitutive Kras signaling in *Ndst* mutant mice. Notice that phospho-ERK was recovered to the wild type level (I, arrow) and Pax2-positive cells were detected in the optic disc and stalk regions (J and K, arrows). Partially restored formation of the optic nerve could therefore be observed in *Ndst*/*Kras* compound mutants (L).

Table 1

Ocular phenotypes in *Ndst* and *Hs6st/Hs2st* mutants

	Ocular Phenotypes			
	Coloboma	**ON aplasia**	**ON hypoplasia**	***n***
Control	0%	0%	0%	20
Six3-Cre;Ndst1$^{flox/flox}$	0%	0%	0%	20
Six3-Cre;Ndst1$^{flox/flox}$;Ndst2$^{KO/KO}$	0%	90%	10%	40
Six3-Cre;Hs2st$^{flox/flox}$	0%	0%	0%	20
Six3-Cre;Hs6st1$^{flox/flox}$;Hs6st2$^{KO/KO}$	0%	0%	0%	20
Six3-Cre;Hs2st$^{flox/flox}$;Hs6st1$^{flox/flox}$;Hs6st2$^{KO/KO}$	0%	25%	75%	20
Rx-Cre;Ndst1$^{flox/flox}$;Ndst2$^{KO/KO}$	100%	100%	0%	10
Six3-Cre;Ndst1$^{flox/flox}$;Ndst2$^{KO/KO}$;LSL-Kras^{G12D}	0%	5%	95%	40

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Table 2

Quantification of the optic disc defects.

The number of Pax2, Netrin-1 and Brn3b positive cells were counted on immunostained sections. ImageJ software was used to measure the area of NF-165 positive region at the optic stalk and the average pixel intensity of phospho-ERK (pERK) immunofluorescence in the retina.

	pERK (average pixel intensity/retina)	Pax2 (cells/section)	NF-165 (area/section)	Netrin-1 (cells/section)	Brn3b (cells/section)
Control	24 ± 2	129 ± 7	205 ± 7	113 ± 4	527 ± 14
Six3-Cre; Ndst1$^{flox/flox}$*; Ndst2*$^{ko/ko}$	15 ± 1*	22 ± 4*	13 ± 2*	21 ± 4*	515 ± 23$^{N.S.}$
Six3-Cre; Ndst1$^{flox/flox}$*; Ndst2*$^{ko/ko}$*; LSL-Kras*^{G12D}	28 ± 2**	49 ± 5**	45 ± 4**	N.D.	N.D.
n	*5*	*10*	*10*	*10*	*5*

Value are mean ± SEM and statistical significance determined by one-way ANOVA analysis.

* *P*<0.01, compared to wild type control.

** *P*< 0.01, compared to *Ndst1/Ndst2* mutants.

N.D., not determined. N.S., not statistically significant.