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## **Developmental regulation of notochord-derived repulsion for dorsal root ganglion axons**

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## **Abstract**

During the initial stages of development, the notochord provides repulsive signals for dorsal root ganglion (DRG) axons via semaphorin 3A/neuropilin-1, axonin-1/SC2, and other unknown repulsive molecules. The notochord is known to produce aggrecan, one of the chondroitin sulfate proteoglycans (CSPGs). We report here that adding aggrecan to the culture medium cannot only induce DRG growth cone collapse, but also inhibit DRG axonal growth. Using cocultures composed of tissues derived from chick embryos or *neuropilin-1*-deficient mice treated with chondroitinase ABC, we show the direct evidence that CSPGs are involved in notochord-derived repulsion for DRG axons. At later developmental stages, CSPGs are involved in perinotochordal sheath-derived axon repulsion, but not in notochord core-derived repulsion. We further demonstrate that TAG-1/axonin-1/SC2 is not involved in mediating repulsive activities by CSPGs, but is required for notochord core-derived axon repulsion. Thus, notochord-derived multiple axon repulsions act in a spatiotemporal-specific manner to shape the initial trajectories of DRG axons.

## Introduction

The notochord is an organizing center in the early development of vertebrate embryos and plays an important role in morphogenesis, cell fate determination, cell survival, and cell migration via a variety of molecules. In the early stages of development, the notochord participates in cell fate determination of the ventral neural tube through Sonic hedgehog (Shh; reviewed in [Jessell and Lumsden, 1997](#)). When the neural tube is formed, the notochord is then involved in the regulation of neural crest cell migration through chondroitin sulfate proteoglycans (CSPGs; e.g., [Oakley et al., 1994](#); [Perissinotto et al., 2000](#)). The notochord also promotes myotome formation through Shh, and regulates the differentiation of sclerotomal cells via Shh and Noggin (reviewed in [Borycki and Emerson, 2000](#); [Christ et al., 2000](#); [Dockter, 2000](#)).

The notochord also plays an important role in axonal guidance ([Keynes et al., 1997](#); [Nakamoto and Shiga, 1998](#)). We previously showed that the notochord regulates DRG axonal guidance through two cell surface receptors, neuropilin-1 and axonin-1/SC2, expressed on DRG axons ([Masuda et al., 2000, 2003](#)). Neuropilin-1 constitutes a receptor complex for semaphorin 3A (Sema3A/SEMA3A; hereafter called Sema3A) with plexin-A1 and L1 ([Castellani et al., 2000](#); [Dickson, 2002](#); [He and Tessier-Lavigne, 1997](#); [Kolodkin et al., 1997](#); [Takahashi et al., 1999](#)). Coculture studies have shown that neuropilin-1 on DRG axons mediates Sema3A-induced axon repulsion from the notochord ([Anderson et al., 2003](#); [Masuda et al., 2003](#)). Axonin-1/SC2, a member of the cell adhesion molecules of the immunoglobulin superfamily (Ig CAMs; [Sakurai et al., 1994](#); [Zuellig et al., 1992](#)), mediates unknown repulsive activities from the notochord. In addition, coculture experiments in which both neuropilin-1 and axonin-1/SC2 were perturbed suggest the presence of another signal involved in notochord-derived repulsion of DRG axons ([Masuda et al., 2003](#)).

CSPGs have been shown to inhibit the axonal growth in vitro (e.g., [Friedlander et al.,](#)

1994; Snow et al., 1990, 1991). The developing notochord produces CSPGs, aggrecan, and PG-M/versican (Bundy et al., 1998; Domowicz et al., 1995; Perissinotto et al., 2000), and Oakley and Tosney (1991) demonstrated that chondroitin sulfates are absent from the pathways of DRG axons, suggesting that CSPGs play a role in barriers to axon advance. However, there has been no direct evidence that the notochord utilizes CSPGs to repel DRG axons (Keynes et al., 1997). In the present study, using cocultures and growth cone collapse assays composed of tissues derived from chick embryos or mutant mice treated with chondroitinase ABC (Ch'ase ABC) or aggrecan, we found that multiple repellents including CSPGs are involved in notochord-derived axon repulsion and that TAG-1/axonin-1/SC2 may not be involved in mediating CSPGs-induced axon repulsion. We further demonstrated that these repulsive events are spatiotemporally regulated.

## **Results**

### *Expression pattern of CSPGs during the initial stages of DRG axonal growth*

The chick notochord is known to express CSPGs including aggrecan and PG-M/versican at the early stages of development (Bundy et al., 1998; Domowicz et al., 1995; Perissinotto et al., 2000). To address the distribution patterns of CSPGs during the initial stages of DRG axonal outgrowth in the chick embryo, we first examined CSPGs expression of thoracic regions using the CS56 antibody, which recognizes chondroitin sulfate chains of CSPGs. Neural crest cells begin to migrate out from the neural tube and have not yet differentiated into DRG neurons at stage 15 (Rickmann and Fawcett, 1985). The notochord is composed of notochordal cells (notochord core) and the perinotochordal sheath around them. Initially, the perinotochordal sheath consists of the basal lamina and microfibrills (Bancroft and Bellairs, 1976), and subsequently mesenchymal cells migrate to join the perinotochordal sheath. At stage 15, intense

CSPGs immunoreactivity was detected in the perinotochordal sheath, whereas moderate immunoreactivity occurred in the notochord core (Fig. 1A). At stage 19, DRG axons were extending both peripherally away from the notochord and centrally toward the spinal cord (Hollyday, 1995; Shiga et al., 2000). CSPGs immunoreactivity was weak in the notochord core, whereas the perinotochordal sheath retained strong immunoreactivity (Fig. 1B). At stage 24 at the thoracic level, DRG axons and motor axons formed the thick fiber bundles of the spinal nerve, and sclerotomal cells are accumulating in the neighborhood of the notochord. CSPGs immunoreactivity was localized in the perinotochordal sheath, was greatly reduced in the notochord core, but had now spread to the perinotochordal sclerotome (Fig. 1C).

#### *Soluble aggrecan inhibits DRG axonal growth*

Aggrecan is one of the major CSPGs expressed in the chick notochord at early stages (Bundy et al., 1998; Domowicz et al., 1995; Perissinotto et al., 2000). Although several studies have demonstrated that substrate-bound aggrecan can inhibit DRG axonal outgrowth (Borisoff et al., 2003; Condic et al., 1999; Snow et al., 1996), little is known about the inhibitory effect of soluble aggrecan on DRG growth cones. To address this question, we first examined the effects of aggrecan on DRG axons using the growth cone collapse assay. As previous studies have shown that 50  $\mu\text{g/ml}$  substrate-bound CSPGs was effective enough to inhibit DRG axonal growth (Borisoff et al., 2003; Snow et al., 1996), growth cone collapse of stage 30 chick DRG explants occurred in a dose-dependent manner when varying amounts of aggrecan were added to the culture medium, and many growth cones exhibited greatly reduced filopodial spreading in the presence of 100  $\mu\text{g/ml}$  aggrecan (Figs. 2A–C). These data indicate that soluble aggrecan acts on DRG neurons directly to induce growth cone collapse.

We next investigated the effects of aggrecan on DRG axonal growth using collagen-gel cultures (Figs. 2D,E). Adding aggrecan to the culture medium at a

concentration of 100  $\mu\text{g/ml}$  significantly reduced DRG axonal growth to 47.2% compared to the growth of the control group. These results indicate that soluble aggrecan can inhibit DRG axonal growth (Fig. 2G).

Because CSPGs are known to exert inhibitory effects by their chondroitin sulfate chains (e.g., Margolis and Margolis, 1997; Snow et al., 1990), we examined whether Ch'ase ABC can diminish the inhibitory effect of aggrecan on DRG axons (Fig. 2F). Cotreatment with 1 U/ml of Ch'ase ABC and 100  $\mu\text{g/ml}$  of aggrecan neutralized the inhibitory effect of aggrecan on DRG axonal growth (110% of the control group) (Fig. 2G). Ch'ase ABC treatment alone had no effect on DRG axonal growth (data not shown).

*Ch'ase ABC treatment diminishes stage 18–19 notochord-derived repulsive activities for DRG axons*

Based on the above results that CSPGs are expressed by the notochord at early stages and that Ch'ase ABC treatment neutralizes aggrecan-induced inhibition of DRG axonal growth, we examined the involvement of CSPGs in notochord-derived repulsion for DRG axons. Currently, there is no direct evidence showing that the notochord utilizes CSPGs to repel DRG axons.

As shown previously, stage 18–19 chick notochord exhibits strong repulsive activities on stage 26–27 chick DRG axons (Masuda et al., 2000, 2003). The addition of Ch'ase ABC significantly increased  $p/d$  values compared to the control group, indicating that Ch'ase ABC treatment reduced stage 18–19 notochord-derived repulsive activities for chick DRG axons (Figs. 3A,B,E). Similarly, Ch'ase ABC treatment in cocultures of embryonic day 13.5 (E13.5) wild-type mouse DRG explants with stage 18–19 chick notochords increased  $p/d$  values (data not shown). Ch'ase ABC treatment had no effect on the survival of notochord cells (data not shown). Because the Ch'ase ABC we used in the present study also digests hyaluronate, we further examined the

involvement of hyaluronate in notochord-derived axon repulsion. The addition of 100 TRU/ml hyaluronidase to cocultures of stage 18–19 notochords with stage 26–27 DRG explants had no effect on *p/d* values, which allows us to exclude the possibility that the effects of Ch'ase ABC were due to the digestion of hyaluronate (Fig. 3F). Together, our data provide the direct evidence for the first time that CSPGs including aggrecan may be involved in notochord-derived long-range repulsion for DRG axons.

*Perturbation of both CSPGs and *Sema3A/neuropilin-1* signaling leads to a complete loss of notochord-derived axon repulsion*

We previously reported that stage 18–19 chick notochord exerts repulsive activities via *Sema3A/neuropilin-1* (Masuda et al., 2003). Then, we examined the roles of *Sema3A* and CSPGs in the notochord-derived axon repulsion. When DRG explants from E13.5 *neuropilin-1*<sup>-/-</sup> mice were cocultured with stage 18–19 chick notochords for 48 h, *neuropilin-1*<sup>-/-</sup> DRG axons grew toward the notochord, but did not reach it, suggesting the existence of notochord-derived repulsive molecules other than *Sema3A* (Masuda et al., 2003) (Fig. 3C). Adding Ch'ase ABC to long-time cocultures of *neuropilin-1*<sup>-/-</sup> DRG explants with notochords inhibited notochord-derived repulsion in 9 out of 16 cocultures (Fig. 3D). These results strongly suggest that notochord-derived CSPGs and *Sema3A* may play a critical role in short- and long-range notochord-derived axon repulsions, respectively.

*Aggrecan-induced axon repulsion is not mediated by *TAG-1/axonin-1/SC2**

Neural cell adhesion molecule TAG-1 is a rodent homologue of axonin-1/SC2 (Zuellig et al., 1992) and is expressed on DRG axons during early stages of development (Wolfer et al., 1994). CSPGs are known to bind Ig CAMs including TAG-1/axonin-1/SC2 to inhibit Ig CAMs-mediated cell adhesion and axonal growth (Friedlander et al., 1994; Grumet et al., 1993; Milev et al., 1994, 1996; Retzler et al.,

1996). Because our in vitro results showed that axonin-1/SC2 is involved in mediating stage 18–19 chick notochord-derived repulsive activities (Masuda et al., 2000), TAG-1/axonin-1/SC2 is possibly involved in mediating CSPGs-induced axon repulsion. To test this, we investigated the effects of aggrecan on *TAG-1*-deficient DRG axonal growth. E13.5 mouse DRG explants were cultured for 24 h in the presence or absence of 100 µg/ml aggrecan in the medium. Exogenous aggrecan markedly reduced *TAG-1*<sup>-/-</sup> DRG axonal growth to 42.8% of control (Figs. 4A–C). Similar results were obtained in the case of *TAG-1*<sup>+/+</sup> DRG axonal growth (38.2%) (Fig. 4C). These results suggest that TAG-1/axonin-1/SC2 is not required for CSPGs-induced axon repulsion. Furthermore, in a collapse assay, aggrecan induced growth cone collapse of E17.5 *TAG-1*<sup>-/-</sup> DRG axons as well as that of *TAG-1*<sup>+/+</sup> DRG axons (Figs. 4D–F). These findings further indicate that TAG-1/axonin-1/SC2 may not be involved in mediating the CSPGs-induced repulsive activity. Next, we examined thoracic DRG axonal trajectories in *TAG-1*<sup>-/-</sup> mice from E10.5 to E14.5. No aberrant DRG axonal projection in relation to the notochord was observed in these mice (Figs. 4G,H, and data not shown). These data also support the idea that multiple repellents are involved in the notochord-derived axon repulsion. Sema3A and CSPGs in the notochord and perinotochordal mesenchyme may prevent DRG axons from innervating these structures in the absence of TAG-1/axonin-1/SC2.

*Before stage 18 CSPGs-induced repulsive activities are produced by the notochord*

We have previously shown that stage 13–14 chick notochord repels DRG axons (Nakamoto and Shiga, 1998). We have now examined whether CSPGs, Sema3A/neuropilin-1, and axonin-1/SC2 may participate in notochord-derived axon repulsion before stage 18. The Ch'ase ABC treatment of cocultures of stage 14 notochord explants with stage 26–27 chick DRG explants significantly increased *p/d* values, indicating that by stage 14 the notochord acquires CSPGs-induced repulsive

activities (Figs. 5A–C).

Our previous results showed that stage 14 notochord does not express *Sema3A* (Masuda et al., 2003). Next, we examined the involvement of *Sema3A/neuropilin-1* by culturing DRG explants from E13.5 *neuropilin-1*<sup>+/+</sup>, *neuropilin-1*<sup>+/-</sup>, or *neuropilin-1*<sup>-/-</sup> mice together with stage 14 chick notochords. DRG neurons derived from these three groups extended shorter axons toward vs. away from stage 14 notochord explants, indicating that *neuropilin-1*<sup>+/-</sup> and *neuropilin-1*<sup>-/-</sup> DRGs responded to notochord-derived repulsion (Fig. 5D). These results indicated that *Sema3A/neuropilin-1* is not involved in stage 14 notochord-derived repulsion. Similarly, DRG explants from *TAG-1*<sup>-/-</sup> mice responded to the repulsive activity from stage 14 chick notochords similar to *TAG-1*<sup>+/+</sup> DRG explants, suggesting that *TAG-1/axonin-1/SC2* is not involved in stage 14 chick notochord-derived repulsion (Fig. 5E). Taken together, these results suggest that CSPGs-induced axon repulsion, but not *neuropilin-1*- or *TAG-1/axonin-1/SC2*-mediated axon repulsions may occur in the notochord by stage 14.

#### *Stage 24 notochord core and perinotochordal sheath use different repulsive signaling*

Because DRG neurons are known to continue extending axons at later stages (stage 23 and later) (Carr and Simpson, 1978; Sharma et al., 1995), it is quite significant that the older notochord retains repulsive activities for DRG axons. We investigated whether the same molecules may contribute to notochord-derived repulsive activities at later stages (stage 24). As embryonic age progresses, the expression of CSPGs is down-regulated in the notochord core and is localized in the perinotochordal sheath (Bundy et al., 1998; Pettway et al., 1996 and in this study). Because the notochord core isolated from the perinotochordal sheath is known to repel DRG axons (Keynes et al., 1997), we isolated the perinotochordal sheath from the notochord core by mechanical microdissection, and examined detailed contributions of CSPGs to stage 24 chick

notochord-derived repulsive activities. Both the perinotochordal sheath and the notochord core showed repulsive activities for DRG axons (Figs. 6A,D). Ch'ase ABC treatment reduced perinotochordal sheath-derived axon repulsion in a dose-dependent manner (Figs. 6B,C), but had no significant effect on notochord core-derived axon repulsion (Figs. 6E,F). The present coculture experiments together with the immunohistochemical observation above suggest that CSPGs-induced axon repulsion may contribute to the perinotochordal sheath-derived repulsion but not to notochord core-derived repulsion.

We also cocultured E13.5 *neuropilin-1*<sup>+/+</sup> or *neuropilin-1*<sup>-/-</sup> mouse DRG explants with the perinotochordal sheath or the notochord core to investigate the involvement of Sema3A/neuropilin-1 in stage 24 chick notochord-derived repulsion. Quantitative analyses indicate that *neuropilin-1*-deficient DRG axons had significantly reduced responsiveness to both perinotochordal sheath- and notochord core-derived repulsions (Figs. 7A,B). These results suggest that both sheath- and core-derived axon repulsions depend partially on neuropilin-1.

Finally, we examined the contribution of TAG-1/axonin-1/SC2 in stage 24 notochord-derived repulsion of DRG axons. Coculture experiments revealed that E13.5 *TAG-1*<sup>-/-</sup> DRG axons significantly reduced responsiveness to core-derived but not sheath-derived repulsions (Figs. 7C-F). These results suggest the involvement of TAG-1/axonin-1/SC2 in mediating notochord core-derived axon repulsion. Together, our data suggest that the perinotochordal sheath utilizes CSPGs and Sema3A/neuropilin-1 to repel DRG axons, whereas the notochord core utilizes Sema3A/neuropilin-1 and an unknown axon repellent(s) via TAG-1/axonin-1/SC2.

## Discussion

In the present study, we examined notochord-derived repulsive activities at early

stages of development. By combining *neuropilin-1*-deficient DRG explants with Ch'ase ABC treatment, we provided the direct evidence that the notochord utilizes CSPGs to repel DRG axons. In addition, we showed that soluble aggrecan, one of major CSPGs that are produced by the notochord, can directly induce DRG growth cone collapse independent of other associated cues in the substrate. Finally, we demonstrate that the notochord secretes at least three repulsive molecules for DRG axons: CSPGs, Sema3A, and a TAG-1/axonin-1/SC2-mediated repellent (Figs. 8A,B). These repulsive molecules are produced by the notochord in a stage-specific developmentally regulated manner.

#### *The notochord secretes multiple repellents for DRG axons*

We have previously reported that neuropilin-1 and axonin-1/SC2 on DRG axons are required for mediating notochord-derived repulsive activities for DRG axons (Masuda et al., 2000, 2003). In coculture experiments in which both neuropilin-1 and TAG-1/axonin-1/SC2 were perturbed, we failed to completely prevent responsiveness to notochord-derived axon repulsion, suggesting the presence of additional repulsive signals (Masuda et al., 2003). In the present in vitro study, CSPGs induced the collapse of DRG growth cones and inhibited DRG axonal growth. Furthermore, we demonstrated that DRG axons innervate the notochord in the absence of both neuropilin-1 and CSPGs. Together, these results suggest that Sema3A/neuropilin-1 and CSPGs play crucial roles in notochord-derived axon repulsion. A previous in vitro study failed to find a contribution of CSPGs to notochord-derived repulsion for DRG axons (Keynes et al., 1997). It is highly likely that this failure is due to the strong diffusible repulsive activities of Sema3A, which masked axon repulsion exerted by CSPGs.

There seem to be obvious differences in the repulsive activities of Sema3A and CSPGs. When DRGs were cultured with the notochord in the absence of Sema3A/neuropilin-1 signaling, repulsive activities were greatly diminished but not completely lost, leaving an apparent axon-free zone around the notochord (Masuda et

al., 2003 and the present study). These results suggest that Sema3A/neuropilin-1 may be involved in long-range axon repulsion in a collagen gel (see also Messersmith et al., 1995), although Sema3A seems to act as a short-range repellent within the developing spinal cord (Fu et al., 2000). The difference may be due to the limited availability of the extracellular space within the spinal cord. It is interesting that collapsin/Sema3A was originally purified from membrane fractions of chick brains (Luo et al., 1993). Studies with a specific antibody that recognizes Sema3A may help to clarify the diffusible properties of Sema3A. Based on our in vitro studies that the response of chick DRG axons to notochord-derived repulsion was weakened and that *neuropilin-1*-deficient DRG axons could reach the notochord when chondroitin sulfate chains of CSPGs were enzymatically digested, CSPGs seem to act not only as a short-range chemorepulsive cue but also as a long-range chemorepellent in notochord-derived axon repulsion in vitro. Our present study, however, has revealed that CSPGs are mainly distributed within and near the notochord in the chick embryo, suggesting the possibility that CSPGs may have limited diffusibility in vivo.

In the present study, we demonstrated that soluble aggrecan can induce DRG growth cone collapse on laminin. Our result seems to contradict the previous result that soluble CSPGs had little effect on DRG axonal growth on laminin (Snow et al., 1996). Taken together with the fact that several studies have shown that soluble CSPGs can inhibit DRG axonal growth on a laminin-free substrate (Katoh-Semba et al., 1995; Snow et al., 1996 and the present study), the discrepancy between our results and others may be explained by acute treatment of soluble CSPGs or not. That is, our growth cone collapse assay was carried out by acute treatment of soluble aggrecan, while DRG axonal growth assay of Snow et al. (1996) was carried out by chronic treatment of soluble CSPGs. It is possible that chronic treatment of soluble CSPGs on a laminin substrate may change DRG axonal response to CSPGs-induced axon repulsion. It has been shown that DRG axons cultured on laminin adapt to the inhibitory activity of aggrecan by increasing the

expression of the integrin receptor during culture (Condic et al., 1999). In addition, the balance between the concentrations of CSPGs and growth-promoting molecule laminin may be important. We coated a lower concentration of laminin than Snow et al. (1996) did (10 µg/ml vs. 20–25 µg/ml).

#### *Notochord-derived repellents are developmentally regulated*

The present study demonstrates that CSPGs were expressed in the chick notochord as early as stage 15. In subsequent stages, the expression of CSPGs is down-regulated within the notochord core but is up-regulated in and around the perinotochordal sheath. By contrast, *Sema3A* mRNA was not expressed in the notochord until stage 18 and then continued to be expressed as late as stage 27 (Masuda et al., 2003; T. Masuda and T. Shiga, unpublished observation). Thus, the expression of CSPGs and *Sema3A* in the notochord appears to be temporally regulated. The developmental regulation of *Sema3A* was also shown in the repulsion of DRG axons by the ventral spinal cord where *Sema3A* is involved in the repulsion at older stages but not younger stages (Masuda et al., 2003; Messersmith et al., 1995).

Previous studies have suggested that CSPGs act as a barrier preventing the migration of neural crest cells around the notochord (e.g., Oakley et al., 1994; Perissinotto et al., 2000). Because DRG neurons do not begin to extend their pioneer axons until stage 18 (Shiga et al., 2000), this is a likely function of the early expression of CSPGs in stage 15 notochord.

At stage 18–19 when DRG neurons are extending pioneer axons to peripheral targets, the notochord produces both *Sema3A* and CSPGs. As noted above, *Sema3A* and CSPGs are thought to act as long-range and short- and long-range repellents, respectively, for DRG axons. At later stages (stage 23 and later), the notochord continues to express *Sema3A* and now the perinotochordal sheath produces CSPGs. Because late-generated DRG neurons may continue to extend axons at stage 29 (E6,

Carr and Simpson, 1978; Sharma et al., 1995), these two repellents may work together to prevent late-growing DRG axons from entering the perinotochordal regions. In mature higher vertebrates, the notochord and perinotochordal sclerotomes contribute to the formation of intervertebral disc. These repellents may be involved in the creation of a sensory nerve-free intervertebral disc (McCarthy et al., 1992). The older notochord (stage 24–27) retains the repulsive activity for DRG axons (the present study and unpublished observation), whereas it loses the repulsive activity for neural crest migration (Pettway et al., 1996). These results suggest that repulsive systems may be different between for DRG axonal growth and for neural crest migration.

#### *Roles of TAG-1/axonin-1/SC2 in notochord-derived axon repulsion*

Although we have previously demonstrated that the expression of axonin-1/SC2 on DRG axons is required to mediate notochord-derived repulsion, its ligand is unknown (Masuda et al., 2000). TAG-1/axonin-1/SC2 is anchored to the axonal membrane by glycosylphosphatidylinositol and is associated with the nonreceptor tyrosin kinase Fyn in the axoplasm (Kunz et al., 1996). Recently, it was reported that Fyn mediates Sema3A signaling used for the dendrite guidance of cortical neurons (Sasaki et al., 2002). By contrast, we failed to show that TAG-1/axonin-1/SC2 is a functional receptor for Sema3A (Masuda et al., 2003). In the present study, we found that CSPGs act as the notochord-derived repellent for DRG axons. Although the putative receptor for CSPGs has not been identified (but see Li et al., 2000), CSPGs interact with Ig CAMs including TAG-1/axonin-1/SC2 and inhibit the neuronal adhesion and the axonal growth (Friedlander et al., 1994; Grumet et al., 1993; Milev et al., 1994, 1996; Retzler et al., 1996). We therefore hypothesized that TAG-1/axonin-1/SC2 may be involved in CSPGs-induced repulsion for DRG axons. However, our results showed that TAG-1/axonin-1/SC2 is not required for the repulsive activity by aggrecan because aggrecan can induce DRG growth cone collapse and inhibit the axonal growth even in

the absence of TAG-1. Therefore, the notochord-derived repellent for TAG-1/axonin-1/SC2 remains unknown. Finally, it is especially interesting that stage 24 chick notochord produces several distinct repellents: Sema3A is secreted by both the notochord core and the perinotochordal sheath; CSPGs by the perinotochordal sheath; and an unknown repellent that acts via TAG-1/axonin-1/SC2 expressed by the notochord core (see Fig. 8B).

## **Experimental methods**

### *Animals*

Fertilized chicken eggs were obtained from a local farm and incubated at 37.6°C, until they reached the appropriate ages between stage 14 (E2) of [Hamburger and Hamilton \(1951\)](#) and stage 30 (E7). The generation and the identification of *neuropilin-1*-deficient mice and *TAG-1*-deficient mice were performed as described ([Fukamauchi et al., 2001](#); [Kitsukawa et al., 1997](#)). E0.5 was defined midday of the day of the presence of a vaginal plug.

### *Collagen-gel cultures*

Notochord explants were dissected from stage 14–24 (E2–4) chick embryos. DRG explants from thoracic segments were dissected out from stage 26–27 (E5) chick embryos and E13.5 mouse embryos. Pioneer DRG axons begin to extend by avoiding the notochord, the dermamyotome, and the ventral spinal cord, at around stage 18–19 (E3) and E10.5 in chick and mouse embryo, respectively ([Ozaki and Snider, 1997](#); [Shiga et al., 2000](#)). We used stage 26–27 chick and E13.5 mouse DRG explants because it was difficult to isolate younger DRG explants from surrounding mesenchymal tissues, and younger DRG explants were not suitable for quantitative analyses because of highly fasciculated axon bundles ([Anderson et al., 2003](#); [Masuda et al., 2003](#)). DRG explants

were embedded in a collagen gel approximately 300  $\mu\text{m}$  distant from the notochord explants. Cultures were incubated at 37°C for 24–36 h in Dulbecco's modified Eagle's medium (DMEM; Celox Laboratories, St. Paul, MN) containing 10% heat-inactivated fetal calf serum (FCS; Invitrogen Corp., Carlsbad, CA), 50 ng/ml 7S nerve growth factor (NGF; Chemicon, Temecula, CA), and 50 ng/ml neurotrophin-3 (NT-3; Sigma, St. Louis, MO). In some cases, cocultures were incubated for 48 h to examine whether DRG axons actually reach the notochord explants. For enzymatic treatment, chondroitinase ABC (Seikagaku Corp., Tokyo, Japan), or streptomyces hyaluronidase (Seikagaku Corp.) was added to the culture medium. To examine the effects of aggrecan on the outgrowth of DRG axons, stage 26–27 chick or E13.5 mouse DRG explants were cultured alone for 24 h in the presence of 100  $\mu\text{g}/\text{ml}$  aggrecan from bovine cartilage (Sigma) in a medium.

#### *Analysis of DRG axonal growth*

For the visualization of DRG axons, cultures were fixed for several days with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Collagen gels containing tissue explants were then excised and processed for whole-mount immunohistochemistry using an anti- $\beta$ -tubulin antibody (1:2000; Promega Corp., Madison, WI) as previously described (Masuda et al., 2000). After staining DRG axons, the length and the trajectory of axons were traced using a camera lucida (Nikon, Tokyo, Japan). Axons from DRG explants cocultured with notochords were grouped into four quadrants: proximal, distal, and two lateral quadrants (see Figs. 3, 5–7). Lengths of the five longest axons were measured in the proximal ( $p$ ) and distal ( $d$ ) quadrants for each culture (Lumsden and Davies, 1983). The axonal growth ratio  $p/d$  value is a measure of the repulsive activity, with a ratio of 0 and 1 indicating complete or no repulsion, respectively. To evaluate effects of aggrecan and Ch'ase ABC on the outgrowth of DRG axons, the axon area outside the DRG explant core region was measured using NIH

Image software. Data were statistically analyzed using Student's unpaired *t* test and ANOVA.

### *Immunohistochemistry*

For immunohistochemical detections of CSPGs in vivo, stage 15–24 (E2–4) chick thoracic segments were immersed in an ice-cold acid alcohol fixative (95% ethanol + 5% acetic acid) overnight at 4°C. Then, they were transferred to 100% ethanol and xylene and embedded in paraffin. Transverse sections (10 µm) were cut and collected onto gelatin-coated slides. The CS56 antibody (1:500; Sigma) was used for immunohistochemistry as previously described (Fukuda et al., 1997). For staining DRG axons in vivo, E10.5–14.5 *TAG-1*<sup>-/-</sup> mouse thoracic segments were fixed with 4% PFA overnight. After immersion in graded sucrose solutions in PB, tissues were frozen in Tissue-Tek OCT compound (Miles Inc., Elkhart, IN), and transverse sections (14 µm) were cut using a cryostat and collected onto gelatin-coated slides. The anti-β-tubulin antibody (1:2000) was used for immunohistochemistry as previously described (Masuda et al., 2003).

### *Collapse assay*

Stage 30 (E7) chick or E17.5 mouse DRG explants were cultured in a medium containing 90% DMEM, 10% FCS, 50 ng/ml NGF, and 50 ng/ml NT-3 on 12-mm glass coverslips coated with a mixture of poly-L-lysine (10 µg/ml; Sigma) and laminin (10 µg/ml; Invitrogen Corp.). After 24 h of culture, varying amounts of aggrecan were added to the medium. The cultures were incubated at 37°C for 45 min before fixing them with 4% PFA. For the visualization of growth cones, cultures were incubated for 30 min with 2% normal goat serum (Invitrogen Corp.) in 0.1 M PB. They were processed for the immunohistochemistry using an anti-axonin-1/SC2 antibody (1 µg/ml; a gift from Dr. M. Grumet, the State University of New Jersey) (Masuda et al., 2000) or

an anti-neurofilament 160-kDa antibody (10 µg/ml; Zymed Laboratories, South San Francisco, CA) for 4 h, followed by a biotinylated secondary anti-mouse or anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA) for 1 h and a peroxidase-conjugated avidin-biotin complex (Vector Laboratories) for 30 min. They were then reacted with diaminobenzidine (DAB) using the ImmunoPure metal enhanced DAB substrate kit (Pierce, Rockford, IL). All incubations were carried out at room temperature. After staining, preparations were scored as to whether their growth cones had normal spread-like morphology (i.e., had filopodia) (Raper and Kapfhammer, 1990). Data were statistically analyzed using Student's unpaired *t* test.

### **Acknowledgments**

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## Figure legends

Fig. 1. Expression pattern of CSPGs at the thoracic level in the chick embryo. (A) At stage 15 (St. 15), strong CSPGs expression was detected in the perinotochordal sheath (arrowhead), whereas only moderate expression occurs in the notochord core (c). sc, spinal cord. (B) At stage 19 (St. 19), CSPGs expression was decreased in the notochord core, whereas the perinotochordal sheath retained strong expression (arrowhead). (C) At stage 24 (St. 24), CSPGs expression has spread to the perinotochordal sclerotome (arrowheads) but continues to be expressed in the perinotochordal sheath (arrows). Scale bars: 25  $\mu\text{m}$  (A, B); 100  $\mu\text{m}$  (C).

Fig. 2. The effects of aggrecan on DRG axonal growth. (A–C) The effects of aggrecan on growth cones of stage 30 chick DRG neurons. DRG growth cones stained by an anti-axonin-1/SC2 antibody are shown in the presence (B) or absence (A) of aggrecan (100  $\mu\text{g}/\text{ml}$ ). (C) Aggrecan dose-response curve for DRG growth cone collapse. Data are mean  $\pm$  SEM for three or four separate experiments (20–50 growth cones in each treatment). (D–F) Stage 26–27 chick DRG explants were cultured alone for 24 h in the presence (E) or absence (D) of aggrecan (100  $\mu\text{g}/\text{ml}$ ), or in the presence of both aggrecan and chondroitinase ABC (Ch'ase ABC; 1 U/ml) (F). (G) Addition of aggrecan significantly reduced DRG axonal growth. Ch'ase ABC treatment neutralized the inhibitory effect of aggrecan on DRG axonal growth. Results are expressed as % control values. The bars represent mean + SEM and the number of cocultures is shown in the bar. \* $P < 0.001$ , compared to the control group. Scale bars: 50  $\mu\text{m}$  (B); 200  $\mu\text{m}$  (D).

Fig. 3. Perturbation of both CSPGs and Sema3A/neuropilin-1 removes a notochord-derived repulsive signal for DRG axons. (A, B) Stage 26–27 chick DRG explants were cocultured with stage 18–19 chick notochord (E3NC) in the presence (B)

or absence (A) of 1 U/ml Ch'ase ABC. In the Ch'ase ABC-treated group, longer axons extended toward the notochord. (C) In 48-h cocultures, E13.5 *neuropilin-1*<sup>-/-</sup> DRG axons did not reach notochord explants, leaving an axon-free zone (arrowheads). (D) Addition of Ch'ase ABC to long-time cocultures lead to a complete loss of stage 18–19 chick notochord-derived axon repulsion. (E, F) Quantification of effects of Ch'ase ABC and hyaluronidase (Hy'ase) on notochord-derived repulsive activities for DRG axons. Addition of 1 U/ml Ch'ase ABC significantly increased *p/d* values, but Hy'ase had no effect on *p/d* values. The bars represent mean + SEM and the number of cocultures is shown in the bar. \**P* < 0.0001, compared to the control group. Scale bars: 200 μm (A, B); 100 μm (C, D).

Fig. 4. Aggrecan-induced axon repulsion is not mediated by TAG-1/axonin-1/SC2. (A, B) E13.5 *TAG-1*<sup>-/-</sup> DRG explants were cultured in a collagen gel in the presence (B) or absence (A) of 100 μg/ml aggrecan. (C) Addition of aggrecan significantly reduced *TAG-1*<sup>-/-</sup> DRG axonal growth. Similar results were obtained in the case of *TAG-1*<sup>+/+</sup> DRG axonal growth. The bars represent mean + SEM and the number of cocultures is shown in the bar. \**P* < 0.05, compared to the control group. (D, E) The effects of aggrecan on growth cones of E17.5 *TAG-1*<sup>-/-</sup> DRG axons. *TAG-1*<sup>-/-</sup> growth cones stained by an anti-neurofilament antibody are shown in the presence (E) or absence (D) of aggrecan (100 μg/ml). (F) Aggrecan dose-response curve for *TAG-1*<sup>+/+</sup> DRG or *TAG-1*<sup>-/-</sup> DRG growth cone collapse assays. The collapse rate induced by aggrecan in *TAG-1*<sup>-/-</sup> DRG growth cones did not differ significantly from *TAG-1*<sup>+/+</sup> DRG growth cones. Data are mean SEM for three or four separate experiments (20–50 growth cones in each treatment). (G, H) Transverse sections of E11.5 mice embryos stained with an anti-β-tubulin antibody. There are no aberrant DRG axonal projections to the notochord in *TAG-1*<sup>-/-</sup> mice (H) compared to *TAG-1*<sup>+/+</sup> mice (G). Arrowheads indicate DRG axons. drg, DRG cell bodies; n, notochord; sc, spinal cord. Scale bars: 200 μm (A, B,

H); 50  $\mu\text{m}$  (E).

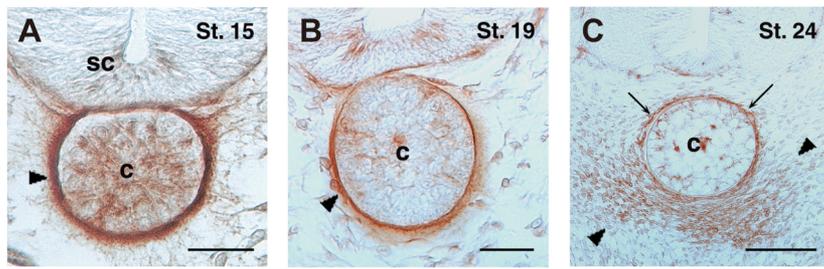
Fig. 5. CSPGs-induced axon repulsion is exerted by the notochord before stage 18. (A, B) Stage 14 chick notochord (E2NC) was cocultured with stage 26–27 chick DRG explants in the presence (B) or absence (A) of 1 U/ml Ch'ase ABC. (C) Quantification of effects of Ch'ase ABC on notochord-derived repulsive activities for DRG axons. Addition of 1 U/ml Ch'ase ABC significantly increased  $p/d$  values. (D)  $p/d$  values in neither *neuropilin-1*<sup>+/-</sup> nor *neuropilin-1*<sup>-/-</sup> DRG axons differed significantly from those of *neuropilin-1*<sup>+/+</sup> DRG axons, suggesting that neuropilin-1 is not required for stage 14 notochord-derived repulsion. (E) Similarly, the response of *TAG-1*<sup>-/-</sup> DRG axons to stage 14 notochord-derived repulsion was comparable to that of *TAG-1*<sup>+/+</sup> DRG axons. The bars in C, D, and E represent mean + SEM and the number of cocultures is shown in the bar. \* $P < 0.01$ , compared to the control group. Scale bars: 200  $\mu\text{m}$ .

Fig. 6. Ch'ase ABC treatment reduces perinotochordal sheath-derived axon repulsion of stage 24 chick embryos. (A) Stage 24 perinotochordal sheath explants exerted repulsive activities for DRG axons. (B, C) Ch'ase ABC treatment significantly reduced perinotochordal sheath-derived axon repulsion in a dose-dependent manner. (D) Stage 24 notochord core explants also exerted repulsive activities for DRG axons. (E, F) Ch'ase ABC treatment had no effect on notochord core-derived repulsion for DRG axons. The bars in C and F represent mean + SEM and the number of cocultures is shown in the bar. \* $P < 0.05$ , \*\* $P < 0.001$ , \*\*\* $P < 0.0001$ , compared to the control group. Scale bars: 200  $\mu\text{m}$ .

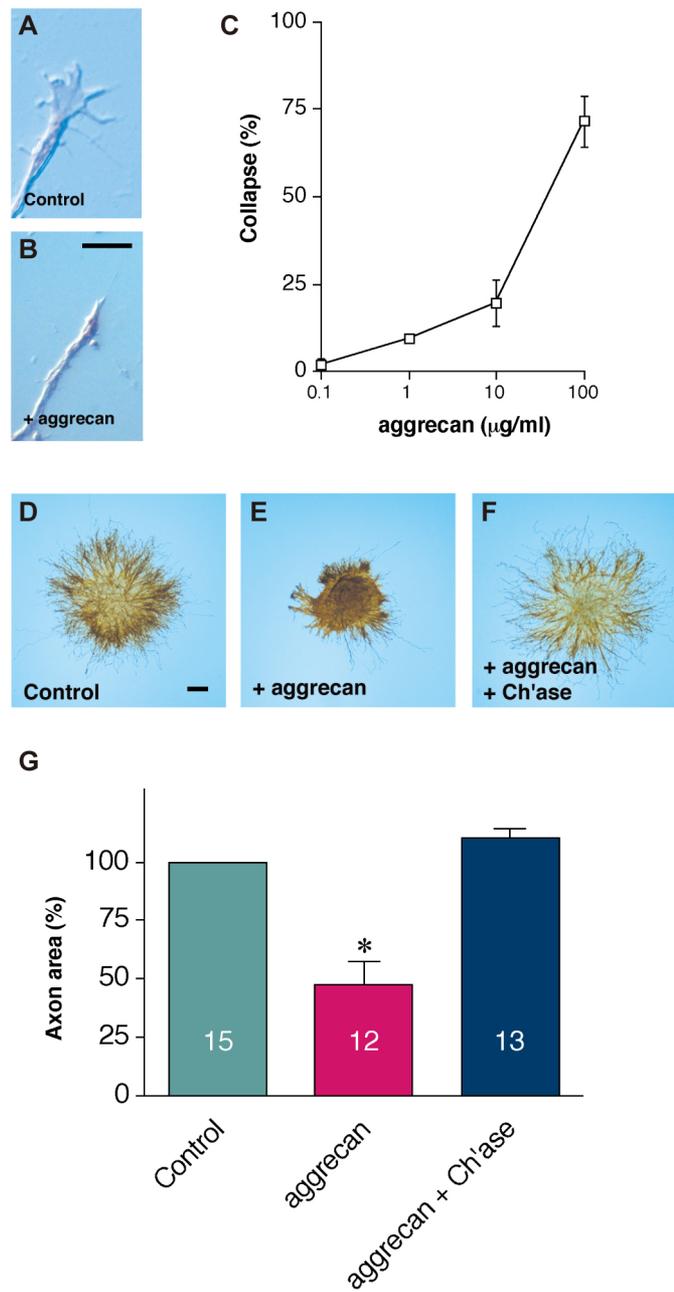
Fig. 7. Differential contribution of neuropilin-1 and TAG-1/axonin-1/SC2 to notochord core-derived axon repulsion. (A, B) *Neuropilin-1*<sup>+/+</sup> or *neuropilin-1*<sup>-/-</sup> DRG explants were cocultured with stage 24 perinotochordal sheath or notochord core explants.

*Neuropilin-1*<sup>-/-</sup> DRG axons significantly decreased responsiveness to both sheath- and core-derived repulsion. (C, D) *TAG-1*<sup>+/+</sup> DRG explants were repelled by notochord core explants (C), while *TAG-1*<sup>-/-</sup> DRG explants decreased responsiveness to notochord core-derived repulsion (D). (E, F) Quantification of repulsive activities derived from the perinotochordal sheath and the notochord core for *TAG-1*<sup>+/+</sup> and *TAG-1*<sup>-/-</sup> DRG axons. The bars in A, B, E, and F represent mean + SEM and the number of cocultures is shown in the bar. \**P* < 0.01, compared to the control group. Scale bars: 200 μm.

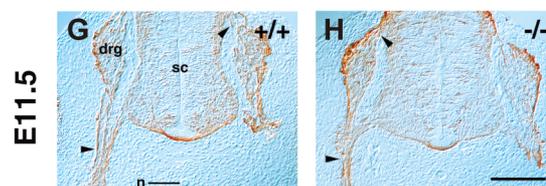
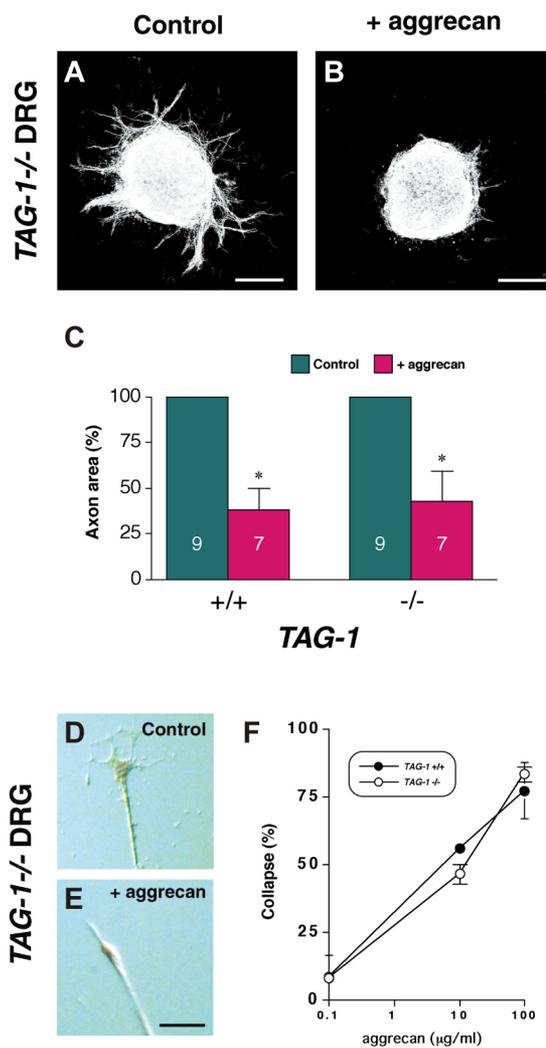
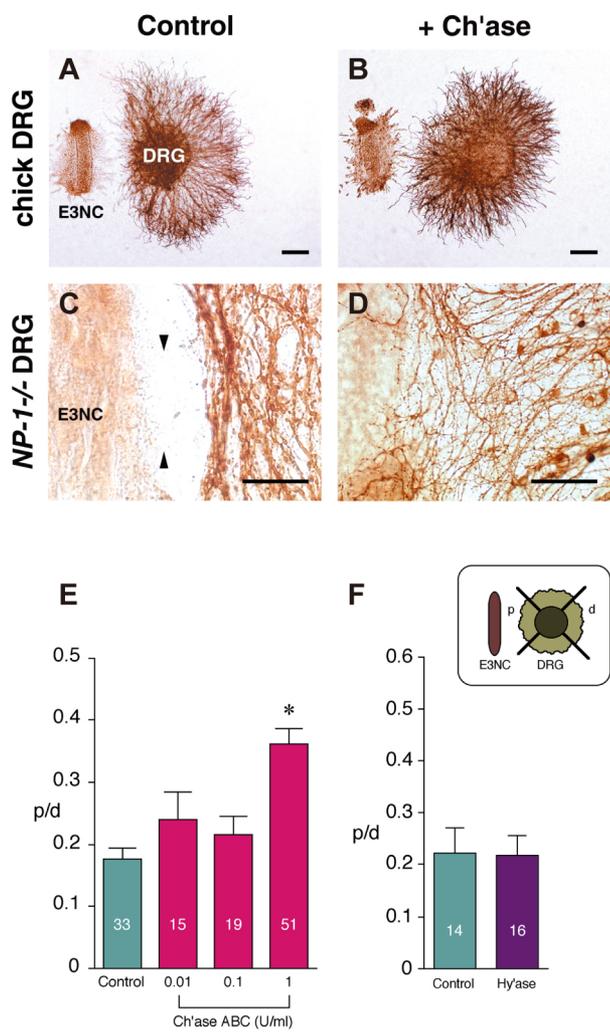
Fig. 8. A schematic diagram summarizing the multiple molecular mechanisms mediating notochord-derived repulsion of DRG axons. (A) Stage 18 notochord produces at least three repulsive molecules to repel DRG axons, namely, CSPGs, *Sema3A*, and an unknown repellent. CSPGs-induced axon repulsion is thought to be mediated by unknown receptor(s) on DRG axons. *Sema3A*-induced repulsion is mediated by a receptor complex comprised of *neuropilin-1/plexin-A1/L1* on DRG axons. *TAG-1/axonin-1/SC2* is thought to mediate axon repulsion by an unknown notochord-derived repulsive signal. (B) Differential contribution of repellents derived from stage 24 perinotochordal sheath- and notochord core-derived repulsion. The perinotochordal sheath utilizes CSPGs and *Sema3A* to repel DRG axons, whereas the notochord core utilizes *Sema3A* and an unknown repellent(s) that acts independent of CSPGs-induced repulsion of DRG axons.



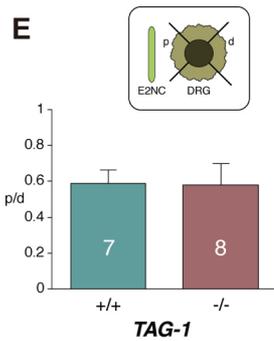
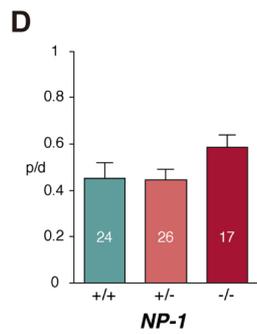
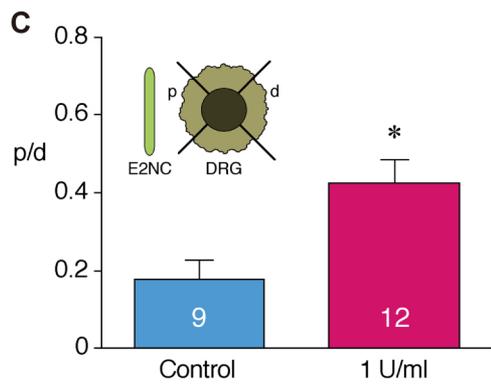
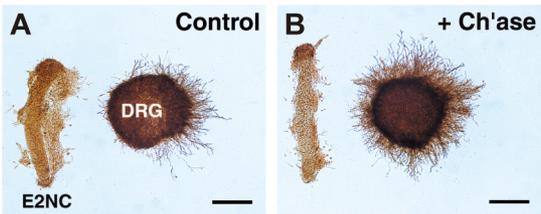
**Fig. 1**



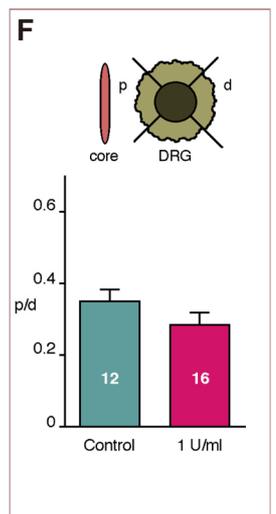
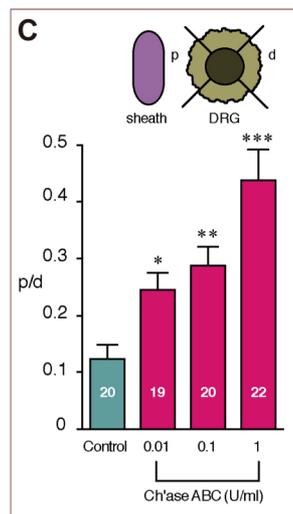
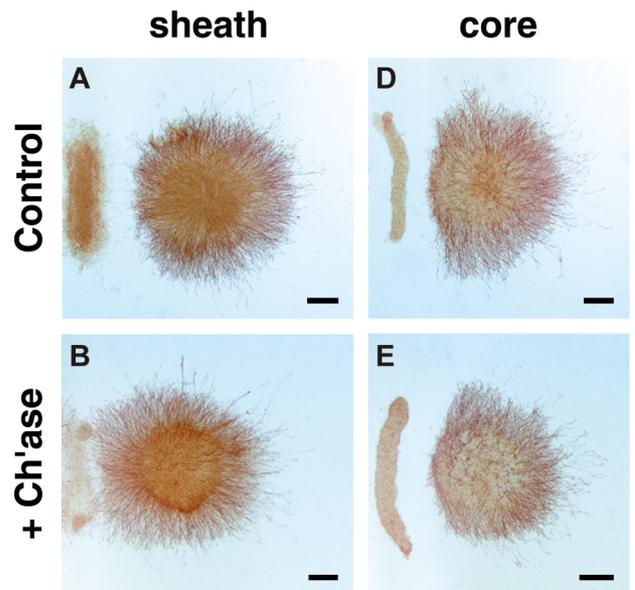
**Fig. 2**



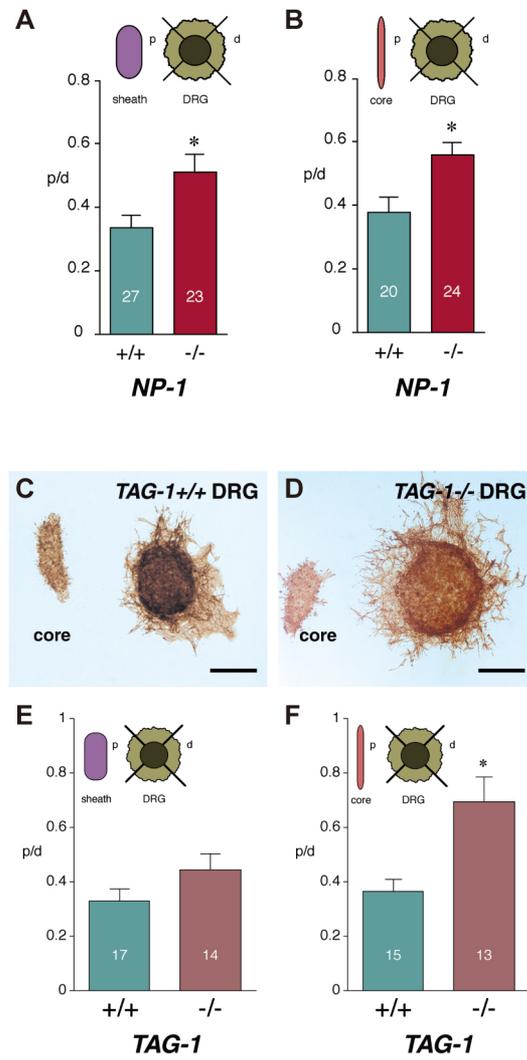
**Fig. 4**



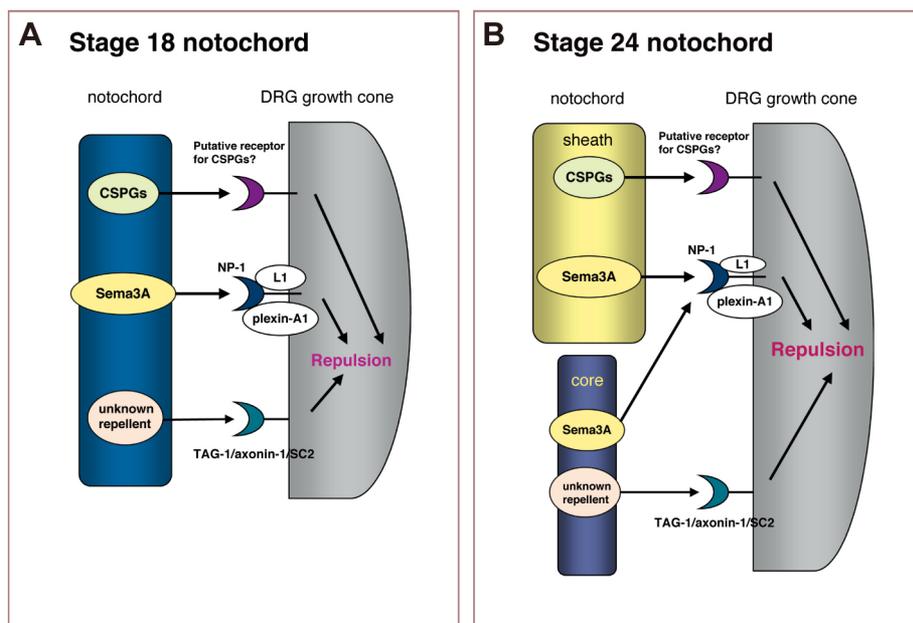
**Fig. 5**



**Fig. 6**



**Fig. 7**



**Fig. 8**