Mutations affecting the formation of the notochord in the zebrafish, *Danio rerio*

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SUMMARY

In a large scale screen for mutants with defects in the embryonic development of the zebrafish we identified mutations in four genes, *floating head* (*flh*), *momo* (*mom*), *no tail* (*ntl*), and *doc*, that are required for early notochord formation. Mutations in *flh* and *ntl* have been described previously, while *mom* and *doc* are newly identified genes. Mutant *mom* embryos lack a notochord in the trunk, and trunk somites from the right and left side of the embryo fuse underneath the neural tube. In this respect *mom* appears similar to *flh*. In contrast, notochord precursor cells are present in both *ntl* and *doc* embryos.

In order to gain a greater understanding of the phenotypes, we have analysed the expression of several axial mesoderm markers in mutant embryos of all four genes. In *flh* and *mom*, Ntl expression is normal in the germ ring and tailbud, while the expression of Ntl and other notochord markers in the axial mesodermal region is disrupted. Ntl expression is normal in *doc* embryos until early somitic stages, when there is a reduction in expression which is first seen in anterior regions of the embryo. This suggests a function for *doc* in the maintenance of *ntl* expression. Other notochord markers such as *twist, sonic hedgehog* and *axial* are not expressed in the axial mesoderm of *ntl* embryos, their expression parallels the expression of *ntl* in the axial mesoderm of mutant *doc*, *flh* and *mom* embryos, indicating that *ntl* is required for the expression of these markers. The role of *doc* in the expression of the notochord markers appears indirect via *ntl*.

Floor plate formation is disrupted in most regions in *flh* and *mom* mutant embryos but is present in mutant *ntl* and *doc* embryos. In mutant embryos with strong *ntl* alleles the band of cells expressing floor plate markers is broadened. A similar broadening is also observed in the axial mesoderm underlying the floor plate of *ntl* embryos, suggesting a direct involvement of the notochord precursor cells in floor plate induction.

Mutations in all of these four genes result in embryos lacking a horizontal myoseptum and muscle pioneer cells, both of which are thought to be induced by the notochord. These somite defects can be traced back to an impairment of the specification of the adaxial cells during early stages of development. Transplantation of wild-type cells into mutant *doc* embryos reveals that wild-type notochord cells are sufficient to induce horizontal myoseptum formation in the flanking mutant tissue. Thus *doc*, like *flh* and *ntl*, acts cell autonomously in the notochord.

In addition to the four mutants with defects in early notochord formation, we have isolated 84 mutants, defining at least 15 genes, with defects in later stages of notochord development. These are listed in an appendix to this study.

Key words: notochord, floor plate, muscle pioneer cells, *floating head*, *no tail*, *doc*, *momo*

INTRODUCTION

The notochord is an early embryonic structure which is the source of signals important for axis formation and neural differentiation. Grafting and ablation experiments mainly performed in the chick embryo have shown that the notochord can induce ventral cell fates, such as the floor plate and motor neurons, in the neural tube (reviewed by Smith, 1993). The floor plate is a morphologically visible structure in the ventral-most part of the neural tube. In the chick, transplantation of an ectopic notochord into a region lateral to the prospective neural tube leads to the formation of an additional floor plate in the

region in contact with the transplanted notochord, while removal of the notochord prevents floor plate formation (Yamada et al., 1991; Placzek et al., 1991 and references therein).

The notochord also plays a crucial role in somite patterning (reviewed by Bumcrot and McMahon, 1995). Grafting of an ectopic notochord to a position adjacent to presomitic mesoderm causes the development of extra sclerotome adjacent to the graft and prevents development of dermomyotome in the vicinity of the graft. Conversely, removal of the notochord prevents the formation of sclerotome and results in the fusion of somites, of exclusively dermomyotomal origin, beneath the neural tube in place of the notochord (Münsterberg and Lassar, 1994, and references therein). In the zebrafish, the presence of a differentiated notochord appears to be required for the induction of muscle pioneer cells (Halpern et al., 1993), a set of early differentiating muscle cells which are thought to be descendants of the adaxial mesoderm (Felsenfeld et al., 1991).

In the zebrafish, two genes that are required for notochord development, *no tail* (*ntl*) and *floating head* (*flh*), have previously been identified. Loss-of-function mutations in the *flh* gene cause a lack of notochord along the entire length of the embryo, with somites in the trunk fused below the neural tube (Talbot et al., 1995). *flh* was recently shown to be the zebrafish homologue of the *Xenopus* gene *Xnot* (Talbot et al., 1995 and references therein). This gene is expressed in the notochord and floor plate of early zebrafish embryos (Talbot et al., 1995). Expression studies performed in *flh* embryos suggest that cells lacking *flh* function differentiate into muscle rather than notochord (Halpern et al., 1995). A fate map analysis of the dorsal marginal region in gastrula stage *flh* mutant embryos supports this finding (Melby et al., 1996).

ntl is required for notochord and tail formation (Halpern et al., 1993; Schulte-Merker et al., 1994). Molecular analysis has shown that *no tail* (*ntl*) is the zebrafish homologue of the mouse *T* (*Brachyury*) gene, which encodes a transcription factor (Schulte-Merker et al., 1994; Kispert and Herrmann, 1993). The *ntl* gene is expressed in the germ ring of gastrulating embryos, in the developing notochord during somitogenesis (axial mesoderm) and in the prospective mesodermal cells of the tailbud (Schulte-Merker et al., 1992). In contrast to *flh*, mutant *ntl* embryos have undifferentiated axial mesodermal cells separating flanking somitic mesoderm (Halpern et al., 1993). The floor plate is present in *ntl* embryos, whereas muscle pioneer cells in the somites are not formed and the embryos do not develop a horizontal myoseptum which normally separates the dorsal and ventral parts of the somites (Halpern et al., 1993).

We have performed a systematic mutational analysis of early development in the zebrafish (Haffter et al., 1996). In this screen we have isolated alleles of *ntl* and *flh*, as well as of two new genes, *mom* and *doc*, which are also required for early notochord formation. This paper presents a preliminary characterisation of the four genes by comparing the expression pattern of various genes specifically expressed in the notochord, the floor plate and the adaxial mesoderm in mutant embryos. The *mom* phenotype is similar to *flh*, while *doc* resembles *ntl*. In both *mom* and *flh*, the formation of the floor plate is reduced, whereas in *ntl* it is enlarged, and in *doc* a normal floor plate is formed. *mom* and *doc* appear to be required predominantly in the trunk as mutant embryos exhibit a normal notochord in the posterior part of the tail. Mutations in all four genes disrupt the formation of the adaxially derived muscle pioneer cells and the horizontal myoseptum. Our analysis suggests that *flh* and *mom* have a common function in the early specification of the axial versus paraxial mesoderm, while *ntl* and *doc* are required for the development of the axial mesoderm into notochord.

MATERIALS AND METHODS

Fish raising, screen and crosses

Fish stocks were maintained as previously described by Mullins et al. (1994). The screen was performed as described by Haffter et al. (1996).

Antibody staining

Antibody staining was done as described by Bernhardt et al. (1990) except that the embryos were fixed in 4% PFA in PBS and permeabilised with acetone (described by Schulte-Merker et al., 1992). The detection was performed using a peroxidase kit (Vektor) according to the manufacturer's instructions.

In situ hybridization

In situ hybridization was performed as described by Hammerschmidt and Nüsslein-Volhard (1993).

Transplantation

The protocol of Ho and Kane (1990) was used for transplantation, with modifications described by van Eeden et al. (1996). About 10-15 cells from sphere stage wild-type donor embryos, previously marked with the lineage tracer dye rhodamine dextran, were transplanted to the margin of unlabeled host embryos at the same stage. The fate of the transplanted wild-type cells was scored at 24 hours of development under a compound microscope with fluorescence illumination. By transplanting labeled wild-type cells into *doc* recipients, offspring of donor cells were found in the region of the notochord in 30 *doc* recipient embryos. In 10 of these, big vacuolated notochord cells were obtained in trunk regions. 12 other transplants were not informative, because the wild-type cells were in the posterior part of the embryo where a normal appearing notochord forms in mutant embryos. The number of labeled wild-type cells in the remaining 7 mutant embryos was too large for a meaningful interpretation.

RESULTS

Mutations in four genes cause defects in notochord formation

Mutations in any of the four genes *floating head* (*flh*), *momo* (*mom*), *no tail* (*ntl*) and *doc* lead to the failure of proper notochord formation. *flh* and *ntl* have been described before (Talbot et al., 1995; Halpern et al., 1993), whereas *mom* and *doc* are newly identified genes. Although the absence of a differentiated notochord in the trunk region is common to strong alleles of all four genes, mutations in each of the four genes display a distinct phenotype. The characteristic features of the four genes are summarized in Table 1.

In *floating head* and *momo*, notochord precursors are absent

We have isolated two *flh* alleles whose sequence alterations

Table 1. Summary of the phenotypes of embryos mutant in the four genes required for early notochord formation

Gene	Alleles	Trunk phenotype	Tail phenotype
floating head (flh)	tm229, tk241	No notochord formed, somites fused in the midline	No notochord formed, somites separated by a large blood sinus
momo (mom)	th211	No notochord formed, somites fused in the midline	Notochord present
no tail (ntl)	tb244e, tc41,ts260	Notochord precursor cells present, somites separated	No tail formed
doc (doc)	tc233a, tt202, tt258	Notochord precursor cells present, somites separated	Notochord present

suggest that they are most likely to be functional null alleles (Talbot et al., 1995). The resulting phenotype is indistinguishable from that of the previously described allele *flh^{n1}*, that is the notochord is completely absent in both trunk and tail. In the trunk region, the left and right somites are fused underneath the neural tube by early somite stages, whereas tail somites are separated by a large blood sinus (Fig. 1B). Discontinuous groups of floor plate cells can be seen in the ventral spinal cord (arrowhead in Fig. 1B).

Only one allele of *mom* was isolated in our screen. In *mom* mutant embryos, the trunk notochord is missing while the notochord in the tail region appears to be normal (Fig. 1C). As in *flh* embryos, somites are fused underneath the neural tube in the trunk of *mom* embryos. This phenotype is not fully penetrant and a few notochord cells can be detected in the trunk region at early somite stages of some *mom* embryos (Fig. 1H). As revealed by the counts of mutant embryos in individual egg lays, a variable fraction, averaging 20%, of genotypically mutant embryos even display a normal notochord and are indistinguishable from wild type. In *mom* embryos displaying a strong notochord phenotype, disruption of the floor plate appears more severe compared to *flh*, whereas in the tail tip both a floor plate and a notochord is formed (Fig. 1C). In addition to the defects in the trunk, about 15% of *mom* embryos also display variable head defects such as small eyes (Fig. 1J,K).

In *no tail* and *doc,* notochord precursors are present

Two alleles of the *ntl* gene of equal strength, *ntl^{b160}* and *ntl^{b195}*, have previously been identified and characterized (Halpern et al., 1993). In *ntl* mutant embryos notochord precursor cells are present in the trunk but fail to differentiate (Halpern et al., 1993; Schulte-Merker et al., 1994). In addition, the tail does not form and the notochord is completely missing from the posterior part of the embryo, resulting in fusion of the somites (Halpern et al., 1993, Fig. 1D). We have isolated three new alleles of *ntl* that differ in phenotype strength. In embryos with the weakest allele, *ntl^{ts260}*, small vacuolated cells in the axial region are detectable over the entire length of the embryo, and a tail is formed whose length is reduced by only one somite (Fig. 1E). *ntl^{tb244e}* is of intermediate strength; it results in failure to form vacuolated notochord cells, and mutant embryos are reduced in length by four somites (data not shown). The phenotype of *ntl^{tc41}* mutants is stronger than that of the previously described *ntl^{b160}* and *ntl^{b195}* embryos. The post anal region in *ntl^{tc41}* is shorter than in *ntl^{b160}*, even though the same number of somites are formed (Fig. 2A, and see below).

To define the molecular lesion of the strongest allele *ntl^{tc41}*, we PCR amplified and cloned genomic DNA from the *ntl* region of homozygous mutant embryos. The sequence analysis revealed a single nucleotide exchange (T to A transversion) changing a leucine (TTG) at position 156 into a premature stop codon (TAG). The truncated protein is shorter than the predicted product of *ntl^{b160}* (245 amino acids) but slightly longer than that of *ntl^{b195}* (103 amino acids plus 35 amino acids encoded by an insertion; Schulte-Merker et al., 1994). From the sequence analysis, it is not obvious why the three alleles differ in strength. The molecular nature of the other two alleles, *ntl^{tb244e}* and *ntl^{ts260}*, is so far not clear.

Three alleles of *doc* have been isolated and all of them cause the axial mesoderm to remain undifferentiated in most parts of the embryo (Fig. 1F). In embryos with the strongest allele, *doc^{tt258}*, vacuolated notochord cells are only formed in a few posterior segments (Fig. 1L). In embryos with the weaker allele, *doc^{tt202}*, a thin notochord forms in both the tail and the trunk (data not shown). The amount of differentiated notochord formed in the tail region is variable and depends on the strength of the allele. The phenotype is slightly variable in all three alleles, even between embryos of the same clutch.

At high temperature (29°C), *doc* heterozygous individuals may display a dominant phenotype, that is at day six of development they are shorter, and the notochord has an irregular shape, with smaller cells (Fig. 2C). Most of these heterozygous fish survive to adulthood and are fertile.

Expression of notochord markers in mutant embryos

To gain a better understanding of the individual function of the four genes at early stages of development, we have carried out in situ hybridization and antibody staining with a number of molecular probes expressed early in the axial mesoderm of wild-type embryos (*ntl*, *twist (twi)*, *sonic hedgehog (shh)*, and *axial (axl)*).

Antibodies to the Ntl protein label nuclei in cells of the presumptive mesoderm of the germ ring and, later, the tailbud of wild-type embryos, as well as the axial mesoderm, which later forms the notochord (Schulte-Merker et al., 1992; Fig. 3A). During early gastrulation stages (shield stage to 60% epiboly) *flh* mutant embryos labeled with an antiserum against the Ntl protein are indistinguishable from wild-type embryos (data not shown). At later gastrulation stages mutant embryos exhibit persistent *ntl* expression in the tailbud, whereas in the midline mesoderm expression is restricted to a few cells in the posterior axis near the tailbud (Fig. 3B).

In *mom* embryos with a strong phenotype, Ntl protein distribution is similar to that of *flh* embryos. Expression is normal in cells of the presumptive tailbud mesoderm, but in the axial mesoderm Ntl-expressing cells are largely confined to posterior regions, with only a few anterior cells exhibiting

reduced levels of Ntl protein (Fig. 3C). In *mom* embryos with a weak phenotype, Ntl expression in the notochord may be detectable over the entire length in a thin stripe, or even appears normal (data not shown). These data indicate that *flh* and *mom* are both required for *ntl* expression in the more anterior axial mesodermal region, but not for the early expression in the presumptive mesoderm of germ ring and tailbud, of the embryo.

We analyzed *ntl* expression of all *ntl* alleles at the RNA and protein levels in tailbud stage embryos. Ntl protein is strongly reduced in embryos mutant for the two strong alleles ntl^{b160} and ntl^{b195}, whereas transient weak RNA expression can be observed in the tailbud and in the axial mesoderm (Schulte-Merker et al., 1994). Ntl protein is present at low levels in the notochord of mutants with the intermediate and weak alleles, ntl^{tb244e} and ntl^{ts260}. The strongest allele, ntl^{tc41}, results in neither Ntl protein nor RNA being detectable in the axial mesoderm (data not shown). These data confirm the morphological observations on a molecular level and suggest that ntl^{tc41} is a null allele.

In *doc* mutant embryos Ntl expression is normal until the tailbud stage. The expression in the axial mesoderm decreases during early somite stages and it is no longer detectable in the anterior axial mesoderm by the 10-somite stage (Fig. 3H). In heterozygous *doc* embryos displaying a dominant phenotype (see above), Ntl staining reveals that at the 10-somite stage the nuclei in the anterior notochord cells are not arranged as regularly as in wild-type embryos (Fig. 3G). Some nuclei have lost Ntl protein completely (enlargement in Fig. 3G). These findings suggest that *doc* is required for the maintenance of *ntl* expression in the axial mesoderm in a dose dependent manner after gastrulation.

We also analyzed *twist* (*twi*), which is expressed in the axial mesoderm during gastrulation (Halpern et al 1995). *twi* is not expressed in *ntl* embryos (Fig. 3D,E). In *flh* and *mom* embryos expression of *twi* is only detectable in axial cells near the tailbud that also express *ntl* (data not shown) suggesting a requirement of *ntl*

function for the expression of *twi* in the axial mesoderm. *twi* expression in *doc* embryos is indistinguishable from wild-type expression until the tailbud stage, it then decreases in the anterior notochord during early somite stages in parallel with the decreasing expression of Ntl. These data suggest that the initial *twi* expression is not dependent on *doc* function, while
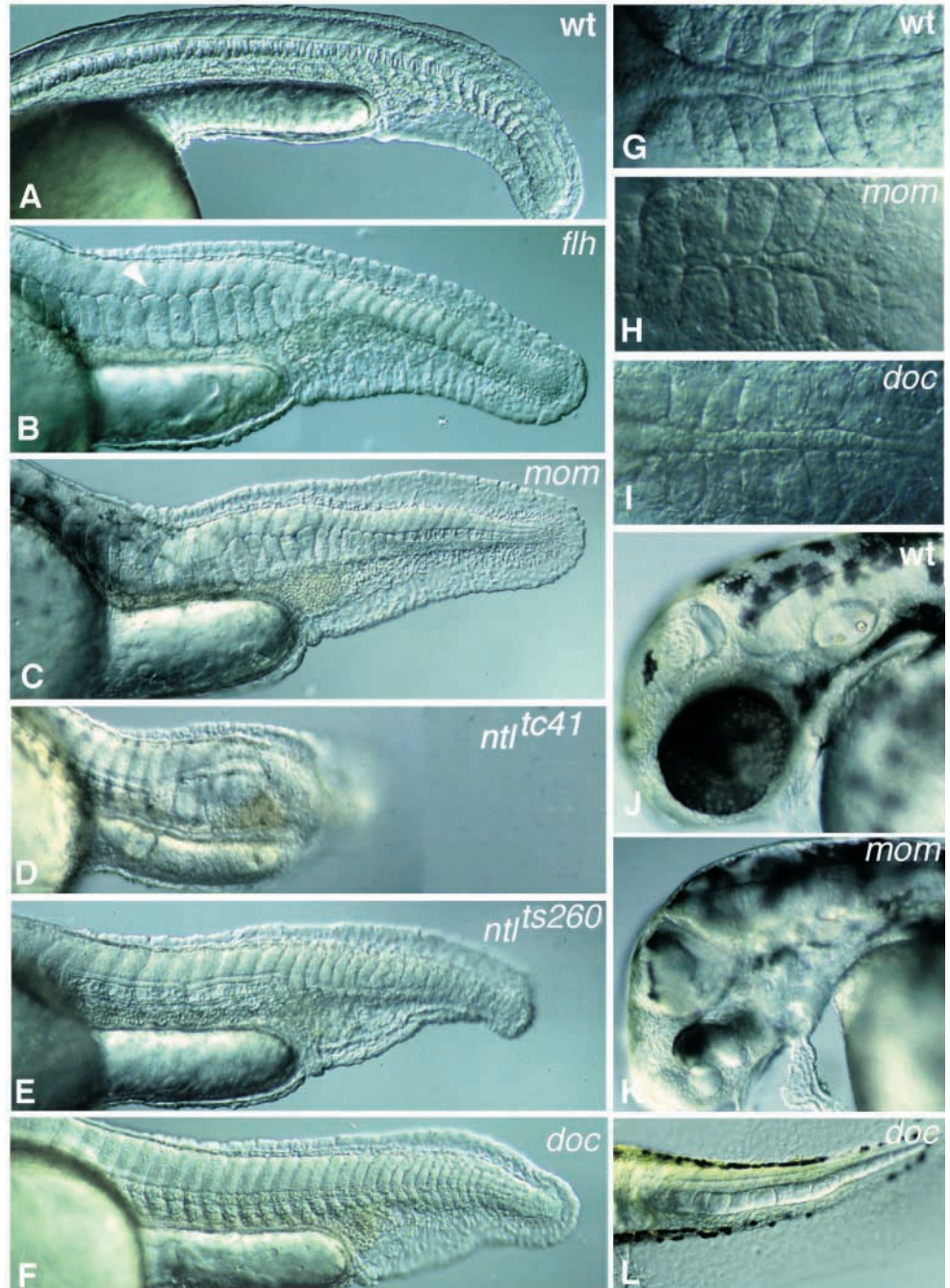

Fig. 1. Images of live mutant embryos. Anterior is left, dorsal is top in all pictures, except in G-I, which are dorsal views. (A) 24-hour old wild-type embryo. (B) flh^{tk241} embryo; a small patch of floor plate in the ventral neural tube is indicated by an arrowhead. (C) mom^{th211} embryo. (D) *ntl* embryo with the strongest allele, ntl^{tc41}. (E) ntl^{ts260}, the weak allele produces a reduced ventral tailfin. (F) doc^{tt258} embryo. (G-I) Dorsal view of 10-somite stage embryos: (G) wild type, (H) mom^{th211} and (I) doc^{tt258}. (J-K) Heads of 48-hour (J) wild-type and (K) *mom* larvae in a lateral view. (L) Tailtip of a 60 hour doc^{tt258} larva.
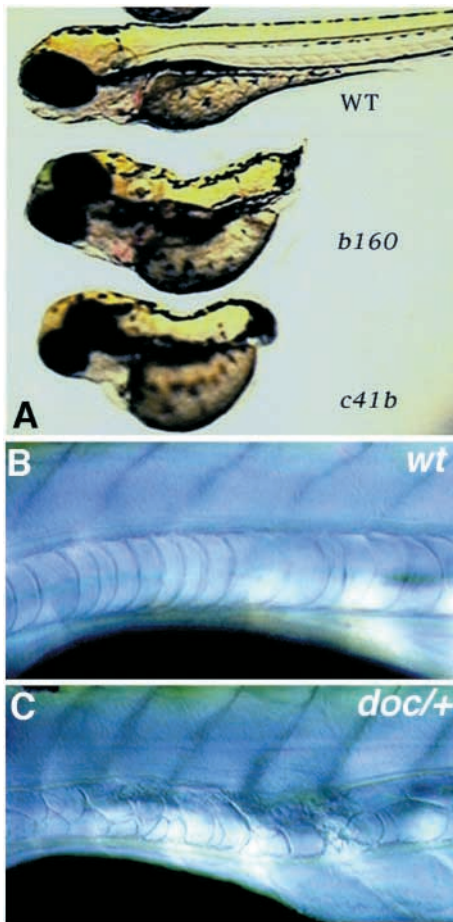
Fig. 2. (A) 3-day old wild-type, *ntl*^{b160} and *ntl*^{tc41} larvae. (B,C) The anterior notochord in (B) a wild-type and (C) a heterozygous *doc/+* larva after 5 days of development.

for its maintenance it requires *doc*, probably through its function in the maintenance of *ntl* expression.

The two genes *shh* (Krauss et al., 1993) and *axl* (Strähle et al., 1993) are expressed in both the notochord and the overlying floor plate. Similarly to the pattern of expression observed for *twist*, in the midline mesoderm of mutant embryos *shh* and *axl* transcripts are only detected in cells that also express Ntl. This is illustrated for *shh* in *doc* in Fig. 3J, and *mom* in Fig. 4C.

We conclude that *flh* and *mom* are required for the expression of *ntl* in the axial mesodermal region while the function of *doc* is to maintain this expression. The expression of other notochord markers, such as *twi, shh*, and *axl* depends on high levels of Ntl in the axial mesoderm.

Floor plate development is disrupted in *flh* and *mom*

We investigated a possible correlation between notochord and floor plate formation in mutant embryos. The floor plate is reduced to small patches (arrowhead in Fig. 1B) in embryos mutant for *flh* and *mom*, whereas a floor plate is formed in *ntl* and *doc* embryos (Fig. 1D-F). We also investigated the expression of the floor plate marker *shh*. In wild-type embryos, *shh*, in addition to its expression in the developing notochord, is expressed in a one-cell-wide stripe in the ventral neural tube (the midline floor plate cell; Krauss et al., 1993; Fig. 4A). At

the 15-somite stage short interrupted stretches of *shh* expression are found in the ventral neural tube of *flh* embryos, although there are no notochord cells in the underlying mesoderm (Fig. 4B). In *mom* embryos at the 15-somite stage, *shh* expression in the ventral neural tube is often detectable in single dispersed cells (Fig. 4C). Double labeling with the Ntl antiserum reveals that these *shh*-expressing cells often, but not always, overlie residual notochord cells, expressing both *shh* and Ntl (arrows in Fig. 4C and data not shown). Overall, we observe that in *mom* the floor plate defects are stronger than in *flh*, despite its weaker effect on the notochord, suggesting overlapping but distinct functions for the two genes.

In *ntl* embryos, in contrast to *flh* and *mom* embryos, the *shh*-expressing domain in the ventral neural tube is enlarged. In embryos mutant for the strongest allele, *ntl*^{tc41}, *shh* is expressed in a two to three cell-wide stripe in the ventral neural tube (Fig. 4D, note that *shh* expression in the axial mesoderm is completely abolished). In contrast, only a single row of cells expresses *shh* in the ventral spinal cord in embryos with the weak *ntl* allele, *ntl*^{ts260} (Fig. 4E), and in *doc* embryos (Fig. 4F). Together with the observation of a broadened domain of axial mesoderm in strong, but not weak *ntl* alleles (see below), this effect of *ntl* on the floor plate supports an involvement of the axial mesoderm in floor plate induction. This induction does not require *ntl* or *doc* (see Discussion).

Adaxial mesoderm development depends on *flh*, *mom*, *ntl* and *doc*

Somites in mutant embryos of *flh*, *mom*, *ntl* and *doc* appear u-shaped instead of showing the normal v-shape (shown for *doc* in Fig. 5B). The horizontal myoseptum, which divides the somites into dorsal and ventral parts, is missing in mutants of all four genes. We analyzed the somite phenotype with an antibody against Engrailed-like proteins (4D9, or Eng), which labels the nuclei of a subset of the paraxial cells, called the muscle pioneer cells, from early somite stages on (Patel et al., 1989; Hatta et al., 1991 and Fig. 5C). Eng expression is not detectable in the trunk somites of mutant embryos of *flh*, *mom*, *ntl* and *doc* (shown for *doc* in Fig. 5D). However, Eng-expressing cells can be detected in regions where the notochord is present in *mom* and *doc* embryos (data not shown). These observations are in agreement with findings of Halpern et al. (1993) that a differentiated notochord is required for the development of muscle pioneer cells in the adjacent somites.

Muscle pioneer cells are thought to be derived from a single cell-wide band of cells bounding the notochord, called the adaxial cells (Felsenfeld et al., 1991). At the tailbud stage, *myoD* is expressed in these adaxial cells (Weinberg et al., 1996 and Fig. 6A). This adaxial *myoD* expression appears unaffected in *flh* and *mom* mutants. In addition, in these mutants, axial cells between the two adaxial stripes also express *myoD* (Halpern et al., 1995 and Fig. 6B,C). These results are consistent with findings of Melby and coworkers (1996), who have shown that the notochord fate in axial cells of *flh* mutants is altered into muscle fate. The similarity of *myoD* staining in *flh* and *mom* mutants suggests that these two genes have similar functions in the specification of the axial mesoderm.

In *ntl* embryos, expression of *myoD* is strongly reduced in the adaxial cells at the tailbud stage (Weinberg et al., 1996). In embryos with the strongest allele, *ntl*^{tc41}, expression is barely detectable in two faint stripes flanking a region of axial
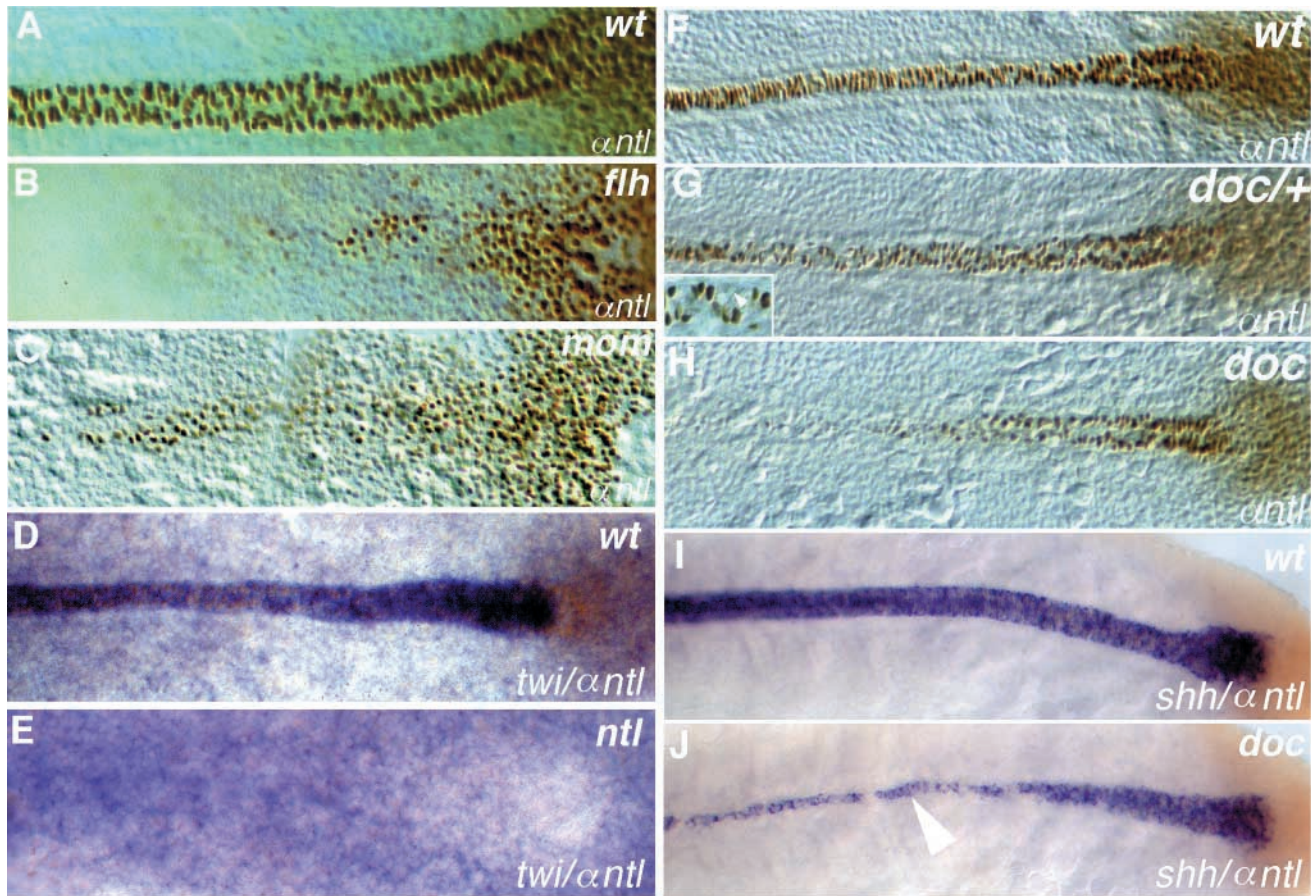
Fig. 3. Dorsal views of (A-E) tailbud stage and (F-J) 10-somite stage embryos. (A-C, F-H) Whole-mount Ntl antibody staining; (D,E) *twi* in situ hybridization (blue) and Ntl antibody staining (brown); (I, J) *shh* in situ hybridization (blue) and Ntl antibody staining (brown). Anterior is to the left. (A,D,F,I) Wild type, (B) *flh*^{tk241}, (C) *mom*^{th211}, (E) *ntl*^{tc41}, (G) heterozygous and (H, J) homozygous *doc*^{tt258} embryos. Ntl expression in the axial mesoderm is reduced in (B) *flh* and (C) *mom* embryos at the tailbud stage. Expression of *twi* in (D) wild-type and (E) *ntl*^{tc41} embryos, note that expression in the notochord is not detectable. (G) In heterozygous *doc*^{tt258}/+ embryos, Ntl is not detectable in a few nuclei of the notochord (arrowhead in the enlargement). In (J) *doc*^{tt258} embryos notochord expression of *shh* (blue) is only detectable in notochord cells which also express Ntl (brown, compare to Ntl expression in H). In regions to the left of the arrowhead (anterior), *shh* expression is restricted to the floor plate.

mesoderm that is broader than normal (arrows in Fig. 6D). Levels of *myoD* expression increase with decreasing strength of the *ntl* allele (Fig. 6E,F). These data suggest that *ntl* is required to induce adaxial cell development and *myoD* expression in the paraxial mesoderm (see Discussion). Early expression of *myoD* in adaxial cells of *doc* embryos is only slightly reduced (Fig. 6G), suggesting that *doc* has no, or only a weak role in the initial formation of the adaxial cells.

A differentiated notochord is required to maintain adaxial development

At later somite stages *myoD* continues to be expressed in adaxial cells, and it is also expressed in the posterior part of each somite (Weinberg et al., 1996 and Fig. 6H). No adaxial expression of *myoD* is detectable at the 15-somite stage in embryos with the strong *ntl* allele, *ntl*^{tc41}, while *myoD* expression appears normal in the somitic mesoderm (Fig. 6K). In *flh* and *mom* embryos, adaxial expression of *myoD* is disrupted in regions anterior to the tailbud (Fig. 6I,J), while it appears normal close to the tailbud where *myoD*-expressing cells of the right and left side are separated by *ntl*-expressing

cells. This suggests that axial mesodermal cells initially present in *flh* and *mom* embryos are able to initiate adaxial *myoD* expression, but that adaxial development in somitic regions is not maintained in the absence of axial mesoderm. In embryos with the weak allele, *ntl*^{ts260}, and in *doc*, *myoD* expression is reduced in the anterior adaxial mesoderm during somitic stages. These observations reveal a correlation between *ntl* expression in the axial mesoderm and the development of adaxial cells.

In summary, we have found for all four genes that the failure to form a horizontal myoseptum correlates with the absence of the muscle pioneer cells. It can be traced back to the specification of the adaxial cells of the paraxial mesoderm. *ntl* expressing cells in the axial mesoderm appear to be required for the development of adaxial cells. Furthermore, development of the notochord, or a signal downstream of *doc*, is necessary for the development of muscle pioneer cells from a subset of the adaxial cells (see Discussion).

doc functions cell autonomously in the notochord

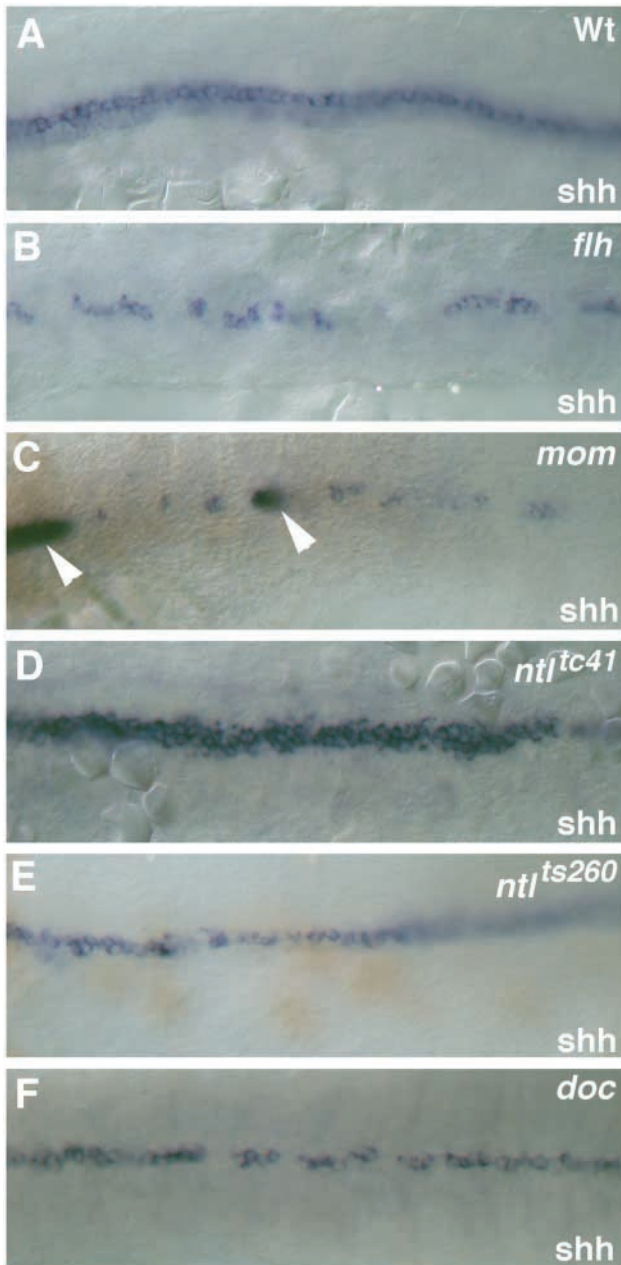Genetically mosaic embryos produced by cell transplantation

Fig. 4. Dorsal view (anterior to the left) of the expression pattern of the floor plate marker *shh* at the 15-somite stage in (A) wild-type, (B) *flh^{tk241}*, (C) *mom^{th211}*, (D) *ntl^{tc41}*, (E) *ntl^{ts260}* and (F) *doc^{tt258}* embryos. (A) At the 15-somite stage *shh* is expressed in the notochord (weak, out of focus) and in the midline floor plate cell of the spinal cord (strong). (C) In *mom^{th211}* embryos *shh* is expressed in a few faintly labeled cells in the ventral spinal cord. These cells frequently overlie cells in the dorsal mesoderm expressing both *shh* and *ntl* (arrowheads, out of focus). (D) Note that in embryos with the strong allele, *ntl^{tc41}*, no expression of *shh* in the axial mesoderm is detectable. (E) Axial mesodermal expression of *shh* is strongly reduced in mutant embryos with the weak allele, *ntl^{ts260}*.

can give important information about the requirement and function of gene expression in particular tissues. Such chimeric embryos have been produced for *ntl* and *flh* (Halpern et al., 1993, 1995). These experiments showed that *ntl* and *flh*

function are autonomously required in the notochord. In addition, it was shown that *ntl* function is required in the notochord for the formation of muscle pioneer cells in the somites, indicating an inductive signal from the notochord (Halpern et al., 1993).

We have carried out similar experiments for *doc*. Labeled wild-type cells were transplanted into *doc* embryos. The recipient embryos were analyzed during the pharyngula period (Kimmel et al., 1995). Descendants of labeled wild-type cells differentiated into large vacuolated notochord-like cells in an anterior notochord region (Fig. 7A-D). However, the wild-type cells were not able to induce neighboring *doc* mutant cells to form notochord. Mutant *doc* cells transplanted into wild-type recipients never formed vacuolated cells in the notochord. These transplantation experiments therefore show that the *doc* gene product acts cell autonomously in the notochord for its formation.

Some of the transplanted wild-type cells that formed a stretch of notochord in *doc* embryos were found to be associated with a horizontal myoseptum in the neighboring somites, which consisted entirely of *doc* mutant cells (arrow in Fig. 7E,F). This illustrates that *doc* gene activity is required in the notochord for the formation of the horizontal myoseptum. Transplanting cells from *doc* embryos into wild-type hosts support this finding. Large areas of *doc* mutant cells in the somites of wild-type hosts were able to form a morphologically normal horizontal myoseptum, indicating that *doc* mutant cells in the somites are able to respond to the signal from the notochord (arrows in Fig. 7G,H). This experiment indicates that the formation of the horizontal myoseptum depends on the function of *doc* in the notochord, resulting in the production of the inducing signal, and not in the somites where the signal from the notochord is received.

DISCUSSION

We have isolated multiple alleles of two genes previously known to be required for notochord formation: 3 alleles for *ntl* (Halpern et al., 1993) and 2 alleles for *flh* (Talbot at al., 1995; Halpern et al., 1995). Only one allele of *mom* was isolated. The variability observed in the *mom* phenotype suggests that the allele we isolated is not a null allele. For *doc* three alleles of different strength were isolated. *doc* has a temperature dependent dominant phenotype that may lead to a reduced viability of heterozygous individuals. Dominant lethality, even with low penetrance, reduces the number of heterozygous carriers and biases against the recovery of strong alleles. No dominant effects have been observed for *flh*, *mom* and *ntl*.

The *flh* alleles are all of the same strength and probably reflect the amorphic state of the gene. During the time of this study, *flh* has been shown to encode the zebrafish homologue of *Xnot*, a homeobox-containing transcription factor from *Xenopus laevis* (Talbot et al., 1995 and references therein). Two alleles isolated in our screen, *flh^{tm229}* and *flh^{tk241}*, carry point mutations causing premature stop codons in the coding region upstream of the homeodomain. It is likely that these two alleles represent a complete loss of function (Talbot et al., 1995). For *no tail*, we have isolated three alleles, all of different strength. One of them, *ntl^{tc41}*, results in a phenotype that is stronger than that of *ntl^{b160}* and *ntl^{b195}*. This is surprising, as
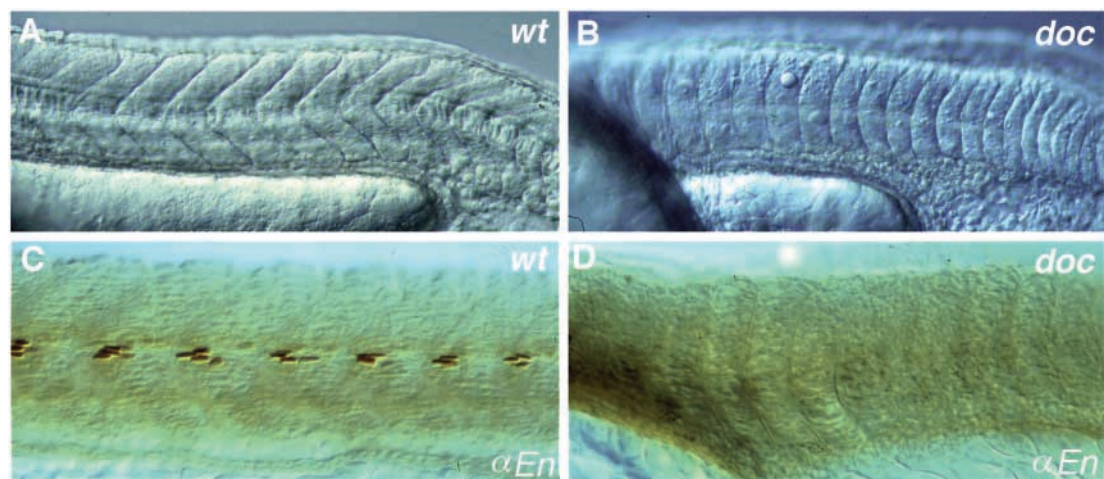
Fig. 5. Formation of the horizontal myoseptum is affected in *doc*[tt258] embryos. (A) Somites of a wild-type embryo showing the characteristic v-shape at 24 hours of development. (B) In *doc*[tt258] embryos, as in *flh*, *mom* and *ntl* embryos, the somites are u-shaped instead of v-shaped. (C) In the apex of the v, the nuclei of muscle pioneer cells stain with the antibody against Eng. (D) Eng staining in the somites is not detectable in *doc*[tt258] embryos, whereas expression in the midbrain-hindbrain boundary is normal (not shown). Lateral views, anterior is to the left.

Table 2. The expression of markers for the tailbud and axial mesoderm, for the floor plate and for the somites in early notochord formation mutants

	Marker						
	Tailbud and axial mesoderm				Floor plate	Somites	
Mutant	αNtl	*axial*	*twist*	*shh*	*shh*	*myoD*	αEng
flh	Present near tailbud	Present near tailbud	Present near tailbud	Present near tailbud	Patchy	Axial and adaxial, not maintained	Absent
mom	Present near tailbud	Present near tailbud	Present near tailbud	Present near tailbud	Patchy, individual cells	Axial and adaxial, not maintained	Absent in trunk
ntl	Absent in embryos with strong alleles	Absent in embryos with strong alleles	Absent in embryos with strong alleles	Absent in embryos with strong alleles	Broader (3 cells)	Very weak	Absent
doc	Normal early, later not maintained	Normal early, later not maintained	Normal early, later not maintained	Normal early, later not maintained	Normal (1 cell)	Initially adaxial, not maintained	Absent in trunk

all three alleles introduce stop codons in the N-terminal DNA binding domain and are expected to be amorphic.

In *flh* embryos, axial mesodermal cells appear to form somitic muscle cells instead of notochord. *myoD*, which normally marks the paraxial cells, is ectopically expressed in the axial mesodermal cells of *flh* (Halpern et al., 1995). Fate map studies support this finding (Melby et al., 1996). *mom* resembles *flh* with respect to the fate of the axial mesodermal cells. In contrast, axial mesodermal cells are present in *ntl* and *doc* embryos but remain undifferentiated. This suggests a role for *flh* and *mom* in the early specification of the axial mesoderm, preventing it becoming paraxial mesoderm instead. In *ntl* and *doc* mutant embryos, however, axial mesodermal development is initiated and notochord precursor cells are formed that fulfill some of the functions of the notochord, such as signalling to the ventral neuroectoderm, while signals required for the formation of a horizontal myoseptum by the paraxial mesoderm is impaired in embryos mutant of all four genes.

A phenotype reminiscent of the *flh* and *mom* phenotype has been described in mice. Mutant *HNF-3β* embryos do not form an organized node and notochord (Ang and Rossant, 1994; Weinstein et al., 1994). In addition, mutant embryos show marked defects in the organization of the somites and the neural tube. *axial* is the zebrafish homologue of *HNF-3β*

(Strähle et al., 1993). A linkage analysis between *mom* and *axial* is currently being performed.

The role of *ntl* for the expression of axial mesodermal markers

In order to investigate the function of the individual genes in notochord formation, we analyzed the expression of several notochord markers in *flh, mom, ntl* and *doc* mutants (summarized in Table 2). We found that *ntl* is expressed at the beginning of gastrulation in the axial mesoderm of *flh* and *mom* embryos. During later stages, however, *ntl* expression remains restricted to a small region close to the tailbud. Only in this *ntl* expressing region are other notochord specific markers, such as *twi*, *shh* and *axial* detectable. In more anterior regions, cells of the axial mesoderm now express adaxial markers (*myoD*, *snail*; Halpern et al. 1995). These findings suggest that *flh* and *mom* suppress adaxial development in these cells. In both mutants, disruption of axial mesoderm development is not absolute and faint expression of axial mesodermal markers such as *ntl* can be detected anterior to the tailbud in *flh* and *mom* embryos (Talbot et al., 1995; this study). It is possible that *flh* and *mom* have partially redundant functions in the specification of the axial mesoderm. Double mutant analysis will clarify this point.
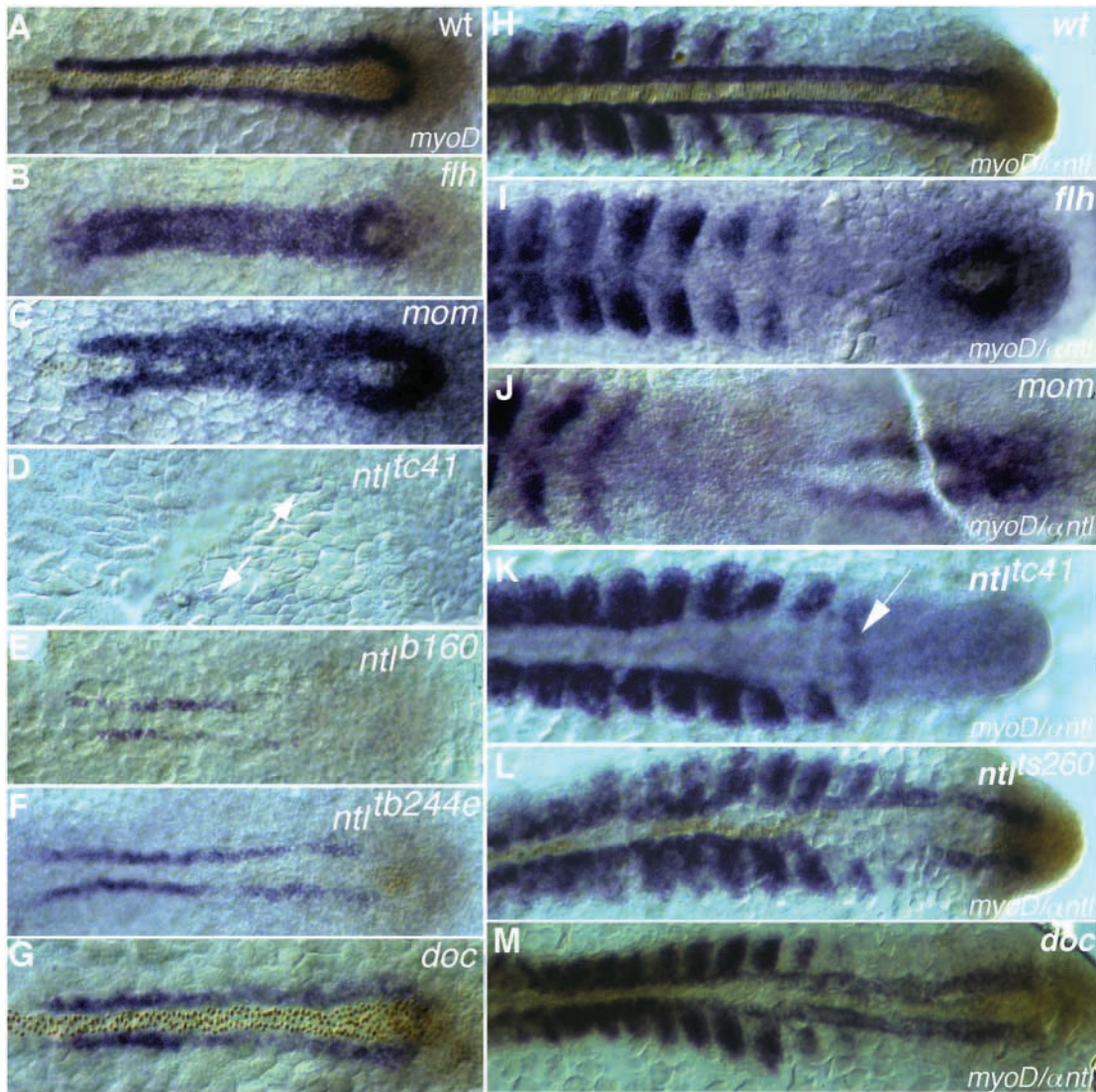
Fig. 6. Dorsal view of *myoD* RNA (blue staining) and Ntl antibody staining (brown nuclear staining) at (A-G) tailbud stage and (H-M) 15-somite stage of (A,H) wild-type, (B,I) *flh^tk241*, (C,J) *mom^th211*, (D,K) *ntl^tc41*, (E) *ntl^b160*, (F) *ntl^tb244e*, (L) *ntl^ts260* and (G,M) *doc^tt258* embryos. (A) In wild type at the tailbud stage *myoD* expression is restricted to a line of cells left and right of the notochord. (B,C) The gap between the two stripes is filled with *myoD* expressing cells in (B) *flh^tk241* and (C) *mom^th211* embryos. *ntl* expression is detectable only in the tailbud and sometimes in a few cells anteriorly, which do not express *myoD* (C). (D) Strongly reduced *myoD* expression a greater distance from the midline is detectable in embryos with the strong allele, *ntl^tc41* (arrows). *myoD* expression at the 15-somite stage in (H) wild type is expressed in the adaxial cells and in the posterior part of the somites. In (I) *flh^tk241* embryos and (J) *mom^th211* embryos *myoD* expression in the adaxial cells is not maintained. (K) In embryos with the strong allele, *ntl^tc41*, somites are separated by a big gap of nonexpressing cells, whereas the 15th somite is fused in the midline (arrow). (L) In embryos with the weak allele, *ntl^ts260*, adaxial cells next to *ntl*-expressing cells express *myoD* at a higher level than the somites on the opposite side. (M) In *doc^tt258* embryos adaxial expression in anterior regions is reduced and closer together.

In *doc* embryos, Ntl expression is initiated in the axial mesoderm but it is not maintained beyond early somitic stages. The expression of other notochord markers parallels the presence of Ntl. These notochord markers are not expressed in the axial mesoderm of strong *ntl* alleles. We propose that the similarity in phenotype of *doc* and *ntl* is explained by a function of *doc* in maintaining Ntl at high levels in the axial mesoderm, which in turn is required for the further development of the notochord, and the expression of *twi, shh* and *axial*.

Floor plate formation is dependent on the axial mesoderm in the zebrafish

The notochord in mutant *flh* embryos is missing, whereas the floor plate is disrupted in most regions of the embryo (Talbot et al., 1995). *flh* is expressed in the notochord and in the floor plate (Talbot et al., 1995) and therefore could have a function in both structures. However, descendants of transplanted mutant cells into wild-type embryos contribute to a morphologically normal floor plate, indicating that *flh* function in the floor plate is not necessary for its proper formation (Halpern
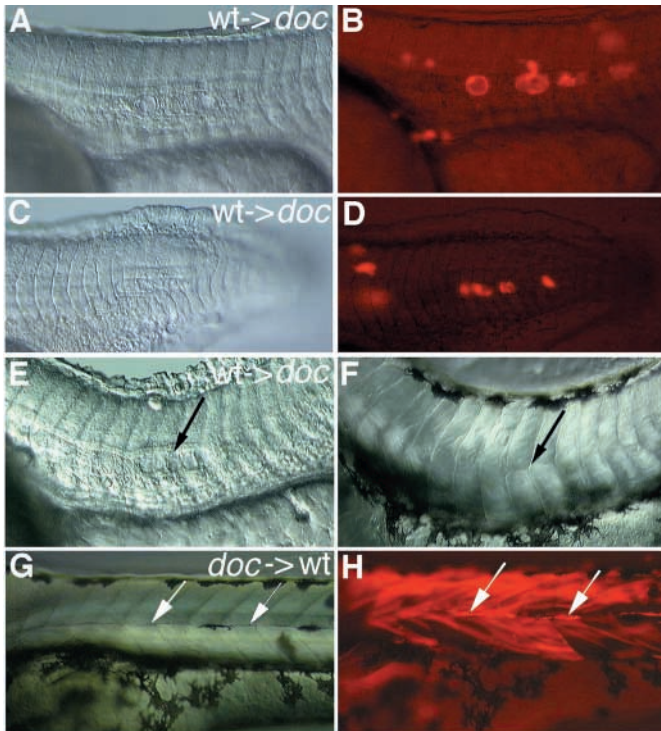
Fig. 7. (A,C,E,F,G) Nomarski optics and (B,D,H) fluorescence pictures of transplantation experiments performed in *doc^tt258* mutant embryos. Lateral view, anterior to the left. (A-D) Only wild-type derived donor cells in a mutant environment give rise to vacuolated notochord cells. Two different experiments are shown (A,B and C,D). (E) Wild-type cells in a mutant environment at 24 hours of development induce a horizontal myoseptum in some neighboring somites on day 3 (arrow in F). Mutant donor cells in wild-type somites give rise to normal horizontal myosepta (arrows in G,H), which can be identified by their strong fluorescence. In some regions this strong fluorescence is quenched by melanophores populating the horizontal myoseptum.

et al., 1995). Although the axial mesoderm is less affected in mutant *mom* than in *flh* embryos, the floor plate is affected more strongly. In *mom* embryos, floor plate cells often overly residual notochord cells, which is consistent with a direct induction of floor plate by the notochord. This is not the case for *flh* embryos in which the notochord is almost completely absent (as seen by the failure of the midline mesoderm to express notochord markers) while floor plate is formed to a substantial degree. It has been suggested that the midline mesoderm in *flh* retains some of its capacity to induce floor plate development, despite the absence of further notochord differentiation (Halpern et al., 1995). Alternatively, floor plate development in *flh* embryos could be induced early on while the presumptive ventral neural tube cells are still in contact with the axial mesoderm close to the shield. In *flh* and *mom* embryos these cells do express axial mesodermal markers. We suggest that floor plate development in normal embryos depends on both the initial specification of ventral neural tube during shield stage, and later, on the adjacent axial mesodermal cells, as has been shown for other vertebrate systems (Yamada et al., 1991; Placzek et al., 1991).

Evidence for a direct role of the axial mesoderm in floor

plate induction is provided by the examination of the floor plate in *ntl* embryos. In embryos with the strong *ntl* alleles the floor plate is broadened as indicated by the enlarged expression domains of *shh* in the ventral spinal cord (Fig. 4D,H). The enlargement of the *shh* domain correlates with the region occupied by undifferentiated axial mesoderm cells in *ntl* embryos (dependent on the phenotypic strength of the *ntl* allele). This region is also enlarged in *ntl* embryos as seen by *flh* expression (Talbot et al., 1995) and by the increased distance between the two bilateral stripes of adaxial cells expressing *myoD* (Fig. 6D,E) and *snail* (Hammerschmidt and Nüsslein-Volhard, 1993). We propose that cells of the ventral neural tube are induced to develop floor plate in *ntl* mutants by their contact with undifferentiated axial mesodermal cells. This induction does not require *ntl* (Halpern et al., 1993) or *doc*.

The broadened axial mesoderm in *ntl* embryos suggests that convergent extension in the axial mesoderm during gastrulation is affected. Our results are consistent with the finding that the *brachyury* gene, the mouse homologue of *ntl*, is required for convergent extension movements in the axial mesoderm (Yamada, 1994 and references therein).

Somite patterning is affected in mutant embryos of all four genes

The notochord in vertebrates plays a crucial role in somite patterning by inducing sclerotome in the presomitic mesoderm (reviewed by Bumcrot and McMahon, 1995). In the zebrafish the embryonic sclerotome is a rather small structure and specific markers expressed in this structure are not yet available. However, signals from the notochord seem to be required for the formation of muscle pioneer cells and the horizontal myoseptum (Halpern et al., 1993).

Somites are abnormally shaped in mutant embryos of *flh*, *mom*, *ntl* and *doc*. A morphologically distinct horizontal myoseptum does not form and Eng expression in the nuclei of muscle pioneer cells is not detectable in regions where a differentiated notochord is missing. Normal levels of adaxial *myoD* expression in *flh* and *mom* embryos early on indicate that a differentiated notochord is not necessary for the initiation of *myoD* expression. Strongly reduced levels of *myoD* expression in the adaxial cells were found in *ntl* mutant embryos (Weinberg et al., 1996). We find that the level of *myoD* expression in *ntl* embryos is dependent on the allele strength. Therefore *ntl* itself appears to have a function in the initiation of adaxial *myoD* expression to full levels. *ntl* is transiently expressed in the presumptive mesoderm (Schulte-Merker et al., 1992), and therefore could in principle function early in the paraxial precursor cells to activate *myoD* expression. However, *ntl* has been shown to be required cell autonomously in the notochord for the patterning of the adjacent paraxial mesoderm (Halpern et al. 1993). Furthermore, *shh* expressed ubiquitously in the embryo induces *myoD* in the entire paraxial mesoderm, even in *ntl* embryos (Weinberg et al., 1996). This suggests that the requirement for *ntl* in the induction of *myoD* is via its role in the expression of *shh*.

Reduced levels of *myoD* expression in the adaxial cells at later stages of development in *flh*, *mom* and *doc* embryos correlates with the loss of *ntl* expression in the axial mesoderm. In *doc* embryos Eng-expressing muscle pioneers are missing, even though the adaxial expression of *myoD* is maintained until later somite stages. As *doc* is also required autonomously in
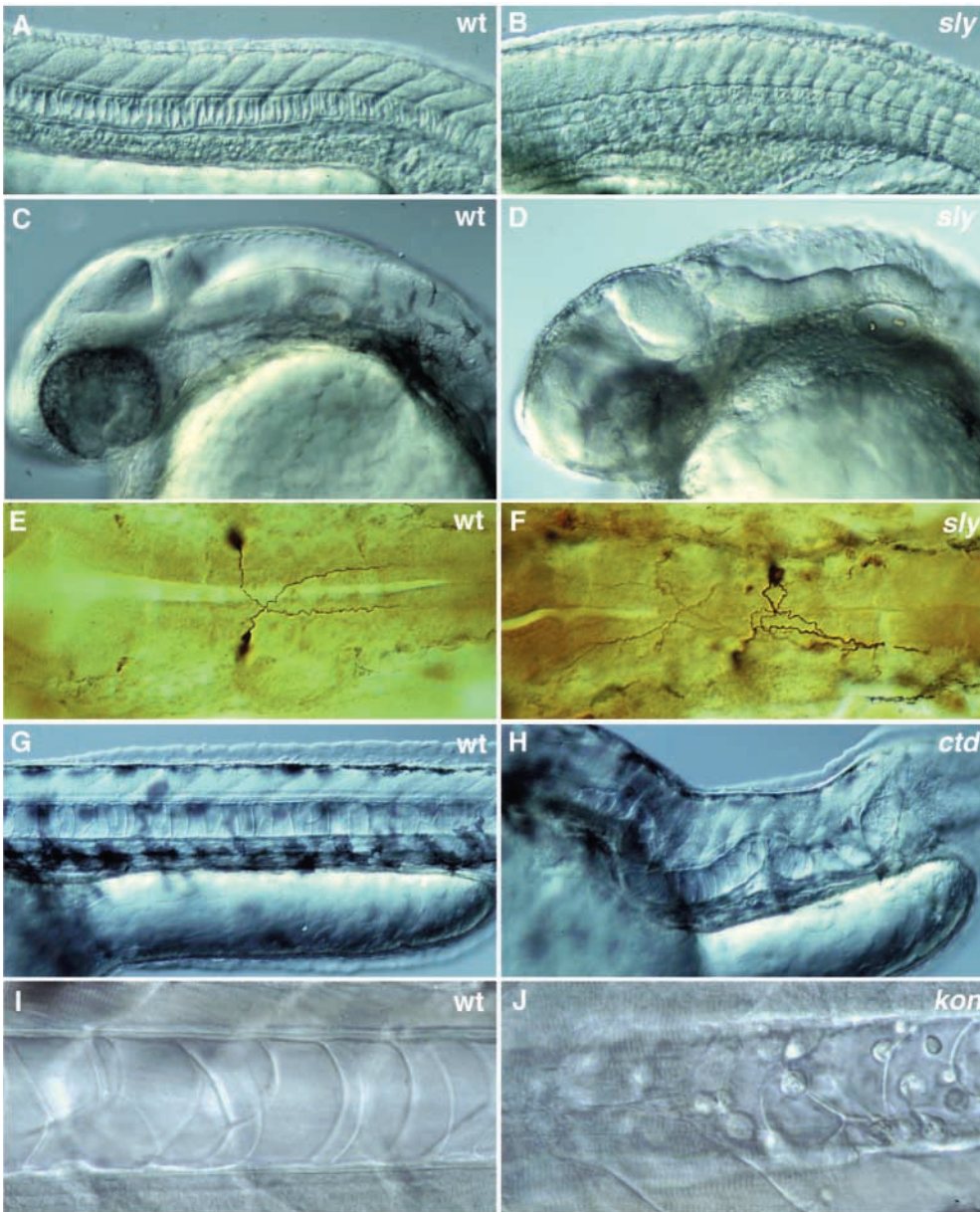
Fig. 8. Photographs of a variety of selected mutants with late notochord defects. (A-D) Lateral views of 1-day old wild-type and mutant zebrafish embryos. (A,C) 1-day old wild-type embryo. (B,D) 1-day old sly^{ti263a}. (A) In wild type, the notochord cells are vacuolated and the notochord has a 'stack-of-pennies' appearance. (B) In embryos with strong alleles of *sly*, the notochord is thinner and poorly vacuolated. In a lateral view, the brain of *sly* embryos looks very disorganized when compared to wild type (C,D). (E,F) Antibody staining of the Mauthner neurons in 1-day old embryos using the monoclonal antibody 3A10. (E) In wild type, the axons of the Mauthner neurons cross the midline and grow towards the posterior. (F) In *sly*, the axons of one or both Mauthner neurons frequently fail to cross the midline and grow posteriorly along the ipsilateral side. (G,H) Lateral views of 2-day old wild-type and mutant embryos. (G) Lateral view of wild type with a normal, straight notochord. (H) Lateral view of *ctd*, in which the notochord extends in a random fashion throughout the embryo. (I) Notochord of a 40-hour old wild-type embryo with an intact notochord sheath. (J) Notochord of a 40-hour old kon^{tc230} embryo. The vacuolated notochord cells are surrounded by undifferentiated round cells that failed to become notochord sheath cells.

the notochord, maintained notochord development or a signal downstream of *doc* appears to induce muscle pioneers in a subset of the *myoD*-expressing adaxial cells. Mutations affecting this signal or its reception are expected to display a somite phenotype similar to that observed in *flh*, *mom*, *ntl* and *doc* mutants, while retaining a normal notochord. During the course of this screen, six other genes were identified with defects in the horizontal myoseptum and the muscle pioneers (*chameleon* (*con*), *choker* (*cho*), *sonic-you* (*syu*), *u-boot* (*ubo*), *you* and *you-too* (*yot*); van Eeden et al., 1996). In contrast to the notochord mutants described in this study, these *you*-type mutants do not show any morphological notochord defects. However, *myoD* expression is severely reduced or absent in adaxial cells of embryos mutant for *you*, *yot*, *syu*, and *con* (van Eeden et al., 1996). While *ntl* and *doc* are shown by mosaic analysis to be required in the notochord, similar experiments performed in *yot* and *ubo* indicate a requirement for these two genes in the somites, suggesting a function in the reception of

the signal from the notochord. The similarity between the somite phenotypes of *you*-type mutants and that of *flh*, *mom*, *ntl* and *doc* indicate that our screen has identified genes involved in different steps of a signaling pathway from the notochord to the somites.

APPENDIX

Mutants with late notochord defects

In addition to the mutants with defects in early notochord formation we have identified a number of mutants with later notochord defects (Tables 3 and 4, Fig. 8B). Mutations in six genes, *sleepy (sly)*, *grumpy (gup)*, *bashful (bal)*, *happy (hap)*, *sneezy (sny)* and *dopey (dop)*, produce embryos without fully differentiated notochords. The defects in notochord differentiation result in short embryos with thin notochords that are poorly vacuolated. Although the somites of mutant embryos

Table 3. Mutations resulting in undifferentiated notochords or undulating notochords

Gene	Alleles	Notochord phenotype	Other phenotypes	References
sleepy (sly)	*te223, te333, tf215b, ti263a, ti272a, to216a, tm89, tp16, ts33a*	Undifferentiated notochord	Disorganized brain, abnormal somites, retinotectal pathfinding	a, b
grumpy (gup)	*tg210, ti228b, tj229a, tx221, tl17b, tm61, tp42*	Undifferentiated notochord	Disorganized brain, abnormal somites, retinotectal pathfinding	a, b a, b
bashful (bal)	*tb244f, tc245a, tc248f, tf209, tf235, tm220a, tm267a, to265, tq210, tr203, tr259, tt206, tp82, tp86, tv36*	Undifferentiated notochord	Disorganized brain, abnormal somites, retinotectal pathfinding	a, b
happy (hap)	*tc229, te239, tm285, tr278, ty230, tk56a*	Undifferentiated notochord	Abnormal somites	a
sneezy (sny)	*td204a, tn211, tq249b, tm11, tm75*	Undifferentiated notochord	Abnormal somites	a
dopey (dop)	*tr222b, tz226, tm18a*	Undifferentiated notochord	Abnormal somites	a
crash test dummy (ctd)	*tc36, tl43b, tw38g*	Undulating notochord	Distorted body axis	a
zickzack (ziz)	*tq286b, tf4d*	Undulating notochord	None	a
quasimodo (qam)	*ta81, tb244c, tf208, tm138b, tw25a, ty41*	Undulating notochord	Pale melanin pigmentation	a, c
kinks (kik)	*tl209*	Kinky, wavy notochord		a
wavy tail (wat)	*tm303a*	Undulating notochord in tail, homozygous viable		a

References: a, this paper; b, Karlstrom et al., 1996; c, Kelsh et al., 1996.

Table 4. Mutations causing a variety of later notochord defects

Gene	Alleles	Phenotype	References
blobbed (blo)	*tm289*	Notochord curved up in tip of tail, neurocoel ending in a blob, homozygous viable	a
punkt (pun)	*tl39, tk22, te380, tp219d*	Degenerating notochord, reduced melanophore and iridophore pigmentation, lethal	b
korken (kon)	*tc230*	No notochord sheath, reduced motility, jump up once after touch, lethal	a
lucky (luc)	*tm95c, ty18*	Degenerating notochord, reduced motility, homozygous viable	a
	tc248b	Local notochord degeneration	a
	tc265b	Patchy notochord degeneration, reduced motility, homozygous viable	a
	tc313	Notochord degeneration, homozygous viable	a
	tc323a	Irregular notochord edges, homozygous viable	a
	te353	Anterior local notochord degeneration, homozygous viable	a
	tf244	Notochord degeneration, lethal	a
	tg209f	Patchy notochord degeneration, homozygous viable	a
	tk259	Degenerating notochord, reduced motility, lethal	a
	tm70d	Interrupted notochord	a
	tm97d	Patchy notochord degeneration, variable amounts of melanophores on lateral stripe, lethal	a,b
	tm130e	Patchy notochord degeneration, homozygous viable	a
	tm339	Notochord degeneration, reduced body length, lethal	a
	tn21	Patchy notochord degeneration, homozygous viable	a
	to2a	Variable notochord degeneration, pale melanophore pigmentation, lethal	a, b
	tp65	Irregular notochord edges	a
	tu33	Local notochord degeneration	a
	tu46	Notochord degeneration, pale melanophore and xanthophore pigmentation, lethal	a, b
	tv214a	Degenerating notochord	a

References: a, this paper; b, Kelsh et al., 1996.

appear disorganized, Eng-expressing muscle pioneer cells are present. In addition, three of these mutants (*sly*, *gup* and *bal*) also show a severe disorganization of the overall brain morphology (Fig. 8D) and a number of axons that normally cross the midline grow abnormally in *sly*, *gup* and *bal*. In particular retinotectal axons frequently turn rostrally after crossing the midline or project to the tectum on the ipsilateral side instead of crossing the midline (Karlstrom et al., 1996). Similarly, we found that in *sly* the Mauthner cell axons frequently fail to cross the midline and grow along the ipsilateral side (Fig. 8F).

We have isolated mutations in five genes, which lead to undulations in the normally straight and rod-like notochord. In *crash test dummy* (*ctd*), the notochord is bent variably in many positions, causing severe distortions of the body axis (Fig. 8H). In *zickzack* (*ziz*), the notochord specifically undulates sideways at regular intervals along the body axis. In *quasimodo* (*qam*), there are irregular bulges and thickenings of the notochord that increase in size over time. In addition to the notochord phenotype, *qam* also displays reduced melanophore pigmentation in the body and in the eyes. Mutations of *kinks* and *wavy tail* lead to undulations and kinks in the notochord predominantly in the tail.

The vacuolated notochord cells are surrounded by an epithelial monolayer of cells termed the notochord sheath. The notochord sheath is thought to keep the notochord cells in a linear array, resulting in the rod-like stiff structure of the notochord. Embryos mutant for *korken* (*kon*) do not form the notochord sheath. Instead, round cells that look similar to undifferentiated notochord cells are present all around the periphery of the notochord at 40 hours of development whereas

the vacuolated cells of the notochord appear normal (Fig. 8J). Embryos homozygous for *kon* also have reduced motility and only jump once after touch.

26 mutants display a variety of other notochord defects, mostly notochord degeneration (Table 5). 10 of these mutants are homozygous viable.

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