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The mouse gene *Noto* is expressed in the tail bud and essential for its morphogenesis

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Running title: *Noto* in the tail bud

ABSTRACT

The mouse transcription factor *Noto* is expressed in the notochord and involved in its development. *Noto* mouse mutants, $Noto^{tc/tc}$ (*truncate*) and $Noto^{GFP/GFP}$ (*Noto* null-mutant), exhibit a segmental lack of the notochord in the caudal part of the embryo, and subsequent tail truncation in the adult animals. In order to address the relationship between the tail bud (the undifferentiated mesenchymal cells in the tip of the embryo tail) and the caudal notochord, $Noto^{GFP/GFP}$ a loss of function mutant was analyzed. Taking advantage of the $Noto^{GFP/+}$ heterozygotes we could track *Noto-GFP* expressing cells from the tail bud, over the tail cord, to the caudal notochord, and confirm a morphological continuum from the tail bud mesenchyme to the caudal notochord. Loss of *Noto* disturbed the tail bud morphogenesis: *Noto-GFP* expressing cells were scattered in the tail bud mesenchyme and instead at the tail cord they segregated in the notochord-like structure within the medullary cord, which subsequently disappeared. In the tail cord, instead of the notochord, additional lumen of the tail gut was formed. These findings suggest that *Noto* is involved in both rearrangement and morphogenesis of the tail bud during notochord formation.

Key words: *Noto*, tail bud, notochord, tail, mouse

INTRODUCTION

The vertebrate body plan is established during gastrulation when the three germ layers are formed. Nevertheless, after gastrulation is completed and the primitive streak has disappeared, considerable axial elongation of the embryo is taking place giving rise to the caudal part of the embryo. However, the origin of nascent cells in the caudal region, and their differentiation potential remain unclear. One of the suggested sources for the caudal structures is the group of mesenchymal cells in the caudal tip of the embryo (the tail bud). Development of the caudal structures from the tail bud is referred to as secondary body formation [Holmdahl, 1925; Catala et al., 1995; Davis and Kirschner, 2000; Mitrecic et al., 2004; McGrew et al., 2008]. Another concept proposes that the caudal structures are extensions of the corresponding cell populations predetermined during the gastrulation process (referred to as primary body formation). Although pluripotent potential of the tail bud was suggested [Tam, 1984; Hall, 2000; Cambray and Wilson, 2002; McGrew et al., 2008], it was shown that the tail bud is not a uniform blastema, but it is regionalized by distinct domains of gene expression [Gofflot et al., 1997; Cambray and Wilson, 2007].

During rodent morphogenesis, development of the posterior part of the body includes formation of the three axial tail structures: the neural tube, the notochord, and the tail gut, while differentiation of the paraxial mesenchyme leads to the formation of the somites. Morphological analyses showed that the mesenchymal cells of the tail bud aggregate in the medullary and the tail cord. The medullary cord gives rise to the secondary neural tube by rearrangement of cells which exhibit mesenchymal – epithelial transformation (a process referred to as secondary neurulation) [Schoenwolf, 1984]. Within the tail cord, the notochord and the tail gut formation takes place [Gajovic et al., 1989, 1993; Mitrecic et al., 2004].

Despite the morphological continuum of three tail axial structures (the neural tube, the notochord, and the tail gut) and the tail bud, their origin from the tail bud remains questionable.

To address the question whether in the mouse the tail notochord originates from the tail bud, we have analysed the expression of *Noto-GFP* and the consequences of *Noto* loss of function during tail bud development. *Noto* is a gene responsible for *truncate* (*Noto*$^{tc/tc}$) mutation, characterized by segmental loss of the notochord in the caudal part of the mouse embryo, which subsequently leads to the tail truncation in the adult mice [Abdelkhalek et al., 2004; Mitrecic et al., 2004]. During gastrulation, *Noto* is involved in node morphogenesis and migration of nodal and notochordal precursors [Beckers et al., 2007; Yamanaka et al., 2007]. As morphological analyses of *truncate* (*Noto*$^{tc/tc}$) hypomorphic mutant indicated that the notochord malformations were related to the changes in the tail bud [Mitrecic et al., 2004], newly developed *Noto*$^{GFP/GFP}$ mouse null-mutant [Abdelkhalek et al., 2004] was used to address the given question. Its advantage was that *Noto* activity is completely abolished and that *Noto* expression could be traced using in frame GFP expression.

The analyses showed that the loss of *Noto* disturbed tail bud morphogenesis, suggesting the function of *Noto* in the notochord formation from the tail bud cells.

MATERIALS AND METHODS

Isolation of embryos and genotyping

Noto$^{GFP/GFP}$ homozygous and heterozygous embryos aged 11.5 days were used. (morning of plug discovery = 0.5 days). Embryos were isolated from the uterus and the extra-embryonic membranes were removed. Embryo genotyping was performed according to Abdelkhalek [Abdelkhalek et al., 2004]. For the purpose of this work 30 homozygous, 30 heterozygous, and 10 wild type embryos from 10 different litters were used.

Analyses of semithin sections

The posterior parts of the embryos were separated and immersed in a mixture of 1% paraformaldehyde and 1% glutaraldehyde in 0.1M phosphate buffer. After fixation for 2 h the specimens were washed in the buffer and postfixed for 1 h in 1% osmium tetroxide. The specimens were dehydrated in ascending concentrations of ethanol and embedded in Durcopan (Fluka). Serial semithin sections (perpendicular to the longitudinal tail axis) were obtained on a Reichert-Jung UltracutE ultramicrotome. They were stained with toluidine blue and analyzed by light microscopy.

Confocal microscopy

Embryos used for direct confocal visualization of GFP flourescence were isolated in phosphate buffer. Caudal region of the body was carefully transferred to the slide and attached

with Aquatex (Merck). Zeiss LSM 510 Meta confocal microscope and Zeiss software Axio Vision 4.7.1 for photo analyses were used.

Imunoflourescence against GFP

Immunohistochemical reaction on semithin sections was performed using direct imunflouresecence visualizations. As immunohistochemistry on plastic sections is burdened by nonpenetrable material, two approaches from literature were tested. First approach was based on etching, bleaching and proteolysis [D'Alessandro et al., 2004] and another one on etching and citrate buffer antigen retrieval [Groos et al., 2001]. As the second protocol yielded satisfactory results, data presented in this publication were obtained using this approach. Briefly, sections were immersed in 10-50% NaOH in absolute alcohol for 30 – 60 minutes. After rinsing in phosphate buffer, sections were transferred to 0.01 M citrate buffer pH 6.0 and boiled in microwave oven around 15 minutes. After cooling in phosphate buffer, routine immunohistochemitry protocol using primary antibody against GFP (polyclonal rabbit, A6455, Invitrogen), and Alexa 594 (A11012, Invitrogen) secondary antibody, both in 1:100 dilution, was applied. Obtained immunohistochemical signals were analyzed and photographed using Zeiss Axiovert 200M fluorescent microscope.

RESULTS

Expression of Noto-GFP is present in the tail bud

In order to get insight into the relation between notochord and the tail bud, the expression of notochordal marker *Noto* was addressed. As the tail bud region is rather small and a very precise expression localization analyses was needed, three levels of its visualization were used with increasing spatial resolution: whole mount embryos, sections of the whole mounts, and the immunochemistry of the GFP protein on serial semi-thin sections embedded in epoxy resin. These three levels of analyses clearly revealed that *Noto-GFP* is expressed in the caudal notochord and that its expression extends to the tail bud region (Figs. 1, 2 and 3). In the tail bud region, the expression is wider when compared to those in the narrow notochord. Analyses of serial sections using both direct confocal visualization of GFP fluorescence and immunohistochemistry against GFP revealed that *Noto-GFP* expressing cells reside in the tail bud, in the dorsal portion of the tail cord (Fig. 4) and in the notochord (Fig. 5). In addition, some single *Noto-GFP* expressing cells were found in the surrounding mesenchyme (arrow in Fig. 1B).

Noto mutation affects the morphology of the tail bud and its relation to the notochord

The important function of *Noto* in the development of the caudal notochord is revealed in the mutant phenotype, i.e. notochord is missing specifically in the tail region [Abdelkhalek et al., 2004; Mitrecic et al., 2004]. Our hypothesis was that if the notochord develops from the tail bud, a mutant embryo should exhibit changes in the tail bud phenotype. Moreover, we expected to find more pronounced phenotype in the null-mutants $Noto^{GFP/GFP}$ compared to *tc*

hypomorphs. To test this hypothesis, tail bud phenotype in *Noto*$^{GFP/GFP}$ mutants was analyzed, combining the ability to identify *Noto-GFP* expressing cells by GFP activity and the insight in detailed morphology using 1 µm thick serial semi-thin epoxy resin embedded sections.

Lack of *Noto* caused disturbances of notochord development in the caudal part of *Noto*$^{GFP/GFP}$ strain (Figs 7, 8). Although in homozygotes the morphology of undifferentiated cells of the tail bud appeared comparable to the wild type control embryos (Figs. 7A and 8A vs. 6A), the rearrangement and differentiation of the tail bud cells was different in the mutants (Figs. 7B, 8B). The additional group of cells was found in the ventral part of the medullary cord (marked by X in the lower third of the structure marked by M in Fig. 7B). In the dorsal part of the tail cord another lumen of the tail gut was formed (Figs. 7C,D; 8C,D). The group of cells situated in the ventral region of the medullary cord formed a small group of cells toward the base of the tail resembling the notochord (arrow in Figs. 7C,D; 8C,D). This structure disappeared in the more cranial sections (Figs. 7E, 8E). The ventral group of cells which formed the additional lumen of the tail gut fused with the principal tail gut lumen and/or disappeared (Figs. 7E, 8E). In 3 analyzed embryos (10% of homozygous), two medullary cords forming two secondary neural tubes were found (arrowheads in Figs. 8C,D,E). In comparison to *Noto*$^{tc/tc}$ hypomorphs, *Noto*$^{GFP/GFP}$ null-mutants exhibited more pronounced phenotype: 1) the additional group of cells found in the ventral region of the medullary cord was much bigger in *Noto*$^{GFP/GFP}$ than in *Noto*$^{tc/tc}$ mutants, 2) the number of notochord fragments in *Noto*$^{tc/tc}$ mutants ranged from 2 to 5, while in *Noto*$^{GFP/GFP}$ scattered *Noto* expressing cells did not form additional fragments, and 3) two medullary cords/neural tubes were found only in *Noto*$^{GFP/GFP}$ mutants.

In Noto null-mutant Noto-GFP expressing cells are irregularly distributed within the tail bud

In order to reveal the consequences of *Noto* loss of function on the *Noto* expressing cells, they were traced in homozygous embryos with the help of GFP. Whole mount analysis revealed that *Noto-GFP* expressing cells can be found in the tail bud of the homozygous embryo (Figs. 9 and 10). However the cells which express *Noto-GFP* were not confined to strictly demarcated region but rather irregularly distributed within the tail bud mesenchyme (Fig. 10). In the heterozygotes during tail bud morphogenesis the expression of *Noto* was present in the dorsal part of the tail cord, but this was not the case in the homozygotes. *Noto-GFP* expression was confined only to the small group of cells which on the toluidine stained semi-thin sections resembled notochord (arrow in Fig. 11). This notochord-like structure, which readily disappeared in the cranial direction, developed not within the tail cord, but within the medullary cord. In addition, *Noto-GFP* positive cells were found in the paraxial mesenchyme adjacent to the notochord (arrowheads in Figs. 9B, 11, 12). Compared to the heterozygotes, in homozygotes clearly more *Noto-GFP* positive cells were found outside the notochord region.

DISCUSSION

This work intended to analyze the function of *Noto* during the tail bud development and to address the question of the potential of the tail bud mesenchyme. As previous analyses of *tc* hypomorph suggested that the disturbed tail bud morphogenesis is responsible for disturbed development of the notochord [Mitrecic et al., 2004], $Noto^{GFP/GFP}$ offered the possibility to further analyse the observed phenotype using the null-mutant. The continuity of *Noto-GFP* expressing cells from the undifferentiated tail bud mesenchyme to the notochord supported our hypothesis that mesenchymal cells in the tip of the embryo could represent a source of the caudal notochord: *Noto-GFP* expressing cells from the tail bud mesenchyme in the more cranial sections segregated in the dorsal portion of the tail cord, and consequently formed *Noto*-expressing-notochord parallel with the tail gut formation in the ventral part of the tail cord. Thus, the notochord identity in the caudal part of the embryo seemed to correlate with the expression of *Noto*. A similar finding was obtained regarding expression of *T* gene: there is continuity from the notochord to the tail bud [Dietrich et al., 1993].

The *Noto* function during differentiation of the tail bud mesenchyme toward notochord was revealed in $Noto^{GFP/GFP}$ homozygous. There were three main findings in $Noto^{GFP/GFP}$ homozygotes: (1) irregularly distributed *Noto-GFP* expressing cells in the tail bud mesenchyme, (2) *Noto-GFP* expressing cells were located in the notochord-like structure originating from the medullary cord, and (3) these cells were missing in the tail cord, where instead of the notochord the additional lumen of the tail gut appeared. The morphology of the notochord development in $Noto^{GFP/GFP}$ was similar, but the phenotype was more severe,

compared to the one caused by *truncate* (*Noto*$^{tc/tc}$) mutation [Mitrecic et al., 2004]. Lack of *Noto* on the tail bud morphology was accompanied by the improper location of *Noto-GFP* expressing cells during the tail bud morphogenesis, hence *Noto* function in the tail bud seems to be clearly related to the formation of the tail notochord. We could speculate that the tiny notochord-like structure which indeed expressed *Noto* as a notochordal marker represents a result of *Noto* independent developmental mechanism, which is more similar to those in the trunk of the embryo, where notochord and the neural plate develop in a close relationship [Le Douarin and Halpern, 2000]. Nevertheless, these notochord-like cells represent not a complete substitute for the notochord, they were discontinuous, and as a consequence, the loss of notochord and the tail truncation developed in these animals [Abdelkhalek et al., 2004; Mitrecic et al., 2004]. This could be compared to the switch from the axial toward paraaxial fate reported in *flh*, zebrafish counterpart of *Noto*. *Flh* is expressed in the notochord and floor plate of early zebrafish embryos [Talbot et al., 1995] its mutants lack notochord, and the cells expressing *flh* in the *flh* mutants contribute to the differentiation of the muscle [Halpern et al., 1995].

The idea of the secondary body development as an alternative mechanism of body formation was previously questioned as well at the level of universal applications of biology principles [Handrigan, 2003; McGrew et al., 2008]. The opponents claimed the existence of the unique mechanism, i.e. gastrulation, extending into the embryo tail [Catala et al., 1995; Gofflot et al., 1997]. However, the uniqueness of the mechanism clearly does not exist in the case of notochord – not only at the tail vs. trunk level, but as well not in other regions of the embryo body. The analysis of gene expression patterns suggested that at least 3 parts of the notochord corresponding to the 3 ways of notochord formation can be distinguished: anterior head process, trunk notochord, and the tail notochord [Yamanaka et al., 2007]. The fourth way of

the notochord formation was actually suggested long ago and it corresponds to secondary notochord development from the tail bud mesenchyme [Holmdahl, 1925; Gajovic et al., 1989; Mitrecic et al., 2004]. Our findings are in line with the fourth way of the notochord formation, although the presented descriptive data need further experimental support. The secondary notochord formation would include differentiation of the tail bud mesenchyme, formation of the tail cord, and subsequent separation of the notochord and the tail gut. The notochord in the tail would develop by two different mechanisms related to the switch from the primary (i.e. gastrulation) toward secondary body development. While the primary tail notochord develops from the node derived cells which migrate to the caudal part of the embryo before the tail bud stage, the secondary tail notochord would be derived from the tail bud mesenchyme. Analogous to the secondary neurulation, the secondary (tail bud derived) notochord formation would denote one more tail specific developmental mechanism. The existence of the tail-specific (i.e. secondary) morphogenetic mechanisms is indeed supported by the tail phenotype of the *Noto*$^{GFP/GFP}$ mutants and the corresponding tail bud-specific function of *Noto*.

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FIGURE LEGENDS

Figures 1-5.

Noto expression in the caudal part of 11.5 days old *Noto*$^{GFP/+}$ heterozygous embryo.

1A-C. Longitudinal whole mount confocal photos reveal that Noto-GFP reaches the tip of the embryo (tail bud region). In addition, some Noto-GFP expressing cells not belonging to the notochord were found (arrow in 1B).

2A-C. Higher magnification of longitudinal whole mount confocal photos visualized Noto-GFP cells. The red signal corresponds to red blood cells within blood vessels.

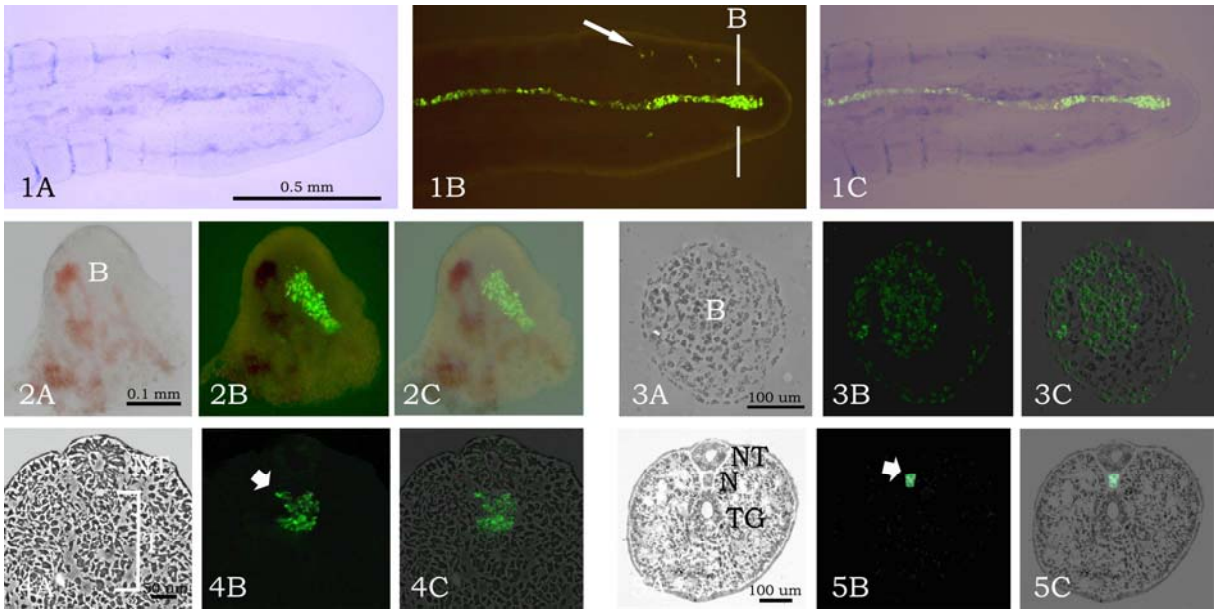
3-5. GFP immunohistochemistry performed on semithin sections of the tail bud region at progressively more cranial levels.

3A-C. Noto-GFP positive cells in the tip of the embryo are clearly visible.

4A-C. In more cranial sections, Noto-GFP expressing cells were present in the dorsal portion of the tail cord (broad arrow in 4B).

5A-C. Even further cranial, Noto-GFP expressing cells were present in the notochord (broad arrow in 5B).

B – tail bud, **T** – tail cord, **NT** – neural tube, **N** – notochord, **TG** – tail gut.


1A
0.5 mm
1B
B
1C
B
2A
0.1 mm
2B
2C
B
3A
100 um
3B
3C
4B
4C
NT
N
TG
100 um
5B
5C

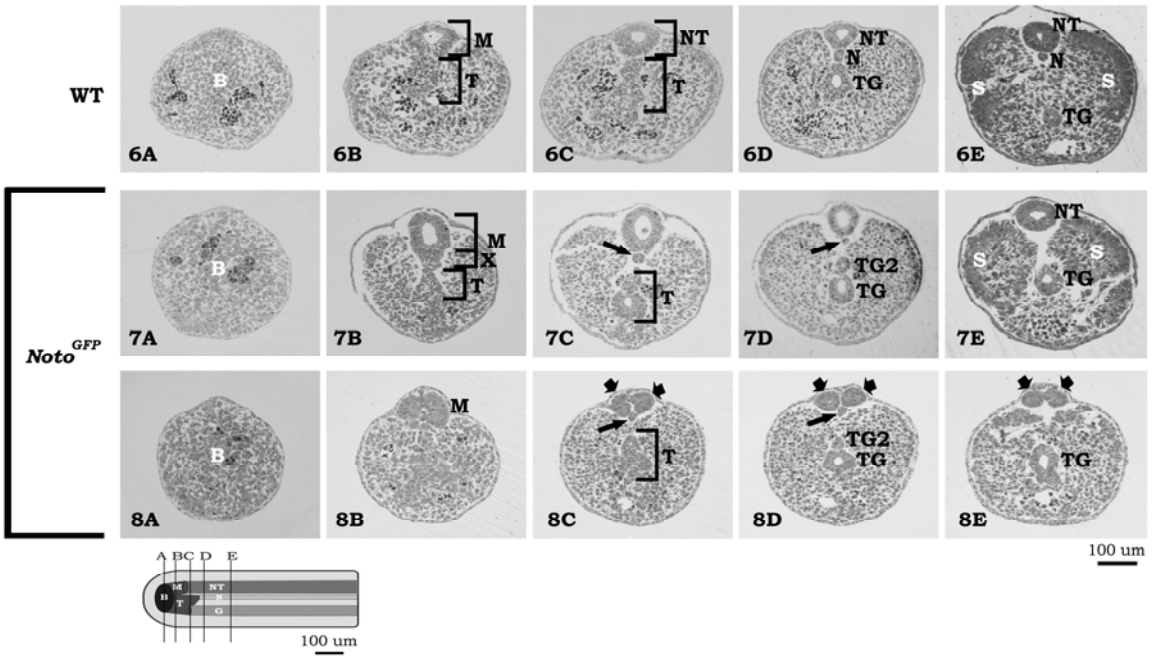
Figures 6-8.

Selected serial cross sections through the tip of the tail arranged in direction toward the base of the tail.

6A-E. In the tip of the 11.5 days old wild type mouse embryo (Fig. 6A), the tail bud mesenchyme (B) is visible. In the more cranial sections, the tail bud is continuous via the tail cord (T) and the medullary cord (M) to the notochord (N) and the tail gut (TG). In paraaxial mesenchyme, somites are formed (S).

7A-E and 8A-E. In *Noto*$^{GFP/GFP}$ embryos (Figs. 7A-E, 8A-E), differentiation of the tail bud is disturbed: notochord-like cells are found in the region of the ventral medullary cord (region X in Fig. 7B). In the region where in the wild type embryo notochord is found, small irregularly shaped group of cells resembling notochord are present (arrows in Figs. 7C,D and 8C,D). These cells disappear in the more cranial sections (Figs. 7E, 8E). Instead of the notochord, additional lumen of the tail gut is formed (TG2 in Figs. 7D and 8D). In some embryos, two medullary cords/neural tubes were found (arrowheads in Figs. 8C-8E).

B – tail bud, **M** – medullary cord, **T** – tail cord, **NT** – neural tube, **TG** – tail gut, **TG2** – additional tail gut, **S** – somites.


WT
Noto GFP
6A
6B
6C
6D
6E
7A
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7C
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8C
8D
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TG2
100 um
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100 um

Figures 9-12.

Expression of *Noto* in the caudal part of 11.5 days old *Noto*$^{GFP/GFP}$ homozygous embryo.

9A-C. Longitudinal whole mount confocal photo reveals that *Noto* reaches the tip of the embryo (tail bud region) (Figs. 9B,C).

10A-C. Immunohistochemistry performed on semithin sections reveals that the morphogenesis of the tail bud region is disturbed.

11. Immunohistochemistry performed on semithin sections reveals that instead of the notochord, additional lumen of the tail cord is formed, and *Noto-GFP* positive cells were present only in the small portion of this group of cells (arrow in Fig. 11). *Noto-GFP* positive cells were also found in adjacent paraaxial mesenchyme (wide arrows).

12. Longitudinal whole mount confocal photo reveals *Noto-GFP* expressing cells within differentiating somites. The same can be seen marked by wide arrows in Figs. 9B and 11.