A role for N-cadherin in mesodermal morphogenesis during gastrulation

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Abstract

Cell adhesion molecules mediate numerous developmental processes necessary for the segregation and organization of tissues. Here we show that the zebrafish biber (bib) mutant encodes a dominant allele at the N-cadherin locus. When knocked down with antisense oligonucleotides, bib mutants phenocopy parachute (pac) null alleles, demonstrating that bib is a gain-of-function mutation. The mutant phenotype disrupts normal cell–cell contacts throughout the mesoderm as well as the ectoderm. During gastrulation stages, cells of the mesodermal germ layer converge slowly; during segmentation stages, the borders between paraxial and axial tissues are irregular and somite borders do not form; later, myotomes are fused. During neurulation, the neural tube is disorganized. Although weaker, all traits present in bib mutants were found in pac mutants. When the distribution of N-cadherin mRNA was analyzed to distinguish mesodermal from neuroectodermal expression, we found that N-cadherin is strongly expressed in the yolk cell and hypoblast in the early gastrula, just preceding the appearance of the bib mesodermal defects. Only later is N-cadherin expressed in the anlage of the CNS, where it is found as a radial gradient in the forming neural plate. Hence, besides a well-established role in neural and somite morphogenesis, N-cadherin is essential for morphogenesis of the mesodermal germ layer during gastrulation.

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Introduction

Here we report on the phenotype and the molecular identity of the biber (bib) mutant identified in the Tübingen large-scale screen for mutations that affect morphogenesis in the zebrafish, carried out at the Max Planck Institut für Entwicklungsbiologie (Haffter et al., 1996). In the initial characterization of the mutant, it was placed into the gastrulation and tail class, where it was described briefly as a simple homozygous recessive lethal, having indistinct somites and a disorganized tail (Hammerschmidt et al., 1996). Phenotypically unique amongst the mutants found in the screen, bib was never successfully placed in a complementation group.

In the first portion of this work, we demonstrate that the bib mutation is in a single gene that encodes the zebrafish homolog of N-cadherin. One of the earliest calcium-dependent cell adhesion molecules identified, N-cadherin was first described in the CNS of the mouse and chicken (Hatta and Takeichi, 1986), and is now grouped with E-, P- and R-cadherins into a subfamily of cadherins known as the classic cadherins (Takeichi et al., 1988). In its most well-known role, N-cadherin is strongly expressed in the neural ectoderm (Hatta and Takeichi, 1986; Iwai et al., 1997; Radice et al., 1997; Redies et al., 1993; Redies and Takeichi, 1993), where its expression pattern is complementary to the expression of E-cadherin in the epidermal ectoderm (Takeichi, 1995). In general, the pattern of expression during embryogenesis seems to correlate with changes in cell shape and motility, suggesting a potential role in the epithelial to mesenchymal transition (Duband et al., 1988; Hatta et al., 1987; Oda et al., 1998). N-Cadherin tends to be expressed in mesenchymal cell types whereas E-cadherin is expressed in epithelial cell types. This complementary expression and involvement in tissue integrity is also seen in the progression of many cancers, for very often E-cadherin expression is lost in metastastic cells, while the expression of N-cadherin is elevated (Derycke and Fracke, 2004).

The N-cadherin protein is characterized by five extracellular cadherin (EC) repeats, a single pass transmembrane domain and a cytoplasmic domain. The EC repeats are extremely conserved among their homologs in other species, and are necessary for the specific adhesion properties of the cadherins (Blaschuk et al., 2007).
The cytoplasmic domain of the protein binds alpha- and beta-catenin, and indirectly actin. This anchors the protein to the cytoskeleton of the cell and connects the molecule to the WNT and other signaling pathways (Christofori, 2003; Derycke and Fracke, 2004; Gumbiner and McCrea, 1993; Herrenknecht et al., 1991; Kintner, 1992; McCrea et al., 1991; Sanson et al., 1996). The protein is thought to function as a dimer, and the EC repeats are necessary for this dimerization (Briehler et al., 1996; Nagar et al., 1996; Shapiro et al., 1995). Based on EC domain switching studies, the adhesive specificity of cadherins is primarily conferred by the first EC domain (Blaschuk et al., 1990; Nose et al., 1990). The protein is thought to function as a dimer, and the EC repeats are necessary for this dimerization (Briehler et al., 1996; Nagar et al., 1996; Shapiro et al., 1995). Based on EC domain switching studies, the adhesive specificity of cadherins is primarily conferred by the first EC domain (Blaschuk et al., 1990; Nose et al., 1990), a specificity that is mediated through a twofold symmetric interaction between EC1 domains on opposing cells (Boggon et al., 2002; Harrison et al., 2005).

In the second portion of this work, we examine the role of N-cadherin in the development of the mesoderm during gastrulation and early segmentation. Although N-cadherin is expressed in the mesoderm, targeted knockout studies in the mouse suggest that it is not essential for mesodermal morphogenesis during gastrulation; defects are seen only later during somitogenesis where they are limited to malformations of somitic and cardiac structures (Radice et al., 1997; Horikawa et al., 1999). When N-cadherin function is rescued in the heart, allowing the mutant embryos to develop further, dramatic defects occur in brain development (Luo et al., 2001). Even more subtle are targeted N-cadherin mutations in Drosophila. Null mutant embryos are partly viable with no defects in mesodermal structures and, when examined more closely, the mutants show axonal pathfinding errors in the central nervous system (Iwai et al., 1997).

Recently N-cadherin mutations have been identified in the zebrafish at the parachute (pac) locus (Birely et al., 2005; Lele et al., 2002; Masai et al., 2003; Wielllette et al., 2004). Although N-cadherin expression is reported as fairly ubiquitous throughout the zebrafish embryo during early development (Bitzer et al., 1994; Lele et al., 2002), the phenotypes observed in pac alleles suggest that N-cadherin is only essential for neural development, where it is necessary for the convergence of the ectoderm during neurulation, and later, the cellular organization of the brain and retina (Lele et al., 2002; Malicki et al., 2003; Masai et al., 2003). Outside the nervous system, N-cadherin seems only required during late somitogenesis for the radial migration of a select population of slow muscle fibers to the surface of the myotome (Cortes et al., 2003). Hence, to date, the studies in zebrafish and other animals support the idea that N-cadherin has no role in mesodermal morphogenesis during gastrulation.

In this report, we demonstrate that the zebrafish mutant bib is a dominant gain-of-function mutation in the N-cadherin gene. We focus primarily on cellular defects that are observed in the bib mutant mesoderm, especially during gastrulation, and show that pac mutants exhibit similar albeit more subtle defects in the same cell types. Analysis of RNA expression during the blastula and gastrula stages correlates the presence of N-cadherin RNA in the hypoblast with the time of appearance and location of the bib mutant phenotypes, showing a surprisingly delicate control of N-cadherin expression in the early mesoderm. The gain-of-function nature of the bib allele reveals that N-cadherin is playing a far greater role in morphogenesis of the mesoderm during gastrulation than previously thought.

Methods
Zebrafish strains

The bib allele was isolated in a large-scale mutagenesis screen (Haffer et al., 1996) and initially outcrossed to the polymorphic WIK (L11) strain of wild-type fish for mapping. Subsequent generations were out-crossed to various wild-type strains of fish that were polymorphic for the appropriate microsatellite markers surrounding the bib locus. The pac allele of N-cadherin (Lele et al., 2002) was used for complementation studies. The trp allele of Van Gogh like 2 (Jessen et al., 2002), the kmy allele of glypican heparan sulfate proteoglycan 4/6 (Topczewski et al., 2001) and the spt allele of tbx16 (Griffin et al., 1998b) were used for double mutant studies.

Mapping of the bib locus

bib was initially mapped to one arm of Linkage Group 20 by half tetrad analysis (Johnson et al., 1995). Fine-resolution mapping was obtained using a larger panel of 353 EP-diploid embryos to identify two closely linked microsatellite markers (Knapik et al., 1998). An independent panel of 657 related diploid embryos was used to further characterize recombination frequencies between closely linked microsatellite markers and the bib locus. Microsatellite markers were mapped as simple sequence length polymorphisms and genes were mapped as single-stranded conformation polymorphisms as in McFarland et al. (2005).

Isolation of the bib mutation and genotypic characterization

To locate the site of the bib mutation we used the RT–PCR approach described in Kane et al. (2005) using five overlapping primer pairs designed from the published cDNA. We confirmed the mutation by isolating genomic DNA from individual mutant and wild-type embryos to generate sequence for the site of lesion (F-5’ CAT CGT GCT CAT GCA G-3’, R-5’ CTG GTT TGG CAC CCT CAT C-3’). Characterization of individual embryos to determine genotype in subsequent experiments was performed using the above reverse primer and one of the two following forward primers (bib: F-5’ CCC ATT GAC ATC AA 3’, 55 °C annealing; wild type: F-5’ CCC ATT GAC ATC ATC 3’, 60 °C annealing) which took advantage of a polymorphism based on a silent nucleotide change in the mutant background as well as the mutant lesion, see examples in Fig. 2C. Alternatively, embryos were genotyped by the closely linked markers z6425 or z78809 (distal) and z20582, z61014, z59566 or z9343 (proximal).

Time-lapse and data analysis

For in vivo observations, embryos derived from bib heterozygotes were mounted and recorded in multi-plane as previously described (Warga and Kane, 2003). Embryos were recorded from 60° epiboly until tailbud stage at the region between 30° and 60° lateral from the shield. Afterwards, the recordings were analyzed using Cytos Software, looping half hour segments from the time lapse recording. In the case of Fig. 6, black and white images from one plane of the time-lapse video recording were imported into Adobe Photoshop and pseudo-colored to aid in presentation.

Embryo manipulation, immunohistochemistry and RNA in situ hybridization

Morpholino antisense oligonucleotides (5’TCT GTA TAA AGA AAC CGA TAG AGT T) corresponding to ~40 to ~16 of the N-cadherin cDNA (Lele et al., 2002) were injected into 1-cell stage embryos at a final concentration of 50 μM. At the 18-somite stage embryos were photographed and genotyped.
Transplantation was carried out between doming and 30% epiboly stage as described elsewhere (Ho and Kane, 1990) with the following modification: donor embryos were injected with either fixable FITC–dextran or a combination of neutral rhodamine–dextran and fixable biotinylated–dextran (Molecular Probes). Hosts were examined at 30 h and recorded live as described (Warga and Kane, 2003), or fixed in 4% paraformaldehyde and processed for the fixable tracers.

To detect the FITC–tracer we used a peroxidase conjugated anti-FITC antibody (Roche) and a DAB enzyme substrate, giving a brown reaction product. Detection of the biotin tracer was performed using the Vectastain Elite ABC (peroxidase) kit and Vector SG enzyme substrate (Vector laboratories), giving a blue reaction product.

RNA in situ hybridization was carried out as described in Thissé et al. (1994) with modifications from a more up to date protocol by the same authors at http://zfinfo/monitor/vol5.1/vol5.1.html. Embryos were cleared in 70% glycerol, photographed on a Zeiss Axioskop equipped with a Sony F-707 Digital Camera, and in most instances, genotyped.

Results

Cell contact is perturbed in the bib mesoderm

The bib phenotype first becomes apparent near the end of gastrulation, when the anlage of the notochord begins to characteristically kink in the mutant (Figs. 1A, A′) and cells in the paraxial mesoderm appear to loose cell contact, phenotypes easily observed on the stereo microscope. By the 3-somite stage, the paraxial mesoderm of the mutant has disaggregated into a loose association of spherical cells (Figs. 1B, B′). Curiously, where paraxial cells contacted the notochord they appeared to adhere to it, accentuating its knobby and uneven appearance. In the notochord region itself, cell adhesion defects did not appear as severe, although cells were not as closely packed as usual and did not organize into the uniform rows as seen in normal embryos (Figs. 1B, B′). All these presumptive adhesion defects were particularly severe in the tailbud of the mutant (which can be seen nicely below in Figs. 3A, C). Later, during tail formation, mesodermal tissues remained disorganized, somite boundaries were fused (Figs. 1C–E′), and the tail appeared vacuolated, flatter, and often curled up, the basis for the moniker “biber”, German for “beaver”.

Although cellular defects were first detected within mesodermal structures, by the late somite stages, the neural anlagen of the mutant became disorganized and it was difficult to define brain anatomy based on morphological landmarks (Figs. 1D, D′). Eventually, by 30 h, both the brain and spinal cord were filled with rounded cells and both structures acquired an uneven appearance (Figs. 1E, E′). Despite these morphological abnormalities, the basic body plan was normal at the gross level, and, at the cellular level, mutant tissues differentiated into histotypes appropriate for their location in the embryo. For example, striated muscle formed in the myotomal regions (Figs. 1G, G′) and differentiated neurons formed in the CNS (data not shown).

bib is a mutation in N-cadherin

Using microsatellite markers and half-tetrad analysis, we mapped bib to one arm of Linkage Group 20 (Fig. 2A) close to several centromeric markers (Fig. 2B; Table 1). This placed bib in the vicinity of the gene N-cadherin (also termed cdh2). Ironically, this gene was initially rejected as a candidate because the bib phenotype was distinct from the phenotype of alleles of parachute (pac) (compare Figs. 1E′ to 1F), mutations in the zebrafish N-cadherin locus (Birely et al., 2005; Lele et al., 2002; Malicki et al., 2003; Masai et al., 2003; Wiellette et al., 2004). Nevertheless, fine mapping placed bib very close to N-cadherin (0 recombinants in 192 meioses), and subsequently, matings between bib/+ and pac″m101/+ adults revealed that bib and pac failed to complement. Interestingly, bib/pac transheterozygotes resembled bib homozygous mutants, the more extreme phenotype. Normally a transheterozygote phenotype resembles an intermediate phenotype close to the weaker of the two phenotypes.

The bib allele was sequenced from cDNA generated by RT–PCR. The mutation is a transversion in a nucleotide within the coding region of the first EC domain of the N-cadherin protein, changing the well-conserved isoleucine242 to an asparagine (Figs. 2D–F). To confirm that the bib mutation was in N-
cadherin, we sequenced individual embryos and designed and tested primers specific for either the wild-type or the bib N-cadherin variants. In all cases, the mutation always segregated with the bib phenotype (Fig. 2C). To determine if the bib phenotype was the result of a co-segregating second mutation that was exasperating a pac phenotype, we tested linkage of the bib phenotype to several markers closely linked to N-cadherin (Table 1), looking for changes in the bib phenotype. Recombination events were found on either side of the bib locus between closely linked proximal and distal markers (see mutant 6 in Figs. 2B and C; also, and Table 1), and in these cases, the bib phenotype was unchanged. Hence, it is unlikely that the bib phenotype is the result of an additional mutation.

bib is a dominant gain-of-function mutation

The complementation analysis between bib and pac suggested that bib was not acting as a simple recessive mutation: pac is thought to be a null phenotype and yet the bib phenotype was more extreme. The analysis of mutations in E-cadherin (Kane et al., 2005) showed that non-synonymous changes in conserved amino acids of the EC1 domain often result in dominant mutations.

After sorting 190 embryos into normal, intermediate and strong classes, we found on genotyping that the intermediate phenotype correlated with the heterozygous genotype (with the exception of two heterozygous individuals misidentified as normal). Analysis of expression of papc in the paraxial mesoderm (Yamamoto et al., 1998) followed by genotyping showed that the tailbud of the homozygous mutants (Figs. 3D–F). Hence, the bib phenotype segregated as a semi dominant trait (Fig. 3G). Comparison of pac clutches revealed a similar cellular disorganization in the tailbud of pac homozygotes (Figs. 3H, I). However, analysis of papc expression (Figs. 3J, K) followed by genotyping revealed that the pac phenotype segregated as a simple recessive trait (Fig. 3L). Note the striking similarity of the bib heterozygous and pac homozygous phenotypes.

Table 1

<table>
<thead>
<tr>
<th>Interval</th>
<th>bib/bib</th>
<th>bib/+</th>
<th>+/+</th>
<th>cM</th>
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</thead>
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<tr>
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<td>0/17</td>
<td>5/65</td>
<td>3.16</td>
</tr>
<tr>
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<td>1/30</td>
<td>3/162</td>
<td>0.69</td>
</tr>
<tr>
<td>bib–Z3964</td>
<td>0/241</td>
<td>2/30</td>
<td>0/162</td>
<td>0.23</td>
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<tr>
<td>Z3964–61014</td>
<td>1/161</td>
<td>14/30</td>
<td>1/162</td>
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<td>3/128</td>
<td>7/28</td>
<td>2/122</td>
<td>2.16</td>
</tr>
<tr>
<td>Z21067–dsg2</td>
<td>13/41</td>
<td>0/7</td>
<td>9/38</td>
<td>12.79</td>
</tr>
</tbody>
</table>

a Centromere is in this interval.
To directly test if the bib gene product was producing the extreme phenotype of bib mutants, we blocked the bib product using morpholino antisense oligonucleotides (Fig. 4). The oligonucleotides were originally designed to knock down the normal N-cadherin gene product to phenocopy the pac mutant in the study by Lele et al. (2002). We injected the oligonucleotides into one-cell stage embryos produced from a cross of two bib heterozygotes, a cross that would normally yield 25% bib mutants. However, 100% of the injected embryos exhibited the pac phenotype (Figs. 4C, D). In particular, none of the embryos had fused somites, an obvious phenotypic trait that distinguishes bib from pac mutants (Figs. 4A, B). To verify that bib homo-
zygotes were represented in the pac phenocopies, we genotyped all the embryos using closely linked markers (Figs. 4E, F), and found the expected numbers of +/+, bib/+, and bib/bib individuals (Fig. 4G). Hence, when the bib mutant gene product was blocked in bib/bib individuals, a pac phenotype was produced.

In both bib and pac mutants, morphogenesis of both neuroectoderm and mesoderm is impaired

Whereas pac mutants are known for defects in brain development (Jiang et al., 1996; Lele et al., 2002; Masai et al., 2003), the most notable features of bib mutants are their striking mesodermal defects. To cross-check these phenotypes, we examined neural and mesodermal defects in bib/bib, bib/+ , pac/pac, bib/pac and +/+ allelic combinations. (For brevity and clarity in these sections we refer to the mutant phenotype by putative genotype. In the cases of bib/+ and +/+ combinations, the genotype was determined using closely linked markers, with the exceptions noted in the figure legend.)

To assay defects in the neuroectoderm of the mutants, we first compared the width and shape of the neural keel. In pac/pac mutants neural defects can be identified during the mid-somite stages by an uneven neural keel and small bulges in the brain (Jiang et al., 1996; Lele et al., 2002); bib/bib mutant embryos have similar traits (Fig. 5A). However, in both bib/bib and bib/pac mutants morphological defects in neurulation first became apparent much earlier, at the end of epiboly, when the anterior neural keel appeared ventrally flatter in cross-section, as seen for bib/bib in Fig. 1A’. By later stages, the anterior neural keel was both flatter and broader than normal (Fig. 5B). This is consistent with the interpretation that convergence of neural cells to the dorsal midline is already delayed or slowed by the beginning of segmentation. At about the 5-somite stage similar defects were discerned in bib/+ and pac/pac mutants, in which the anterior neural keel appeared only slightly broader than in +/+ . Also, whereas the width of the neural keel in the tail is almost normal in pac/pac mutants, it appeared wider in bib/bib, bib/pac, and bib/+ mutants. Markers that label the neural plate such as notch5, notch1b and dld (Haddon et al., 1998; Westin and Lardelli, 1997) agreed with these observations (Fig. 5D and data not shown) and data not shown) and the idea that the bib mutation slows convergence of the neural keel along the entire A–P axis. Of note, many early traits of the pac/pac mutant were identical to that of the bib/+ mutant.

In the mesodermal germ layers, the somite territory was noticeably broader in all allelic combinations compared to +/-, even in the pac/pac mutants (Fig. 5C). However, whereas pac/pac mutants made normal epithelial somites, bib/+ made mildly disorganized somites, and bib/pac and bib/bib made none (Fig. 5C’). Metameric mesodermal patterns of notch5 and myoD expression (Weinberg et al., 1995; Westin and Lardelli, 1997) revealed a somite prepattern in bib/pac and bib/bib (Figs. 5D, E). These markers, along with the expression of papc (Fig. 5G), confirmed that all allelic combinations possessed a wider somite territory than +/-, particularly in the posterior region of the embryo. For example, normally the width of the somite territory is about 80 µm from the edge of the notochord to the outer edge of the somite. This interval is about 90 to 95 µm in pac/pac and bib/+ mutants, and about 120 µm in bib/bib and pac/bib mutants. In general, the width of the somite territory formed an allelic series similar to that seen in the morphological analysis of the neural keel, with one exception: while overall mesodermal phenotype of the pac/pac mutant was very like the bib/+ mutant (including the cell contact defect visible in the tailbud; compare Figs. 3B to I), the width of the paraxial territory in the pac/pac tailbud was intermediate between that of the bib/+ and bib/bib trait (see Fig. 5G and compare Figs. 3G to L).

Defects in mesodermal border formation in bib and pac mutants

The expression of notch5, myoD and papc indicated that a segmentation pre-pattern occurred in bib/bib and bib/pac mutants, both mutants that later have fused somites. However, the stripes of expression in these mutants were thinner, irregularly placed and markedly disorganized compared to +/- embryos. Note that pac/pac and bib/+ somites were subtly irregular even though they displayed no obvious morphological defects in later epithelial organization (Fig. 5E).

In all of the allelic combinations, borders separating mesodermal tissues were perturbed. In bib/bib, bib/pac and, to a lesser degree, pac/pac mutants, the border between the intermediate and paraxial mesoderm was ragged and diffuse as revealed by the outermost edge of papc expression (Fig. 5G); this could correlate with the abnormal positioning of the pronephric tubule in older mutant embryos (data not shown). In all the allelic combinations, defects along the axial–paraxial border were visible. Comparing myoD and papc expression in the muscle territory with ml expression in the notochord (Schulte-Mmerker et al., 1994) revealed that the notochord–muscle border was uneven in the pac/pac and bib/+ mutants and chaotic in the bib/bib and bib/pac mutants (Figs. 5E–G’). Furthermore, individual cells in the notochord domain often expressed paraxial genes ectopically (Figs. 5E, G’). In sectioned material, such cells were often attached by thin protrusions to the somite mesoderm (data not shown), suggesting that they might be an extreme elaboration of this border disruption.

Defects in mutant mesodermal cell behavior appear during gastrulation

To identify the origin of the widened axis in the early somite stages, we examined expression patterns in the gastrula. Markers that label the paraxial mesoderm, such as deltaC (Smithers et al., 2000), or outline the tailbud and posterior notochord, such as wnt8 (Kelly et al., 1995), indicated that these territories were already wider in the mutants by 90–100% epiboly (Figs. 6A–D). Markers such as axial, that label endoderm, did not show such widening (Figs. 6E, F). Examination with DIC optics of the midgastrula at 80% epiboly demonstrated that mesodermal cells in pac and bib mutants already appeared rounder and less polarized (Figs. 6G–J). Wild-type mesodermal cells adjacent to
Fig. 5. Comparison of the bib and pac phenotypes. Rows (1–5) indicate genotype of the individual. In the case of all bib and wild-type individuals, all which are siblings, this was determined by linkage mapping, with the exception of ntl and notch5 expression, where the genotype was inferred by phenotype. Also, for the pac homozygotes and bib/pac transheterozygotes, genotype was based on phenotype. Columns (A–C) show the same live embryo from a different view. Columns (D–G) show whole-mount in situ hybridizations. (A) Asterisk indicates uneven neural keel; arrowhead indicates absence of somite furrows. (B) Optical cross section at level of midbrain–hindbrain, arrows indicate width of neural plate. (C) Superficial view of somite region, arrows indicate width of paraxial mesoderm at level of 4th somite. (C′) Magnified view showing epithelial organization. (D) Dorsal view, arrows indicate width of neural plate; arrowheads indicate width of paraxial mesoderm. (E) Dorsal view, arrows indicate width of paraxial mesoderm, arrowhead indicates cells in notochord domain. (F) Dorsal view, arrowhead indicates knobby notochord protrusions. (G) Vegetal view, arrows indicate width of tailbud, arrowhead indicates cells in notochord domain. (G′) Magnified view showing irregular paraxial–notochord border and ectopic cells.
the notochord normally appear oriented and confluent with one another at this stage; in the mutants, this was not the case.

To determine if cells were moving abnormally in the gastrula, we recorded mutant and wild-type embryos using time lapse video, focusing on the paraxial mesoderm layers (Figs. 6K, L). Normally at 60% to 70% epiboly, cells in this region of the embryo take a common trajectory towards the axis, with individual cells moving as a cohort as shown in Figs. 6K and K′. Hence, summary roses of the individual wild-type trajectories appeared similar to each other (Fig. 6K″), and showed that the cells move a single general direction. In general, wild-type cells move at a net rate of about 150 ± 5 μm/h (n = 27 cells). In mutant embryos, cells tended to behave as individuals, frequently moving out of register with each other (Figs. 6L and L′). Mutant cells move at a net rate of about 90 ± 7 μm/h (n = 33 cells). Often individual trajectories cross one another and cells were often difficult to trace because they did not stay in the same focal plane. Mutant cells also blebbed occasionally and stopped erratically. Hence, the summary roses of individual cells were quite different when compared to each other or when compared to wild-type cells (Fig. 6L″). Interestingly, although these cells take longer to reach the dorsal side of the embryo, there was an impression that they did not slow as much as expected, seen in an analysis of their instantaneous speed, which was about 120 ± 9 μm/h, compared to about 160 μm/h for wild-type cells. Hence, although mutant cells were slower, they also spent more time dilly dallying.

**Interactions of the bib phenotype with the tri, kny and spt phenotypes**

The cell convergence defects resemble those seen in *trilobite* (tri) and *knypek* (kny) (Solnica-Krezel et al., 1996). Alleles of tri are mutations in the gene that encodes a homolog of the *Drosophila melanogaster Van Gogh* gene and human VANGL2 (Jessen et al., 2002); alleles of kny are mutations in the gene that encodes a member of the glypican family of heparan sulfate proteoglycans (Topczewski et al., 2001). Both of these genes are necessary essential components of the non-canonical Wnt/planar cell polarity pathway in zebrafish. Hence we compared the bib mutant to both of these mutants and examined the double mutant phenotypes.

Figs. 7A–D show a comparison of the bib, tri and bib;tri double mutant phenotypes at 24 h. Both the tri and bib mutants
were short with truncated tails. The bib;tri double mutant was shorter than either of the single mutants, and was very similar in stature to the tri;kny double mutant (Marlow et al., 1998). However, there was no cyclopia in the double mutant, as reported for the tri;kny double. Also, there was a conspicuous group of detached cells along the dorsal midline of the bib;tri double mutant that resembled the detached dorsal cells that are seen in half baked mutants of the E-cadherin locus (Kane et al., 2005).

Comparison of myoD expression at the 7-somite stage revealed that bib had a similar segmentation pattern to tri: the somites were compressed in the AP direction and progressively wider in the more posterior somites. Measurements showed that the width of bib somites was about 120 μm from

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![Image](image_url)

Fig. 7. Interactions of bib with tri and spt. (A–D) Sibling embryos: 24 h phenotype, arrow indicates dorsal detached cells and 7-somite phenotype visualized with the myoD or dlD probes, arrows indicate lateral row of neurons. (E–H) Sibling embryos 24 h phenotype and 7-somite phenotype visualized with the ntl and pax2.1 or dlD probes, arrows indicate area of paraxial mesoderm expression. For bib and bib;spt in G/H, we could not distinguish the 24 h phenotypes.

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![Image](image_url)

Fig. 8. Autonomous and non-autonomous effects of bib in the nervous system and muscle. 24 h mosaic embryos after transplantation of wild-type and/or bib mutant cells into wild-type or bib mutant hosts as indicated. (A–C) Neural transplants into the hindbrain; dorsal view. (A) Arrow indicates typical wild-type neuroepithelial cell. (B) Arrow indicates aggregate of >10 wild-type cells; inset shows magnification of cluster and white dots indicate individual cells. (C) Arrowheads indicate aggregate mutant cells, white arrow indicates isolated mutant cell, black arrow indicates normal wild-type cell. (D–E) Myotomal transplants into the trunk; lateral view. (D) Low magnification of chimera; dotted lines indicate myotome boundaries; asterisk indicates normal-shaped myotome. (E) Region magnified from panel D, showing UV, white light and composite images; solid arrow indicates typical wild-type fiber, hollow arrow indicates isolated normal-looking mutant fiber, solid arrowheads indicate abnormal wild-type fibers, hollow arrowheads indicate typical mutant aggregates; solid outline indicates abnormal myotome boundaries and dotted outline indicates unusual borders within the myotome.
the edge of the notochord to the lateral edge of the most posterior somite, compared to about 80 μm in wild-type embryos and 120 μm in the tri mutant. The bib;tri double mutants have the widest somites, at about 180 μm, and the AP compression is extreme. We used the expression pattern of deltaD at the 7-somite stage to examine the widening of neural plate width measuring between the most lateral rows of neurons (Haddon et al., 1998). Here we found that bib mutants have a neural plate width of about 120 μm, compared to about 50 μm in wild-type embryos and 100 μm in the tri mutant. The bib;tri double mutants have the widest neural plate, at about 220 μm. Results with the bib;kny double mutants were very similar, and are not shown. In summary, addition of the bib phenotype into either the wild-type, kny or tri backgrounds tended to widen structures by about 50% in the mesoderm and about 100% in the neuroectoderm. We interpret this to mean the double mutant phenotype was the simple addition of mutant phenotypes.

To ask if bib affected the motility of mesodermal cells we examined the bib phenotype in double mutant combination with spadetail (spt), a mutation in the tbx16 gene (Griffin et al., 1998a; Ruvinsky et al., 1998). In the spt mutant, paraxial cells move to the tail of the embryo rather than moving to the trunk (Kimmel et al., 1989), and this altered cell movement seems to be active (Ho and Kane, 1990). We found that when double heterozygous fish were mated, the 24 h phenotypes segregated with a 9:3:4 ratio of WT:spt:bib (Figs. 7E–H), demonstrating that bib was epistatic to spt at least in regards to this late phenotype: clearly, the double mutants had not formed the spade of cells at the end of the tail.

When we examined fixed embryos at the 7-somite stage (Figs. 7E–H), we were able to distinguish double mutants based on the expression pattern of pax2.1, which marks intermediate mesoderm (Majumdar et al., 2000). In spt mutants, paraxial mesoderm was missing in the trunk and intermediate mesoderm collapsed towards the notochord (marked by ntl expression). Conversely, in bib mutants, intermediate mesoderm was further from the axis, consistent with the idea that cells move toward the axis slower. In the double mutant, pax2.1 expression was almost at the wild-type width, suggesting that, like in the spt mutant, paraxial cells are missing, but like in bib, cells are moving slowly. The lack of paraxial expression in both the spt and the double mutant was confirmed using the expression pattern of deltaD which marks paraxial mesoderm (Haddon et al., 1998). Hence, in bib:spt double mutants, cells were missing from the expected spade at end of the tail and cells were also missing from the trunk. We suspect that these missing cells may be distributed broadly in the trunk–tail region of the double mutant, a region which is thickened in the bib mutant and in the double mutants as well.

To crosscheck our double mutant analysis, we checked for N-cadherin expression in spt mutants and tbx16 expression in bib mutants. In both cases, we saw the expected results: the expression pattern of N-cadherin was fairly normal in spt mutants and the expression pattern of tbx16 was wider in bib mutants (data not shown). In summary, these results, and those above with tri and kny, support the idea that whereas N-cadherin is necessary for cells to move in an efficient manner, other cell movement pathways also act to sustain cell movement.

bib modifies the adhesion of cells in the nervous system and muscle in an autonomous manner

To determine if the bib gene product acts intrinsically to modify the adhesion of cells, we transplanted groups of mutant and wild-type cells into mutant and wild-type host blastulae and examined the mosaic embryos at 24 h (Fig. 8). Wild-type cells found in the CNS of wild-type hosts displayed the typical slender morphology of neuroepithelial cells (Fig. 8A). In mutant hosts, wild-type cells formed coherent spherical aggregates (Fig. 8B) and were never found as single isolated cells. In reciprocal experiments, bib mutant cells also tended to form coherent aggregates when placed in wild-type hosts (Fig. 8C). Occasionally, single isolated bib cells were observed and they resembled the morphology of wild-type cells. These results are very similar to results reported in the analysis of pac mutants (Lele et al., 2002; Malicki et al., 2003).

When wild-type and mutant cells were found together in the myotomes of a wild-type host (Figs. 8D, E), wild-type cells typically formed single elongate muscle fibers which spanned the length of a myotome, whereas mutant cells tended to form loose aggregates of short thick fibers that did not mix into the surrounding host muscle. Single, isolated mutant cells tended to form normal looking fibers. Interestingly, when wild-type fibers were near large groups of mutant cells they acquired a shorter and more rounded appearance. Moreover, aggregates of mutant cells sometimes caused formation of multiple ectopic boundaries within a host myotome, and when near a myotome border, they caused disruption of the border (Fig. 8E). Thus, in the muscle, bib has cell-autonomous as well as cell-non-autonomous effects: both wild-type and bib cells retain their phenotypic characteristics when in groups but change their morphology and behavior when isolated.

Differential N-cadherin expression in the mesoderm and ectoderm of the early embryo

To better understand the mesodermal basis for the bib phenotype, we analyzed the timing and distribution of N-cadherin during early development, concentrating on the blastula and gastrula stages. RT–PCR showed that N-cadherin transcripts appeared just after the midblastula transition in the sphere stage embryo, before morphological germ layers form; these transcripts were maintained through 2 days of development (Fig. 9A).

Using the probe made to the N-cadherin cDNA by Bitzer et al. (1994), we analyzed N-cadherin expression in the early embryo, focusing on activity in individual cell layers. N-cadherin transcripts were first detected in the syncytial layer of the yolk cell during doming stage with the highest expression in a crescent near the blastoderm margin on the presumptive dorsal side (Fig. 9B). Before germ ring formation, transcripts also began to accumulate in cells at the dorsal margin (Fig. 9C). At this location, there was a very subtle gradient of expression, with
the highest expression in the interior of the blastoderm adjacent to the yolk cell. By late shield stage, N-cadherin was robustly expressed throughout the entire yolk syncytial layer, in the dorsal forerunner cells (Fig. 9D), and within the forming hypoblast layer (Fig. 9E). In the epiblast layer, N-cadherin was weakly expressed in the presumptive neural plate, where it formed a steep radial gradient with expression highest in the cells just superficial to the hypoblast (Fig. 9F). At 70% epiboly, N-cadherin expression was maintained in the hypoblast and the forerunner cells but almost completely cleared from the yolk syncytial layer (Fig. 9G); the gradient of expression in the neural plate still persisted (Fig. 9H). In all of these stages, we never observed expression in the enveloping layer.

By the end of epiboly, the pattern of N-cadherin expression became more complex. Examining sections taken through the different levels of the embryo shown in Fig. 9I allowed us to look at regional differences in expression and compare the registration between the neuroectodermal and mesodermal tissues. In the anterior neural plate (Fig. 9J), transcripts were robustly expressed, displaying a very minor gradient of expression with a peak adjacent to the mesoderm. (At this level, the mesendoderm is very thin, and it is difficult to distinguish from the overlying neuroectoderm.) At the level of the trunk (Fig. 9K), transcripts were expressed in all cells of the neural plate and in the mesoderm. In the trunk neural plate, expression of N-cadherin was less robust compared to more anterior; however, the gradient of expression was steeper. In the trunk mesoderm, expression was extremely high in the notochord, similar to the expression found in the anterior neural plate. In a stepwise manner, transcripts in the paraxial mesoderm were weaker than in the notochord, and transcripts in the intermediate mesoderm were weaker still. At the level of the tailbud (Fig. 9L), expression was very weak in the posterior neural plate, but here the gradient of expression was the steepest. In the posterior mesoderm, expression of N-cadherin was extremely high in comparison to the overlying neuroectoderm, and tended to equal or surpass that in the anterior neural plate. Here transcript levels in the notochord, paraxial mesoderm and intermediate mesoderm retained their stepwise gradient of expression with less of a difference between the notochord and paraxial regions.

We noted a close alignment of the N-cadherin expressing regions of the paraxial mesoderm with the neural plate in both the trunk and tailbud sections. For comparison, Fig. 9Li shows a comparable section that shows the expression of papc, which marks the paraxial mesoderm (Fig. 9M).

We also examined the expression patterns of N-cadherin in the bib and pac mutants (data not shown). In the bib allele, a missense mutation, we observed that transcript levels remained longer in individual tissues. In the pac tm101 allele, a nonsense mutant, we found almost complete absence of transcript, suggesting that nonsense-mediated transcript degradation is occurring, a common consequence of premature stop codon mutations.

Discussion

Based on genetic linkage data, identification of the mutant sequence and lack of complementation with pac, we have shown
that *bib* encodes the zebrafish homolog of N-cadherin. Our analysis of the *bib* and *pac* phenotypes demonstrate that N-cadherin is required in the mesoderm during gastrulation, where it is necessary for convergence of the mesodermal germ layer, and after gastrulation, where it is necessary for somitogenesis and border formation; this complements earlier analysis of the *pac* phenotype where it was established that N-cadherin is necessary for cell adhesion of the neural ectoderm during neurulation (Hong and Brewster, 2006; Jiang et al., 1996; Lele et al., 2002; Malicki et al., 2003; Masai et al., 2003). Here we discuss the nature of the *bib* mutation, and its recessive and dominant phenotypes, and then consider the multiple functions of N-cadherin in the morphogenesis of the mesoderm.

**biber is a dominant gain-of-function mutation in N-cadherin**

The *bib* allele possesses a number of genetic features that suggest that the allele acts as a gain-of-function mutation. First, the allele has a semi dominant effect whereas *pac* nulls do not, the classic “morph” definition of Muller for gain-of-function alleles. Our data is consistent with the notion of *pac* nonsense alleles being null mutations as already argued by Lele et al. (2002). To elaborate, most of the *pac* alleles are nonsense mutations in the EC4 domain, eliminating the entire carboxy half of the protein, including the transmembrane and intracellular portions. Also, in situ hybridization detects almost no RNA (our observations) and antibody staining or Western blots detect no protein in the embryo (Lele et al., 2002; Malicki et al., 2003) suggesting that nonsense-mediated degradation of the RNA is occurring.

Second, the recessive phenotype of *bib* is stronger than all other alleles at the *pac* locus, and, third, the phenotype of the *bib/pac* transheterozygote resembles the stronger *bib* allele, both common features of gain-of-function alleles. Last, to convincingly demonstrate that *bib* was a gain-of-function allele, we “suppressed” the strong *bib* phenotype by shifting it to the weaker null phenotype using antisense oligonucleotides. To our knowledge, this is the first time that such methodology has been used to demonstrate a gain-of-function effect. It is notable that *glass onion* (*glo*) has some similarities to *bib*, including a possible dominant phenotype (Malicki et al., 2003), and it was suggested that *glo* had the true null phenotype. In light of our findings, this proposal must be reexamined.

In general, cells in *bib* mutants seem less adhesive than those in *pac* mutants. Because the recessive *bib* mutant is homozygous, we cannot invoke an inter-allelic dominant negative interaction. Clearly, the *bib* gene product produces a stronger phenotype than the null. The mutation is a nucleotide transversion in the EC1 domain, a region not only highly conserved among N-cadherin homologues, but conserved among all the classic cadherin paralogs. Of the five EC domains that are necessary for N-cadherin function, the first has long been known to be of particular importance for homophilic binding specificity (Nose et al., 1990). Hence, one would imagine that the *bib* gene product should be present in cells that normally express N-cadherin, presumably properly processed and inserted into the cell membrane placing this mutant EC1 domain on the outside of the cell membrane, where it exerts its phenotype.

The *bib* mutation could possess hypermorph or neomorphic activity, either of which being gain-of-function mutations that would behave genetically in a manner shown here for *bib*. A hypermorph *bib* mutation could represent a N-cadherin molecule with a stronger homophilic binding activity, a view that does not seem to fit with the idea of a less adhesive *bib* phenotype. Furthermore, such a mutation would not be expected to produce the strong *bib*-like phenotype in *pac/bib* transheterozygotes, but rather exhibit a weaker phenotype. We favor the notion that *bib* is a neomorphic mutation, conferring on N-cadherin an ability to interact with other molecules, possibly in a heterophilic fashion, weakening adhesive interactions.

The dominant phenotype of *bib* could be based on this second interpretation. In this case, we imagine that the wild-type and *bib* gene products would both be present in the cell membrane and the *bib* gene product would retain its neomorphic effect. However, when heterozygous, *bib* could certainly be acting in an antimorphic, dominant negative fashion, dimerizing and interfering with the wild-type gene product (Gumbiner, 1996).

Whereas the early phenotypes of *bib* heterozygotes and *pac* homozygotes are similar, only the *pac* phenotype is lethal. Early in development, the *bib* heterozygote is sensitive to the mutant gene product but not late? There are a number of possible reasons for this difference; we suggest that in early development N-cadherin is dynamically regulated whereas in later development N-cadherin participates in the stable cell—cell structural junctions typical of more mature tissues. The mutant protein may not integrate into such structures, and hence be targeted for degradation. If so, the dominant effect would be attenuated.

**Expression of N-cadherin in the early embryo**

Part of the motivation for our analysis of N-cadherin expression in the blastula and gastrula stages was to carefully compare early N-cadherin expression with attributes of the *bib* and *pac* phenotypes, focusing carefully on which germ layers were expressing N-cadherin. In general, the details of N-cadherin expression in the mesoderm of the blastula and gastrula have not been well described because it is partially occluded by the neural expression.

Early N-cadherin expression is much more dynamically regulated than expected, and forms an interesting complement to E-cadherin expression. The gene is first expressed in the yolk cell at sphere stage, the time when the blastoderm uniformly expresses E-cadherin (Kane et al., 2005). As epitholy commences, N-cadherin expression spreads into the deep cells of the organizer region of the embryo and displays a radial gradient of expression, peaking against the yolk cell. Meanwhile, E-cadherin begins to down-regulate in the blastoderm near the surface of the yolk cell, forming a radial gradient of expression that peaks in cells against the enveloping layer, exactly opposite the N-cadherin gradient. The function of these gradients and their correlation with future fates is not known. One might imagine that N-cadherin could provide an opposing substrate on the yolk cell to aid in the morphogenesis of epitholy. In the case...
of E-cadherin, interior epiblast cells intercalate into the exterior layer of the epiblast and remain in close apposition to the enveloping layer, which is strongly E-cadherin positive. By analogy, N-cadherin-based adhesion could be driving epiboly of the inner layers. Alternatively, N-cadherin could be aiding in compaction of the organizer region, a cell movement at the dorsal margin that precedes shield formation (Warga and Nüsslein-Volhard, 1998). We are currently examining N-cadherin mutants for such defects.

As expression of N-cadherin is up-regulated in the neuroectoderm, another radial gradient of expression emerges, with the peak of expression adjacent to the mesoderm of the hypoblast, which by this stage is strongly N-cadherin positive. Interestingly, the patterns of N-cadherin expression in the two layers are in rather close alignment. Again, by analogy with the function of E-cadherin, the phenotypic consequences in half baked mutants is that interior epiblast cells intercalate into the exterior layer of the epiblast but lack the ability to remain there (Kane et al., 2005). Analogous to the role of the enveloping layer for the epiblast (and in lieu of the yet to be formed basal lamina), the mesoderm may be performing an “organizing” role for the morphogenesis of the neural plate as it undergoes radial intercalation to transform into a columnar epithelium. In support of this idea, recent work has demonstrated that cells of the forming neural epithelium are disorganized in pac mutants, perhaps leading to the delay in keel formation (Hong and Brewster, 2006).

**N-cadherin is necessary for morphogenesis of the mesoderm**

Here we show that mesodermal cells in bib mutants converge slowly to the dorsal side of the embryo during gastrulation. Normally after formation of the germ ring, mesendodermal precursors internalize and begin moving on the surface of the yolk cell. At the approximate time that these interactions are occurring, N-cadherin is expressed both in the migrating cells and in the yolk cell. N-cadherin must be necessary for the attachment of cells to neighboring cells and perhaps to the yolk cell. When these interactions are lost, cells become misdirected, which in turn slows their dorsawards progression. It is interesting that the widening of mesodermal expression patterns – about 50% wider – is so similar to the slowing of the mesoderm cells – about 40% slower. This suggests that the slowing of convergence causes the widened expression patterns we show in the early part of this work. A detailed analysis of these movement defects is to be published elsewhere.

Note that as the hypoblast segregates into independent germ layers and the yolk cell becomes covered by endodermal cells, the yolk cell down-regulates expression of N-cadherin. Whereas from our observations we cannot determine the degree of N-cadherin expression in the thin endodermal layer, we show that endoderm is not effected in the bib phenotype based on expression of axial. This would be in line with the traditional view of the endoderm, which is more known for expression of E-cadherin than N-cadherin.

Nevertheless, mesodermal cells still continue dorsawards. In lieu of N-cadherin, what other adhesion systems might be acting to partially rescue convergence in the embryo? Our experiments that examine bib in combination with other mutants affecting mesodermal cell movement indicate that N-cadherin is not an essential component of the planar cell polarity (PCP) pathway. Both kny and tri are necessary for the PCP pathway (Jessen et al., 2002; Topczewski et al., 2001). When mutants in either of these genes are in combination with bib, we see not an epistatic, but an additive interaction: convergence is slowed in the double mutants relative to either tri or kny mutant controls by about the same degree as convergence is slowed in bib mutants relative to wild-type controls. We interpret this to mean that N-cadherin and the PCP pathway act in parallel to drive convergence. Given that convergence still occurs in the bib double mutants, and that kny and tri are also reported to be in separate pathways (Jessen et al., 2002), it would be interesting to create the triple mutant, which we have not yet done. The bib; spt double mutant supports the idea that N-cadherin is necessary for movement of the mesodermal germ layer in a general way: in spt, paraxial cells that normally move to the trunk become misdirected to the tail of the embryo; in the double mutant cells move slower and the spt “spade” never forms. Rather than their normal destination in the spt tail, these cells most likely only reach the trunk–tail border, which is normally quite thick in bib mutants.

At the end of gastrulation, the bib mutation has profound effects on tissue boundaries. By this time N-cadherin mRNA is expressed at differing concentrations in various mesodermal tissues, with a higher concentration of N-cadherin mRNA expressed in the notochord than in the paraxial mesoderm. In the bib mutant, the border between these tissues show defects, and sometimes, based on ectopic expression of paraxial markers, individual paraxial mesoderm cells are positioned ectopically in the notochord region. Similar defects are present in pac mutants. Later, although pre-patterning of the somites occurs in bib mutants, paraxial mesoderm fails to complete the segmentation process. This supports a role for N-cadherin in formation and maintenance of the somite border in zebrafish, similar to that suggested from studies in mammals (Radice et al., 1997; Horikawa et al., 1999).

It seems that N-cadherin is distributed in a manner that has been proposed to drive self-assembly of embryonic tissues. According to Steinberg’s differential adhesion theory (Steinberg, 1970), sorting occurs when cells with different activities of the same cadherin are mixed (Friedlander et al., 1989; Steinberg and Takeichi, 1994). Our transplantation experiments fit well with this idea. Wild-type cells always separate from mutant cells in bib hosts, and, with some exceptions, mutant cells do the same in wild-type hosts. Interestingly, one of the most disruptive locations for donor mutant cells is where a border will form: there they non-autonomously cause defects in the organization of the wild-type cells forming the borders. Borders might be expected to be sensitive indicators of adhesive interactions because defects at such locations could arise from too much adhesion as well as too little.

In summary, we have presented evidence that besides its well-known role in the neuroectoderm and in somite formation, N-cadherin is necessary for the morphogenesis of the mesoderm during gastrulation. Furthermore, we have shown that the ex-
pression pattern of N-cadherin in the early gastrula coincides with the bib and pac mutant phenotypes. Understanding the detailed role that N-cadherin plays in balancing the subtle forces that underlie adhesion and de-adhesion will be essential for a complete understanding of vertebrate morphogenesis. Mutants such as bib, that change that balance, will be valuable tools in such an investigation.

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References


ishmus reveals a role for pax2.1 in tubule differentiation and patterning events in the pronephric primordia. Development 127, 2089–2098.


