Probing Cadherin Interactions in Zebrafish with E- and N-Cadherin Missense Mutants

Rachel M. Warga¹ and Donald A. Kane

Department of Biological Sciences, Western Michigan University, Kalamazoo, Michigan 49008

ABSTRACT Cadherins are cell adhesion molecules that regulate numerous adhesive interactions during embryonic development and adult life. Consistent with these functions, when their expression goes astray cells lose their normal adhesive properties resulting in defective morphogenesis, disease, and even metastatic cancer. In general, classical cadherins exert their effect by homophilic interactions via their five characteristic extracellular (EC) repeats. The EC1 repeat provides the mechanism for cadherins to dimerize with each other whereas the EC2 repeat may facilitate dimerization. Less is known about the other EC repeats. Here, we show that a zebrafish missense mutation in the EC5 repeat of N-cadherin is a dominant gain-of-function mutation and demonstrate that this mutation alters cell adhesion almost to the same degree as a zebrafish missense mutation in the EC1 repeat of N-cadherin. We also show that zebrafish E- and N-cadherin dominant gain-of-function missense mutations genetically interact. Perturbation of cell adhesion in embryos that are heterozygous mutant at both loci is similar to that observed in single homozygous mutants. Introducing an E-cadherin EC5 missense allele into the homozygous N-cadherin EC1 missense mutant more radically affects morphogenesis, causing synergistic phenotypes consistent with interdependent functions being disrupted. Our studies indicate that a functional EC5 repeat is critical for cadherin-mediated cell affinity, suggesting that its role may be more important than previously thought. These results also suggest the possibility that E- and N-cadherin have heterophilic interactions during early morphogenesis of the embryo; interactions that might help balance the variety of cell affinities needed during embryonic development.

KEYWORDS cadherin; extracellular repeat; adhesion; affinity; homophilic; heterophilic; homodimer; heterodimer

CADHERINS are a large family of transmembrane glycoproteins that regulate cell affinity. During embryonic development, their expression in different cell populations has been associated with multiple aspects of tissue morphogenesis including cell adhesion, cell sorting, cell movement, cell polarity, and synapse formation. Later in life, cadherins are responsible for the homeostasis of many tissues. Hence, changes in their expression are linked to many types of cancer [reviewed in Redies and Takeichi (1996) and Halbleib and Nelson (2006)]. The best understood of these adhesion molecules, whose members are defined by a common structural repeat of ~110 residues in their extracellular (EC) domain, are the vertebrate classical cadherins, E- and N-cadherin (also known as *cdh1* and *cdh2*) [reviewed in Brasch *et al.*

(2012)]. These were the first cadherins discovered and are developmentally the earliest to be expressed in most vertebrate embryos [reviewed in Takeichi (2018)]. Both belong to the type I classical cadherin subfamily of vertebrates (which we henceforth refer to as simply cadherins unless necessary). In this subfamily, all cadherins have an ectodomain possessing five characteristic EC repeats, responsible for their Ca^{++} -dependent cell affinity, a transmembrane domain, and a highly conserved cytoplasmic tail that anchors cadherins to the cell cortex through their association with p120 catenin, β -catenin, and α -catenin. Invertebrates also have classical cadherin orthologs that perform similar functions, but their ectodomain structure differs radically with up to 16 EC repeats, as well as other repeats such as EGF domains [reviewed in Brasch *et al.* (2012)].

How vertebrate cadherins mediate cell affinity has been well studied. Using their most membrane-distal EC1 repeat, cadherin protomers on opposed cells swap strands with each other to form adhesive dimers. Once *trans*-dimerization happens, *cis* interactions can occur between the EC1 repeat of one cadherin protomer and the EC2 repeat of a parallel

Copyright © 2018 by the Genetics Society of America
doi: <https://doi.org/10.1534/genetics.118.301692>

Manuscript received August 2, 2018; accepted for publication October 16, 2018; published Early Online October 25, 2018.

¹Corresponding author: Department of Biological Sciences, Western Michigan University, 1903 W Michigan Ave., Kalamazoo, MI 49008. E-mail: rachel.warga@wmich.edu

cadherin protomer on the same cell. This combination of *trans* and *cis* interactions is initiated and mediated solely by the ectodomain, and results in the clustering of cadherins at points of cell contact [reviewed in Brasch *et al.* (2012) and Ishiyama and Ikura (2012)]. Whether EC repeats three to five contribute to these adhesive interactions is unclear. Molecular force measurements suggest that they do, yet more conventional methods are unable to detect binding interactions outside the EC1 and EC2 repeats [reviewed in Leckband and Prakasam (2006)]. Because the cytoplasmic tail of classical cadherins interacts with the actin cytoskeleton, this further clusters and stabilizes cadherins onto the cell surface [reviewed in Ratheesh and Yap (2012)]. Once enough stable interactions occur, adherens junctions form.

The binding properties of vertebrate cadherins such as E- and N-cadherin are classically thought to be homophilic, forming only homodimers with themselves. Early studies found that dissociated cells taken from different places in the animal, if mixed, would preferentially sort and reaggregate with cells of the same type (Townes and Holtfreter 1955). Once discovered, cadherins provided a molecular basis for this homotypic cell affinity in a number of classical models of tissue segregation. Not only were different cadherins expressed on particular cell types, but cells transfected with one type of cadherin quickly aggregated with each other and could not aggregate with cells transfected with a different cadherin (Hatta and Takeichi 1986; Nose *et al.* 1988), reinforcing the belief that cadherins interact homophilically. However, in a number of *in vitro* settings, heterophilic adhesive interactions between the ectodomains of different cadherins have also been reported to occur (Volk *et al.* 1987; Shan *et al.* 2000; Shimoyama *et al.* 2000; Niessen and Gumbiner 2002; Duguay *et al.* 2003; Patel *et al.* 2006; Prakasam *et al.* 2006; Katsamba *et al.* 2009; Ounkomol *et al.* 2010). Whether this is a widespread phenomenon *in vivo* is less clear, as cadherins interacting heterophilically in *cis* or in *trans* have only been demonstrated in the adult endoderm-derived tissues of mammals where the vast majority of adherens junctions are composed of E- and N-cadherin heterodimer complexes (Straub *et al.* 2011).

In the zebrafish, E-cadherin, which is supplied maternally and expressed zygotically, is required for epiboly, the first movement of morphogenesis (Kane *et al.* 2005). N-cadherin is not supplied maternally (Bitzur *et al.* 1994), but its zygotic expression is necessary for the convergence of cells to the dorsal midline (Lele *et al.* 2002; Warga and Kane 2007). By the time cell movements begin, the zebrafish embryo is composed of three spatially distinct cellular domains: an embryonic deep cell domain, the enveloping layer, and the extraembryonic yolk syncytial layer (Kane *et al.* 1992). All three domains take part in the process of epiboly [reviewed in Kane and Adams (2002) and Lepage and Bruce (2010)], but only the deep domain is required for cell convergence [reviewed in Yin *et al.* (2009)]. The deep cell domain gives rise to all three germ layers and eventually the embryo proper, while the enveloping layer is an epithelial monolayer

that covers the deep cells of the blastoderm, acting as the embryo's first skin. The yolk syncytial layer is a membrane-enclosed group of nuclei lying on top of the yolk cell that aids in the epiboly process [reviewed in Kimmel *et al.* (1995)]. Although E-cadherin is initially found in all cells of the blastoderm, by gastrulation its expression is concentrated in the enveloping layer and the deep cells that become the nonneural ectoderm. N-cadherin expression on the other hand, is concentrated in the yolk syncytial layer, and the deep cells that become neurectoderm and mesendoderm (Kane *et al.* 2005; Warga and Kane 2007). Thus, there is little overlap in E- and N-cadherin expression once germ layers form and the embryo's body plan begins to emerge.

We and others have identified a number of E- and N-cadherin mutants in zebrafish (Lele *et al.* 2002; Malicki *et al.* 2003; Masai *et al.* 2003; Wiellette *et al.* 2004; Birely *et al.* 2005; Kane *et al.* 2005; Shimizu *et al.* 2005; von der Hardt *et al.* 2007; Warga and Kane 2007; Barresi *et al.* 2010; Yamaguchi *et al.* 2010), many of which are nonsense or missense mutations in one of the five EC repeats (Figure 2A). In general, nonsense mutations have loss-of-function phenotypes because translation of their transcripts is prematurely terminated, leading to nonsense-mediated decay; thus, they tend to behave as strictly recessive null alleles. However, because they have an altered amino acid, missense mutations may cause gain-of-function phenotypes if their product acts in opposition to the normal product, or engages in novel interactions or activities. Hence, their homozygous phenotype can be more severe than that of a null allele and they often display dominant-negative effects when heterozygous. Such is the case at the E-cadherin locus where all the alleles are formally known as *half baked* (*hab*), though in the past because of problems with interpretation of complementation, each allele had its own unique name (Kane *et al.* 1996). Three of the five *hab* alleles are missense mutations in EC1 or EC5 that display unique phenotypes when heterozygous, which indicate a dominant-negative character for these alleles (Kane *et al.* 2005; McFarland *et al.* 2005). Furthermore, when homozygous, these missense mutants arrest earlier in epiboly and are more likely to die during epiboly than mutants for either of the two nonsense alleles (McFarland *et al.* 2005). Thus, at the E-cadherin locus, nonsense mutants and missense mutants have distinguishable traits. Further, the observation that missense mutants in EC5 behave similar to missense mutants in EC1 and have phenotypes that strongly resemble each other suggests that adhesive binding may in some way also require the function of EC5.

The N-cadherin locus likewise has a confusing array of names because different N-cadherin mutants were found in different screens or in some cases assigned to different complementation groups, although, in retrospect, all bear similarity to one another when homozygous with striking defects in brain morphology (Hammerschmidt *et al.* 1996; Jiang *et al.* 1996; Malicki *et al.* 2003; Masai *et al.* 2003) and, when examined more carefully, pronounced defects in mesoderm and tail development (Cortés *et al.* 2003; Birely *et al.* 2005;

Harrington *et al.* 2007; Warga and Kane 2007) due to their cells being less adhesive (Masai *et al.* 2003). Here, they are formally known as *biber* (*bib*), *glass onion* (*glo*), or *parachute* (*pac*). The two missense mutations in EC1 (*bib* and *glo*) are gain-of-function mutations that have dominant-negative features (Malicki *et al.* 2003; Warga and Kane 2007), as determined by knocking down the mutant protein by antisense oligonucleotide (Warga and Kane 2007). Both alleles have more severe defects in cell adhesion and cell movement when homozygous than any of the *pac* alleles that are either nonsense or missense mutations in EC4 or EC5 (Lele *et al.* 2002; Masai *et al.* 2003; Birely *et al.* 2005; Hong and Brewster 2006; von der Hardt *et al.* 2007; Warga and Kane 2007; Yamaguchi *et al.* 2010). However, unlike the E-cadherin locus, the N-cadherin missense alleles in EC4 or EC5 have no reported dominant features.

Here, we report that a N-cadherin missense mutation in EC5 has dominant effects. Furthermore, we show that when homozygous, like the N-cadherin EC1 missense mutation, the N-cadherin EC5 missense mutation expresses a more severe phenotype than N-cadherin nonsense mutants, in keeping with the equivalent mutations in E-cadherin. We also show that the N-cadherin EC1 and EC5 missense mutants genetically interact with the E-cadherin EC1 and EC5 missense mutants. Together, these results indicate both E- and N-cadherin require a functional EC5 repeat for cadherin-mediated cell adhesion. They also suggest the possibility that heterophilic binding between E- and N-cadherin is not just a phenomenon in differentiated endodermal tissues, but perhaps a normal occurrence during formation of the embryo.

Materials and Methods

Zebrafish crosses

For reference, the various EC repeat mutations in E- and N-cadherin are illustrated in Figure 2A; the alleles we use are shown in red and Table 1 gives a brief description of their phenotypic traits. Embryos shown in figures were derived from heterozygous parents except for those in Figure 1, A–R, where the parents were wild-type. For Figure 1, S–U, Figure 2, Figure 3, Figure 4, Figure 5, Figure 6, A–C, Figure 7, B'–F', and Figure 8, A'–D', parents were heterozygous at one locus; for Figure 6, D–I, females were heterozygous at the N-cadherin locus and males were heterozygous at the E-cadherin locus; for Figure 6, J–L, females were heterozygous at the E-cadherin locus and males were heterozygous at the N-cadherin locus; for Figure 7 and Figure 8, females were heterozygous at both the E- and N-cadherin loci and males were heterozygous at the N-cadherin locus; and for Figure 9, parents were heterozygous at both the E- and N-cadherin loci.

Genotyping

Genotypes of the embryos were determined before fixation by scoring the characteristic traits displayed by individual alleles

(see Table 1), which allow one to separate homozygous mutant from heterozygous mutant from homozygous wild-type. This method was verified by DNA genotyping, as in shown in Figure 3D and previous publications (Kane *et al.* 2005; McFarland *et al.* 2005; Warga and Kane 2007). The exceptions where embryos were too young to identify were: Figure 2A, where we did not genotype because there was no phenotype; Figure 5, where embryos were genotyped afterward by simple sequence length polymorphism using the closely linked microsatellite markers, as in McFarland *et al.* (2005) and Warga and Kane (2007); and Figure 9, A–D and M, where we did not genotype for the N-cadherin allele at 75% epiboly.

Embryo recording, *in situ* hybridization, and staining

Embryos were dechorionated, anesthetized if necessary with 0.4% Tricaine (MS222; Sigma [Sigma Chemical], St. Louis, MO), mounted in 3% Methyl Cellulose, and then photographed live on a Zeiss ([Carl Zeiss], Thornwood, NY) Axioskop equipped with a Sony F-707 Digital Camera or on a Nikon (Garden City, NY) SMZ-U stereomicroscope equipped with a Canon EOS Rebel T5 Digital Camera.

Embryos were fixed in 4% paraformaldehyde in PBS buffer overnight. Whole-mount RNA *in situ* hybridization was carried out using digoxigenin-labeled riboprobes as in Thisse and Thisse (2008). Whole-mount antibody staining was performed using an anti- β -catenin antibody (1:2000; Sigma), an N-cadherin antibody (1:200; GeneTex, Irvine, CA), or an E-cadherin antibody (1:200; GeneTex). Embryos were cleared in 70% glycerol, and photographed as above or on a Nikon C2 confocal microscope.

Number of forerunner clusters and left–right asymmetry

For the number of forerunner clusters in Figure 2, we examined fixed embryos at 80% epiboly, before most homozygous mutants die, and for the number of rosettes embryos were examined at the one-somite stage, when it is simple to segregate the homozygous mutant and wild-type from the heterozygous mutant before fixation. Of note, the heterozygous mutant in the nonsense mutation is phenotypically wild-type. For the phenotype of forerunner clusters and Kupffer's vesicle in Figure 9, except for 75% epiboly where we only segregated for the E-cadherin mutation, embryos were segregated for either the homozygous E-cadherin mutant or the homozygous E- and N-cadherin mutant genotype before fixation.

To visualize the forerunner cluster, we used the expression of *ntl*, *sox17*, *sox32*, and *lrd1*, which have identical expression in the forerunner cell cluster. For heart looping, we examined live embryos at 48 hr. For gut and brain symmetry, we sorted by genotype before fixation and used expression of *foxa3* at 30 hr for the gut, and expression of *lft1* at 24 somites for the brain.

Data availability

All zebrafish strains as well as plasmids are available upon request. The authors affirm that all data necessary for

Table 1 E- and N-cadherin mutant strains used in this study

Mutant allele	Locus	Mutation	Where	Renamed:	Dominant traits ^a	Allelic strength ^{b,c}
<i>hab</i> <i>tx230</i>	E-cadherin	Nonsense ^d	EC4	<i>tx230</i> (<i>ecad</i> ; 0)	— ^{d,e,f}	4 ^{d,e,f,g}
<i>hab</i> <i>tm94</i>	E-cadherin	Missense ^d	EC1	<i>tm94</i> (<i>ecad</i> ; EC1)	MD, ZMD ^{d,e,f}	2 ^{d,e,f,g}
<i>hab</i> <i>dtv43</i>	E-cadherin	Missense ^d	EC5	<i>dtv43</i> (<i>ecad</i> ; EC5)	PD, MD, ZMD ^{d,e,f}	1 ^{d,e,f,g}
<i>pac</i> <i>tm101</i>	N-cadherin	Nonsense ^h	EC4	<i>tm101</i> (<i>ncad</i> ; 0)	— ⁱ	4 ^{g,i,j,k}
<i>bib</i> <i>tb08</i>	N-cadherin	Missense ⁱ	EC1	<i>tb08</i> (<i>ncad</i> ; EC1)	ZD ^j	1 ^{g,i}
<i>pac</i> <i>p79emcf</i>	N-cadherin	Missense ^l	EC5	<i>p79</i> (<i>ncad</i> ; EC5)	ZD ^g	2 ^{g,i,l}

EC, extracellular; MD, maternal dominant effect: embryos have multiple forerunner clusters regardless of genotype; ZMD, zygotic maternal dominant effect: epiboly in the heterozygotes derived from a heterozygous mother is slower than normal and cells later detach from the dorsal neural keel, in many of these embryos the neural keel also does not converge together properly causing gaps to form and resulting in lethality; PD, paternal dominant effect: the hatching gland in heterozygotes derived from a heterozygous father exhibit an enlarged and ragged appearance; ZD, zygotic dominant effect: heterozygous embryos exhibit a phenotype in the neurectoderm and mesoderm that is intermediate between that of the homozygous wild-type and homozygous mutant.

^a —, no dominant effects, embryos exhibit only the recessive mutant phenotype.

^b The strength of the E-cadherin alleles, rated on a scale of 1–5 (most severe to normal), is based on: (1) the maternal, zygotic maternal and paternal dominant effects, (2) the β-catenin localization presented in this paper, and (3) the severity of the homozygous recessive phenotype, where epiboly of the deep cells slows relative to the enveloping layer and dorsal forerunner cells, followed by eventual retraction of the blastoderm and dissociation of the embryo usually by the end of epiboly. In addition, in all the homozygous E-cadherin mutants, dorsal forerunner cells do not always coalesce together properly. For the nonsense mutants this is a strictly recessive trait, but for the missense mutants it is also a maternal effect, see **MD** above.

^c The strength of the N-cadherin alleles, rated on a scale of 1–5 (most severe to normal), is based on: (1) the zygotic dominant effects previously published and presented in this paper, (2) the β-catenin localization presented in this paper, and (3) the severity of the homozygous recessive phenotype, where cells in the paraxial mesoderm round up and dissociate, neurectoderm and mesoderm converge more slowly to the midline, whether or not somite boundaries form, the notochord kinks, or slow muscle cells migrate to the periphery. In addition, the morphology of the brain and tail are highly abnormal.

^d Kane *et al.* (2005).

^e Kane *et al.* (1996).

^f McFarland *et al.* (2005).

^g This paper.

^h Lele *et al.* (2002).

ⁱ Warga and Kane (2007).

^j Hong and Brewster (2006).

^k Cortés *et al.* (2003).

^l Birely *et al.* (2005).

confirming the conclusions of this article are represented fully within the article, and its tables and figures.

Results

E- and N-cadherin are mostly expressed in complementary patterns during embryogenesis

To better understand the phenotypes of E- and N-cadherin mutants, we did a careful side-by-side comparison of E- and N-cadherin expression starting at 30% epiboly (4.6 hr), when maternal E-cadherin expression is still present and zygotic N-cadherin expression is starting to emerge (Figure 1). At this time, E-cadherin is still expressed uniformly throughout the blastoderm in the enveloping layer and in the deep cells, but not in the yolk syncytial layer (Figure 1A), N-cadherin, on the other hand, is expressed in the yolk syncytial layer and in a subset of deep cells, but not in the enveloping layer (Figure 1G). Some deep cells weakly express N-cadherin throughout the blastoderm, though at the blastoderm margin its expression is robust, and based on their position in the blastoderm these cells are fated to become endoderm (Warga and Nusslein-Volhard 1999), whose adult derivatives in mammals are known to form adherens junctions composed of E-N-cadherin heterodimers (Straub *et al.* 2011). Once gastrulation commences and germ layers begin to form (5.3 hr), E-cadherin expression continues to remain robust in the enveloping layer. However, in the deep cells, which give rise to the embryo proper, its expression diminishes and by midgastrulation

(7 hr) it is only found in the nonneural ectoderm, in the prechordal mesendoderm (open arrowheads), and in a group of cells ahead of the blastoderm known as dorsal forerunners (arrows; Figure 1, B–D). In contrast, N-cadherin expression continues to remain robust in the yolk syncytial layer, while in the deep domain its expression is now localized in the neurectoderm and in cells that have internalized to become mesendoderm; like E-cadherin, N-cadherin is also expressed in prechordal mesendoderm (open arrowheads) and in the dorsal forerunner cells (arrows; Figure 1, H–J). As development proceeds, these expression patterns for the most part do not change (Figure 1, E, F, K, and L). Thus, with the exception of prechordal mesendoderm and dorsal forerunner cells, E- and N-cadherin are expressed in complementary domains.

Of the two embryonic tissues where E- and N-cadherin are coexpressed, we examined more carefully the time course of expression in dorsal forerunner cells, as they are easy to identify once they segregate from the enveloping layer to migrate in front of the deep cell margin (Oteíza *et al.* 2008; Warga and Kane 2018). Like all enveloping layer cells, the precursors to dorsal forerunner cells strongly express E-cadherin. However at ~50% epiboly (5.3 hr), as these cells change their morphology (Figure 1, B and M) and begin undergoing an epithelial to mesenchymal transition (Oteíza *et al.* 2008; Warga and Kane 2018), they also begin expressing N-cadherin (Figure 1, H and P). By 60% epiboly (6.5 hr), when dorsal forerunner cells have migrated ahead of the deep cells, E- and N-cadherin are both strongly coexpressed (Figure 1, N and Q), but by 70% epiboly (7 hr), E-cadherin

Figure 1 E- and N-cadherin tend to be expressed in a complementary pattern. Expression of: E-cadherin (A–F, M–O, and T and U) and N-cadherin (G–L, P–R, and S). (A–L) Low-magnification view of wild-type embryos at similar stages of development; shown in side view, except (B, F, H, and L) shown in dorsal view and (D) shown in an oblique view. Prechordal mesendoderm (white arrowheads) and the dorsal forerunner cluster (black arrows) both express E- and N-cadherin. (M–R) Higher-magnification view of the dorsal forerunner cluster in wild-type embryos at similar stages of development, (M and P) are higher-magnification views of embryos shown in (B and H). Note that the forerunner cluster is tightly opposed to the blastoderm margin. Bar, 50 μ m. (S–U) Low-magnification view of homozygous mutant embryos and their siblings at similar stages of development shown in dorsal view: (S) the E-cadherin nonsense allele, (T) the N-cadherin nonsense allele, and (U) the N-cadherin EC1 missense allele. RNA transcripts are comparable between homozygous mutants and wild-type siblings, allowing for differences in the embryos shape. Arrow in S indicates the forerunner cluster, which in all the epiboly mutants continues to migrate vegetalward. Arrows in (T) show the wider anterior axis typical of all N-cadherin mutants due to the impaired convergence of cells to the dorsal midline, and arrows in (U) show that migration of the epidermis over the neural keel is defective in the N-cadherin missense mutant compared to its wild-type sibling. e-cad, E-cadherin; n-cad, N-cadherin; WT, wild-type.

expression is mostly gone (Figure 1, C and O) leaving only N-cadherin expression (Figure 1, I and R). Thus, for almost 2 hr, both cadherins are coexpressed in this cohort of cells.

Missense alleles of E- and N-cadherin affect forerunner cell development

We asked whether the various EC repeat mutations in E- and N-cadherin (Figure 2A) had patterning defects in dorsal forerunner cells or their derivative Kupffer's vesicle, a ciliated structure in the tail that confers left-right asymmetry in the zebrafish (Melby *et al.* 1996; Amack and Yost 2004). Using localization of β -catenin, we first examined forerunner cell morphology shortly after they formed. Then, using forerunner-specific markers such as *sox32* and *no tail* (Alexander and Stainier 1999; Amack and Yost 2004), we examined the forerunner

cells once they had polarized and compacted prior to forming a vesicle. β -catenin is normally enriched in the cytoplasm and nuclei of all forerunner cells (Figure 2, B and C), and in the homozygous recessive mutants at the N-cadherin locus—*bib* *tb08*, a missense allele in EC1, hereon referred to as *tb08* (*ncad*; EC1); *pac* *p79^{emcf}*, a missense allele in EC5, hereon referred to as *p79* (*ncad*; EC5); and *pac* *tm101*, a nonsense allele in EC4 and hereon referred to as *tm101* (*ncad*; 0)—forerunner patterning was essentially wild-type at 60% epiboly (6.5 hr). Both in terms of β -catenin localization, cell number, and position of the cluster (Figure 2B) juxtaposed to the deep cells (Figure 1, Q and R). For reference, we provide Table 1 for help with renaming of mutant alleles and their individual attributes. However, by the one-somite stage (10.3 hr), there were consistently fewer forerunner cells in the compacted rosettes of the *tb08* (*ncad*; EC1) mutant compared to wild-type siblings (Figure 2D), and later, Kupffer's vesicle was smaller and often asymmetrically placed (Figure 2F). In agreement with these defects, laterality of the heart, endodermal organs, and brain were frequently reversed or abnormal (Figure 2, H and J). While neither the *p79* (*ncad*; EC5) nor *tm101* (*ncad*; 0) mutants appeared to have defects in forerunner cell morphogenesis, the EC5 missense mutant also displayed defects in left-right asymmetry not observed in the nonsense mutant (Figure 2H).

In the homozygous recessive mutants at the E-cadherin locus—*hab* *tm94*, a missense allele in EC1, hereon referred to as *tm94* (*ecad*; EC1); *hab* *dtv43*, a missense allele in EC5, hereon referred to as *dtv43* (*ecad*; EC5); and *hab* *tx230*, a nonsense allele in EC4, hereon referred to as *tx230* (*ecad*; 0)—overt forerunner patterning was not normal at 60% epiboly. In all these homozygous mutants, cells in the forerunner cluster did not always coalesce into a single aggregate (Figure 2I), nor did they remain in contact with the deep cell margin (Figure 2C). Instead, forerunner cells advanced far ahead with the enveloping layer as seen in Figure 1S, leaving the deep cells far behind, perhaps because forerunner cells derive from the enveloping layer, which continues to contribute cells to the cluster throughout epiboly (Warga and Kane 2018). However, in addition to these phenotypes, in both missense mutants, but not the nonsense mutant, β -catenin expression was elevated at 60% epiboly (compare phenotypic wild-type in Figure 2B to heterozygous and homozygous siblings in Figure 2C). Nonetheless, even with this difference in β -catenin expression, all homozygous mutant alleles of E-cadherin appeared to have the correct number of forerunner cells at the one-somite stage, only they did not always form a single rosette (Figure 2, E and I). Although these homozygous mutants do not generally survive much past epiboly (McFarland *et al.* 2005), occasional *dtv43* (*ecad*; EC5) mutants sometimes survive to five-somites and rare individuals to 10-somites (McFarland *et al.* 2005). In one surviving individual, Kupffer's vesicle formed, but there was more than one vesicle (Figure 2G). Though impossible to score left-right asymmetry because of this early lethality, missense mutations at the E-cadherin locus also display a

Figure 2 Forerunner cell development and left-right asymmetry are perturbed in E- and N-cadherin missense mutants. (A) Location of E- and N-cadherin zebrafish mutants as determined from reported sequences (Lele et al. 2002; Malicki et al. 2003; Masai et al. 2003; Birely et al. 2005; Kane et al. 2005; von der Hardt et al. 2007; Warga and Kane 2007; Yamaguchi et al. 2010). Only the EC repeat alleles are shown. At the E-cadherin locus, all homozygous mutants display a common phenotype where epiboly of the deep cells slows relative to the enveloping layer and dorsal forerunner cells (see Figure 1S), followed by retraction of the blastoderm and disassociation of the embryo, usually before onset of somitogenesis. At the N-cadherin locus, all homozygous mutants display a common phenotype where convergence of the deep cells is defective (see Figure 1, T and U). Embryos eventually display pronounced defects in brain morphology, including cells detaching from the neuroepithelial surface and, where examined, abnormalities in muscle, eye, and tail development. Solid arrows denote a nonsense mutation, open arrows denote a missense mutation, asterisk denotes a mutation reported to have domi-

zygotic-maternal dominant (ZMD) phenotype when both the mother and her progeny are heterozygous, where embryos display more subtle aspects of the homozygous mutant cell adhesion defects (Table 1). These embryos also frequently have multiple forerunner clusters (Figure 2I) and later multiple vesicles (Figure 2G). Both *dtv43* (*ecad*; EC5) and *tm94* (*ecad*; EC1) heterozygous ZMD missense mutants displayed defects in laterality of the heart and gut (Figure 2, H and J). In sum, E- and N-cadherin missense mutants, but not nonsense mutants, affect different aspects of forerunner cell morphogenesis, and if vesicles form in these mutants this vesicle does not coordinate left-right asymmetry properly.

A possible explanation for the weaker phenotypes observed above in the cadherin nonsense mutants is genetic compensation, where changes in RNA or protein levels of related

nanot effects, and red denotes the alleles used in this study. For further information see Table 1. Abbreviations: Cyt, cytoplasmic domain; EC1–5, extracellular repeats; Pro, prodomain; S, signal sequence; TM, transmembrane domain. (B and C) High-magnification view of the dorsal forerunner cells (arrows), showing localization of β-catenin. (B) A representative embryo from a *tb08* (*ncad*; EC1) clutch, exhibiting a phenotypic wild-type appearance (39/39). (C) Representative embryos from a *dtv43* (*ecad*; EC5) clutch showing more intense staining in the forerunner cells of the homozygous mutant (8/8) compared to the heterozygous zygotic-maternal dominant (ZMD) mutant (27/28), even in isolated cells (arrowheads). Wild-type embryos had less intense staining in the forerunner cells (12/12) and resembled embryo shown in (B). Note also that the forerunner cluster in the homozygous mutant is at the leading edge of the enveloping layer, but already far from the deep cell margin (17/17). In the heterozygous ZMD mutant the forerunner cluster is closer to the deep cell margin, but not completely touching (47/47). In the wild-type sibling, the forerunner cluster abuts the blastoderm margin (23/23), as seen in (B). Bar, 25 μm. (D and E) High-magnification view of the forerunner cluster once cells have begun to aggregate into rosettes, visualized with sox32. (D) Showing reduced cell number in the homozygous *tb08* (*ncad*; EC1) mutant (11/11) compared to phenotypic wild-type (WT) (30/30). (E) Showing range of phenotypes for the forerunner cluster in E-cadherin mutants. A homozygous *tx230* (*ecad*; 0) mutant, its wild-type sibling, and a homozygous *dtv43* (*ecad*; EC5) mutant are shown. For the *tx230* (*ecad*; 0) allele, this phenotype is strictly a recessive trait, but for the *dtv43* (*ecad*; EC5) allele, this phenotype is a maternal trait (Table 1); homozygous wild-type and heterozygous ZMD mutant siblings (not shown) also frequently have more than one cluster. For quantification of the range of phenotypes in the different alleles and genotypes see graphs in (I) below. Bar, 40 μm. (F and G) The morphology of Kupffer's vesicle in live embryos, Kupffer's vesicle is the zebrafish organ of left-right asymmetry, and the derivative of dorsal forerunner cells. (F) Showing smaller vesicle in the homozygous *tb08* (*ncad*; EC1) mutant (9/9) compared to phenotypic wild-type (13/13), and (G) showing multiple vesicles in a single surviving homozygous *dtv43* (*ecad*; EC5) mutant and its ZMD mutant sibling (9/34). Bar, 50 μm. (H) Quantification of left-right asymmetry using looping of the heart. A minimum of five clutches was examined for each category; number of embryos is shown below. (I) Quantification of the number of forerunner clusters in the different alleles of E-cadherin (left), and number of forerunner rosettes for the different genotypes of *dtv43* (*ecad*; EC5) and *tx230* (*ecad*; 0) (right). A minimum of three clutches was examined for each category; number of embryos is shown below. (J) Quantification of left-right asymmetry using looping of the gut or left-sided expression in the diencephalon. A minimum of two clutches was examined for each category; number of embryos is shown below.

Figure 3 The N-cadherin EC5 missense allele has a dominant phenotype. (A–C) Phenotype of embryos from a *p79* (*ncad*; EC5) mutant clutch, shown in: side view (A–C), optical cross section (A'–C'), and outline (A''–C'') traced from optical cross section; br, brain; n, notochord; nk, neural keel; and s, somites. Note in optical cross section how the brain goes from oval in the wild-type (55/55) to triangular in the heterozygote (113/113) to mushroom-shaped in the homozygous mutant (55/55). In addition, the neural tube and somites are wider posteriorly in both the heterozygous and homozygous mutant. (D) Genotype of embryos sorted by phenotype using microsatellite marker z6425 closely linked to N-cadherin (Warga and Kane 2007). Arrow indicates product segregating with the wild-type allele; asterisk denotes an individual sorted as phenotypically wild-type, but possessing both products indicating either a recombination event or sorting error.

genes can functionally compensate for the loss-of-function gene [reviewed in El-Brolosy and Stainier (2017)]. However, examination of N-cadherin expression in the E-cadherin nonsense mutant, and of E-cadherin expression in the N-cadherin nonsense mutant, revealed no obvious change in levels of gene expression allowing for differences in the embryo's architecture (Figure 1, S and T). Further, there was no difference in levels of E-cadherin expression in the N-cadherin EC1

missense mutant (Figure 1U), as would be expected. Thus, other than revealing their characteristic phenotypes, the arrest in epiboly of deep cells for the E-cadherin mutants, or the defective convergence of deep cells in the N-cadherin mutants, we could discern no increase in RNA transcripts. In support of these results, the phenotype of both E- and N-cadherin nonsense mutants is identical to their knockdown phenotype (Lele *et al.* 2002; Kane *et al.* 2005; Warga and Kane 2007), which is not subject to genetic compensation [reviewed in El-Brolosy and Stainier (2017)].

The N-cadherin EC5 missense allele has a dominant effect

The results above demonstrate that the N-cadherin EC5 missense mutant is more like the N-cadherin EC1 missense mutant than the N-cadherin nonsense mutant. Although EC1 and EC2 are generally thought to be the only repeats necessary for the adhesive interactions of classical cadherins, comparison of the missense mutations in EC1 and EC5 of E-cadherin suggest otherwise. Both display zygotic dominant (ZD)-negative phenotypes when heterozygous that are akin to one another, suggesting that their mutant product interacts with the normal product in these individuals to poison cell adhesion (Kane *et al.* 2005). Thus, adhesive interactions are possibly also contingent on EC5. However, to date, no missense mutations in any of the N-cadherin EC repeats, other than the ones in EC1 (Figure 2A), have been reported to have dominant effects. To investigate if perhaps a dominant character had been overlooked for the EC5 missense mutations (Figure 2A), we examined a *p79* (*ncad*; EC5) mutant clutch more carefully (Figure 3).

Due to defects in coordinated cell movements (Hong and Brewster 2006; Warga and Kane 2007), homozygous mutants for all alleles at the N-cadherin locus can be identified by midsomite stages by a widening of their axis (Figure 1T), particularly in the anterior and posterior of the embryo where cell contact is most perturbed (Lele *et al.* 2002; Harrington *et al.* 2007; Warga and Kane 2007), and a neural keel that in optical cross section appears mushroom-shaped rather than oval (Jiang *et al.* 1996; Lele *et al.* 2002; Harrington *et al.* 2007; Warga and Kane 2007). A similar but less severe phenotype is also observed in a *tb08* (*ncad*; EC1) clutch when the embryo is heterozygous mutant (Warga and Kane 2007), which hereon we refer to as a ZD phenotype (Table 1). We examined a *p79* (*ncad*; EC5) clutch at 10-somites (14 hr) for equivalent phenotypes and found, as expected, that 25% of the clutch (55/223) displayed the severe morphological defects of the homozygous mutant. However, exactly one-half of the clutch (113/223) exhibited a phenotype that was more intermediate, where the brain in optical cross section appeared triangular-shaped and, in the posterior of the embryo, the neural keel and somites were conspicuously wider than normal (Figure 3, A–C). Sorting by these phenotypes followed by genotyping showed that the intermediate phenotype segregated with the heterozygous genotype (Figure 3D). Hence, the N-cadherin EC5 missense allele displays a ZD

Figure 4 N-cadherin missense mutants are more severe than N-cadherin nonsense mutants. Only homozygous mutants and homozygous wild-type (WT) are shown. (A–D) High-magnification view of the eye showing N-cadherin protein (red) and DAPI nuclear staining (blue) in: *tm101* (*ncad*; 0) mutants (12/12), *p79* (*ncad*; EC5) mutants (11/11), and *tb08* (*ncad*; EC1) mutants (12/12). Bar, 30 μ m. (E–H) Embryo morphology of the different N-cadherin alleles. (I–N) High-magnification view of: (I–K) the enveloping layer showing E-cadherin protein (red) and DAPI nuclear staining (blue); and (L–N) the presumptive mesoderm showing N-cadherin protein (red) and DAPI nuclear staining (blue). Bar, 20 μ m. (O–Q) Expression of *dIA* in neuroblasts in: (O, O') *tm101* (*ncad*; 0) mutants (16/16), (P, P') *p79* (*ncad*; EC5) mutants (16/16), and (Q, Q') *tb08* (*ncad*; EC1) mutants (14/14); arrowheads denote width of the neural keel. (R–T) Expression of *dIC* in the somitic mesoderm of: (R, R') *tm101* (*ncad*; 0) mutants (12/12), (S, S') *p79* (*ncad*; EC5) mutants (41/41), and (T, T') *tb08* (*ncad*; EC1) mutants (15/15). (U–W) Expression of *fgf8a* in the somitic mesoderm of: (U, U') *tm101* (*ncad*; 0) mutants (22/22), (V, V') *p79* (*ncad*; EC5) mutants (26/26), and (W, W') *tb08* (*ncad*; EC1) mutants (23/23). Both *dIC* and *fgf8a* are also expressed in the nervous system. Embryos are shown in side view except (O–Q') shown in dorsal view.

phenotype previously only reported for N-cadherin EC1 missense mutants.

The homozygous phenotypes of N-cadherin missense mutants are stronger than N-cadherin nonsense mutants

The dominant character of the *p79* (*ncad*; EC5) allele suggests that it makes a mutant product that interferes with the wild-type product in a dominant-negative fashion, as demonstrated previously for the *tb08* (*ncad*; EC1) allele (Warga

and Kane 2007). However, it has been reported that the N-cadherin protein is not localized to the cell membrane in the *p79* (*ncad*; EC5) mutation (Harrington *et al.* 2007). We reexamined this using an N-cadherin antibody that recognizes repeats EC3–EC5 of zebrafish. As expected, the polyclonal antibody did not recognize a protein product in the *tm101* (*ncad*; 0) allele (Lele *et al.* 2002; Malicki *et al.* 2003), but in both the *p79* (*ncad*; EC5) and *tb08* (*ncad*; EC1) alleles, membrane staining in the eye was equivalent to that of wild-type embryos (Figure 4, A–D), indicating that not only is the

Table 2 Localization of β -catenin is altered in the missense alleles of E- and N-cadherin

Locus	Allele	Genotype	Membrane	Cytoplasmic	Incongruent ^a	Number	Clutches	Figure panel
Wild-type	—	+/+	+			81/81	2	Figure 5A
E-cadherin	<i>tx230^b</i>	-/-	+			11/11	1	Figure 5B
	<i>tm94</i>	+/+	+			23/23	2	—
		+/-	+	+		39/39		—
		-/-	+	+	+	20/21 ^c		Figure 5C
	<i>dtv43</i>	+/+	+			9/9	1	Figure 5D
		+/-	+	+		20/20		Figure 5D'
N-cadherin		-/-	+	+	+	8/8		Figure 5D''
	<i>tm101^b</i>	-/-	+			21/21	2	Figure 5E
	<i>p79^d</i>	-/-	+	+		15/15	1	Figure 5F
	<i>tb08</i>	+/+	+			39/39	4	—
		+/-	+	+		78/80 ^e		—
		-/-	+	+	+	36/37 ^f		Figure 5G

^a Incongruent refers to how cell-cell contact sites visualized with β -catenin staining are no longer uniform. Cells in normal embryos possess numerous contact sites and are uniformly spaced, whereas cells in missense mutants exhibit aberrant contact in which cells are bunched together and staining appears more intense, or fewer contact sites because of extracellular gaps where staining is not visible.

^b In these crosses, all β -catenin staining of embryos appeared wild-type: *tx230* (47/47) and *tm101* (71/71) total embryos.

^c The one exception was a heterozygous mutant.

^d In these crosses, we did not note an intermediate phenotype in the β -catenin staining of embryos, only a mutant pattern (15/15) and a normal pattern (43/43), perhaps because β -catenin mislocalization is less severe in this allele.

^e The two exceptions were homozygous mutants.

^f The one exception was a heterozygous mutant.

protein made but it is properly inserted into the membrane. We further confirmed that N-cadherin is not maternally encoded as previously reported (Bitzur *et al.* 1994; Warga and Kane 2007), unlike E-cadherin, which is (Kane *et al.* 2005). Typically, maternal products in the zebrafish are still present in the gastrula and not exhausted until somitogenesis (Kane *et al.* 2005; Warga *et al.* 2016). In the E-cadherin nonsense mutant, E-cadherin protein was visible in the enveloping layer at 80% epiboly (8 hr), at a level not discernably different from wild-type embryos or the missense mutant (Figure 4, I–K), but in the N-cadherin nonsense mutant, there was no N-cadherin protein at 80% epiboly, although it was visible in the mesoderm of wild-type embryos and the missense mutant (Figure 4, L–N). Hence, the phenotypes of the dominant N-cadherin mutants are entirely due to the action of their zygotic product.

Missense alleles of E-cadherin not only have dominant features, but display more extreme recessive phenotypes than nonsense alleles (Kane *et al.* 1996; McFarland *et al.* 2005). Previously, we showed the same for the EC1 missense and nonsense alleles of N-cadherin (Warga and Kane 2007). Therefore, we suspected that the EC5 missense allele of N-cadherin might behave in a similar fashion. Comparing the homozygous phenotypes of the three N-cadherin alleles shows that cell adhesion defects in the *p79* (*ncad*; EC5) mutant are more like the *tb08* (*ncad*; EC1) mutant and less like the *tm101* (*ncad*; 0) mutant. Not only was cell contact in the brain and tail more disordered (Figure 4, E–H), but neuroblasts visualized with *deltaA* (*dla*) (Haddon *et al.* 1998) were spaced further apart (Figure 4, O–Q') at 10 somites (14 hr) because cells in these mutants move slower to the dorsal midline (Lele *et al.* 2002; Warga and Kane 2007).

Homozygous *tb08* (*ncad*; EC1) mutants also possess cell adhesion defects in the somitic mesoderm that are less evident in homozygous *tm101* (*ncad*; 0) mutants. Thus, while gene expression indicates some segmental patterning in homozygous *tb08* (*ncad*; EC1) mutants, albeit irregular, morphological boundaries never form (Warga and Kane 2007) (Figure 4H). In contrast, homozygous *tm101* (*ncad*; 0) mutants have subtle defects in segmental patterning and somite boundaries appear normal (Warga and Kane 2007) (Figure 4F). We found that in homozygous *p79* (*ncad*; EC5) mutants, morphological boundaries were absent in the more posterior somitic mesoderm (compare Figure 4, E and G). Hence, here as well, homozygous *p79* (*ncad*; EC5) mutants bear more similarity to homozygous *tb08* (*ncad*; EC1) mutants than to homozygous *tm101* (*ncad*; 0) mutants.

We studied these differences further by examining gene expression. Normally, *deltaC* (*dlc*) is expressed in the tailbud and in oscillating stripes in the presomitic mesoderm. Afterward, it is maintained in the posterior half of each somite (Jiang *et al.* 2000; Smithers *et al.* 2000). At 18-somites (18 hr), all three alleles of N-cadherin expressed *dlc* normally in the presomitic mesoderm and tailbud, but not in the somites (Figure 4, R–T'). In *tm101* (*ncad*; 0) mutants, only the posterior “younger” somites kept normal metamerical *dlc* expression; in the anterior somites, metamerical expression was present only ventrally. However, in *p79* (*ncad*; EC5) and *tb08* (*ncad*; EC1) mutants, even the posterior-most somites had very little metamerical *dlc* expression. A similar phenomenon was observed with *fgf8a* (Figure 4, U–W'), which is normally expressed in the tailbud and maintained in the anterior half of each somite (Reifers *et al.* 1998). In the *tm101* (*ncad*; 0) mutant, metamerical *fgf8a* expression was reduced, though present in most every somite, whereas the *p79* (*ncad*; EC5) or *tb08* (*ncad*; EC1)

Figure 5 β -catenin mislocalizes to the cytoplasm in E- and N-cadherin missense mutants. (A–G) High-magnification view of the blastoderm, showing localization of β -catenin at 40% epiboly in: (A) WT, (B) *tx230* (*ecad*; 0), (C) *tm94* (*ecad*; EC1), (D–D'') *dtv43* (*ecad*; EC5), (E) *tm101* (*ncad*; 0), (F) *p79* (*ncad*; EC5), and (G) *tb08* (*ncad*; EC1) alleles. In wild-type or homozygous nonsense mutants (A, B, D, and E) β -catenin is membrane-bound, but in heterozygous or homozygous missense mutants (C, D', D'', F, and G), β -catenin is also cytoplasmic. Note that for the homozygous missense mutants in (C, D'', and G), cells are also more disorganized and staining appears more intense where cells aggregate together, but absent where there is extracellular space. (D–D'') are siblings. Bar, 20 μ m. For quantification of phenotypes please refer to Table 2. WT, wild-type; ZMD, zygotic–maternal dominant.

mutant had little metameric expression as well as differences in their patterns. Perhaps this is due to different EC repeats possessing different functions in different regions of the embryo, as has been posited for specific aspects of the dominant phenotypes in E-cadherin missense mutants (Kane *et al.* 2005). Regardless, these results suggest that maintaining these transcripts likely depends on cell interactions that are perturbed in *tm101* (*ncad*; 0) mutants, but nearly absent in *p79* (*ncad*; EC5) and *tb08* (*ncad*; EC1) mutants, where cell adhesion is less normal. In sum, it is far more deleterious to express a N-cadherin gene product possessing a mutated EC repeat than no gene product whatsoever, comparable to equivalent mutations in E-cadherin (Kane *et al.* 2005).

Missense alleles of E- and N-cadherin mislocalize β -catenin

Cadherins both modulate and are modulated by their intracellular binding partners. Normally, β -catenin is enriched at the cell membrane, where it helps anchor the cadherin complex to the cytoskeleton [reviewed in Ratheesh and Yap (2012)]. What with β -catenin being a Wnt signal transducer, typically little is found in the cytoplasm unless Wnt signaling

is activated. Thus, in general, cells clear cytoplasmic β -catenin, unless they are unable to as sometimes occurs when cadherin-based cell adhesion is dysfunctional [reviewed in McCrea *et al.* (2015) and Zhan *et al.* (2017)]. To better understand the missense alleles of E- and N-cadherin, and the severity of their phenotypes, we examined the subcellular distribution of β -catenin at 40% epiboly (5 hr), a time after zygotic E- and N-cadherin transcripts appear (Figure 1), but before detectable morphological defects are present in any of these mutants.

When comparing the different alleles of E-cadherin, we found little difference in β -catenin localization between embryos that were homozygous mutant for the nonsense allele of E-cadherin or embryos that were homozygous wild-type, whether or not the mother was heterozygous mutant for a missense allele of E-cadherin. In all three cases, β -catenin was enriched at the cell membrane and not in the cytoplasm (Figure 5, A, B, and D and Table 2). However, if embryos were heterozygous or homozygous mutant for a missense allele of E-cadherin this pattern changed. In embryos heterozygous mutant for *dtv43* (*ecad*; EC5) or *tm94* (*ecad*; EC1), β -catenin localized to the cell membrane and also to the cytoplasm (Figure 5D' and Table 2), whereas in embryos homozygous mutant for *dtv43* (*ecad*; EC5) or *tm94* (*ecad*; EC1), β -catenin staining additionally revealed that cells were no longer uniformly spaced (Figure 5, C and D'' and Table 2). In some areas, cells aggregated together causing staining to appear more intense, whereas in other places there were extracellular gaps with no staining whatsoever. Whether there was more cytoplasmic staining overall was difficult to tell. These phenotypes are consistent with the more aberrant cell adhesion observed in the missense mutants (Kane *et al.* 1996; McFarland *et al.* 2005).

A similar pattern was observed when comparing the different alleles of N-cadherin. Homozygous *tm101* (*ncad*; 0) mutants looked normal (Figure 5E and Table 2); heterozygous *tb08* (*ncad*; EC1) mutants and homozygous *p79* (*ncad*; EC5) mutants resembled the less severe phenotype of the *dtv43* (*ecad*; EC5) and *tm94* (*ecad*; EC1) heterozygotes, where β -catenin was localized to both the cell membrane and cytoplasm (Figure 5F and Table 2); and homozygous *tb08* (*ncad*; EC1) mutants looked like the more severe phenotypes of the *dtv43* (*ecad*; EC5) or *tm94* (*ecad*; EC1) homozygote showing loss of uniformity of β -catenin staining because of altered cell contact (Figure 5G and Table 2). Hence, if there is no E- or N-cadherin product, the subcellular distribution of β -catenin is normal. However, if either cadherin has a mutated EC repeat, not only is cell contact more severely affected consistent with dysfunctional adhesion, but the subcellular distribution of β -catenin shifts into the cytoplasm, indicating that the cadherin–catenin complex is not functioning properly.

E- and N-cadherin missense alleles genetically interact

The dominant nature of the E- and N-cadherin missense alleles make them unique tools to explore possible cadherin

interactions. To test the idea that E- and N-cadherin genetically interact with each other, we crossed females heterozygous for either the EC1 or EC5 missense allele of N-cadherin to males heterozygous for the EC5 missense allele of E-cadherin, as it is the strongest dominant allele (see Table 1). We then examined the resulting progeny at midsomite stages, when these heterozygous N-cadherin missense mutants can be unambiguously identified based on their ZD phenotype (Figure 3B and Table 1). We performed the cross in this direction to avoid confusion in assigning genotypes as the phenotype of the heterozygous E-cadherin ZMD missense mutant (Figure 6A) is similar: a wide, misshapen neural keel (McFarland *et al.* 2005). For brevity and clarity in this section and those that follow, we refer to the mutant phenotype by its respective genotype based on the characteristic traits that each allele displays (Table 1).

If females heterozygous for the *p79* (*ncad*; EC5) allele were crossed to males heterozygous for the *dtv43* (*ecad*; EC5) allele, their embryos exhibited either a wild-type or heterozygous *p79* (*ncad*; EC5) ZD mutant phenotype (Figure 6, D–D’). No embryo had a phenotype as severe as a homozygous *p79* (*ncad*; EC5) mutant (Figure 6B), but 12.5% of the total embryos ($n = 43/360$; three crosses) exhibited not only the ZD brain phenotype of *p79* (*ncad*; EC5), but also an additional phenotype in the tailbud, where cells along the dorsal midline of the tailbud detached (Figure 6D’), much like they do in the heterozygous *dtv43* (*ecad*; EC5) ZMD mutant (Figure 6A). The identification of such embryos as doubly heterozygous was confirmed by the eventual appearance of the *dtv43* (*ecad*; EC5) paternal dominant effect (PD) hatching gland trait (Table 1).

Crossing females heterozygous for the *tb08* (*ncad*; EC1) allele to males heterozygous for the *dtv43* (*ecad*; EC5) allele gave similar results (Figure 6, E–E’); only the additional phenotype was more penetrant, 21% of the total embryos, and closer to the expected 25% for the double heterozygote. While heterozygous *tb08* (*ncad*; EC1) ZD mutants with the detached cell phenotype ($n = 176/855$; six crosses) were not phenotypically as severe as homozygous *tb08* (*ncad*; EC1) mutants (Figure 6C), they even more strongly resembled the heterozygous *dtv43* (*ecad*; EC5) ZMD mutant. Generally, in the ZMD mutant, cells round up and detach from the trunk and tail (Figure 6A), but in more severe mutants, cells above the head also detach (McFarland *et al.* 2005). This occurred in the heterozygous *tb08* (*ncad*; EC1) ZD mutants (Figure 6E’’) that we later determined were also heterozygous for *dtv43* (*ecad*; EC5). Thus, the combination of these two loci being heterozygous mutant introduces a phenotype previously observed only when both the zygotic and maternal genomes were heterozygous mutant at the E-cadherin locus.

Conversely, if we crossed females heterozygous for the *tm101* (*ncad*; 0) allele to males heterozygous for the *dtv43* (*ecad*; EC5) allele, embryos only exhibited a wild-type appearance barring the PD hatching gland trait of *dtv43* ($n = 461/461$; five crosses), as the *tm101* (*ncad*; 0) allele has no ZD mutant phenotype (Table 1). Therefore, if N-cadherin

Figure 6 Missense alleles of E- and N-cadherin genetically interact. (A–C) Morphology of the heterozygous *dtv43* (*ecad*; EC5) ZMD mutant, and the homozygous *p79* (*ncad*; EC5) and *tb08* (*ncad*; EC1) mutant; black arrows indicate cells detaching from the neural keel, and white arrows indicate disaggregated cells in the brain and tail. (D–D’’) Embryos derived from a female heterozygous for the *p79* (*ncad*; EC5) allele and a male heterozygous for the *dtv43* (*ecad*; EC5) allele. (E–E’’) Embryos derived from a female heterozygous for the *tb08* (*ncad*; EC1) allele and a male heterozygous for the *dtv43* (*ecad*; EC5) allele. (F–F’’) Embryos derived from a female heterozygous for the *tm94* (*ecad*; EC1) allele and a male heterozygous for the *tb08* (*ncad*; EC1) allele. ZMD, zygotic–maternal dominant.

activity is reduced, the detached cell phenotype in the double heterozygote disappears.

Next, we crossed females heterozygous for the *tm94* (*ecad*; EC1) allele, which has a weaker ZMD phenotype (Kane *et al.* 1996), to males heterozygous for the *tb08* (*ncad*; EC1) allele. Here, we examined the resulting embryos slightly later, when the heterozygous *tm94* (*ecad*; EC1) ZMD mutant phenotype is more easily distinguished from the heterozygous *tb08* (*ncad*; EC1) ZD mutant phenotype by gaps in the neural keel (Table 1). In addition to wild-type embryos, heterozygous *tm94* (*ecad*; EC1) ZMD mutants, and heterozygous *tb08* (*ncad*; EC1) ZD mutants (Figure 6, F and F’ and data not shown), there was a fourth distinguishable category in which 21% of the total embryos displayed attributes of both mutations (Figure 6F’). Like the heterozygous *hab tm94* (*ecad*; EC1) ZMD mutant (Figure 6F’), these embryos

Figure 7 The EC5 missense allele of E-cadherin exacerbates the homozygous phenotype of the EC1 missense allele of N-cadherin. (A–F) embryos obtained from a female heterozygous for *dtv43* (*ecad*; EC5) and *tb08* (*ncad*; EC1), and a male heterozygous for *tb08* (*ncad*; EC1), in these crosses the expected ratio for +/+, ZMD*dtv43*/+, *tb08*/*tb08*, and ZMD*dtv43*/+; *tb08*/*tb08* is 1/8. For comparison (B'–F') embryos were obtained from a male and female heterozygous for *tb08* (*ncad*; EC1), in these crosses the expected ratio for +/+ and *tb08*/*tb08* is 1/4. The proportion of embryos exhibiting each phenotype is indicated for each genotype. Expression of: (A) *notch1b* in the neural keel and notochord; (B, B') *notch5* in the neural keel, notochord, and somitic mesoderm; (C, C') *deltaD* in the neural keel and somitic mesoderm; (D, D') *myoD* in the somitic mesoderm; (E, E') *no tail* in the notochord; and (F, F') *foxa2* in the endoderm and notochord. Embryos are shown in dorsal view; designations: open circles, notochord and arrowheads, extent of neural keel. ZMD, zygotic–maternal dominant.

had gaps in the neural keel, but much like the homozygous *tb08* (*ncad*; EC1) mutant (Figure 4H), these embryos also had cells detaching from the brain, and their tail was thicker and cavitated ($n = 130/628$; five crosses). Furthermore, 75% of the mutants in this category ($n = 98/130$) were also cyclopic (Figure 6F"), a phenotype that occurs when prechordal mesendoderm—which coexpresses E- and N-cadherin (Figure 1, D and J)—does not move properly forward to split the eye field (Heisenberg *et al.* 2000). Hence, adding a maternal component to the double heterozygote increases the severity of its phenotype. If we performed the same cross, only using instead the null allele of E-cadherin (*tx230*), embryos exhibited only a wild-type phenotype ($n = 163/321$; three crosses) or the heterozygous N-cadherin ZD mutant phenotype ($n = 158/321$). Thus, as above, if normal activity of E-cadherin is reduced both maternally and zygotically, these unexpected phenotypes disappear.

The E-cadherin locus alters the phenotype of the homozygous N-cadherin mutant

To further explore this genetic interaction, we created embryos that were homozygous mutant at the N-cadherin locus

and heterozygous mutant at the E-cadherin locus using two missense alleles. Crossing females heterozygous for both the *dtv43* EC5 missense allele of E-cadherin and the *tb08* EC1 missense allele of N-cadherin to males heterozygous for only *tb08*, we compared the phenotype of homozygous N-cadherin mutants derived from this cross to homozygous N-cadherin mutants derived from females not carrying a E-cadherin allele.

As previously demonstrated (McFarland *et al.* 2005; Warga and Kane 2007), in both homozygous *tb08* (*ncad*; EC1) mutants and heterozygous *dtv43* (*ecad*; EC5) ZMD mutants, convergence of the ectoderm, mesoderm, and endoderm to the dorsal midline is compromised (Figure 7). For example, at seven somites, the expression of *notch1b*, *notch5*, and *deltaD* in the neural keel (Bierkamp and Campos-Ortega 1993; Westin and Lardelli 1997; Haddon *et al.* 1998); metameric expression of *myoD*, *notch5*, and *deltaD* in the somitic mesoderm (Weinberg *et al.* 1996; Westin and Lardelli 1997; Haddon *et al.* 1998); expression of *no tail* in the notochord (Schulte-Merker *et al.* 1994); and expression of *foxa2* in the nonaxial endoderm (Strähle *et al.* 1993) are all noticeably wider than normal. However, only homozygous *tb08* (*ncad*; EC1) mutants have difficulty forming and maintaining somite

Figure 8 The EC5 missense allele of E-cadherin does not affect the homozygous phenotype of the N-cadherin nonsense allele. (A–D) embryos obtained from a female heterozygous for *dtv43* (*ecad*; EC5) and *tm101* (*ncad*; 0), and a male heterozygous for *tm101* (*ncad*; 0); in these crosses the expected ratio for +/+, ZMD*dtv43*/+, *tm101/tm101*, and ZMD*dtv43*/+; *tm101/tm101* is 1/8. For comparison (A'–D') embryos were obtained from a male and female heterozygous for *tm101* (*ncad*; 0); in these crosses the expected ratio for +/+, ZMD*dtv43*/+, *tm101/tm101*, and ZMD*dtv43*/+; *tm101/tm101* is 1/4. The proportion of embryos exhibiting each phenotype is indicated for each genotype. Expression of: (A, A') *notch5* in the neural keel, notochord, and somitic mesoderm; (B, B') *deltaD* in the neural keel and somitic mesoderm; (C, C') *myoD* in the somitic mesoderm, and (D, D') *no tail* in the notochord. Embryos are shown in dorsal view; designations: open circles, notochord and arrowheads, extent of neural keel. ZMD, zygotic–maternal dominant.

borders (Figure 7, B'–D'), as well as the border between the somitic mesoderm and notochord (Warga and Kane 2007) (Figure 7, D' and E').

When homozygous *tb08* (*ncad*; EC1) mutants are both maternally and zygotically heterozygous for *dtv43* (*ecad*; EC5), all these cell movement defects are more impaired and boundary defects appear in other germ layers. For example, in the ectoderm, cells at the lateral border of the neural keel are disordered (compare *notch1b*, *notch5*, and *deltaD* expression in the neural keel; Figure 7, A–C), and in the endoderm, nonaxial endodermal cells pile up against the midline, suggesting that they form abnormal borders (compare *foxa2* expression; Figure 7F). In addition, in the mesoderm, boundary defects are further amplified: not only is muscle and notochord more highly intermingled (compare *myoD* and *no tail* expression; Figure 7, D and E), but also within the somites, borders are lost (compare metameric expression of *myoD*, *notch5*, and *deltaD*; Figure 7, B and D). Similar, but less severe, perturbations occur in homozygous *tb08* (*ncad*; EC1) mutant siblings zygotically wild-type for

dtv43 (*ecad*; EC5) [compare homozygous *tb08* (*ncad*; EC1) mutants in Figure 7, A–F]. Thus, merely having the mutant E-cadherin product provided maternally affects the homozygous *tb08* (*ncad*; EC1) mutant phenotype [compare homozygous *tb08* (*ncad*; EC1) mutants in Figure 7, B–F to equivalent mutants in Figure 7, B'–F'].

We did not repeat these experiments with a nonsense allele of E-cadherin, because it has no dominant phenotype or maternal effect (Table 1). Instead, we crossed females heterozygous for both the *dtv43* EC5 missense allele of E-cadherin and the *tm101* nonsense allele of N-cadherin to males heterozygous for only *tm101* (Figure 8). Like homozygous *tb08* (*ncad*; EC1) mutants, only less severe, homozygous *tm101* (*ncad*; 0) mutants have defects in convergence of cells to the midline, and maintenance of the border between the muscle and notochord (Lele *et al.* 2002; Warga and Kane 2007). In this background, the *dtv43* (*ecad*; EC5) allele had no effect on the homozygous N-cadherin mutant phenotype: cell movement and boundary defects were no different whether embryos were wild-type for *dtv43* or maternally and zygotically mutant for *dtv43* [compare homozygous *tm101* (*ncad*; 0) mutants in Figure 8, A–D to equivalent mutants in Figure 8, A'–D']. Therefore, with no N-cadherin gene product, the E-cadherin missense allele had no additional consequences. These results support the idea that the E- and N-cadherin missense products interfere with each other.

Forerunner cells are not specified properly, nor do they differentiate in E- and N-cadherin double mutants

Finally, we examined the effect on forerunner cell development when embryos were homozygous mutant at both the E- and N-cadherin locus because of their coexpression in these cells (Figure 1, M–R). Again, we used the two strongest dominant alleles (Table 1), crossing parents heterozygous for both *dtv43* (*ecad*; EC5) and *tb08* (*ncad*; EC1). Of note, *dtv43* (*ecad*; EC5) is the only E-cadherin allele where embryos sometimes live long enough to form a vesicle (Figure 2G). For these experiments, we compared double homozygous mutants to siblings that were homozygous mutant for E-cadherin.

In all alleles of E-cadherin, the homozygous mutant embryo arrests epiboly of the deep cells; however, dorsal forerunner cells and the enveloping layer advance normally over the yolk cell (Kane *et al.* 1996; McFarland *et al.* 2005) (Figure 1S and Figure 2C). We sorted for this phenotype at 75% epiboly (7.5 hr), before the homozygous N-cadherin mutant phenotype is visible (Warga and Kane 2007), and then stained for *no tail* expression (Figure 9, A–D), which marks forerunner cells and the developing notochord. In this group of embryos, the notochord domain did not consolidate properly because convergence to the midline is also perturbed in homozygous E-cadherin mutants (McFarland *et al.* 2005). However, in addition to this phenotype, 35% of these mutant embryos (146/418 and Figure 9M) displayed forerunner defects where cells expressed *no tail* weakly (Figure 9, B and C) and sometimes there were fewer cells (Figure 9D),

Figure 9 Double homozygous E- and N-cadherin missense mutants radically alter forerunner cell patterning. (A–D) Homozygous *dtv43* (*ecad*; EC5) mutants from a double-mutant clutch before the *tb08* (*ncad*; EC1) mutant phenotype can be detected. They are visualized with *no tail* to show dorsal forerunner cell (arrows) phenotypes. For quantification of range of phenotypes see graph (M) below. Note that *no tail* is also expressed in the notochord, which is also perturbed in homozygous *dtv43* (*ecad*; EC5) mutants. (E–H) Homozygous *dtv43* (*ecad*; EC5) single mutants and their (I–L) homozygous *dtv43* (*ecad*; EC5); *tb08* (*ncad*; EC1) double-mutant siblings visualized with *no tail* and anti β -catenin staining; (E and I) are low-magnification view and (E'–H and I'–L) are high-magnification view of Kupffer's vesicle. Bar in (E'–H and I'–L), 60 μ m. (E–G and I–K) Expression of *no tail* in Kupffer's vesicle in: *dtv43* (*ecad*; EC5) single mutants (39/39) and *dtv43* (*ecad*; EC5); *tb08* (*ncad*; EC1) double mutants (17/17). For quantification of range of phenotypes see graph (O) below. Black arrows indicate vesicles and white arrows indicate cells disaggregating from the kinked notochord, a distinguishing feature of the *tb08* (*ncad*; EC1) allele. (H and L) Expression of β -catenin in Kupffer's vesicle in: *dtv43* (*ecad*; EC5) single mutants (29/29) and *dtv43* (*ecad*; EC5); *tb08* (*ncad*; EC1) double mutants (9/9). Dots indicate enveloping layer nuclei showing that β -catenin staining is comparable between siblings. (M) Quantification of range of

forerunner cell phenotype at 75% epiboly shown in (A–D); and (N) at the one-somite stage, 3 hr later after the *tb08* (*ncad*; EC1) notochord phenotype can be seen. (O) Quantification of range of vesicle phenotypes at the five-somite stage shown in (E–G and I–K). For (M) a total of three crosses was examined, for (N and O) a total of four crosses was examined for each category; number of embryos is shown below. dfc, dorsal forerunner cell; KV, Kupffer's vesicle.

suggesting changes in cell specification. If we waited until the one-somite stage (10.5 hr), before staining for *no tail*, we could identify the double mutant by its kinked notochord and cells rounding up in the paraxial mesoderm, characteristic of homozygous *tb08* (*ncad*; EC1) mutants (Warga and Kane 2007). This confirmed that the forerunner-patterning defects observed at 75% epiboly tended to segregate (19/22) with the double mutant (Figure 9N). Similar experiments with embryos doubly homozygous mutant for *tx230* (*ecad*; 0) and *tm101* (*ncad*; 0) showed that they did not have these forerunner-patterning defects (Figure 9N).

Forerunner cells progressively compact together until, by five-somites (11.3–12 hr), they have rearranged themselves into an epithelial ball with a lumen (Melby *et al.* 1996). Examination of embryos at this stage revealed that Kupffer's vesicle(s) in homozygous *dtv43* (*ecad*; EC5) mutants had differentiated properly (Figure 9, E–H), but not in homozygous *dtv43* (*ecad*; EC5); *tb08* (*ncad*; EC1) mutants (Figure 9, I–L). Although the level of *ntl* expression mostly returned to near normal levels, there were frequently not enough cells, or cells formed a grossly irregular or disaggregated structure compared to Kupffer's vesicle(s) in their single-mutant siblings (Figure 9O). Consistent with this apparent loss of cohesivity and change in cell adhesion, β -catenin was barely visible in the cytoplasm and nuclei of these vesicle cells (compare Figure

9, H and L), and the notochord was noticeably more disorganized (compare Figure 9, E and I), together suggesting that cells in the double mutant were less adherent.

Discussion

This study reveals that a functional EC5 repeat is critical for cadherin-mediated cell adhesion, and that E- and N-cadherin appear to interact synergistically. Using missense and nonsense mutants in N-cadherin, we show that embryos with a dysfunctional EC5 repeat have more impaired cell adhesion than embryos without an N-cadherin product. Phenotypically, this EC5 missense mutant resembles an EC1 missense mutant with dominant gain-of-function, in accordance with equivalent mutations in E-cadherin that we previously characterized. We also demonstrate, using missense mutations in both E- and N-cadherin, that when the zygotic genome is mutant at both these loci, or if the maternal genome is mutant, unexpected phenotypes appear. Hence, the EC5 repeat for both E- and N-cadherin is somehow important for cell adhesion, and might participate in cadherin dimerization. Our studies also argue that E- and N-cadherin may possibly dimerize with each other. Thus, in the context of early embryonic development where cells constantly change their adhesive environments, cadherin-mediated cell adhesion may depend on more than just homophilic interactions.

Missense mutations in the cadherin EC5 repeat have gain-of-function dominant-negative effects

Here, we demonstrate that a missense mutation in the EC5 repeat of the zebrafish homolog of N-cadherin is a dominant gain-of-function mutation that acts as an antimorphic allele, much like a missense mutation in the EC1 repeat of N-cadherin that we previously characterized (Warga and Kane 2007). It is widely accepted that the mechanism for adhesive binding by classical cadherins is mediated by the EC1 and EC2 repeats [reviewed in Halbleib and Nelson (2006), Shapiro and Weis (2009), Brasch *et al.* (2012), and Ishiyama and Ikura (2012)]. Nonetheless, it is noteworthy that inherited mutations in any one of the five EC repeats of E-cadherin are the cause for the most aggressive malignant gastric cancers in humans, many of which are missense mutations (Guilford *et al.* 1998; Brooks-Wilson *et al.* 2004; More *et al.* 2007; Mateus *et al.* 2009). The reasons for this malignancy are unclear, but our studies show that cells with missense mutations in either the EC1 or EC5 repeats of E- or N-cadherin alter adhesion and cell interactions far more than cells carrying nonsense mutations. Such cells would be unable to maintain normal tissue homogeneity and, based on our results, might mislocalize β -catenin to the cytoplasm thereby perturbing Wnt signaling, a pathway intimately associated with cancer [reviewed in McCrea *et al.* (2015) and Zhan *et al.* (2017)].

Like each of the two missense mutations in the EC1 repeat of N-cadherin, the missense mutation in the EC5 repeat of N-cadherin occurs in a highly conserved amino acid (Malicki *et al.* 2003; Birely *et al.* 2005; Warga and Kane 2007) and likewise has a dominant-negative effect. Specifically, heterozygous individuals display a phenotype intermediate between homozygous mutant and homozygous wild-type (Figure 3). Our interpretation of this dominant phenotype is that the *p79* (*ncad*; EC5) mutation produces a stable mutant protein that is properly localized (Figure 4) but has a nonfunctional EC5 repeat. This mutant protein could possibly dimerize with wild-type proteins in *trans* as its EC1 repeat is normal and, thus, interfere with gene function in a dominant-negative fashion, as we previously demonstrated with the EC1 missense mutation by knocking down the mutant protein by antisense oligonucleotide (Warga and Kane 2007). Furthermore, when homozygous, the EC5 missense allele expresses a phenotype that is similar to, but more severe than, zebrafish alleles that are nonsense mutations (Figure 2 and Figure 4), an attribute also shared by the EC1 missense mutations (Malicki *et al.* 2003; Warga and Kane 2007). Given the remarkably similar action of these antimorphic alleles and how much they resemble one another phenotypically, it seems unlikely that each participates in a novel abnormal interaction. Therefore, the N-cadherin EC5 repeat must also facilitate cell adhesion.

We found that all missense alleles in E- and N-cadherin, whether in EC1 or EC5, had cytosolic β -catenin during early development (Figure 5). This did not occur in nonsense alleles, where β -catenin was normal and membrane-bound.

These results are consistent with later developmental studies where β -catenin is cytoplasmic in neural cells of the *p79* (*ncad*; EC5) mutant, but not in neural cells of the *tm101* (*ncad*; 0) mutant (Hong and Brewster 2006). Ordinarily, β -catenin is sequestered to the cell membrane through association with the cadherin cytoplasmic tail, where it helps anchor cadherins to the cytoskeleton [reviewed in Ratheesh and Yap (2012)]. In the cytoplasm, where β -catenin is a Wnt signal transducer, it is rapidly destroyed unless Wnt signaling is active [reviewed in McCrea *et al.* (2015), Zhan *et al.* (2017)]. However, the adhesive environment affects activity of the destruction complex that regulates cytosolic β -catenin. In more adhesive sedentary cells β -catenin is destroyed rapidly, but in less adhesive motile cells, it is not, whether Wnt signaling is active or not [reviewed in McCrea *et al.* (2015)]. Thus, the mislocalization of β -catenin in our missense mutants is an indicator of their decreased function. Whether increased Wnt activity contributes to their defective cell adhesion remains to be seen. However, Wnt signaling enhances an epithelial to mesenchymal transition in cancer cells [reviewed in Zhan *et al.* (2017)], a behavior that increases cell scattering and metastasis [reviewed in Kalluri and Weinberg (2009)].

If the EC5 repeat plays a vital role in the adhesive binding of cadherin molecules, how might it act? Classical cadherins cannot form adhesive bonds without first dimerizing with each other in *trans* [reviewed in Brasch *et al.* (2012)]. Given what is known about this process, it seems unlikely that EC5 assists in this process. However, opposite the EC1 strand-swapping site is a highly conserved *cis* interface that orients cadherin protomers in parallel to stabilize *trans* interactions. Less is known about the *cis* interaction, because it is difficult to study [reviewed in Brasch *et al.* (2012) and Mrozik *et al.* (2018)]. Nonetheless, missense products that disrupt the EC1 and EC2 *cis* interface behave much like our missense mutants: they inactivate the wild-type product destabilizing adhesion and, by their self, perturb cell contact more (Harrison *et al.* 2011). Although all EC repeats of classical vertebrate cadherins have a similar “Greek key” topology, EC1 and EC5 are the most unique (Boggon *et al.* 2002). The mutation in the EC5 repeat of zebrafish N-cadherin results in the amino acid substitution of a highly conserved isoleucine (Birely *et al.* 2005), as does the mutation in the EC5 repeat of E-cadherin (Kane *et al.* 2005), suggesting a potential biological role. The *Drosophila* ortholog of N-cadherin requires repeats EC1-9 for dimerization; however, its ectodomain adopts a vastly different folding structure from vertebrate cadherins to align repeat interfaces (Jin *et al.* 2012). Hence, it is also possible that the EC5 repeat contributes to *trans* or *cis* interactions simply by enabling the vertebrate ectodomain to adopt the proper conformation required for cadherin engagement.

Do E- and N-cadherin have heterophilic interactions?

Vertebrate classical cadherins are generally regarded as homophilic adhesion molecules and a large body of evidence

supports this view [reviewed in Takeichi (1995), Halbleib and Nelson (2006), Oda and Takeichi (2011), Takeichi (2011)]. However, there are also numerous reports that classical cadherins form heterophilic interactions (Volk *et al.* 1987; Shan *et al.* 2000; Shimoyama *et al.* 2000; Niessen and Gumbiner 2002; Duguay *et al.* 2003; Patel *et al.* 2006; Prakasam *et al.* 2006; Katsamba *et al.* 2009; Ounkomol *et al.* 2010), although only one shows this *in vivo*, where in the differentiated endodermal organs of mammals, *trans* and *cis* interactions may be quite common (Straub *et al.* 2011). Here, we show that missense alleles of N-cadherin produce a mutated protein that is properly inserted into the membrane (Figure 4), as do the missense alleles of E-cadherin (Figure 4), and that these proteins interfere in a dominant-negative fashion. Hence, these alleles allow us to ask if E- and N-cadherin interact, and perhaps dimerize with each. Our observed genetic interactions suggest they might.

All the missense mutants when homozygous express more severe cell adhesion defects than homozygous nonsense mutants (Figure 4 and Figure 5) (Kane *et al.* 2005; Warga and Kane 2007). Our explanation for this phenotype is that the mutant protein dimerizes and interacts with some other protein. The most likely candidate would be another type I classical cadherin because of the architecture of the *trans* and *cis* interface that is specific for each cadherin subclass [reviewed in Brasch *et al.* (2012)]. As E- and N-cadherin expression tends to be complementary to each other (Figure 1), if heterophilic interactions occur they are likely to be in *trans*, thus juxtaposed tissues would be affected. Dysfunctional *trans* interactions between E- and N-homodimers might explain why, in a homozygous N-cadherin missense mutant (Figure 1U), epidermal cells that express wild-type E-cadherin cannot migrate over neurectoderm cells expressing mutant N-cadherin, even though these epidermal cells are also in contact with the enveloping layer above them, which likewise expresses wild-type E-cadherin.

The phenotypes of embryos that are heterozygous mutant for E- and N-cadherin missense alleles (Figure 6), or heterozygous mutant for an E-cadherin missense allele and homozygous mutant for a N-cadherin missense allele (Figure 7), are also consistent with *trans* interactions between E- and N-homodimers being perturbed between the enveloping layer, the epidermis, and the neurectoderm; phenotypes perhaps compounded by defective *cis* interactions. If heterophilic *trans* interactions cannot occur, via substituting a N-cadherin nonsense allele for the N-cadherin missense allele, these synergistic defects disappear (Figure 8). Finally, consistent with E- and N-cadherin possibly working together in homophilic and heterophilic interactions on some cell types, dorsal forerunner cells in embryos homozygous mutant for either missense allele have defective morphogenesis (Figure 1) and, when doubly homozygous mutant, do not differentiate at all (Figure 9). This could stem from defective *trans* and *cis* interactions between E- and N-heterodimers on the forerunner cells themselves, which express both, and defective *trans* interactions between E- and N-homodimers as

the enveloping layer above expresses E-cadherin, while the yolk syncytial layer below expresses N-cadherin. Perhaps this is why forerunner cells require both cadherins early on in the embryo and why E-cadherin later disappears. For their derivative Kupffer's vesicle must eventually incorporate into mesodermal derivatives, where N-cadherin is expressed, to fulfill its role in left-right asymmetry.

While it is possible that these synergistic phenotypes all stem from interdependent functions being disrupted, and that E- and N-cadherin do not directly or normally interact, why is having a mutant EC repeat more detrimental to cell adhesion than no protein whatsoever? In the case of E-cadherin, it is maternally supplied and the zygotic interfering product likely inactivates maternal wild-type E-cadherin (Kane *et al.* 2005), exacerbating the mutant phenotype. However, N-cadherin is not maternally supplied (Figure 4, L–N), so this explanation is not probable. Perhaps it is because these missense products possess a mutant ectodomain. One possibility is that these mutant ectodomains enable abnormal interactions between E- and N-cadherin; why both EC1 and EC5 would have the same effect is unclear. A second possibility is that post-translational modification on the EC repeats by *N*-glycosylation is perturbed. These modifications are critical for *cis*-dimerization, but while deleting these sites by site-directed mutagenesis alters cell adhesion, it does not affect the cadherin–catenin complex (Guo *et al.* 2009). A third possibility is that signaling pathways controlling cell adhesive behavior are affected, as the ectodomains of E- and N-cadherin form complexes with several cell surface receptors thereby modulating their signaling [reviewed in Christofori (2003), McCrea *et al.* (2015), and Mrozik *et al.* (2018)]. If this were so, this might explain why β -catenin mislocalizes to the cytoplasm in cells of the missense mutants.

The effect of E- and N-cadherin on forerunner cell morphogenesis

Whereas E- and N-cadherin expression is mostly complementary in the embryo (Figure 1), dorsal forerunner cells briefly coexpress both cadherins. In each missense mutant, forerunner cells are specified properly, but each shows a different patterning defect (Figure 2). In E-cadherin missense mutants, forerunner cells have elevated β -catenin levels, and in N-cadherin missense mutants there are fewer cells before Kupffer's vesicle forms. Nonetheless, in both mutants (here we mean the E-cadherin ZMD mutant and the N-cadherin homozygous mutant), Kupffer's vesicle forms, but later left-right asymmetry is perturbed. However, if embryos are mutant for both cadherin missense alleles, forerunner cells are not specified properly nor do they differentiate (Figure 9), in contrast to embryos mutant for both cadherin nonsense alleles, where forerunner cells appear remarkably normal (Figure 9). Perhaps the difference lies in β -catenin no longer being properly localized in the forerunner cells of the double missense mutant (Figure 9), as β -catenin activity is known to be crucial for the activation of downstream target genes in forerunner cell development (Schneider *et al.* 2008; Lin and

Xu 2009; Caron *et al.* 2012; Zhang *et al.* 2012). In the double nonsense mutant, embryos inherit wild-type E-cadherin maternally and possibly this is enough to compensate, free of zygotic interfering products. In any case, the combined effect of two cadherin missense mutations is synergistic, suggesting that cells in the forerunner cluster perhaps utilize a combination of homotypic and heterotypic cellular interactions, which experimental and theoretical studies demonstrate have markedly different adhesive properties (Katsamba *et al.* 2009). However, there are other adhesion molecules that control forerunner cell behavior (Ablooglu *et al.* 2010; Oteiza *et al.* 2010; Zhang *et al.* 2016), whose interactions in the missense mutants might also be impaired.

Animal cells often express multiple cadherins and adhesive affinity is thought to be due to the combinatory action of multiple homophilic interactions [reviewed in Takeichi (1995) and Halbleib and Nelson (2006)]. Yet during early development, most cells only express E- or N-cadherin. While homophilic interactions would be sufficient for cells expressing the same cadherin to retain contact with each other, heterophilic interactions in *trans* would permit juxtaposed tissues expressing E- and N-cadherin to adhere to one another, as our studies suggest. Furthermore, heterophilic interactions in *cis* might allow cells that express both to further modulate their adhesion. It will be of interest to determine to what degree E- and N-cadherin physically interact, or if missense products only promote abnormal interactions. How the EC5 repeat contributes to cadherin-based cell adhesion will also be important. Moreover, it is notable that to date no mutations exist in either EC2 or EC3, while other EC repeats have many (Figure 2A), thus we wonder if such mutations have been missed because of heterozygous lethality or instead they are silent.

Acknowledgments

We acknowledge the late Lyndsay Field for her contribution to this work while an undergraduate at the University of Rochester, and thank Dhara Patel and Darwin Warga Kane for help with experiments while high school students here, our colleague Pamela Hoppe for helpful comments on the manuscript, and our friends Victoria Prince and Thomas Schilling for the kind gift of the E- and N-cadherin antibodies. The research was supported by grants from the Pew Charitable trust (P0215SC), the National Institutes of Health (GM-58513), and start-up funds and grants (08-041) from Western Michigan University.

Literature Cited

- Ablooglu, A. J., E. Tkachenko, J. Kang, and S. J. Shattil, 2010 Integrin α V is necessary for gastrulation movements that regulate vertebrate body asymmetry. *Development* 137: 3449–3458. <https://doi.org/10.1242/dev.045310>
- Alexander, J., and D. Y. Stainier, 1999 A molecular pathway leading to endoderm formation in zebrafish. *Curr. Biol.* 9: 1147–1157. [https://doi.org/10.1016/S0960-9822\(00\)80016-0](https://doi.org/10.1016/S0960-9822(00)80016-0)
- Amack, J. D., and H. J. Yost, 2004 The T box transcription factor no tail in ciliated cells controls zebrafish left-right asymmetry. *Curr. Biol.* 14: 685–690. <https://doi.org/10.1016/j.cub.2004.04.002>
- Barresi, M. J., S. Burton, K. Dipietrantonio, A. Amsterdam, N. Hopkins *et al.*, 2010 Essential genes for astroglial development and axon pathfinding during zebrafish embryogenesis. *Dev. Dyn.* 239: 2603–2618. <https://doi.org/10.1002/dvdy.22393>
- Bierkamp, C., and J. A. Campos-Ortega, 1993 A zebrafish homologue of the *Drosophila* neurogenic gene Notch and its pattern of transcription during early embryogenesis. *Mech. Dev.* 43: 87–100. [https://doi.org/10.1016/0925-4773\(93\)90027-U](https://doi.org/10.1016/0925-4773(93)90027-U)
- Birely, J., V. A. Schneider, E. Santana, R. Dosch, D. S. Wagner *et al.*, 2005 Genetic screens for genes controlling motor nerve-muscle development and interactions. *Dev. Biol.* 280: 162–176. <https://doi.org/10.1016/j.ydbio.2005.01.012>
- Bitzur, S., Z. Kam, and B. Geiger, 1994 Structure and distribution of N-cadherin in developing zebrafish embryos: morphogenetic effects of ectopic over-expression. *Dev. Dyn.* 201: 121–136. <https://doi.org/10.1002/aja.1002010204>
- Boggon, T. J., J. Murray, S. Chappuis-Flament, E. Wong, B. M. Gumbiner *et al.*, 2002 C-cadherin ectodomain structure and implications for cell adhesion mechanisms. *Science* 296: 1308–1313. <https://doi.org/10.1126/science.1071559>
- Brasch, J., O. J. Harrison, B. Honig, and L. Shapiro, 2012 Thinking outside the cell: how cadherins drive adhesion. *Trends Cell Biol.* 22: 299–310. <https://doi.org/10.1016/j.tcb.2012.03.004>
- Brooks-Wilson, A. R., P. Kaurah, G. Suriano, S. Leach, J. Senz *et al.*, 2004 Germline E-cadherin mutations in hereditary diffuse gastric cancer: assessment of 42 new families and review of genetic screening criteria. *J. Med. Genet.* 41: 508–517. <https://doi.org/10.1136/jmg.2004.018275>
- Caron, A., X. Xu, and X. Lin, 2012 Wnt/beta-catenin signaling directly regulates Foxj1 expression and ciliogenesis in zebrafish Kupffer's vesicle. *Development* 139: 514–524. <https://doi.org/10.1242/dev.071746>
- Christofori, G., 2003 Changing neighbors, changing behavior: cell adhesion molecule-mediated signalling during tumour progression. *EMBO J.* 22: 2318–2323. <https://doi.org/10.1093/emboj/cdg228>
- Cortés, F., D. Daggett, R. J. Bryson-Richardson, C. Neyt, J. Maule *et al.*, 2003 Cadherin-mediated differential cell adhesion controls slow muscle cell migration in the developing zebrafish myotome. *Dev. Cell* 5: 865–876. [https://doi.org/10.1016/S1534-5807\(03\)00362-9](https://doi.org/10.1016/S1534-5807(03)00362-9)
- Duguay, D., R. A. Foty, and M. S. Steinberg, 2003 Cadherin-mediated cell adhesion and tissue segregation: qualitative and quantitative determinants. *Dev. Biol.* 253: 309–323. [https://doi.org/10.1016/S0012-1606\(02\)00016-7](https://doi.org/10.1016/S0012-1606(02)00016-7)
- El-Brolosy, M. A., and D. Y. R. Stainier, 2017 Genetic compensation: a phenomenon in search of mechanisms. *PLoS Genet.* 13: e1006780. <https://doi.org/10.1371/journal.pgen.1006780>
- Guilford, P., J. Hopkins, J. Harraway, M. McLeod, N. McLeod *et al.*, 1998 E-cadherin germline mutations in familial gastric cancer. *Nature* 392: 402–405. <https://doi.org/10.1038/32918>
- Guo, H. B., H. Johnson, M. Randolph, and M. Pierce, 2009 Regulation of homotypic cell-cell adhesion by branched N-glycosylation of N-cadherin extracellular EC2 and EC3 domains. *J. Biol. Chem.* 284: 34986–34997. <https://doi.org/10.1074/jbc.M109.060806>
- Haddon, C., L. Smithers, S. Schneider-Maunoury, T. Coche, D. Henrique *et al.*, 1998 Multiple *delta* genes and lateral inhibition in zebrafish primary neurogenesis. *Development* 125: 359–370.
- Halbleib, J. M., and W. J. Nelson, 2006 Cadherins in development: cell adhesion, sorting, and tissue morphogenesis. *Genes Dev.* 20: 3199–3214. <https://doi.org/10.1101/gad.1486806>

- Hammerschmidt, M., F. Pelegri, M. C. Mullins, D. A. Kane, M. Brand *et al.*, 1996 Mutations affecting morphogenesis during gastrulation and tail formation in the zebrafish, *Danio rerio*. *Development* 123: 143–151.
- Harrington, M. J., E. Hong, O. Fasanmi, and R. Brewster, 2007 Cadherin-mediated adhesion regulates posterior body formation. *BMC Dev. Biol.* 7: 130. <https://doi.org/10.1186/1471-213X-7-130>
- Harrison, O. J., X. Jin, S. Hong, F. Bahna, G. Ahlsen *et al.*, 2011 The extracellular architecture of adherens junctions revealed by crystal structures of type I cadherins. *Structure* 19: 244–256. <https://doi.org/10.1016/j.str.2010.11.016>
- Hatta, K., and M. Takeichi, 1986 Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development. *Nature* 320: 447–449. <https://doi.org/10.1038/320447a0>
- Heisenberg, C. P., M. Tada, G. J. Rauch, L. Saúde, M. L. Concha *et al.*, 2000 Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. *Nature* 405: 76–81. <https://doi.org/10.1038/35011068>
- Hong, E., and R. Brewster, 2006 N-cadherin is required for the polarized cell behaviors that drive neurulation in the zebrafish. *Development* 133: 3895–3905. <https://doi.org/10.1242/dev.02560>
- Ishiyama, N., and M. Ikura, 2012 The three-dimensional structure of the cadherin-catenin complex. *Subcell. Biochem.* 60: 39–62. https://doi.org/10.1007/978-94-007-4186-7_3
- Jiang, Y. J., M. Brand, C. P. Heisenberg, D. Beuchle, M. Furutani-Seiki *et al.*, 1996 Mutations affecting neurogenesis and brain morphology in the zebrafish, *Danio rerio*. *Development* 123: 205–216.
- Jiang, Y. J., B. L. Aerne, L. Smithers, C. Haddon, D. Ish Horowicz *et al.*, 2000 Notch signaling and the synchronization of the somite segmentation clock. *Nature* 408: 475–479. <https://doi.org/10.1038/35044091>
- Jin, X., M. A. Walker, K. Felsovalyi, J. Vendome, F. Bahna *et al.*, 2012 Crystal structures of *Drosophila* N-cadherin ectodomain regions reveal a widely used class of Ca(2)+-free interdomain linkers. *Proc. Natl. Acad. Sci. USA* 109: E127–E134. <https://doi.org/10.1073/pnas.1117538108>
- Kalluri, R., and R. A. Weinberg, 2009 The basics of epithelial-mesenchymal transition. *J. Clin. Invest.* 119: 1420–1428. <https://doi.org/10.1172/JCI39104>
- Kane, D., and R. Adams, 2002 Life at the edge: epiboly and involution in the zebrafish. *Results Probl. Cell Differ.* 40: 117–135. https://doi.org/10.1007/978-3-540-46041-1_7
- Kane, D. A., R. M. Warga, and C. B. Kimmel, 1992 Mitotic domains in the early embryo of the zebrafish. *Nature* 360: 735–737. <https://doi.org/10.1038/360735a0>
- Kane, D. A., M. Hammerschmidt, M. C. Mullins, H. M. Maischein, M. Brand *et al.*, 1996 The zebrafish epiboly mutants. *Development* 123: 47–55.
- Kane, D. A., K. N. McFarland, and R. M. Warga, 2005 Mutations in half baked/E-cadherin block cell behaviors that are necessary for teleost epiboly. *Development* 132: 1105–1116. <https://doi.org/10.1242/dev.01668>
- Katsamba, P., K. Carroll, G. Ahlsen, F. Bahna, J. Vendome *et al.*, 2009 Linking molecular affinity and cellular specificity in cadherin-mediated adhesion. *Proc. Natl. Acad. Sci. USA* 106: 11594–11599. <https://doi.org/10.1073/pnas.0905349106>
- Kimmel, C. B., W. W. Ballard, S. R. Kimmel, B. Ullmann, and T. F. Schilling, 1995 Stages of embryonic development of the zebrafish. *Dev. Dyn.* 203: 253–310. <https://doi.org/10.1002/aja.1002030302>
- Leckband, D., and A. Prakasam, 2006 Mechanism and dynamics of cadherin adhesion. *Annu. Rev. Biomed. Eng.* 8: 259–287. <https://doi.org/10.1146/annurev.bioeng.8.061505.095753>
- Lele, Z., A. Folchert, M. Concha, G. J. Rauch, R. Geisler *et al.*, 2002 *parachute/N-cadherin* is required for morphogenesis and maintained integrity of the zebrafish neural tube. *Development* 129: 3281–3294.
- Lepage, S. E., and A. E. Bruce, 2010 Zebrafish epiboly: mechanics and mechanisms. *Int. J. Dev. Biol.* 54: 1213–1228. <https://doi.org/10.1387/ijdb.093028sl>
- Lin, X., and X. Xu, 2009 Distinct functions of Wnt/beta-catenin signaling in KV development and cardiac asymmetry. *Development* 136: 207–217. <https://doi.org/10.1242/dev.029561>
- Malicki, J., H. Jo, and Z. Pujic, 2003 Zebrafish N-cadherin, encoded by the *glass onion* locus, plays an essential role in retinal patterning. *Dev. Biol.* 259: 95–108. [https://doi.org/10.1016/S0012-1606\(03\)00181-7](https://doi.org/10.1016/S0012-1606(03)00181-7)
- Masai, I., Z. Lele, M. Yamaguchi, A. Komori, A. Nakata *et al.*, 2003 N-cadherin mediates retinal lamination, maintenance of forebrain compartments and patterning of retinal neurites. *Development* 130: 2479–2494. <https://doi.org/10.1242/dev.00465>
- Mateus, A. R., J. Simões-Correia, J. Figueiredo, S. Heindl, C. C. Alves *et al.*, 2009 E-cadherin mutations and cell motility: a genotype-phenotype correlation. *Exp. Cell Res.* 315: 1393–1402. <https://doi.org/10.1016/j.yexcr.2009.02.020>
- McCrea, P. D., M. T. Maher, and C. J. Gottardi, 2015 Nuclear signaling from cadherin adhesion complexes. *Curr. Top. Dev. Biol.* 112: 129–196. <https://doi.org/10.1016/bs.ctdb.2014.11.018>
- McFarland, K. N., R. M. Warga, and D. A. Kane, 2005 Genetic locus half baked is necessary for morphogenesis of the ectoderm. *Dev. Dyn.* 233: 390–406. <https://doi.org/10.1002/dvdy.20325>
- Melby, A. E., R. M. Warga, and C. B. Kimmel, 1996 Specification of cell fates at the dorsal margin of the zebrafish gastrula. *Development* 122: 2225–2237.
- More, H., B. Humar, W. Weber, R. Ward, A. Christian *et al.*, 2007 Identification of seven novel germline mutations in the human E-cadherin (CDH1) gene. *Hum. Mutat.* 28: 203. <https://doi.org/10.1002/humu.9473>
- Mrozik, K. M., O. W. Blaschuk, C. M. Cheong, A. C. W. Zannettino, and K. Vandyke, 2018 N-cadherin in cancer metastasis, its emerging role in haematological malignancies and potential as a therapeutic target in cancer. *BMC Cancer* 18: 939. <https://doi.org/10.1186/s12885-018-4845-0>
- Niessen, C. M., and B. M. Gumbiner, 2002 Cadherin-mediated cell sorting not determined by binding or adhesion specificity. *J. Cell Biol.* 156: 389–399. <https://doi.org/10.1083/jcb.200108040>
- Nose, A., A. Nagafuchi, and M. Takeichi, 1988 Expressed recombinant cadherins mediate cell sorting in model systems. *Cell* 54: 993–1001. [https://doi.org/10.1016/0092-8674\(88\)90114-6](https://doi.org/10.1016/0092-8674(88)90114-6)
- Oda, H., and M. Takeichi, 2011 Evolution: structural and functional diversity of cadherin at the adherens junction. *J. Cell Biol.* 193: 1137–1146. <https://doi.org/10.1083/jcb.201008173>
- Oteíza, P., M. Köppen, M. L. Concha, and C. P. Heisenberg, 2008 Origin and shaping of the laterality organ in zebrafish. *Development* 135: 2807–2813. <https://doi.org/10.1242/dev.022228>
- Oteíza, P., M. Köppen, M. Krieg, E. Pulgar, C. Farias *et al.*, 2010 Planar cell polarity signalling regulates cell adhesion properties in progenitors of the zebrafish laterality organ. *Development* 137: 3459–3468. <https://doi.org/10.1242/dev.049981>
- Ounkomol, C., S. Yamada, and V. Heinrich, 2010 Single-cell adhesion tests against functionalized microspheres arrayed on AFM cantilevers confirm heterophilic E- and N-cadherin binding. *Biophys. J.* 99: L100–L102. <https://doi.org/10.1016/j.bpj.2010.11.013>

- Patel, S. D., C. Ciatto, C. P. Chen, F. Bahna, M. Rajebhosale *et al.*, 2006 Type II cadherin ectodomain structures: implications for classical cadherin specificity. *Cell* 124: 1255–1268. <https://doi.org/10.1016/j.cell.2005.12.046>
- Prakasam, A. K., V. Maruthamuthu, and D. E. Leckband, 2006 Similarities between heterophilic and homophilic cadherin adhesion. *Proc. Natl. Acad. Sci. USA* 103: 15434–15439. <https://doi.org/10.1073/pnas.0606701103>
- Ratheesh, A., and A. S. Yap, 2012 A bigger picture: classical cadherins and the dynamic actin cytoskeleton. *Nat. Rev. Mol. Cell Biol.* 13: 673–679. <https://doi.org/10.1038/nrm3431>
- Redies, C., and M. Takeichi, 1996 Cadherins in the developing central nervous system: an adhesive code for segmental and functional subdivisions. *Dev. Biol.* 180: 413–423. <https://doi.org/10.1006/dbio.1996.0315>
- Reifers, F., H. Böhl, E. C. Walsh, P. H. Crossley, D. Y. R. Stainier *et al.*, 1998 Fgf8 is mutated in zebrafish *acerebellar* (*ace*) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. *Development* 125: 2381–2395.
- Schneider, I., D. W. Houston, M. R. Rebagliati, and D. C. Slusarski, 2008 Calcium fluxes in dorsal forerunner cells antagonize beta-catenin and alter left-right patterning. *Development* 135: 75–84. <https://doi.org/10.1242/dev.004713>
- Schulte-Merker, S., F. V. Eeden, M. E. Halpern, C. B. Kimmel, and C. Nüsslein-Volhard, 1994 *No tail* (*ntl*) is the zebrafish homologue of the mouse *T* (*Brachyury*) gene. *Development* 120: 1009–1015.
- Shan, W. S., H. Tanaka, G. R. Phillips, K. Arndt, M. Yoshida *et al.*, 2000 Functional cis-heterodimers of N- and R-cadherins. *J. Cell Biol.* 148: 579–590. <https://doi.org/10.1083/jcb.148.3.579>
- Shapiro, L., and W. I. Weis, 2009 Structure and biochemistry of cadherins and catenins. *Cold Spring Harb. Perspect. Biol.* 1: a003053. <https://doi.org/10.1101/cshperspect.a003053>
- Shimizu, T., T. Yabe, O. Muraoka, S. Yonemura, S. Aramaki *et al.*, 2005 E-cadherin is required for gastrulation cell movements in zebrafish. *Mech. Dev.* 122: 747–763. <https://doi.org/10.1016/j.mod.2005.03.008>
- Shimoyama, Y., G. Tsujimoto, M. Kitajima, and M. Natori, 2000 Identification of three human type-II classic cadherins and frequent heterophilic interactions between different subclasses of type-II classic cadherins. *Biochem. J.* 349: 159–167. <https://doi.org/10.1042/bj3490159>
- Smithers, L., C. Haddon, Y. J. Jiang, and J. Lewis, 2000 Sequence and embryonic expression of *deltaC* in the zebrafish. *Mech. Dev.* 90: 119–123. [https://doi.org/10.1016/S0925-4773\(99\)00231-2](https://doi.org/10.1016/S0925-4773(99)00231-2)
- Strähle, U., P. Blader, D. Henrique, and P. W. Ingham, 1993 *Axial*, a zebrafish gene expressed along the developing body axis, shows altered expression in *cyclops* mutant embryos. *Genes Dev.* 7: 1436–1446. <https://doi.org/10.1101/gad.7.7b.1436>
- Straub, B. K., S. Rickelt, R. Zimbelmann, C. Grund, C. Kuhn *et al.*, 2011 E-N-cadherin heterodimers define novel adherens junctions connecting endoderm-derived cells. *J. Cell Biol.* 195: 873–887. <https://doi.org/10.1083/jcb.201106023>
- Takeichi, M., 1995 Morphogenetic roles of classic cadherins. *Curr. Opin. Cell Biol.* 7: 619–627. [https://doi.org/10.1016/0955-0674\(95\)80102-2](https://doi.org/10.1016/0955-0674(95)80102-2)
- Takeichi, M., 2011 Self-organization of animal tissues: cadherin-mediated processes. *Dev. Cell* 21: 24–26. <https://doi.org/10.1016/j.devcel.2011.06.002>
- Takeichi, M., 2018 Historical review of the discovery of cadherin, in memory of Tokindo Okada. *Dev. Growth Differ.* 60: 3–13. <https://doi.org/10.1111/dgd.12416>
- Thisse, C., and B. Thisse, 2008 High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat. Protoc.* 3: 59–69. <https://doi.org/10.1038/nprot.2007.514>
- Townes, P., and J. Holtfreter, 1955 Directed movements and selected adhesions of embryonic amphibian cells. *J. Exp. Zool.* 128: 53–120. <https://doi.org/10.1002/jez.1401280105>
- Volk, T., O. Cohen, and B. Geiger, 1987 Formation of heterotypic adherens-type junctions between L-CAM-containing liver cells and A-CAM-containing lens cells. *Cell* 50: 987–994. [https://doi.org/10.1016/0092-8674\(87\)90525-3](https://doi.org/10.1016/0092-8674(87)90525-3)
- von der Hardt, S., J. Bakkers, A. Inbal, L. Carvalho, L. Solnica-Krezel *et al.*, 2007 The Bmp gradient of the zebrafish gastrula guides migrating lateral cells by regulating cell-cell adhesion. *Curr. Biol.* 17: 475–487. <https://doi.org/10.1016/j.cub.2007.02.013>
- Warga, R. M., and D. A. Kane, 2007 A role for N-cadherin in mesodermal morphogenesis during gastrulation. *Dev. Biol.* 310: 211–225. <https://doi.org/10.1016/j.ydbio.2007.06.023>
- Warga, R. M., and D. A. Kane, 2018 Wilson cell origin for kupffer's vesicle in the zebrafish. *Dev. Dyn.* 247: 1057–1069. <https://doi.org/10.1002/dvdy.24657>
- Warga, R. M., and C. Nüsslein-Volhard, 1999 Origin and development of the zebrafish endoderm. *Development* 126: 827–838.
- Warga, R. M., A. Wicklund, S. E. Webster, and D. A. Kane, 2016 Progressive loss of RacGAP1/ogre activity has sequential effects on cytokinesis and zebrafish development. *Dev. Biol.* 418: 307–322. <https://doi.org/10.1016/j.ydbio.2016.06.021>
- Weinberg, E. S., M. L. Allende, C. S. Kelly, A. Abdelhamid, T. Murakami *et al.*, 1996 Developmental regulation of zebrafish MyoD in wild-type, no tail, and spadetail embryos. *Development* 122: 271–280.
- Westin, J., and M. Lardelli, 1997 Three novel Notch genes in zebrafish: implications for vertebrate Notch gene evolution and function. *Dev. Genes Evol.* 207: 51–63. <https://doi.org/10.1007/s004270050091>
- Wiellette, E., Y. Grinblat, M. Austen, E. Hirsinger, A. Amsterdam *et al.*, 2004 Combined haploid and insertional mutation screen in the zebrafish. *Genesis* 40: 231–240. <https://doi.org/10.1002/gene.20090>
- Yamaguchi, M., F. Imai, N. Tonou-Fujimori, and I. Masai, 2010 Mutations in N-cadherin and a Stardust homolog, Nagie oko, affect cell-cycle exit in zebrafish retina. *Mech. Dev.* 127: 247–264. <https://doi.org/10.1016/j.mod.2010.03.004>
- Yin, C., B. Ciruna, and L. Solnica-Krezel, 2009 Convergence and extension movements during vertebrate gastrulation. *Curr. Top. Dev. Biol.* 89: 163–192. [https://doi.org/10.1016/S0070-2153\(09\)89007-8](https://doi.org/10.1016/S0070-2153(09)89007-8)
- Zhan, T., N. Rindtorff, and M. Boutros, 2017 Wnt signaling in cancer. *Oncogene* 36: 1461–1473. <https://doi.org/10.1038/onc.2016.304>
- Zhang, J., Z. Jiang, X. Liu, and A. Meng, 2016 Eph/ephrin signaling maintains the boundary of dorsal forerunner cell cluster during morphogenesis of the zebrafish embryonic left-right organizer. *Development* 143: 2603–2615. <https://doi.org/10.1242/dev.132969>
- Zhang, M., J. Zhang, S. C. Lin, and A. Meng, 2012 Beta-catenin 1 and beta-catenin 2 play similar and distinct roles in left-right asymmetric development of zebrafish embryos. *Development* 139: 2009–2019. <https://doi.org/10.1242/dev.074435>

Communicating editor: B. Draper